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Original Article

Effects of Btbd7 knockdown on the proliferation of human dental pulp cells and expression of Dspp

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Abstract: *BTB/POZ domain-containing protein 7* (*Btbd7*) is recognized as a regulatory gene that promotes epithelial tissue remodeling and branching morphogenesis. In cancer cells, it is involved in epithelial-mesenchymal transition and cell invasion. However, the role of Btbd7 in human dental pulp cells (hDPCs) is not clear. The aim of this study is to explore the function of Btbd7 in hDPCs. Expression of Btbd7 in hDPCs was examined by immunocytochemical staining. Lentiviral vectors expressing small interfering RNA (siRNA)-Btbd7 were used to knockdown expression of Btbd7 in hDPCs. Proliferation of Btbd7 knockdown hDPCs was determined using a cell counting Kit-8 assay, and expression of dentin sialophosphoprotein (Dspp) was assessed using real-time quantitative reverse transcription-PCR and Western blot. Btbd7 was mainly expressed in the cytoplasm and nucleus of hDPCs. Suppression of Btbd7 temporarily promoted hDPC proliferation and significantly inhibited expression of Dspp in hDPCs. Our results show that Btbd7 plays a role in hDPC proliferation, and possibly participates in odontoblast differentiation of hDPCs and dentin formation by regulating the expression of Dspp.

Keywords: BTB/POZ domain-containing protein 7, dentin sialophosphoprotein, human dental pulp cell, proliferation, small interfering RNA

Introduction

Dental pulp is an unmineralized oral tissue that lays hidden under a protective shell. The pulp, under appropriate conditions, tends to form dentin, and might also produce a type of reactionary/reparative dentin in response to exogenous stimuli or injury [1]. Human dental pulp cells (hDPCs) play an important role in dentin formation and regeneration throughout life [2, 3]. Several genes have been reported to play a role during dentin formation and regeneration [4-8]. Dentin sialophosphoprotein (Dspp), a major non-collagenous protein found in dentin, has been documented to be critical for odontoblast differentiation and dentin mineralization [7, 8].

The BTB domain, also known as the POZ domain, is a protein-protein interaction motif that is originally identified as a conserved motif and presents in more than 500 proteins throughout eukaryotes [9]. Recently, BTB/POZ domain-containing protein 7 (Btbd7) has been reported as a dynamic regulator that promotes epithelial tissue remodeling and formation of branched organs [10]. Btbd7 plays essential roles in various cancers, and is mainly involved in promoting epithelial-mesenchymal transition and enhancing cancer cell invasion and metastasis [11-14]. We found that Btbd7 is also expressed in hDPCs, however the role of Btbd7 in hDPCs remains unclear.

Materials and methods

Cell culture

Freshly extracted and caries-free third molars were collected from orthodontic patients (18-25 years of age) at Department of Oral and Maxillofacial Surgery, Hospital of Stomatology,

Shandong University, with the approval of the Ethics Committee of the School of Stomatology, Shandong University (No. 20160301) and written consent of each donor. Dental pulp tissues of extracted healthy human third molars were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) and penicillin-streptomycin solution (Hyclone Laboratories, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The primary cultured hDPCs derived from the dental pulp tissues were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin solution at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells used in this study had not undergone more than four passages.

Immunocytochemistry

hDPCs were plated onto coverslips at a density of $2×10^4$ cells/cm^2. The coverslips were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 hours. The cells were then fixed in 4% paraformaldehyde for 30 min at room temperature. After washing three times in phosphate buffer solution (PBS), cells were permeabilized with 0.05% triton for 5 min and blocked with 4% bovine serum albumin for 30 min at 37°C. Subsequently, the coverslips were incubated with rabbit anti-human polyclonal antibody BTBD7 (Novus Biologicals, Littleton, CO, USA) at a dilution of 1:100 at 4°C in a moist chamber overnight. After washing three times in PBS, the coverslips were incubated with goat anti-rabbit antibody (ZSGB-BIO, Beijing, China) for 60 min at 37°C. The coverslips were then washed in PBS three times. Finally, the coverslips were incubated with streptavidin-biotin-horseradish peroxidase (HRP) complex (ZSGB-BIO, Beijing, China) and the signal was developed with diaminobenzidine substrate kit (Solbraio, Beijing, China). Cell nuclei were counterstained with Mayer's hematoxylin. For the control group, PBS was used instead of the primary antibody.

