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Iroquois homeobox 3 regulates odontoblast proliferation and differentiation mediated by Wnt5a expression

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ABSTRACT

Iroquois homeobox (Irx) genes are TALE-class homeobox genes that are evolutionarily conserved across species and have multiple critical cellular functions in fundamental tissue development processes. Previous studies have shown that Irxs genes are expressed during tooth development. However, the precise roles of genes in teeth remain unclear. Here, we demonstrated for the first time that Irx3 is an essential molecule for the proliferation and differentiation of odontoblasts. Using cDNA synthesized from postnatal day 1 (P1) tooth germs, we examined the expression of all Irx genes (*Irx1-Irx6*) by RT-PCR and found that all genes except *Irx4* were expressed in the tooth tissue. *Irx1-Irx3* a were expressed in the dental epithelial cell line M3H1 cells, while *Irx3* and *Irx5* were expressed in the dental mesenchymal cell line mDP cells. Only *Irx3* was expressed in both undifferentiated cell lines. Immunostaining also revealed the presence of IRX3 in the dental epithelial cells and mesenchymal condensation. Inhibition of endogenous *Irx3* by siRNA blocks the proliferation and differentiation of mDP cells. *Wnt3a*, *Wnt5a*, and *Bmp4* are factors involved in odontoblast differentiation and were highly expressed in mDP cells by quantitative PCR analysis. Interestingly, the expression of *Wnt5a* (but not *Wnt3a* or *Bmp4*) was suppressed by *Irx3* siRNA. These results suggest that Irx3 plays an essential role in part through the regulation of Wnt5a expression during odontoblast proliferation and differentiation.
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1. Introduction

Tooth development is highly regulated by the interaction between epithelial and mesenchymal cells, which coordinates the appropriate spatiotemporal expression of various regulatory genes required for proper functional tooth shape. A tooth is composed of two main types of hard calcified tissue: enamel and dentin. Enamel is derived from epithelial cells, and it is hardest tissue in the human body and covers the crown of the tooth. In contrast, dentin is derived from mesenchymal cells, which are close to bone tissue, and it represents the principal component of the entire tooth. Although tooth development occurs in a sequential process, the

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epithelium of the tooth germ can be morphologically classified to describe the process of tooth development. Tooth development is initiated by dental epithelium thickening and subsequent invagination into the mesenchyme, at the tip of which the neural crest-derived dental mesenchyme aggregates and proliferates (bud stage). Then, odontogenic epithelial cells and odontogenic mesenchymal cells form the enamel organ and dental papillae, respectively (cap stage). After that, the inner enamel epithelial cells of the enamel organ differentiate into ameloblasts to form enamel while proliferating dental papilla cells differentiate into preodontoblasts, which eventually differentiate into dentin-forming odontoblasts (bell stage). During this process, elaborate communication occurs between epithelial and mesenchymal cells that regulates the spatiotemporal expression of various growth factors, such as Wnt and bone morphogenetic proteins (Bmps). Wnt, an important secreted protein in embryogenesis, is evolutionarily highly conserved in animals and also involved in many organ developments including teeth tissue [1]. Many Wnt molecules are expressed during tooth development. Wnt5a gene is strongly expressed in the mouse dental papillary mesenchyme from the bud to bell stages [2,3], and Wnt5a-deficient mice show retarded tooth growth and smaller and dysregulated tooth patterning with delayed odontoblast differentiation [3]. In human dental papilla cells, Wnt5a promotes the formation of mineralized nodules and induces the expressions of mineralization-related genes, such as dentine matrix protein-1 [4]. These reports suggest that Wnt5a plays an important role in regulating the differentiation of dental mesenchymal cells into odontoblasts. However, the mechanism underlying Wnt5a expression regulation in dental mesenchymal cells remains unclear.

The homeobox is an evolutionarily well-conserved region that plays an important role in morphogenesis, patterning, and cell fate determination in a wide range of organisms, including animals, fungi, and plants [5]. Homeobox genes encode homeodomain proteins that are characterized by a helix-turn-helix transcription factors [6]. Homeodomain proteins are involved in embryonic development and homeostasis, and their abnormalities have been linked to human diseases and congenital abnormalities [7,8]. Homeobox genes can be divided into two groups: Hox gene families that form chromosomal clusters in the genome and Hox gene families that do not form clusters. HOX genes within a cluster are sequentially regulated during embryogenesis and expressed along the anterior-posterior axis to induce the somite structure [9]. The developmental process of an organism has a complicated biological hierarchy in which a large number of genes work in orchestration, and homeobox genes are considered to act at the top of the hierarchy and play an important role in embryogenesis.

