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Down-regulation of *Wnt10a* affects odontogenesis and proliferation in mesenchymal cells

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ABSTRACT

The *WNT10a* mutation has been found in patients with abnormal odontogenesis. In mice, *Wnt10a* expression is found in the tooth germ, but its role has not yet been elucidated. We aimed to investigate the role of *Wnt10a* in odontogenesis. Mesenchymal cells of the first mandibular molar germ at the bell stage were isolated, transfected with *Wnt10a* SiRNA or plasmid, and reassociated with epithelial part of the molar germ. Scrambled SiRNA or empty vector was used in the control group. The reassociated tooth germs were transplanted into mice subrenal capsules. After gene modification, dental mesenchymal cells cultured *in vitro* were checked for cell proliferation and the expression of *Dspp* was examined. All 12 reassociated tooth germs in the control group resumed odontogenesis, while only 5 of 12 in the *Wnt10a* knockdown group developed into teeth. After *Wnt10a* knockdown, the mesenchymal cells cultured *in vitro* presented repressed proliferation. *Wnt10a* knockdown and overexpression led to both down- and up-regulation of *Dspp*. We conclude that the down-regulation of *Wnt10a* impairs odontogensis and cell proliferation, and that *Wnt10a* regulates *Dspp* expression in mesenchymal cells. These findings help to elucidate the mechanism of abnormal tooth development in patients with the *WNT10A* mutation.

1. Introduction

The tooth is a critical component of the oral system, which performs mastication, assists in pronunciation, and maintains an aesthetic appearance of the face. Abnormalities in odontogenesis include changes in the number, morphology, or structure of the tooth, jeopardizing normal oral function and aesthetics.

Adaimy et al. reported a mutation in *WNT10A* to be associated with an auotosomal recessive ectodermal dysplasia, the odonto-onycho-dermal dysplasia (OODD), of which the phenotype included severe hypodotia [1]. Bohring et al. reported that *WNT10A* mutations caused not only OODD, but also monosymptomatic oligodontia and Schöpf-Schulz-Passarge syndrome, another form of ectodermal dysplasia that presents tooth abnormality [2]. We also found a high prevalence of the *WNT10A* mutation in patients with non-syndrome oligodontia.

The Wnt signal pathway plays an important role in development and disease [3]. Wnt/β-catenin signaling is active at multiple stages of odontogenesis [4]. Deletion of the β-catenin gene in early tooth mesenchymal cells causes developmental arrest at the bud-to-cap transition [5]. Mouse tooth buds expressing stabilized β-catenin in the epithelium results in abnormal odontogenesis [6].

As a member of the canonical Wnt pathway, *Wnt10a* has been reported to regulate adipogenesis and osteoblastogenesis in mesenchymal stem cells [7]. In odontogenesis, *Wnt10a* expression was first detected in the incisor domain, distal to the molar anlagen at embryonic day 12 (E12). Following this, it is expressed at the tip of the epithelial bud at E13.5, and in the enamel knot at E14.5 [8]. From the early bell stage (E16), *Wnt10a* expression shifts from the secondary enamel knot in the epithelium to the underlying mesenchymal cells. In the odontoblast layer, *Wnt10a* shows a similar expression pattern to dentin sialophosphoprotein (*Dspp*). Transient overexpression of *Wnt10a* in fibroblast cell lines cultured on Matrigel induces *Dspp* mRNA [9]. Cell surface heparan sulfate proteoglycan, which is involved in odotogenesis, binds to *Wnt10a*[10]. These reports suggest a potential role of *Wnt10a* in odontoblast cell differentiation.

Cell proliferation is commonly regulated by Wnt signaling [3]. For example, *Wnt9b*$^{-/-}$ mice show significantly retarded outgrowth of the nasal and maxillary processes due to reduced proliferation of mesenchymal cells, leading to cleft lip and palate [11]. *Wnt5a*, a representative of noncanonical Wnts, affects cell proliferation in the tooth germ; *Wnt5a*-deficient mice develop smaller and abnormally patterned teeth [12]. Yet, the effect of *Wnt10a* on proliferation has not been reported to date.