Small interfering RNA (siRNA) transfection

hDPCs were cultured in six-well plates in serum- and antibiotic-free medium for 24 hours until they reached 80% confluence. After that, hDPCs were transfected with siRNA-Btbd7 or the control siRNA (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The optimal concentration of siRNA-Btbd7 was 50 nM. After an incubation period of 48 hours, the extent of gene knockdown was evaluated by real-time quantitative reverse transcription-PCR (qRT-PCR) and western blot.

Real-time qRT-PCR

Total RNA was extracted from hDPCs using TRI Reagent (Sigma, St. Louis, MO, USA) following the manufacturer's protocol. Then, 1 μg of the total RNA was reverse transcribed using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan). Real-time qRT-PCR was performed using the specific primers and SYBR Green Premix EX Taq (Takara, Otsu, Japan). The annealing and extension temperature was set to 56 C for 45 cycles. The following primers were used in this study: Btbd7 (sense 5'-CTGAGCCACTGACAGGAGAGG-3', antisense 5'-GATCCAGCAGCCTCTTTTCATCC-3'), Dspp (sense 5'-TTTGGGCAGTAGCATGGGC-3', antisense 5'-CCATCTTGGGTATTCTCTTGCCT-3'), and GAPDH (sense 5'-GCACCGTCAAGGCTGAGAAC-3', antisense 5'-TGGTGAAGACGCCAGTGGA-3'). Results were normalized to GAPDH expression. Each assay was performed in triplicate. The differential expression of these genes was analyzed based on the ΔCt method and expressed as the fold changes.

Western blot analysis

hDPCs were collected at the indicated time points and lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, supplemented with 2 mM sodium orthovanadate, 0.5 mg/mL leupeptin and 1 mM sodium fluoride at the time of use) for 30 min. After centrifugation at 12,000 rpm for 15 min, the concentration of obtained protein was determined by BCA protein assay kit (Beyotime, Beijing, China). Equal amounts (15 mg) of total protein were loaded on a 10% SDS-polyacrylamide gel and subjected to electrophoresis at 120 V for 1 hour. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane at 100 V for 1 hour. Then, the PVDF membranes were blocked with 5% non-fat dry milk in TBST buffer (Tris-buffered saline with 0.1% Tween) at room temperature

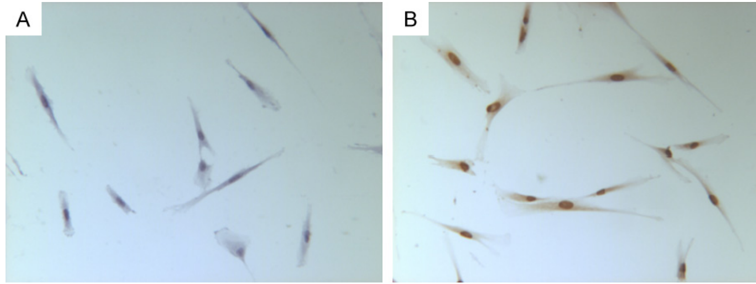

Figure 1. Btbd7 immunocytochemistry (brown) in human dental pulp cells; Mayer's hematoxylin counterstain (blue). Original magnification 200×. A. No Btbd7 staining in the control group. B. Btbd7 staining within the cytoplasm and nucleus of human dental pulp cells.