Iroquois homeobox (Irx) genes are a family of homeodomain transcription factors found in nematodes to humans [10]. The Irxs gene family is composed of *Irx1*, *Irx2*, and *Irx4* in the IrxA gene cluster and *Irx3*, *Irx5*, and *Irx6* in the IrxB gene cluster in mice and humans [11]. The IrxA cluster is present on chromosome 13 in mice and on chromosome 5 in humans. The IrxB cluster is located on chromosome 8 in mice and on chromosome 16 in humans [11]. Irxs genes are found in the central nervous system (CNS), laryngopharyngeal region, lungs, heart, kidneys, pancreas, limbs, and teeth. Expression analysis of Irxs genes in developing tooth buds revealed that Irx1, Irx2, Irx3, and Irx5 are expressed in the dental epithelium. Irx1 is specifically expressed in the outer enamel epithelium (OEE) [12]. Pituitary homeobox 2 (Pitx2), a critical transcription factor in teeth, promotes *Irx1* transcription in dental epithelial stem cells (DESCs) [13], suggesting that Irxs are involved in tooth development. However, the expression and function of Irxs in dental mesenchymal cells remains unclear.

In this study, we report that Irx3 plays a role in odontoblast proliferation and differentiation. The expression of all Irx genes except for *Irx4* was detected in mouse tooth tissue. *Irx3* mRNA was expressed in mouse dental papilla-derived mDP cells and induced during the mineralization process. Inhibition of endogenous *Irx3* by siRNA blocks the proliferation and differentiation. The expression of *Wnt5a* (but not of *Wnt3a* or *Bmp4*) was suppressed by *Irx3* siRNA. Thus, our results suggest that Irx3 regulates proliferation and differentiation via Wnt5a expression in odontoblasts.

2. Material and methods

2.1. Reagents

Anti-mouse IRX3 antibody was purchased from TAKARA Bio (Shiga, Japan), and an Alizarin Red S staining kit was obtained from PG Research (Tokyo, Japan).

2.2. Animal Experiments

Animal experiments were performed in accordance with the Guidelines for Animal Experiments of Tokushima University. This study was conducted in accordance with the Institutional Animal Care and Use Committee of the University of Tokushima (T30-59). Postnatal day 1 (P1) mice were sacrificed, and the molars, brains, lungs, hearts, and kidneys were aseptically extracted. In addition, tooth germs were extracted from embryos at E13.5.

2.3. RT-PCR and quantitative PCR

TRIzol reagent (Life Technologies) and DNase I (Sigma) were used for mRNA extraction from tissues and cells according to the manufacturer's protocol. cDNA synthesis was performed using the PrimeScript^{RT} Master Mix (Takara Bio) [14]. PCR reactions were carried out using KOD plus-ver2 polymerase (Toyobo Biotechnology) and a TaKaRa PCR Thermal Cycler Dice Touch (Takara Bio) system under the following conditions: 98 °C for 2 min and 25-32 cycles of 98 °C for 15 s, 62 °C for 30 s, and 72 °C for 1 min of all *Irxs* and *Panx3*. Moreover, PCR reactions for all *Wnts* and *Bmps* were carried out under the following conditions: 94 °C for 2 min and 25-35 cycles of 94 °C for 15 s, 66 °C for 30 s, and 72 °C for 1 min. PCR products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide and photographed. Quantitative PCR was carried out with SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and the Thermal Cycler Dice Real-Time System (Takara Bio) under the following conditions: 95 °C for 5 s, followed by 40 cycles of 95 °C for 5 s and 63 °C for 15 s. The primer sequences are listed in Supplemental Table 1.

2.4. Immunohistochemistry

Tissue sections (4 μm) of E13.5 tooth embryos were deparaffinized with xylene and rehydrated by passing through ethanol serially diluted with distilled water. Heat-induced antigen retrieval was performed for 20 min in an autoclave. Anti-mouse IRX3 antibody was applied to the sections and incubated overnight at 4 °C. After washing with PBS, the sections were incubated with Alexa Fluor conjugated secondary antibody (Thermo Fisher). Nuclear staining was performed using DAPI (Invitrogen). Immunofluorescent imaging was performed using a BX-X800 microscope (KEYENCE).

2.5. Cell culture

mDP cells, a dental papilla cell line, were cultured in α-MEM medium containing 10% FBS and 1% antibiotic-antimycotic agent

(Nacalai Tesque, Japan) in an incubator at 37 °C and 5% CO_2 conditions. M3H1 cells, a dental epithelial cell line, were grown in the low-Ca^{2+} DMEM with 3.0 mM $CaCl_2$, 1% sodium pyruvate, 1% L-glutamine, 1% penicillin/streptomycin, and 10% Ca^{2+}-free fetal bovine serum (FBS, Gibco-BRL). Cells were passaged when they reached approximately 70–80% confluence.