Bioengineered organ germs made by reassociation of isolated cells from the dental germ resumes odontogenesis and can grow into relatively normal teeth [13,14]. Moreover, after knockdown

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of *Msx1* or *Dlx2* expression by RNA interference (RNAi) in the dental mesenchymal cells, the developmental outcome of reassociated tooth germ generated from these cells recapitulated the phenotype of their specific gene knockout mice [15]. This indicated that reassociated tooth germ after gene knockdown could serve as a method to investigate gene function in odontogenesis.

In our study, we applied *Wnt10a* knockdown and overexpression in dental mesenchymal cells, investigated the influence of *Wnt10a* down-regulation on odontogenesis and cell proliferation, and the effect of *Wnt10a* on *Dspp* expression in dental mesenchymal cells.

2. Materials and methods

2.1. Cell preparation

All animal experiments were approved by the Ethics Committee of Peking University Health Science Center. Embryos at E16 were obtained from time-mated pregnant ICR mice. In sterile phosphate buffered saline (PBS) the first mandibular molar germs were dissected with a fine needle under a stereomicroscope (JSZ6S, Jiangnan Novel, China). Isolation of dental mesenchymal cells was performed as previously described [14]. Briefly, the tooth germs were treated in 1.5 mg/ml Dispase II (Roche, Mannheim, Germany) at 37 °C for 15 min, after which the epithelium was removed with a fine needle. The mesenchyme was then dissociated by gentle pipetting after treatment with 0.25% trypsin (Sigma) at 37 °C for 10 min. Cells were resuspended and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% fetal bovine serum (FBS, Hyclone, Thermo Scientific) with 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin-B (antibiotic antimycotic solution, Sigma, USA) in a humidified incubator at 37 °C with 5% CO_2. Mesenchymal cells were identified by immunohistochemistry using anti-vimentin and cytokeratin-14 antibodies (Bioss, China).

2.2. Genetic modification

pBluescript-*Wnt10a* was kindly provided by Dr. Gregory M. Shackleford of the University of Southern California [16]. Dental mesenchymal cells were trypsinized and washed. Then 1×10^6 cells were transfected with 10 μg of pBluescript-*Wnt10a* or empty vector by Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. To knockdown *Wnt10a*, stealth RNAi duplexes (Invitrogen, USA) were introduced using RNAiMAX (Invitrogen, USA), according to the manufacturer's instructions. Transfection efficiency was assessed by BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen, USA). Cells transfected with scrambled SiRNA (Invitrogen, USA) were used for controls. Twenty-four to 72 h post-transfection, the cells were harvested for RNA and protein extraction or tooth germ reassociation.

2.3. Tooth germ reassociation and in vitro organ culture

Tooth germ reassociation and *in vitro* organ culture were performed as previously described [13], with a slight modification. Briefly, 24 h after RNAi, primary-cultured dental mesenchymal cells were trypsinized, resuspended, and centrifuged at 3000*g* for 2 min into a cell pellet. The cell pellet was transferred onto a semisolid medium made from DMEM (Gibco) containing 0.36% agar (Sigma), 20% fetal bovine serum (Hyclone, ThermoScientific), 0.18 mg/ml ascorbic acid (Sigma), and 2 mM L-glutamine (Gibco). The pellet was then dissected into small parts, using a fine needle under a stereomicroscope. Each piece of the cell pellet contained 2×10^5 cells. Freshly isolated dental epithelium (enamel organ) from first mandibular molar at E16 was overlapped with the mesechymal cell pellet to form a reassociated tooth germ. Twelve reassociated tooth germs were made for each of the control and *Wnt10a*-knockdown groups. The reassociated tooth germ was then cultured on the semisolid medium in a humidified incubator at 37 °C with 5% CO_2 for 24 h before grafting.

