

Original Article

Effects of human bone morphogenetic protein 2 (hBMP2) on tertiary dentin formation

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Abstract: Formation of tertiary dentin to maintain pulp vitality is a major odontoblastic response to dental pulp injury. Human bone morphogenetic protein 2 (hBMP2) can promote proliferation and differentiation of odontoblasts. Current study is interested in evaluating if the hBMP2 can promote the regeneration of tertiary dentin and cure dental pulp injury using the adenoviral vector to deliver hBMP2 cDNA into the pulp. Primary culture of dental pulp cells of exfoliated deciduous teeth (hDPCs) was established. Human serotype 5 adenoviral vector, AdCMV-hBMP2, was created. AdCMV-hBMP2 was used to transduce hDPCs *in vitro* and dental pulp cells in animal model *in vivo*. Data clearly demonstrated that hBMP2 increased ALP and mineralization. Reverse transcription-real time quantitative PCR (RT-QPCR) data showed that hBMP2 dramatically increased gene expressions of Runx2 (Runt-related transcription factor 2), ALP, Col 1 α (Collagen 1a1), SP7 (Osterix), DMP1 (dentin matrix acidic phosphoprotein 1), DSPP (dentin sialophosphoprotein), and BSP (bonesialoprotein), which are normally involved in osteogenesis/odontogenesis. Data from *in vivo* assays demonstrated that hBMP2 promoted pulp cell proliferation and increased formation of tertiary dentin in dental pulp. Our *in vitro* and *in vivo* data suggest that hBMP2 gene can efficiently be delivered into the dental pulp cells by adenovirus, and show potential clinical application for the treatment of dental pulp damage.

Keywords: hBMP2, formation of tertiary dentin, dental pulp cells, gene therapy, mineralization

Introduction

Enamel, dentin, cementum, and pulp are four major components of a tooth. The rigid dentin provides powerful protection for dental pulp and support for enamel. Untreated dental caries can break down the integrity of the normal dentin safe-shield leading to pulp exposure and subsequent inflammation/infection [1]. More than 0.5 mm of residual dentin thickness (RDT) is necessary to avoid causing any evidence of pulp injury in human teeth [2, 3]. Repair of defective dentin tissue remains a great challenge in clinical practice. Previous studies have shown that odontoblastic processes in human teeth can extend to the enamel-dentinal junction [4-6], and can be detected on the dentinal

surface in the cusp region of both rats and monkeys after the age of 2 years old [7]. In rat molars, diameters of dentinal tubules are 1.3-2.7 μ m at the pulp-dentinal junction and 0.5-1.3 μ m at the enamel-dentinal junction [8]. It was reported that microspheres with diameters of 0.02-0.1 μ m could reach the inner third region of the coronal dentin in rat molars after the coronal enamels of the first and the second molars were carefully removed from the occlusal surface (0.2-0.3 mm thick) [9]. Therefore, an ideal regenerative process is to induce odontoblastic differentiation, and reintegrate the dentin shield to protect the pulp.

Bone morphogenetic protein 2 (BMP2), belongs to the TGF- β superfamily of proteins and plays a

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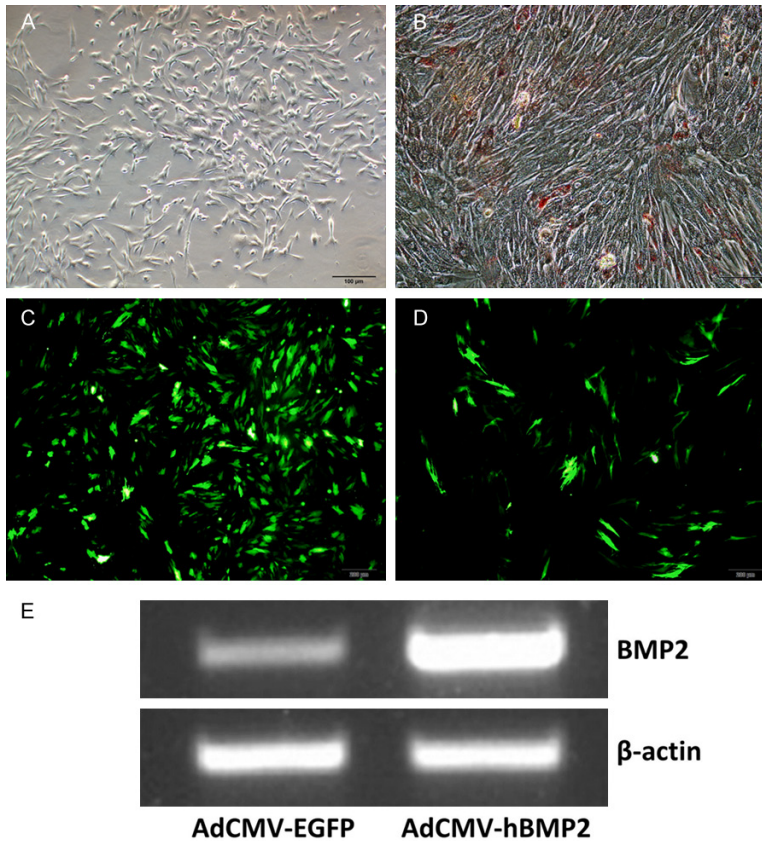