2.6. Cell proliferation

Cell proliferation was assessed using the cell counting method. On the day before cell counting, the cells were plated at 2.0×10^3 cells/well in 35 mm glass-bottomed dishes. Every 24 h, the total number of cells was counted in 20 randomly selected fields of view using an inverted microscope at 20× magnification.

2.7. Cell differentiation

The day before induction, mDP cells were plated at 4.0×10^4 cells/well in 35 mm glass bottom dishes. The cells were cultured in mineralization-inducing medium containing 10 mM β-glycerophosphate, 150 μg/mL ascorbic acid, and 10^{-8} M dexamethasone for cell differentiation. The medium was replaced every two days. Alizarin Red S staining was performed to determine calcium deposition [15].

2.8. siRNA experiments

Cells at 60-80% confluency were transfected with siRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol. The following siRNAs were used: mouse *Irx3* (NM_001253822) Si Genome ON-TARGET plus (LQ- 046747-00-0002; Dharmacon) was used to silence *Irx3* and ON TARGET plus Non-Targeting Pool (D-001810-10-05; Dharmacon) was used as a control.

3. Results

3.1. Irxs expression in tooth tissue and dental cell lines

To examine the expression of Irxs mRNA in tooth tissue, we first extracted brain, heart, lung, kidney, and tooth tissues from postnatal day 1 (P1) mice and performed RT-PCR using RNA isolated from these tissues. RT-PCR showed that *Irx1*, *Irx2*, *Irx3*, *Irx5*, and *Irx6* were expressed in the teeth (Fig. 1A). *Irx1*, *Irx2*, and *Irx3* have also been detected in other tissues, such as the brain, lung, heart, and kidney. Meanwhile, *Irx6* was detected only in the teeth and brain.

Since the interaction between epithelial and mesenchymal cells is essential for tooth development, we used the M3H1 and mDP cell lines to determine the expression of the Irx genes in dental epithelial and mesenchymal cells. M3H1 cells expressed *Irx1*, *Irx2*, and *Irx3*, whereas mDP cells expressed *Irx3* and *Irx5* (Fig. 1B), *Irx4* and *Irx6* expression was not detected in either cell lines (Fig. 1B), while only *Irx3* was expressed in both epithelial and mesenchymal cell lines.

Next, to clarify the IRX3 protein localization in tooth tissue, we performed immunostaining of the E13.5 bud stage of the tooth section with an antibody specific to IRX3. The IRX3 expression was observed in both dental epithelial cells and condensed dental mesenchymal cells (Fig. 2). This result was consistent with *Irx3* mRNA expression in the odontogenic cell lines.

To analyze the role of Irx3 in odontoblast differentiation, mDP cells were used. mDP cells form calcified nodules when cultured in a mineralization-inducing medium. Alizarin Red S staining demonstrated that mDP cells had mineralizing capacity after 10 days of culture (Fig. 3A and B). During this process, the mineralization-inducing medium induced gene expression of the preodontoblast marker pannexin 3 (Panx3) [16], indicating that mDP cells underwent cell differentiation over time (Fig. 3C). We found that both *Irx3* and *Irx5* were expressed during mDP cells differentiation (Fig. 3C). In contrast, no bands were detected for *Irx1*, *Irx2*, *Irx4*, or *Irx6*. These results indicate that Irx3 and Irx5 are involved in mDP cell differentiation, and suggest that they may play a role in odontoblast differentiation.

3.2. Inhibition of cell proliferation and differentiation by suppression of endogenous Irx3 expression

Since mesenchymal cell condensation and proliferation are

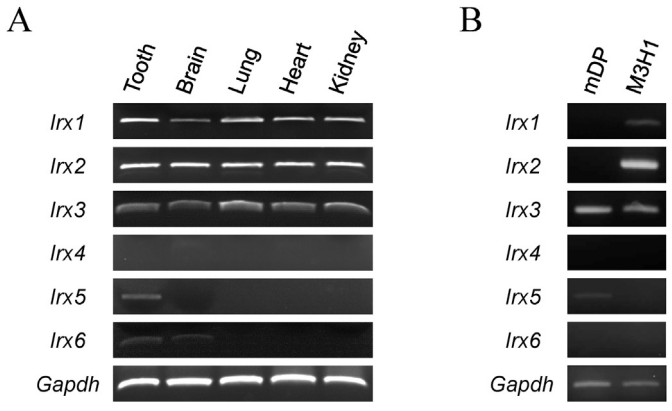

Fig. 1. Expression of *Irxs* mRNA. *Irx1*, *Irx2*, *Irx3*, *Irx4*, *Irx5*, and *Irx6* were analyzed by RT-PCR analyses of RNA from postnatal day 1 (P1) mouse tissues (tooth, brain, lung, heart, and kidney) (A) and mouse dental epithelial cell line M3H1 cells and dental mesenchymal cell line mDP cells (B). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal control. Data were confirmed by three independent experiments, and a representative photograph is shown.