2.4. Tooth germ transplantation and hematoxylin-eosin (HE) staining

After 24 h of *in vitro* culture, the reassociated tooth germs were transplanted into the subrenal capsules of adult ICR male mice as previously described [17]. Briefly, the host mice were anesthetized by intraperitoneal injection of 0.04 mg/g phentobarbital. The lumbar region was shaved and disinfected. The left kidney was exposed and a small breach was made in the capsule membrane. The reassociated tooth germs from the control and *Wnt10a* knockdown groups were then inserted into the same subrenal capsule. For each kidney, no more than three germs were grafted. The kidney was replaced into the abdomen and the incision closed. The grafted tissue was harvested at 3, 5, 7, and 14 days after transplantation, fixed in 4% paraformaldehyde (PFA) for 1 day, and demineralized in 10% ethylenediaminetetraacetic acid (EDTA) for 1 week. Samples were then paraffin-embedded and sectioned in 4-μm slices. Sections were stained with hematoxylin and eosin (HE) for routine histologic examination.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Two micrograms of total RNA was reverse-transcribed to cDNA using M-MLV reverse transcriptase (Promega, USA) reagents. The DNA residual was removed by RNase-free DNase (Promega, USA). Quantitative PCR (qPCR) was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) with SYBR Green reagent (Roche, USA). The corresponding primer sequences were *Wnt10a* (sense: 5′-CATGCTCGAATGAGACTCCAC-3′; anti-sense: 5′-CCCTACTGTGCGGAACTCAG-3′), *Dspp* (sense: 5′-AT TCCGGTTCCCCAGTTAGTA-3′; anti-sense: 5′-CTGTTGCTAGTGGTGCTGTT-3′), *Gapdh* (sense: 5′-AGGTCGGTGTGAACGGATTTG-3′; anti-sense: 5′-GGGGTCG TTGATGGCAACA-3′). *Gapdh* served as the endogenous gene control in the samples. The amplification specificity was confirmed by a melting curve. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Western blot assay

Cells were harvested and lysed in RIPA buffer containing proteinase inhibitors. After concentration measurement, protein samples were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane by western blotting. The membranes were blocked in 5% skim milk for 1 h and incubated with antibodies against Wnt10a (Abcam, USA), Dsp (Santa Cruz, USA), and β-actin (Zhongshan Golden Bridge, China) separately at 4 °C overnight. After incubation with peroxidase-linked secondary antibodies, immunoreactive proteins were visualized by ECL reagent (Thermo, USA). Relative quantification of bands in the western blot was performed by Image J software.

2.7. Proliferation assay

Cell proliferation was assessed by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) (an alternative to the MTT assay) and the 5-Ethynyl-2′-deoxyuridine (EdU, an alternative to BrdU) staining kit (Ribo Bio, China), according to the manufacturer's instructions. For the

CCK-8 test, 5×10^3 cells were seeded into each well of a 96-well plate. At 6, 24, 48, 72, and 96 h after RNAi, the cells were cultured in a medium containing 10% CCK-8 solution for 2 h. The optic density at 450 nm was measured by an Absorbance Microplate Reader (BioTek, USA). For EdU staining, 4×10^4 cells were seeded into each well of a 24-well plate. At 48 and 72 h after RNAi, the cells were cultured in a medium containing 50 μM EdU reagent for 2 h, fixed by 4% PFA, washed, and stained with Apollo staining solution. Nuclear DNA was stained with DAPI. For each sample, eight independent zones were randomly chosen and photographed, and positive staining cells were counted by Image-Pro Plus 6.0 software. In each group, at least 2000 cells were counted and the rate of $EdU^+/DAPI^+$ was used to assess cell proliferation.