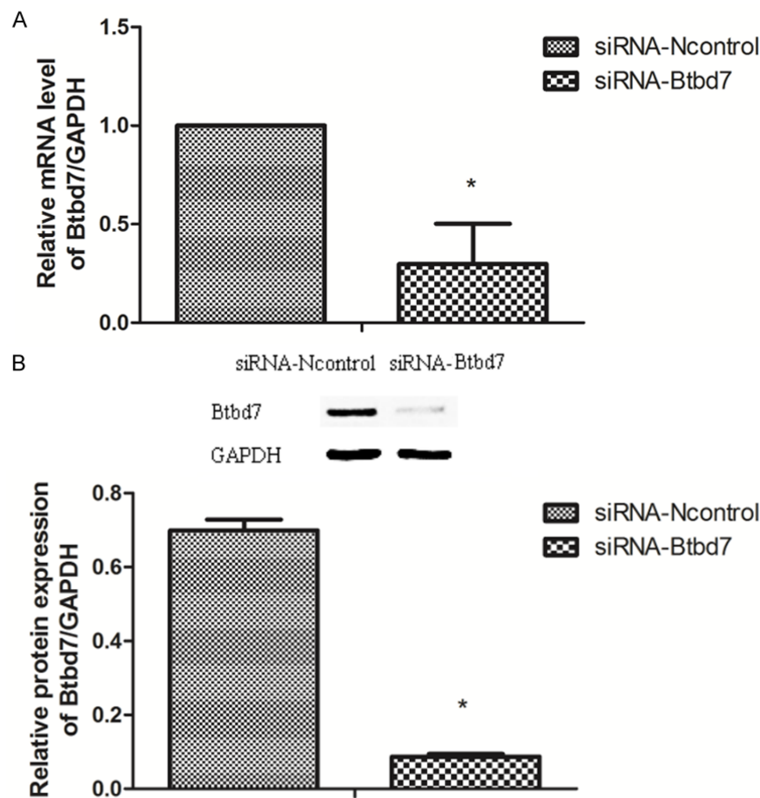

Figure 2. The determination of transfection efficiency. A. The expression level of Btbd7 mRNA was significantly lower in the siRNA-Btbd7 group compared with the control group. B. The expression level of Btbd7 protein in the siRNA-Btbd7 group was significantly lower than that in the control group. Each experiment was repeated in triplicate. siRNA-Ncontrol represents the control siRNA. *$P<0.05$, compared with the control group.

for 1 hour followed by incubation in the primary antibodies (rabbit anti-human BTBD7 antibody and rabbit anti-human DSPP antibody from Thermo Fisher Scientific, Waltham, MA, USA; rabbit anti GAPDH antibody from Protein Tech Group, Chicago, IL, USA) at 4°C overnight. After washing three times in TBST, the membranes were incubated with the secondary antibody conjugated to HRP (Wuhan Boster Biological Engineering Co., Wuhan, China) for 1 hour at 37°C. Proteins were visualized using Enhanced Chemiluminescence reagent (Merck Millipore Co., Billerica, MA, USA) and the Bio-Imaging System (Tanon Science and Technology Ltd., Shanghai, China).

Cell proliferation assay

The effect of Btbd7 on the proliferation of hDPCs was examined with the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Cells were seeded in a 96-well plate at a density of 10^4-10^5 cells/well in 100 μL of culture medium and were cultured in a CO_2 incubator at 37°C for 24 hours. The cells were then transfected with Btbd7 siRNA or the control siRNA (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 1 to 5 days of incubation, 10 μL of CCK-8 solution was added to each well and the cells were further incubated for 3 hours at 37°C. The absorbance at 450 nm was measured using a microplate reader.

Statistical analysis

All results were analyzed using SPSS 17.0 software (IBM, Armonk, NY, USA). Data are presented as mean ± standard deviation (SD) from at least three independent experiments. One-way analysis of variance was used to determine whether there are any statistically significant differences between the groups. Values of $P<0.05$ were considered statistically significant.

Table 1. The number of living hDPCs at different time points after siRNA transfection

	OD (means ± SD, n=4)				
	1 day	2 day	3 day	4 day	5 day
siRNA-Ncontrol	0.802 ± 0.006	1.155 ± 0.007	1.538 ± 0.025	1.904 ± 0.024	2.289 ± 0.228
siRNA-Btbd7	0.814 ± 0.013	1.242 ± 0.015 (*)	1.717 ± 0.015 (*)	2.217 ± 0.035 (*)	2.261 ± 0.119

Note: siRNA-Ncontrol represents the control siRNA. *$P<0.05$, compared with the control group.