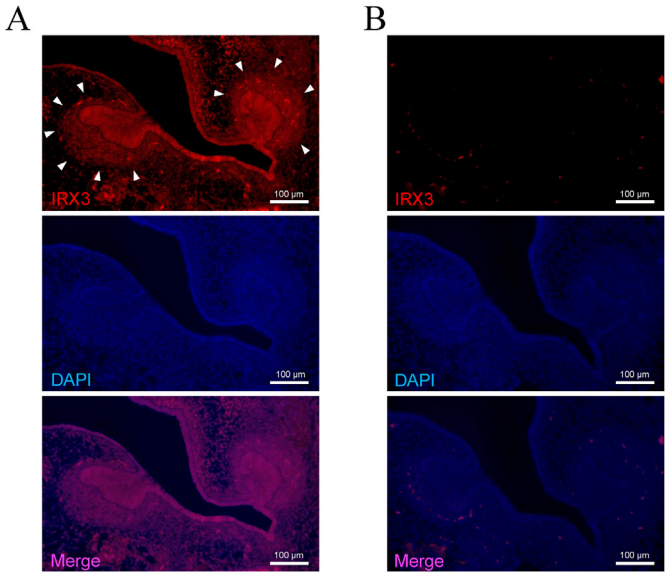

Fig. 2. Expression of IRX3 protein in mouse tooth germ. (A) IRX3 was examined by immunostaining with anti-Irx3 antibody using embryonic day 13.5 (E13.5) tooth sections. Red image and blue image indicate IRX3 expression and DAPI nuclear staining, respectively. IRX3-positive signals were detected in the dental epithelium as well as in the mesenchyme condensation area (arrowheads). (B) Negative control did not include the primary antibody. Scale bar indicates 100 μm. The data shown are representative of three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

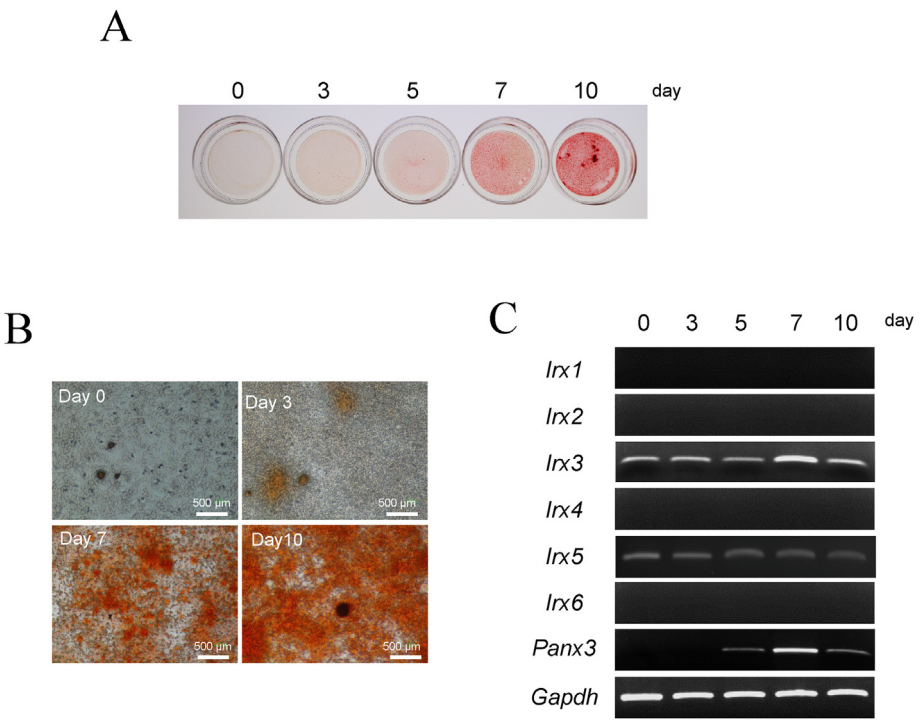

Fig. 3. *Irxs* expression in differentiating mDP cells. The mDP cells were induced to differentiate for 10 days using a mineralization-inducing medium, and the calcified nodules were stained with Alizarin Red S stain (A) and observed at high magnification (B). The gene expression of *Irxs* during the differentiation process of mDP cells was evaluated by RT-PCR (C). *Panx3* and *Gapdh* were used as a differentiation marker of preodontoblasts and an internal control, respectively. Data were obtained by three independent experiments to confirm the data and show a representative photograph. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