2.8. Statistical analysis

Statistical significance was determined using the two-tailed Student's *t*-test, assuming equal variances. The chi-squared test was used to compare rates. The significance was indicated as follows: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Down-regulation of Wnt10a impaired odontogenesis of reassociated tooth germs

All 24 grafts exhibited growth. 3 days post transplantation (3 d), the reassociated tooth germs from the control and *Wnt10a* knockdown groups were almost the same size. However, grafted tissue from the control group grew relatively larger as time progressed (Supplementary figure). On the histologic sections, all reassociated tooth germs resumed odontogenesis and recapitulated classic odontogenesis stages. At 3 d, epithelial and mesenchymal cells formed a well-arranged tooth germ structure (Fig. 1A). At 5 days post transplantation (5 d), mesenchymal and epithelial cells at the basement membrane elongated and formed a single layer, which resembled a normal tooth germ at the cap to bell stage (Fig. 1C). One week posttransplantation (1 w), the reassociated tooth germ formed a well-shaped crown, with odontoblast and ameloblast secreting dentin and enamel matrix (Fig. 1E). Two weeks post transplantation (2 w), the reassociated tooth germ continued to grow and a thicker layer of dentin and enamel emerged (Fig. 1G). However, in the *Wnt10a* knockdown group, the majority of reassociated tooth germs (1/3 in the 3 d group, 2/3 in the 5 d group, 2/3 in the 1 w group, 2/3 in the 2 w group; 7/12 total) failed to form any structurally correct dental tissue, and only grew into irregular cyst and bone-like structures (Fig. 1B, D, F and H).

3.2. Down- and up-regulation of Dspp after Wnt10a-knockdown and overexpression

At 24 and 48 h after *Wnt10a* RNAi, *Wnt10a* was successfully knocked down at the mRNA level to 0.276 ± 0.037 ($P < 0.01$) and 0.307 ± 0.15 ($P < 0.05$) times the control group (Fig. 2A). The same trend was seen at the protein level 48 and 72 h after *Wnt10a* RNAi, as demonstrated by western blotting (Fig. 2C). Quantification of the bands showed that *Wnt10a* protein was down-regulated to 0.347 ± 0.175 ($P < 0.05$) and 0.461 ± 0.136 ($P < 0.05$) times the control group (Fig. 2B).

After *Wnt10a* RNAi, the same down-regulation of *Dspp* could be seen. At the mRNA level, *Dspp* dropped to 0.472 ± 0.153 times the control group ($P < 0.05$) at 24 h, and 0.197 ± 0.122 ($P < 0.05$) at 48 h (Fig. 2A). At 48 and 72 h after *Wnt10a* RNAi, Dsp protein decreased to 0.732 ± 0.125 ($P = 0.065$) and 0.708 ± 0.079 ($P < 0.05$) times the control group (Fig. 2B).

After transfection of *Wnt10a* plasmid, overexpression of *Wnt10a* was seen by qPCR at 24 and 48 h and by western blot at 48 and 72 h (Fig. 2D and F). By relative quantification of the protein bands, *Wnt10a* increased to 1.585 ± 0.362 ($P < 0.05$) and 1.820 ± 0.368 ($P < 0.05$) times the control group at 48 and 72 h (Fig. 2E).

Wnt10a-overexpression led to *Dspp* up-regulation, both at the mRNA and protein levels. Twenty-four hours after transfection, *Dspp* mRNA was almost the same as in the control group, but at 48 h it increased to 6.29 ± 2.18 times the control group ($P < 0.05$) (Fig. 2D). Dsp protein increased by 1.898 ± 0.263 times ($P < 0.05$) at 48 h by relative quantification of western blotting, and by 1.978 ± 0.545 times ($P = 0.09$) at 72 h (Fig. 2E).

3.3. Down-regulation of Wnt10a inhibits proliferation of dental mesenchymal cells

In the cell growth curve detected by CCK-8, proliferation of dental mesenchymal cells after *Wnt10a*-knockdown was inhibited. From the same starting point (6 h), the difference between the control and RNAi groups began to emerge at 24 and 48 h, becoming statistically significant at 72 and 96 h (both $P < 0.05$) (Fig. 3A).