Figure 1. Characteristics of hDPCs and evidence of successful transduction. A. hDPCs were cultured for 10 days. B. hDPCs were stained with Oil Red O after culturing in adipogenic medium for 5 weeks. C. hDPCs were cultured for 3 days after transduction with AdCMV-EGFP at 1000 particles/cells. D. hDPCs were cultured for 28 days after transduction with AdCMV-EGFP at 1000 particles/cells. E. The expression of BMP2 by RT-PCR assay 3 days after hDPCs were transduced with AdCMV-hBMP2 at 1000 particles/cell.

critical role in the development of bone and cartilage [10]. BMP2 is an autocrine protein [10-14]. Previous studies have also demonstrated that BMP2 can promote odontoblastic differentiation and tertiary dentin formation [11-13]. Interestingly, odontoblasts can produce and secrete BMP2 which directly affects the secreting of the adjacent odontoblasts and stimulates their proliferation and differentiation, resulting in dentin formation [14-16].

Actually, recombinant hBMP2 can be used locally. However, repeated injections may be required which makes it inconvenient for local application. Also, the space of the pulp chamber is highly limited. Therefore, tissue engineering, plus gene transfer/gene therapy, is an attractive method to promote odontoblastic differentiation and proliferation to repair or replace damaged cells and tissue. In order to devel-

op novel methods to repair damaged or defective dentin, we delivered hBMP2 into the pulp by gene transduction to evaluate if BMP2 can be used as a bioactive molecule to regenerate tertiary dentin in an *in vivo* rat model.

Materials and methods

Cell culture

Normal human exfoliated deciduous incisors were obtained from children aged from 6 to 8 years old. The protocol was approved by Jilin University under the guidelines of China's bioethics law. The pulp was extracted and washed with H-DMEM (Invitrogen, Carlsbad, CA, USA) containing 1000 units/ml penicillin and 1000 μg/ml streptomycin (Invitrogen), then cut into ~0.5×0.5×0.5 mm mass using sterilized eye scissors in a 1.5 ml Eppendorf tube and immersed in a digestive solution with 3 mg/ml of collagenase type I (Invitrogen) and 4 mg/ml of dispase (Invitrogen) for 15 min at 37°C in 5% CO₂. The hDPCs were

pelleted by spin at 1000 rpm for 5 min. The cell pellet was cultured with H-DMEM containing 20% fetal bovine serum (FBS, Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. After the first passage (~10-15 days), the FBS was reduced to 10%. Cells of passage 3 were used for all further experiments in this study.

Adipogenic induction assay *in vitro*

The hDPCs were plated at 10⁵ cells/well in H-DMEM in 6-well plates and incubated at 37°C in a humidified 5% CO₂ atmosphere. After 36 h, the culture medium was replaced with H-DMEM containing 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 mg/L insulin, and 200 μM indomethacin. The medium was changed every 3 days. On day 35, cells were fixed with 70% ethanol for 1 h, washed with phosphate-

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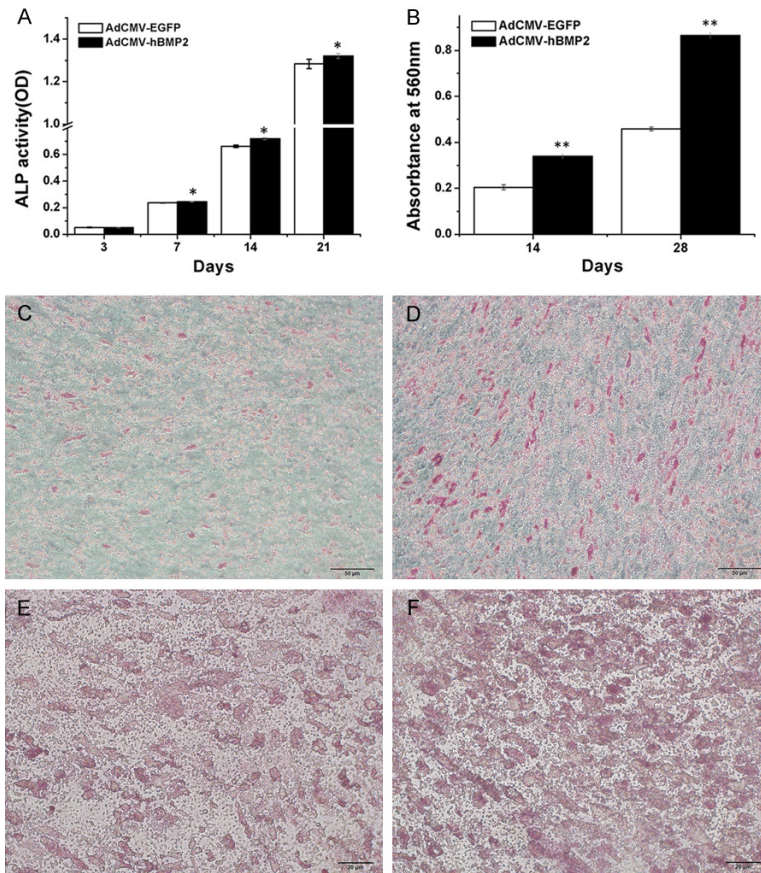