essential for tooth development, we knocked down the expression of endogenous *Irx3* using *Irx3* siRNA to analyze the function of endogenous *Irx3* in mDP cell proliferation. We prepared three different sequences of siRNA against *Irx3* to avoid the effects of off-target effects of siRNA and performed independent experiments for each. When *Irx3* siRNA was transfected into mDP cells, *Irx3* expression was significantly reduced at the mRNA level compared to that in the control siRNA-transfected cells (Fig. 4A). In each *Irx3* siRNA-transfected cell, *Irx3* mRNA expression was ~50% of that in the control. We first investigated whether endogenous *Irx3* affected cell proliferation. mDP cells transfected with *Irx3* siRNA showed a dramatically reduced number of cells after 24 and 48 h of culture compared with the control cells (Fig. 4B). These results suggested that Irx3 is required for mDP cell proliferation.

We examined the effect of *Irx3* siRNA on the differentiation of mDP cells during the mineralization. The results showed that suppression of endogenous *Irx3* inhibited the Alizarin Red S staining area, indicating that Irx3 is responsible for the formation of mineralization nodules in mDP cells (Fig. 4C and D). Similarly, two other siRNAs with different sequences also inhibited the formation of mineralized nodules in mDP cells (data not shown). These results suggest that Irx3 is essential for the differentiation of odontoblasts from dental papilla cells.

3.3. *Wnt5a* expression mediated by Irx3

Wnt and Bmp signals are crucial factors in early development of embryos and the many organs, including the teeth. Since Irx3 is involved in the cell differentiation of mDP cells, we examined the expression of *Wnt2b*, *3a*, *5a*, *7b*, *16*, *Bmp2*, and *Bmp4* in mDP cells by quantitative PCR analysis. We found that mDP cells strongly expressed *Wnt3a*, *Wnt5a,* and *Bmp4* (Fig. 5A). We then examined whether the suppression of endogenous *Irx3* expression affected the expression of these genes and found that *Wnt5a* (but not *Wnt3a* and *Bmp4*) was significantly suppressed in *Irx3* siRNA-transfected mDP cells compared to control cells (Fig. 5B and C). These results suggest that Irx3 regulates Wnt5a expression.

4. Discussion

Irx homeobox genes are expressed in a highly specific and regional pattern in tissues, and they are coordinately regulated at the cluster level. In most tissues, the expression patterns of the clustered genes, especially Irx1 and Irx2 and Irx3 and Irx5 were very closely similar, while the expression patterns of Irx4 or Irx6 are not synchronized [17,18]. *Irx1* and *Irx2* and *Irx3* and *Irx5* have been reported to show similar gene expression patterns in E13.5 tooth embryos, while *Irx4* and *Irx6* expression was not observed [17]. In this study, we analyzed the expression of Irx homeobox genes in the tooth germ and found that all Irx genes except *Irx4* are expressed in teeth. Irx homeobox genes form IrxA and IrxB clusters on chromosomes, and they are characterized by the orientation of their transcription [19]. In the IrxA cluster with *Irx1*, *Irx2*, and *Irx4* in series, *Irx2* and *Irx4* have the same transcription direction, while *Irx1* presents in the opposite direction. Similarly, in cluster B, *Irx3*, *Irx5*, and *Irx6* are transcribed in series but *Irx3* has the opposite transcriptional direction. Bidirectional transcription has been found genome-wide for several genes as an efficient gene expression strategy [20,21]. This system appears to be part of a mechanism that facilitates the transcription of dense genomes and promotes rapid and sophisticated development. These observations suggest that the combinations of Irx1/Irx2 and Irx3/Irx5, which are transcribed in opposite directions, play an important role in tissue development, including tooth tissue.