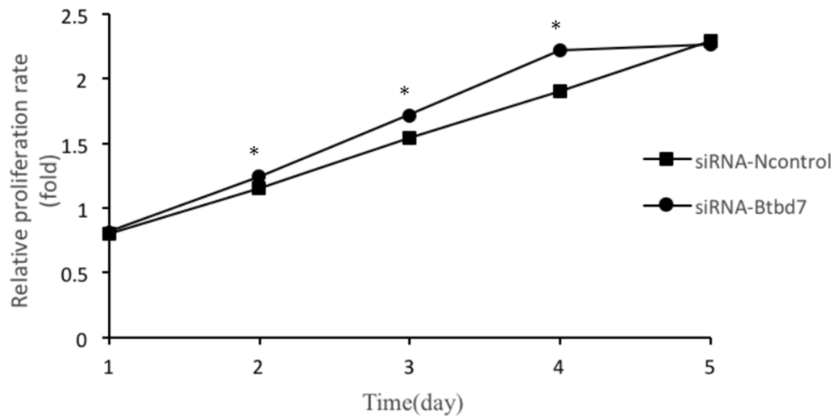

Figure 3. Growth curves of human dental pulp cells transfected with siRNA-Btbd7 or control siRNA. Each experiment was repeated in quadruplicate. siRNA-Ncontrol represents the control siRNA. *$P<0.05$, compared with the control group.

Results

Expression of Btbd7 in hDPCs

The immunocytochemical assay of Btbd7 showed positive staining of the protein mainly in the cytoplasm and nucleus of hDPCs (**Figure 1**).

Establishment of Btbd7 knockdown cells

To investigate the biological functions of Btbd7 in hDPCs, cultured hDPCs were transfected with siRNA-Btbd7 or the control siRNA. As shown in **Figure 2**, the mRNA and protein expression levels of Btbd7 in the siRNA-Btbd7 transfected group were significantly lower than those in the control group. The results demonstrated that Btbd7 knockdown was successful.

siRNA-Btbd7 promotes proliferation of hDPCs

To examine the effects of Btbd7 on proliferation of hDPCs, CCK-8 assays were performed on the cells transfected with siRNA-Btbd7 or the control siRNA at different time points (1 to 5 days) after transfection. As shown in **Table 1** and **Figure 3**, siRNA-Btbd7 significantly promoted proliferation of hDPCs at days 2, 3, and 4, as compared with the control group. This finding demonstrates that Btbd7 temporarily inhibits proliferation of hDPCs.

siRNA-Btbd7 inhibits expression of Dspp in hDPCs

To explore the correlation between Btbd7 and Dspp in hDPCs, hDPCs were transfected with siRNA-Btbd7 or the control siRNA. Afterwards, we used real-time qRT-PCR and Western blot to examine the expression levels of Dspp. The results showed lower Dspp expression levels in the siRNA-Btbd7 group than those in the control group (**Figure 4**), which indicated that expression of Dspp was positively correlated with Btbd7, and Btbd7 enhanced expression of Dspp.

Discussion

In the present study, Btbd7 was detected in hDPCs using immunocytochemical staining. CCK-8 assays on Btbd7 knockdown hDPCs demonstrated that Btbd7 temporarily inhibited the proliferation of hDPCs. Expression of Dspp in hDPCs was inhibited by siRNA-Btbd7 as demonstrated using real-time qRT-PCR and Western blot.