We next applied EdU, which incorporates into dividing cells as a thymidine analogue, to find direct evidence of proliferation. In the merged photo, EdU^+ cells are represented in purple. 48 h after *Wnt10a*-knockdown, fewer cells in the RNAi group were EdU^+;

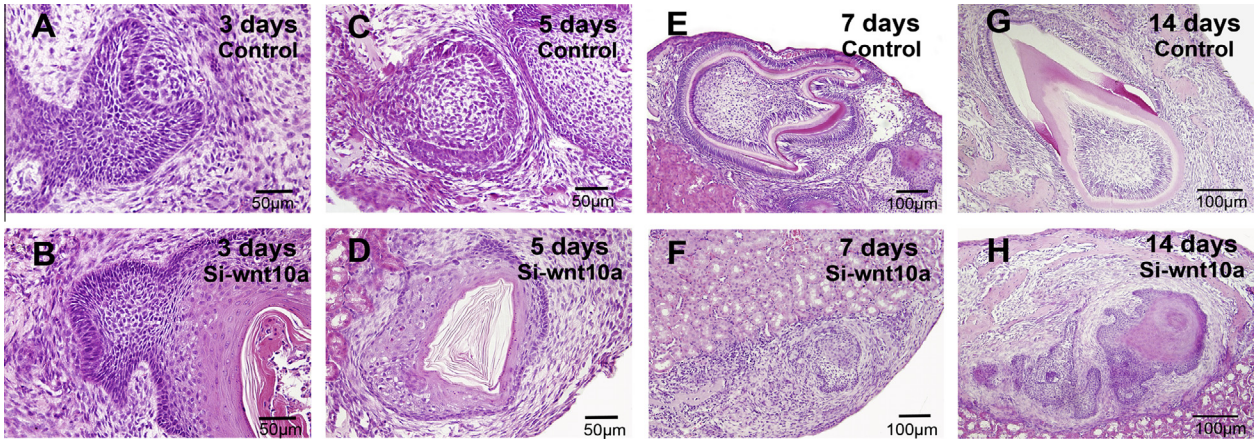

Fig. 1. HE staining of the reassociated tooth germ at 3, 5, 7, and 14 days after grafting. In the control group, all reassociated tooth germs resumed odontogenesis and recapitulated the typical development stages (A, C, E, and G). In the *Wnt10a* knockdown group, the majority of reassociated tooth germs failed to form any structurally correct dental tissue, and only grew into irregular cyst and bone-like structures (B, D, F, and H).