Tooth development is mediated by the interactions between epithelial and mesenchymal cells. To determine the role of Irx

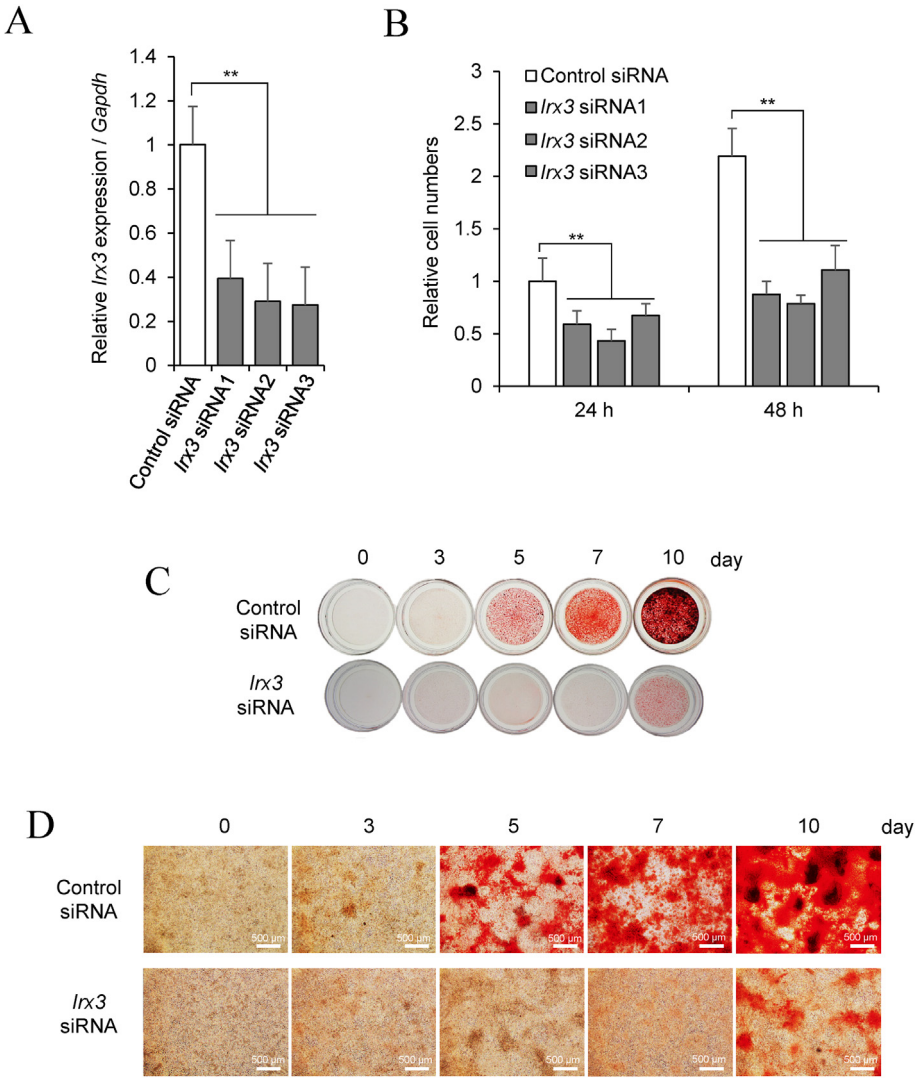

Fig. 4. Inhibition of mDP cell proliferation and differentiation by suppression of endogenous *Irx3*. (A) Real-time PCR was performed to analyze the effects of three different *Irx3* siRNAs on the expression of endogenous Irx3. (B) Cell proliferation was analyzed by the cell counting method after 24 and 48 h. Data are pooled from three independent experiments. The error bars indicate the standard deviation of the mean. Analysis of variance was performed (*P < 0.01). (C) To evaluate the effect of *Irx3* siRNA on mDP cell differentiation by Alizarin Red S staining, the differentiation was induced for 10 days using a mineralization-inducing medium. (D) Microscopic images of Alizarin Red S staining. Three independent experiments were performed to confirm the repeatability of the results. The figure shows a representative picture. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

homeobox genes in teeth, we used the two odontogenic cell lines, M3H1 cells and mDP cells. The M3H1 cells are a dental epithelial cell line and are established from incisor cervical loops derived from the dental epithelium of mice [22]. When cultured in a differentiation-inducing medium, M3H1 cells can mimic the differentiation process of ameloblasts because of their ability to express the enamel matrix proteins, such as ameloblastin and amelogenin, and form calcified nodules. The mDP cell line is composed of dental papilla cells derived from the odontogenic mesenchyme of mice [23]. By inducing differentiation, mDP cells express Panx3, a gap junction protein specifically expressed by preodontoblasts, followed by the expression of dentin matrix protein (Dspp), which is secreted by the terminally differentiated odontoblasts that can then eventually form calcified nodules [16]. Therefore, inducing mDP cell differentiation is useful for analyzing odontoblast differentiation stages. In the current study, we showed that undifferentiated M3H1 cells expressed *Irx1*, *Irx2*, and *Irx3*. In our previous study, *Irx1* expression was reduced in differentiating M3H1 cells, indicating that Irx1 is not involved in the terminal differentiation of ameloblasts [24]. X-gal staining of *Irx1*-LacZ reporter mice revealed that Irx1 is specifically expressed in the outer enamel epithelium (OEE), stellate reticulum, and intermediate layers of developing teeth [12]. A study using *Irx1* null mice showed delayed dental epithelial invagination during initial tooth development and lower incisors in the neonatal stage with lengths that were approximately 85% those of control mice, indicating that Irx1 may be involved in the regulation of tooth morphogenesis [12]. Compared with epithelial cells, undifferentiated mDP cells expressed *Irx3* and *Irx5* based on RT-PCR. Interestingly, we found that only *Irx3* was strongly expressed in undifferentiated M3H1 and mDP cells. Immunostaining also revealed IRX3 expression in both dental epithelial and dental mesenchymal cells in the E13.5 tooth germ. This result was consistent with previous studies on *Irx3* expression, as shown by in situ hybridization [17]. These results suggest that Irx3 plays an important role in initial tooth development. In dental mesenchymal cells, proliferation is necessary