It is well known that functions of the members of the BTB gene family are mainly governed by two prominent mechanisms: BTB domain-based protein-protein interactions and transcriptional regulation by DNA binding domains [9], and *Btbd7* belongs to this family. During embryonic development, the highly focal expression of Btbd7 at cleft-forming sites induces local expression of the cell-scattering gene *Snail2* and suppresses E-cadherin levels,

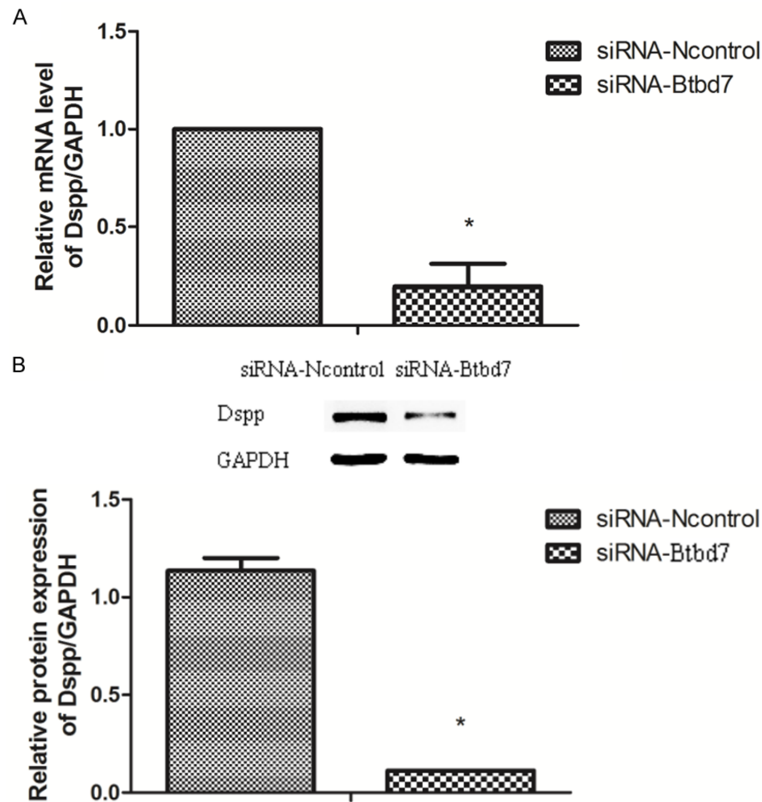

Figure 4. Effects of Btbd7 suppression on the expression of Dspp in human dental pulp cells. A. The expression level of Dspp mRNA was significantly lower in the siRNA-Btbd7 group compared with the control group. B. The expression level of Dspp protein in the siRNA-Btbd7 group was significantly lower than that in the control group. Each experiment was repeated in triplicate. siRNA-Ncontrol represents the control siRNA. **P*<0.05, compared with the control group.

thereby altering epithelial cell mobility [10]. Inhibition experiments show that Btbd7 is required for branching morphogenesis of embryonic organs [10, 15]. hDPCs have odontoblast differentiation potential, and play an important role in dentin formation and regeneration throughout life [2, 3]. Dspp is a key pluripotency gene in hDPCs and involved in epithelial-mesenchymal interactions and branching morphogenesis [7, 8]. Mutations in the *Dspp* gene result in dentinogenesis imperfecta [16-18] and mineralization defects in dentin [19]. Defective dentin secretion and mineralization are associated with significantly decreased Dspp expression [20]. In this study, siRNA-Btbd7 significantly promoted the proliferation of hDPCs at 2, 3, and 4 days after transfection, and dramatically inhibited the expression of Dspp in hDPCs, indicating that Btbd7 plays a role in hDPCs proliferation and differentiation. Btbd7 is also possibly involved in odontoblast differentiation and dentin formation by regulating the expression of Dspp. However, Btbd7 knockdown hDPCs had similar proliferation capability to control cells at 5 days after siRNA transfection, implying that knockdown of Btbd7 only temporarily promoted hDPC proliferation, and that there were other pathways involved in hDPC proliferation. Further investigation on the functions of Btbd7 in hDPCs and the underlying mechanism by which Btbd7 regulates the expression of Dspp is needed.

In conclusion, our results showed that Btbd7 is involved in the proliferation of hDPCs and regulates expression of the key pluripotency gene *Dspp*, suggesting that Btbd7 may participate in odontoblast differentiation of hDPCs and dentin formation by regulating Dspp.

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Disclosure of conflict of interest

None.

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