Figure 2. ALP activity and Alizarin Red S staining. A. ALP activity from hDPCs transduced with AdCMV-EGFP or AdCMV-hBMP2. B. Quantification of absorbance at 560 nm. C-F. Alizarin Red S staining. C. 14 days after transduction with AdCMV-EGFP. D. 14 days after transduction with AdCMV-hBMP2. E. 28 days after transduction with AdCMV-EGFP. F. 28 days after transduction with AdCMV-hBMP2. Data shown are means \pm SEM. These assays were repeated three times.

buffered saline (PBS) once, then stained with Oil Red O for 1 h at 37°C, washed with distilled water for three times, and observed under microscope.

Adenoviral vector preparation

For this study, we constructed two E1 deleted replication deficient adenovirus serotype 5 vectors, AdCMV-EGFP and AdCMV-hBMP2. They were both propagated in 293 cell line and purified using CsCl gradient centrifugation. The titers of vectors were determined by real-time QPCR using transgene-specific primers.

BMP2 expression by RT-PCR

The hDPCs were plated at 5×10^4 cells/well in 12-well plates and cultured for 36 h. Then,

the cultured hDPCs were transduced with AdCMV-EGFP with different particles/cell. Finally, 1000 particles/cell was confirmed as the most appropriate concentration for transduction. The hDPCs were plated at 10^5 cells/well in 6-well plates and cultured for 36 h, then transduced with AdCMV-EGFP or AdCMV-hBMP2 at 1000 particles/cell. After 3 days, total RNA was extracted using Trizol (Invitrogen). One μ g of RNA was used for reverse transcription reaction using PrimerScript[®] RT Reagent Kit (Takara, Dalian, China) to synthesize cDNA. Primers hBMP2F1 (5'-AGTTTGTGATGTCACCCCGC-3') and hBMP2B1 (5'-GATAGCACTGAGTTCTGTCTGGGAC-3') were used for PCR reaction. The PCR products were examined by 2% agarose gel electrophoresis, and imaged with Molecular Imager[®] Gel Doc[™] XR System (BIO-RAD, Hercules, CA, USA).

Alizarin Red S staining and alkaline phosphatase (ALP) activity assays in vitro

The hDPCs were plated at 10^5 cells/well in 6-well plates or at 10^3 cells/well in 96-well plates and cultured for 36 h, then transduced using AdCMV-EGFP or AdCMV-hBMP2 at 1000 particles/cell. One day post-transduction, the culture medium was replaced with H-DMEM containing 10 mM/L sodium β -glycerol phosphate (Sigma-Aldrich, St. Louis, MO, USA), 50 mg/L L-ascorbic acid (Sigma-Aldrich), and 10^{-8} M/L dexamethasone (Sigma-Aldrich) to induce mineralization.

The hDPCs in 96-well plates were used to measure cell ALP activity by alkaline phosphatase substrate (Sigma-Aldrich) on days 3, 7, 14, and 21 using a BioTek Microplate Reader under 520 nm, according to the manufacturer's instructions. On days 14 and 28, cells in 6-well plates were washed with PBS, fixed in 95% ethanol at 4°C for 30 min, and stained with