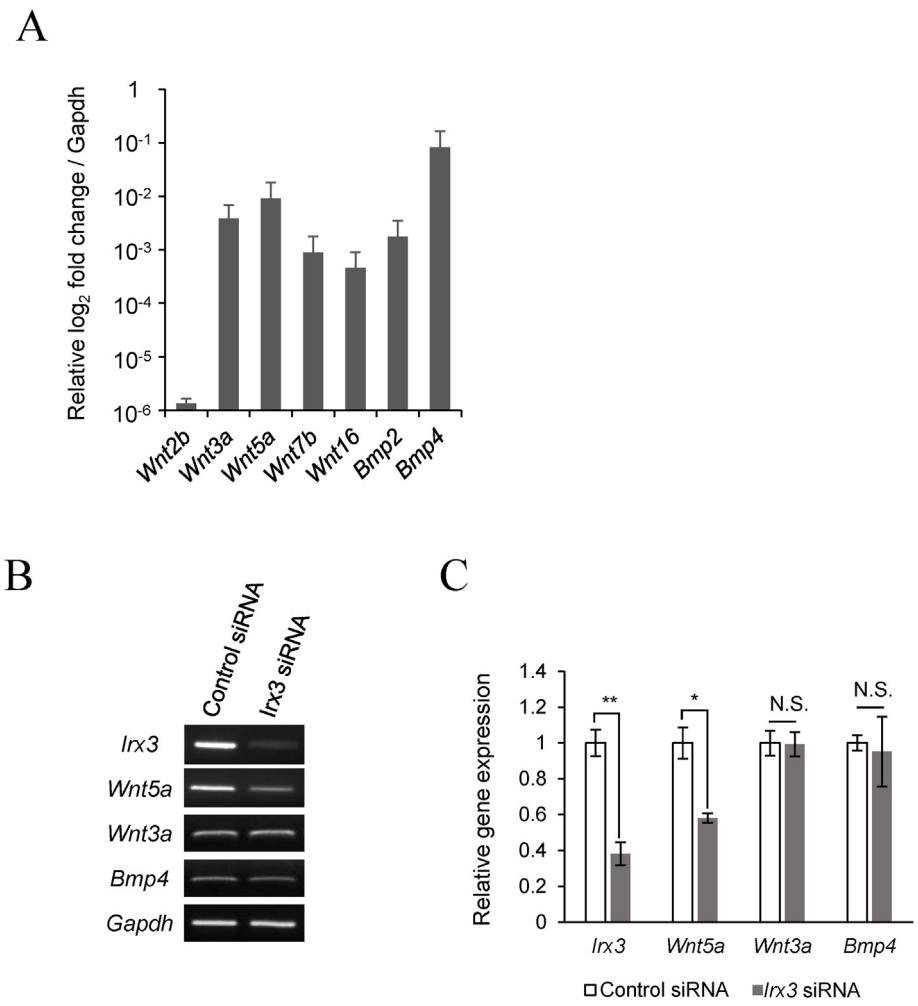

Fig. 5. Wnt5a expression mediated by Irx3. (A) Expression of Wnts in mDP was examined by real-time PCR. (B) Effect of *Irx3* siRNA on the expression of endogenous *Irx3*, *Wnt5a*, *Wnt3a*, and *Bmp4* was analyzed by RT-PCR. Data are pooled from three independent experiments. The error bars indicate the standard deviation of the mean. Analysis of variance was performed (**P < 0.01, *P < 0.05). N.S.: not significant.

during the initial phase of tooth development. The lack of mesenchymal cell proliferation at the bud stage of tooth development results in failed tooth morphogenesis with arrested tooth development [25]. Therefore, to clarify the role of Irx3 in the proliferation of dental mesenchymal cells, we transfected *Irx3* siRNA into mDP cells and examined its effect on proliferation. We found that the knockdown of the endogenous *Irx3* gene expression suppressed mDP cell proliferation. These results suggest that Irx3 is a factor that regulates the proliferation of dental papillary cells in teeth.