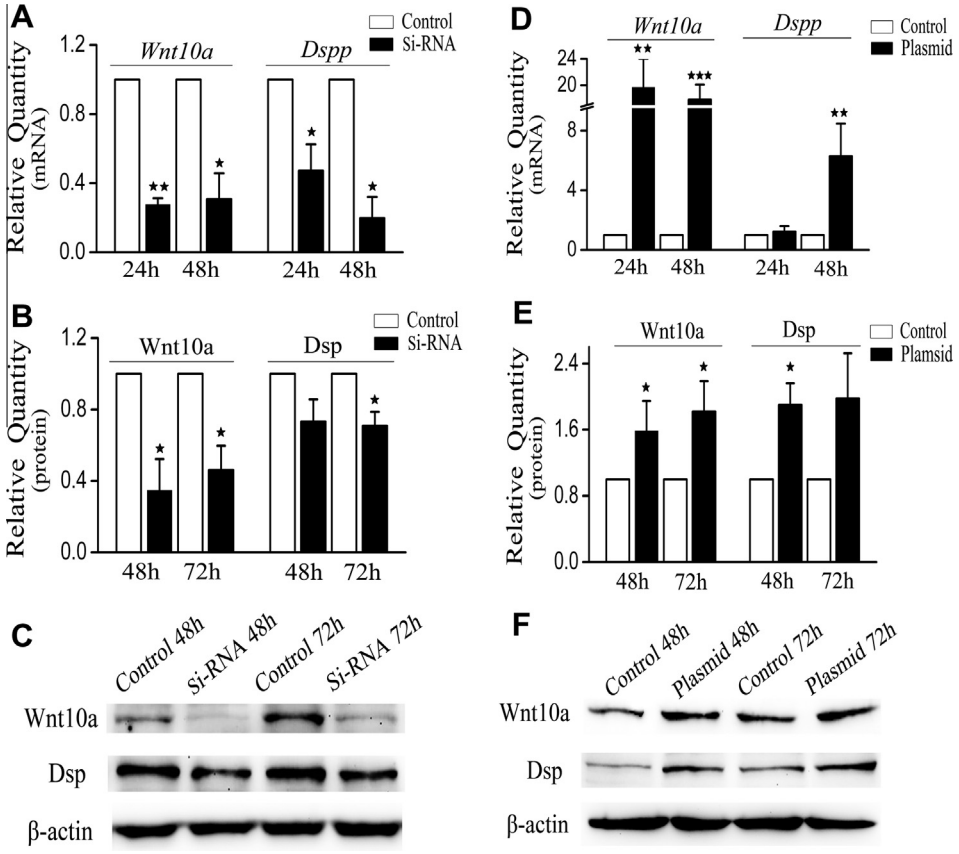

Fig. 2. Regulation of *Dspp* expression by *Wnt10a*. The down-regulation of *Dspp* after *Wnt10a*-RNAi was detected by qPCR at the mRNA level (A), and by western blot both from the band (C) and relative quantification of the band (B). Up-regulation of *Dspp* after *Wnt10a*-transfection could also be seen at the mRNA (D) and protein levels (E and F).

the same trend was seen at 72 h (Fig. 3B). By determining the rate of $EdU^+/DAPI^+$, proliferation in the RNAi group was lower than in controls at both 48 (18.91 ± 0.99% *vs.* 23.83 ± 1.17%) and 72 h (19.65 ± 0.76% *vs.* 25.15 ± 1.78%) (both $P < 0.05$) (Fig. 3C).

4. Discussion

In this study, we first investigated the effect of *Wnt10a* down-regulation on odontogenesis in the reassociated tooth germ model. All the reassociated tooth germs in the control group resumed odontogenesis and developed into structurally correct tooth crowns; this was in accordance with other reports [13–15]. The low percentage of odontogenesis among reassociated tooth germs in the *Wnt10a* down-regulation group corresponded to the oligodontia in the patient with the *WNT10A* mutation. Until now, reports of *WNT10A* focused on cancer research [18] and developmental abnormities [1,2]. To the best of our knowledge, this is the first animal experiment to attribute *Wnt10a*-loss-of-function to abnormal odontogenesis.

However, as a rapid preliminary method to investigate gene function in odontogenesis [15], the reassociated tooth germ model has its drawbacks. Although the reassociated tooth germ could develop dental crown and root, the morphology of the crown is not identical to normal ones [19], so it is not appropriate to assess gene function in determining the crown morphology with this model. Moreover, the subrenal capsule is not the natural physical development niche as compared to alveolar bone. Thus, our work serves as a preliminary study to unveil the effect of *Wnt10a* in odntogenesis; it is necessary to generate *Wnt10a*-knockout mice to further illustrate the function of *Wnt10a* in odontogenesis.

During odontogenesis, odontoblast differentiation starts at the late bell stage in the dental mesenchyme. The expression of *Dspp* by odontoblast is pivotal to dentin formation and tooth morphogenesis [20]. Although *Wnt10a* and *Dspp* have been reported to express a similar pattern in the developing ondotoblast layer by *in situ* hybridization [9], there is no direct link between these two genes in primary cultured dental mesenchymal cells. Our results demonstrate that *Wnt10a* knockdown and overexpression leads to down- and up-regulation of *Dspp* mRNA and Dsp protein (one of the main translation products of the *Dspp* gene) in dental mesenchymal cells. This study provides direct evidence confirming that *Dspp* is the downstream target of *Wnt10a* in dental mesenchymal cells. We thus propose that as an etiological factor, the impaired *WNT10A* affects *DSPP* and odontoblast differentiation, which may jeopardize normal odontogenesis in patients with the *WNT10A* mutation.