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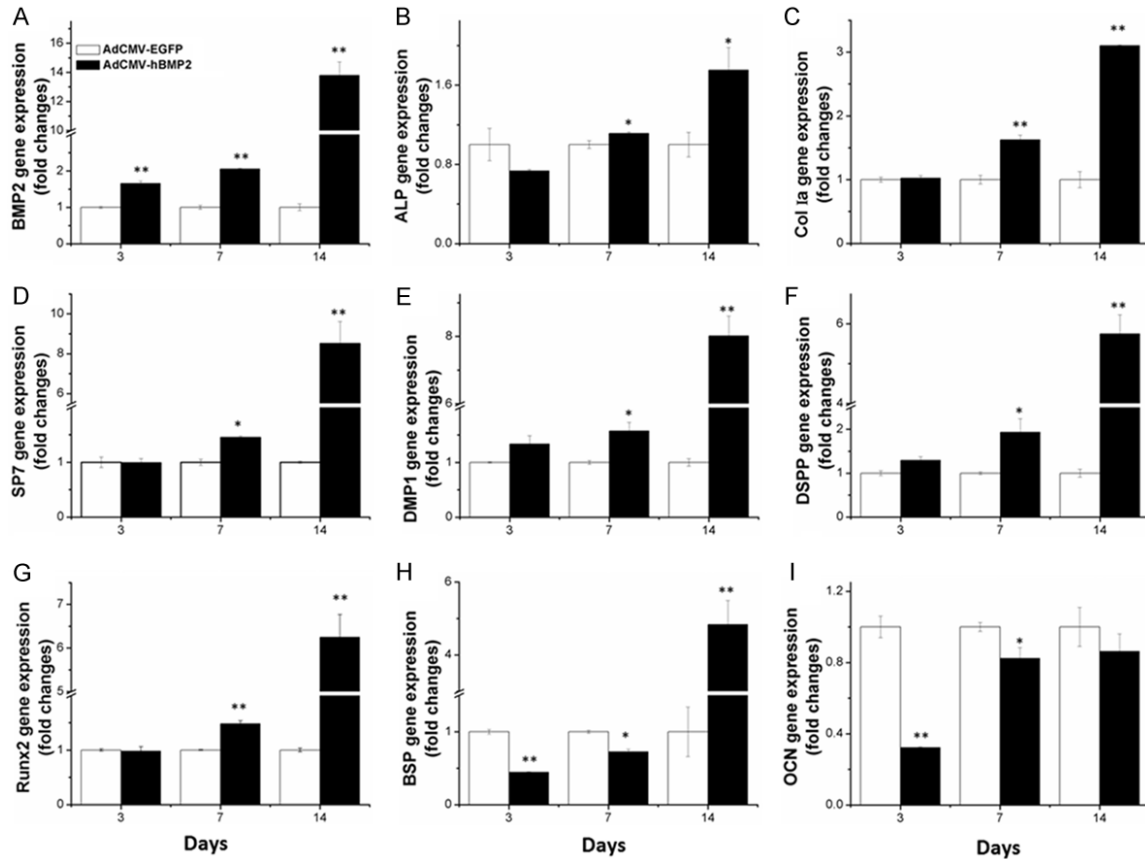


Figure 3. Gene expression profiles of hDPCs transduced with AdCMV-EGFP or AdCMV-hBMP2 3, 7, or 14 days post-transduction. A. BMP2 gene expression. B. ALP gene expression. C. Col 1 α gene expression. D. SP7 gene expression. E. DMP1 gene expression. F. DSPP gene expression. G. Runx2 gene expression. H. BSP gene expression. I. OCN gene expression. Data shown are means \pm SEM. These assays were repeated three times.

0.1% Alizarin Red S (Sigma-Aldrich) at 37°C for 30 min. The stained cells were incubated in 10% cetylpyridinium chloride for 1 h to extract the stain and the absorbance was measured at 560 nm.

Real time quantitative PCR (RT-QPCR) in vitro

The same cultures as mentioned above were used to extract total RNA with Trizol. Six μ g of total RNA was used for reverse transcription reaction with PrimerScript[®] RT Reagent Kit (Takara). One μ l of RT reaction was used for QPCR using the MX3005P system (Agilent, Santa Clara, CA, USA) with TaqMan[®] Universal PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) under the conditions as follows: 50°C for 2 min, 95°C for 10 min, then 95°C for 15 s, and 60°C 1 min for 40 cycles. β -actin was used as the internal control. Primers and probes for BMP2, Runx2, ALP, Col 1 α ,

SP7, DMP1, DSPP, BSP, and OCN were obtained from Life Technologies (Grand Island, NY, USA).

In vivo animal assays

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee and the Ethics Committee of the Faculty of Dentistry, Jilin University. Twenty Wistar rats (~200 g, 2 months old) were used in this study. Animals were anesthetized using ketamine (60 mg/kg) and xylazine (8 mg/kg). The cavities were prepared as follows: the coronal enamel and partial dentin of the first molars was carefully removed from the occlusal surface (~0.5 mm thick). The smear layer was removed with Gluma Etch 20 gel (Heraeus Kulzer GmbH, Hanau, Germany). After washing with saline for 20 s, a gelatin sponge containing AdCMV-EGFP or AdCMV-hBMP2 at 1.25×10^6 particles/0.25 μ L was put on the prepared

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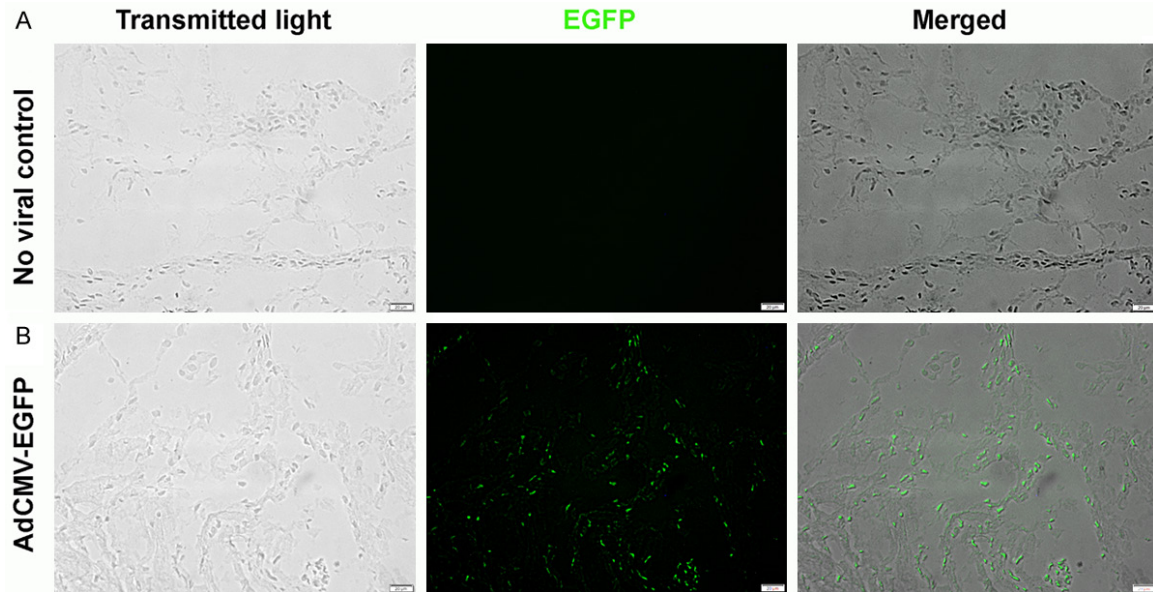