Among Iroquois homeobox genes, *Irx5* is associated with human disease. Homozygous mutations in the *IRX5* gene have been implicated in Hamamy syndrome, a human genetic disorder characterized by craniofacial dysmorphisms, hypodontia, microcytic hypochromic anemia, skeletal abnormalities, and congenital heart defects with intraventricular conduction delay [26,27]. However, although *Irx5*-deficient mice were grossly normal, they showed abnormal differentiation of bipolar cells in the retina [28] and increased arrhythmia susceptibility due to the loss of cardiac repolarization gradients [29]. In contrast, *Irx3*-deficient mice also showed normal development of the craniofacial region and abnormal structure and function of the ventricular conduction system [30]. Interestingly, double knockout mice of *Irx3* and *Irx5* showed several similarities with Hamamy syndrome, including heart defects and craniofacial dysmorphism [31]. Furthermore, deletion of osteoblast-specific *Irx3* with Osterix-Cre in *Irx5*-deficient mice showed abnormal craniofacial mineralization and decreased gene expression of the osteogenic regulators, indicating that Irx3 and Irx5 can cooperatively regulate cranial bone formation [32]. In addition, mice lacking *Irx3* or *Irx5* showed increased bone marrow adipocyte mass [33]. Moreover, deletion of hypertrophic chondrocyte (HC)-specific expression of *Irx3* using *Col10a1*-Cre in *Irx5*-deficient mice increased the amount of HC-derived bone marrow adipocytes and decreased the amount of HC-derived osteoblasts [33]. Genome-wide association studies (GWAS) have shown that *Irx3* is a target for fat mass and obesity-associated genes (*Fto*) and plays an important role in determining body size and composition [34]. Fto is known to promote adipocyte differentiation, and its suppression has been reported to promote osteoblast differentiation of bone marrow-derived mesenchymal stem cells [35,36]. These findings suggest that the function of Irx3 involves the regulation of cell fate determination in mesenchymal stem cells and plays a role in skeletal homeostasis and disease. Dental papilla-derived mDP cells are multipotent cells that differentiate into odontoblasts, adipocytes, and neurons [37]. In the present study, when mDP cells were cultured in mineralization-inducing medium, the formation of calcified nodules was dramatically suppressed in cells transfected with *Irx3* siRNA. This result suggests that Irx3 is an essential molecule for odontogenic differentiation of dental papilla

cells.

The Wnt signaling pathway plays a pivotal role in fundamental cellular processes such as survival, proliferation, differentiation, and fate determination in tissue development, and it is also involved in functional morphogenetic signals in the patterning and development of teeth [1,2]. Wnt signaling and homeobox genes also play an important role in axis patterning during early embryonic development. In the neuroectoderm, Wnt signaling, which is differentially expressed in the polarized tissues, induces genome-wide Irx3+ caudal polarization signals [38]. In the developing brain, Wnt signaling induces Irx3, which plays an important role in determining the anterior-posterior axis of the forebrain [39]. In developing ovaries, Wnt/β-catenin signaling directly stimulates the transcription of *Irx3* and *Irx5* [40]. In cartilage, loss and gain of function of HC-specific β-catenin affected Irx3 and Irx5 expression, suggesting that Irx3 and Irx5 act downstream of WNT signaling. In the kidney, Irx3 interacts significantly with Wnt5a during the driving tubular maturation. Furthermore, an orthotopic xenograft model of Wilms tumors with *Irx3*-and *Irx5*-deficient mice showed that *Irx3*−/− cells had reduced expression of WNT5A compared to *Irx5*−/− cells. These results suggest that Irx3 is associated with Wnt5a [41]. In this study, we found that the knockdown of *Irx3* based on siRNA transfection suppressed the expression of *Wnt5a* in mDP cells, suggesting that Irx3 regulates the expression of Wnt5a in dental papilla cells. These results indicate that Irx3 and Wnt signaling have important reciprocal relationships in tissue development, including in the teeth. The suppression of Irx3 may be involved in the inhibition of Wnt5a expression by Irx3, resulting in the decreased formation of calcified nodules in mDP cells.

In summary, our data suggest that Irx3 is essential for odontoblastic differentiation. Furthermore, Irx3 may play a role in tooth development in part through the Wnt5a expression.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that might affect the research reported in this paper.

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Appendix A. Supplementary data

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