Cell proliferation is necessary for normally sized and patterned organ development. The Wnt signal often involves proliferation. Deficiency in either *Wnt9b* or *Wnt5a* could inhibit cell proliferation and lead to abnormal organ development [11,12]. Small and poorly patterned teeth can be seen in patients with the *WNT10A* mutation [21]. To explore the possible cause of this symptom, we first conducted a CCK-8 assay, which provided numbers of live cells with which to draw a cell proliferation curve. Next, we applied EdU, which incorporates into dividing cells like BrdU, to directly assess the evidence of proliferation. The results consistently demonstrated repressed proliferation of dental mesenchymal cells after *Wnt10a* knockdown. To date, this is the first report on the effect of cell proliferation by *Wnt10a.* This may partially explain the development of small and poorly patterned teeth in patients with the *WNT10A* mutation.

In summary, we have shown impaired odontogenesis after *Wnt10a* knockdown, confirmed *Dspp* as the downstream target of *Wnt10a* in dental mesenchymal cells, and demonstrated repressed

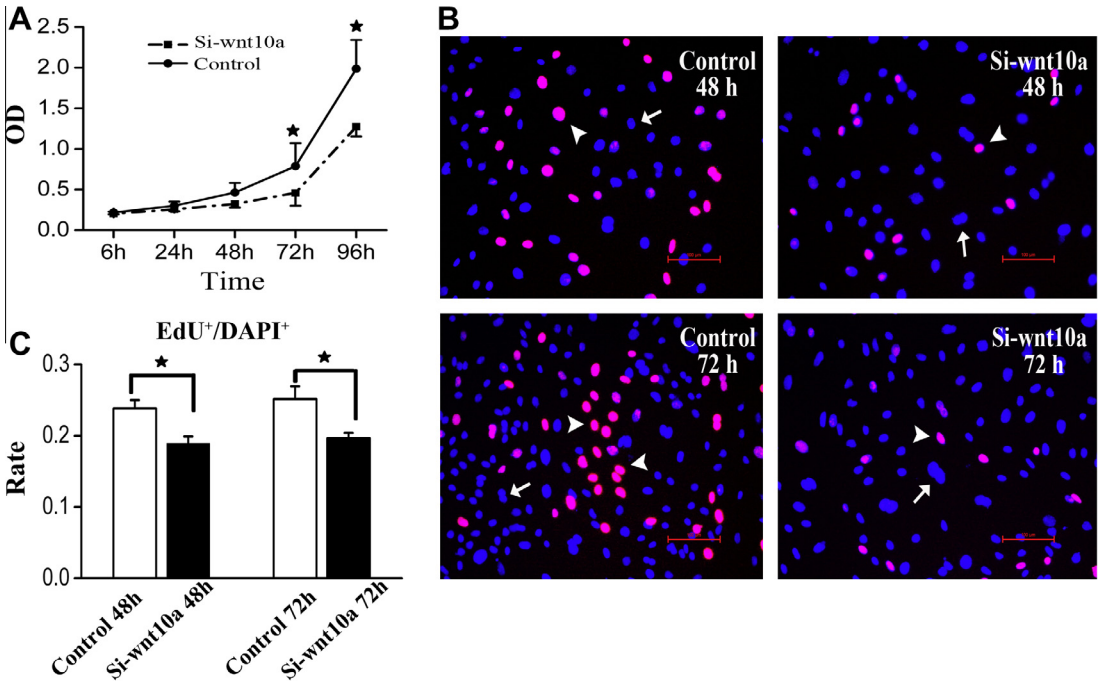

Fig. 3. The effect of *Wnt10a* on mesenchymal cell proliferation. The cells presented repressed proliferation curves after *Wnt10a*-knockdown as detected by CCK-8 assay (A). In the merged photo of EdU staining (B), the *Wnt10a*-knockdown group had fewer EdU^+ cells (arrowhead). The nucleus was stained with DAPI (arrow). The proliferation rate was assessed by $EdU^+/DAPI^+$; the control group demonstrated a higher level of proliferation 48 and 72 h after *Wnt10a*-knockdown (C).

proliferation of dental mesenchymal cells after *Wnt10a* knockdown. Our results may help to elucidate the mechanism of abnormal odontogenesis in patients with the *WNT10A* mutation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.088.

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