Figure 4. Evaluation of transduction efficiency of AdCMV-EGFP in the dental pulp *in vivo*. A. No viral control group. B. AdCMV-EGFP transduced group.

dentin surface for 5 min. Then, the occlusal cavities were sealed with GC Fuji IX GP (Fuji, Tokyo, Japan). On day 3, dental pulp tissues, which were from 3 rats treated only with a gelatin sponge (as background control) or with a gelatin sponge containing AdCMV-EGFP, were separated and prepared for frozen section. Slides were observed under an inverted fluorescence microscope in order to evaluate if the adenoviral vector can efficiently transduce the cells in the dental pulp. On days 3, 7, 14, and 28 post-surgery, rats were anesthetized and euthanized by intracardiac perfusion with saline and 4% paraformaldehyde. The upper molars were extracted and fixed with 4% paraformaldehyde for two days, decalcified in 10% EDTA for three months at room temperature, rinsed in water, dehydrated in a series of increasing concentrations of alcohol, embedded in paraffin, cut into 3 μ m sections, and stained with H&E and immunohistochemistry staining using anti-DMP1-C-8G 10.3 monoclonal antibody and anti-DSP-2C12.3 monoclonal antibody (gifts from Dr. Chunlin Qin, university of texas) at 1:1000 dilution and anti-BMP2 (ab-14933, Abcam, Cambridge, MA, USA) at 1:200 dilution.

Statistical analysis

Data were presented as mean \pm standard error of the mean (SEM) herein. Pair t test was appli-

ed to evaluate the statistical significance of differences between control and BMP-treated groups. $P < 0.05$ was considered to be significant difference.

Results

Characteristics of hDPCs

In this study, hDPCs culture was a full-pulp cell culture [17]. **Figure 1A** shows general morphology of hDPCs on day 10. The hDPCs proliferated rapidly with spindle shape morphology. The hDPCs were cultured for 10-15 days, and then sub-cultured for three passages (5 days/passage). The hDPCs of passage 3 were used to perform all assays for this study. Oil Red O staining demonstrated that there were positive red cells in our culture condition (**Figure 1B**), which indicates that adipogenic differentiation occurred. This data was coincide with previous study [18]. They also found that ~5% of cultured stem cells from human exfoliated deciduous teeth (SHED) differentiated to Oil red O-positive lipid-laden fat cells after 5 weeks of culture with an adipogenic inductive mixture. Data from transduction efficiency assay showed that about 70% of hDPCs were EGFP-positive on day 3 (**Figure 1C**) and the positive cells could be retained till day 28 (**Figure 1D**) after transduced with AdCMV-EGFP at 1000 particles/cell.

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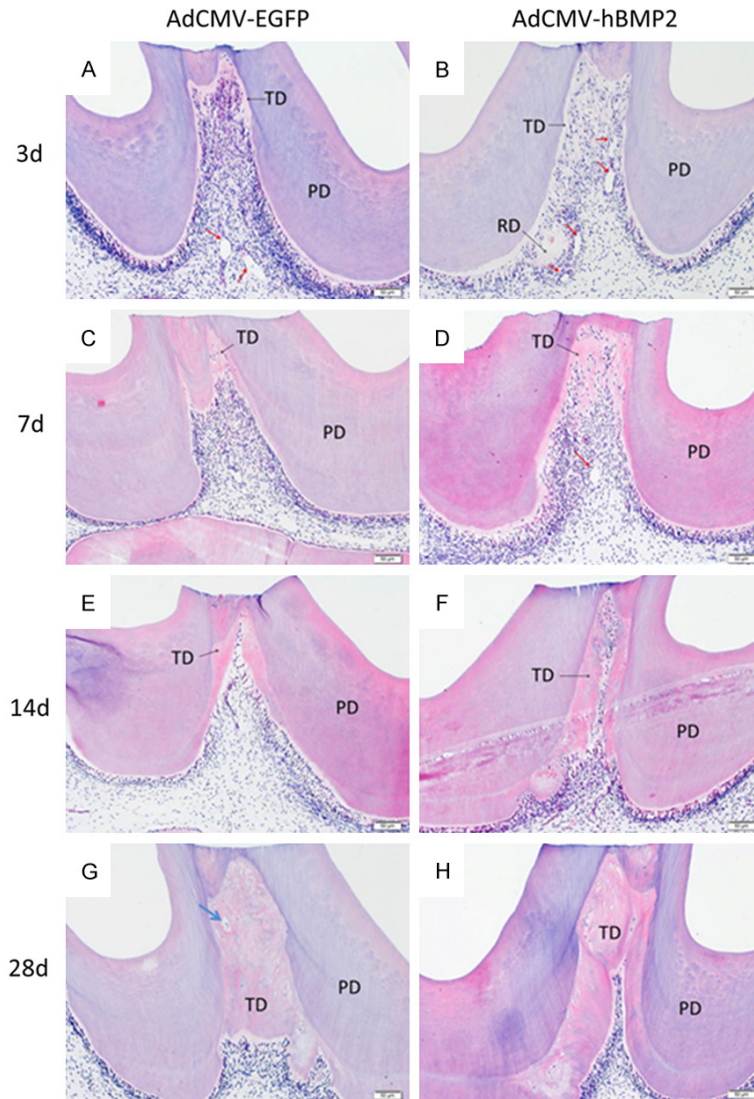


Figure 5. Effects of hBMP2 on tertiary dentin formation *in vivo* by H&E staining. A. 3 days AdCMV-EGFP group. B. 3 days AdCMV-hBMP2 group. C. 7 days AdCMV-EGFP group. D. 7 days AdCMV-hBMP2 group. E. 14 days AdCMV-EGFP group. F. 14 days AdCMV-hBMP2 group. G. 28 days AdCMV-EGFP group. H. 28 days AdCMV-hBMP2 group. PD, primary dentin; TD, tertiary dentin; black arrow indicates TD; red arrow indicates blood vessel; blue arrow indicates pore structure. Bar = 50 μ m.

Effects of hBMP2 on hDPCs differentiation

Real time quantitative PCR (RT-PCR) assay demonstrated that human serotype 5 adenoviral vector (AdCMV-hBMP2) could efficiently transduce hDPCs and increase BMP2 expression in hDPCs without mineralization induction on day 3 (Figure 1E).

Alkaline phosphatase activity and Alizarin Red S staining were two indicators to monitor if hDPCs differentiated in our culture system. Data

from ALP activity assay showed that ALP activity of AdCMV-hBMP2 treated hDPCs was slightly, but significantly increased 7 days post-culture (Figure 2A). Alizarin Red S staining demonstrated that calcium deposition occurred in the hDPCs, whereas the AdCMV-hBMP2 treated group had significantly more Alizarin Red S positive cells or mineralized nodule on days 14 and 28 compared to the AdCMV-EGFP control group (Figure 2B-F). Quantification of alizarin red S staining is statistically significant between the two groups ($P < 0.01$). These results from both assays indicate that BMP2 can promote hDPCs differentiation and mineralization.

Effects of hBMP2 on gene expressions of hDPCs

In this study, we used RT-QPCR assays to evaluate how hBMP affects some osteogenic/dentinogenic gene expressions, such as Runx2, ALP, Col 1 α , SP7, DMP1, DSPP, BMP2, BSP, and OCN. Figure 3A shows that hBMP2 gene expression was markedly increased at all three time points. Gene expression of ALP was obviously increased on days 7 and 14 ($P < 0.05$) (Figure 3B). Runx2 and Col 1 α gene expressions were more distinctly increased in

the AdCMV-hBMP2 treated group on days 7 and 14 ($P < 0.01$) (Figure 3C, 3G). Gene expressions of SP7, DMP1, and DSPP were also significantly increased in the AdCMV-hBMP2 treated hDPCs compared to the AdCMV-EGFP group on day 7 ($P < 0.05$), and especially on day 14 ($P < 0.01$) (Figure 3D-F). Gene expressions of BSP and OCN, however, were dramatically decreased in the AdCMV-hBMP2 treated hDPCs compared to the AdCMV-EGFP group on days 3 ($P < 0.01$), and 7 ($P < 0.05$) (Figure 3H,

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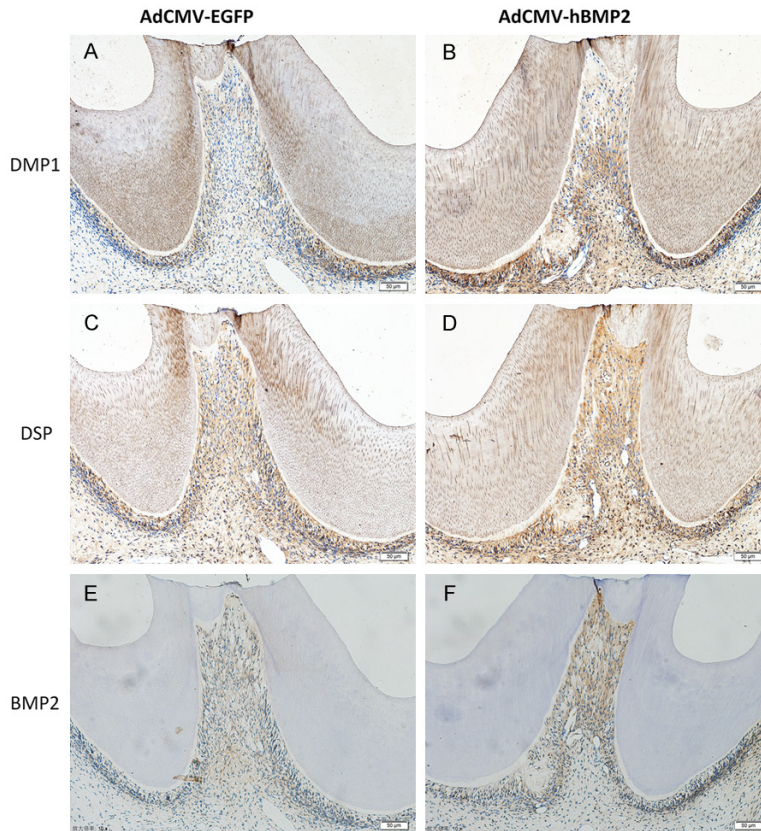


Figure 6. *In vivo* immunohistochemistry staining for DSP, DMP1 and hBMP2 on day 3. A. DMP1 staining for AdCMV-EGFP treated group. B. DMP1 staining for AdCMV-hBMP2 treated group. C. DSP staining for AdCMV-EGFP treated group. D. DSP staining for AdCMV-hBMP2 treated group. E. hBMP2 staining for AdCMV-EGFP treated group. F. hBMP2 staining for AdCMV-hBMP2 treated group.

3I). Interestingly, gene expression of BSP was significantly increased in the AdCMV-hBMP2 treated hDPCs compared to the AdCMV-EGFP group on day 14 ($P < 0.01$), while gene expression of OCN in the AdCMV-hBMP2 treated hDPCs was not different from the AdCMV-EGFP group (**Figure 3H, 3I**). These data indicate that the hBMP2 can influence differentiation of osteoblast/odontoblast.

Effects of hBMP2 on tertiary dentin formation in vivo

Our *in vitro* data indicate that hBMP2 can promote differentiation and mineralization of dental pulp cells. Next, *in vivo* evaluation became necessary to confirm if we could reliably use BMP2 as a therapeutic biomolecule in further clinical application. First, we used the AdCMV-EGFP to evaluate if the adenoviral vector can efficiently transduce dental pulp cells *in vivo*.

Figure 4 clearly showed that the AdCMV-EGFP was successfully transduced to dental pulp cells 3 days after injection *in vivo* compared to the control group. Indeed, *in vivo* data demonstrated that hBMP2 could markedly increase the thickness of tertiary dentin compared to the AdCMV-EGFP treated groups on days 3, 7, and 14 (**Figure 5A-F**). On day 28, the AdCMV-hBMP2 treated groups clearly had more well-organized dentin tubules in the newly formed dentin compared to the AdCMV-EGFP treated group, while more porous conditions was observed in the AdCMV-EGFP treated group (**Figure 5G, 5H**). On day 3, the positive expression of DMP1, DSP and BMP2 in the AdCMV-hBMP2 treated groups were clearly higher than that of the AdCMV-EGFP treated group (**Figure 6A-F**).

Discussion

Repair of defective dentin tissue is a challenge issue to maintain pulp vitality in clinical practice. BMP2 is osteoinductive bone morphogenetic protein which has been demonstrated to potentially induce osteoblast/odontogenic differentiation [1]. BMP2, in an autocrine fashion, produced by odontoblasts, directly signaled themselves and adjacent odontoblasts to stimulate odontoblast differentiation and dentin formation [14]. In tooth development, BMP2 continued to express during the whole process of odontoblast differentiation and maturation [14, 19]. Our study herein demonstrates that hBMP2 can indeed promote differentiation of hDPCs *in vitro* and formation of tertiary dentin *in vivo*.

First generation serotype 5 adenoviral vector can efficiently transduce many kinds of cells. Our data indeed demonstrated that AdCMV-hBMP2 could efficiently transduce hDPCs and express a much higher level of human BMP2 *in vitro* (**Figures 1 and 3**), and also enter the den-

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tinal tubules and transduce dental pulp cells *in vivo* (Figure 4). ALP is a necessary requirement for the differentiation of dental pulp cells. Our results clearly showed that hBMP2 increased ALP activity on days 14 and 21 (Figure 2). Furthermore, Alizarin Red S staining suggested that hBMP2 could promote differentiation of hDPCs (Figure 2).

Runx-2 is one of several important proteins necessary in the early and middle stages of osteoblast differentiation during bone formation, whereas BSP and OCN proteins play critical roles in the late stages of osteoblast differentiation [20]. Runx2 acts as an essential transcription factor for bone and tooth development and controls the expression of mineralization-associated genes, including Dspp [21]. Col 1 α is a major protein for dentin formation and repair. SP7 is a transcription factor that is involved in bone cell differentiation. DSPP gene transcripts and translates two major noncollagenous dentin proteins: dentin sialoprotein and dentin phosphoprotein [22], which are necessary for dentin mineralization [22, 23]. DMP-1 expresses in odontoblast and plays a role in dentinogenesis imperfecta [24]. Our gene expression data demonstrated that hBMP2 could stimulate differentiation of hDPCs (Figure 3) and significantly increase gene expressions of Runx2, ALP, Col 1 α , SP7, DMP1, and DSPP in pulp cells *in vitro* at an early time point, whereas gene expression of BSP and OCN increased at a late time point (Figure 3). Our *in vivo* data demonstrated that the expression of hBMP2 was much higher in the AdCMV-hBMP2 treated groups on day 3, which indicates that adenoviral vector can efficiently penetrate the dental pulp through dentinal tubules and transduce local cells (Figures 3 and 5). Interestingly, hBMP2 could promote differentiation and proliferation of dental pulp cells resulting in formation of tertiary dentin in the dental pulp (Figures 4, 5).

Conclusion

This study clearly demonstrates that the hBMP2 gene can efficiently be delivered into dental pulp cells by first generation serotype 5 adenoviral vectors, and effectively influences proliferation and differentiation of dental pulp cells, as well as mineralization and formation of tertiary dentin *in vitro* and *in vivo*. Our study suggests that a gene therapy scheme can be used

to treat dental pulp diseases. Therefore, current study approves potential therapeutic application of hBMP2 to form tertiary dentin in dental pulp and to help to cure caries.

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Disclosure of conflict of interest

None.

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References

- [1] Asgary S, Nazarian H, Khojasteh A and Shokouhinejad N. Gene expression and cytokine release during odontogenic differentiation of human dental pulp stem cells induced by 2 endodontic biomaterials. *J Endod* 2014; 40: 387-392.
- [2] Murray PE, Smith AJ, Windsor LJ and Mjor IA. Remaining dentine thickness and human pulp responses. *Int Endod J* 2003; 36: 33-43.
- [3] Murray PE, About I, Lumley PJ, Franquin JC, Remusat M and Smith AJ. Cavity remaining dentin thickness and pulpal activity. *Am J Dent* 2002; 15: 41-46.

hBMP2 promotes tertiary dentin formation

- [4] Grotz KA, Duschner H, Reichert TE, de Aguiar EG, Gotz H and Wagner W. Histotomography of the odontoblast processes at the dentine-enamel junction of permanent healthy human teeth in the confocal laser scanning microscope. *Clin Oral Investig* 1998; 2: 21-25.
- [5] Goracci G, Mori G and Baldi M. Terminal end of the human odontoblast process: a study using SEM and confocal microscopy. *Clin Oral Investig* 1999; 3: 126-132.
- [6] Grotz KA, al-Nawas B, Brahm R, Duschner H and Wagner W. Terminal end of the human odontoblast processes. *Clin Oral Investig* 2000; 4: 106-107.
- [7] Tsuchiya M, Sasano Y, Kagayama M and Watanabe M. The extent of odontoblast processes in the dentin is distinct between cusp and cervical regions during development and aging. *Arch Histol Cytol* 2002; 65: 179-188.
- [8] Forssell-Ahlberg K, Brannstrom M and Edwall L. The diameter and number of dentinal tubules in rat, cat, dog and monkey. A comparative scanning electron microscopic study. *Acta Odontol Scand* 1975; 33: 243-250.
- [9] Li Y, Ikeda H and Suda H. Determination of the functional space for fluid movement in the rat dentinal tubules using fluorescent microsphere. *Arch Oral Biol* 2013; 58: 780-787.
- [10] Chen D, Zhao M and Mundy GR. Bone morphogenetic proteins. *Growth Factors* 2004; 22: 233-241.
- [11] Washio A, Kitamura C, Morotomi T, Terashita M and Nishihara T. Possible involvement of smad signaling pathways in induction of odontoblastic properties in KN-3 cells by bone morphogenetic protein-2: a growth factor to induce dentin regeneration. *Int J Dent* 2012; 2012: 258469.
- [12] Qin W, Yang F, Deng R, Li D, Song Z, Tian Y, Wang R, Ling J and Lin Z. Smad 1/5 is involved in bone morphogenetic protein-2-induced odontoblastic differentiation in human dental pulp cells. *J Endod* 2012; 38: 66-71.
- [13] Qin W, Lin ZM, Deng R, Li DD, Song Z, Tian YG, Wang RF, Ling JQ and Zhu XF. p38a MAPK is involved in BMP-2-induced odontoblastic differentiation of human dental pulp cells. *Int Endod J* 2012; 45: 224-233.
- [14] Yang W, Harris MA, Cui Y, Mishina Y, Harris SE and Gluhak-Heinrich J. Bmp2 is required for odontoblast differentiation and pulp vasculogenesis. *J Dent Res* 2012; 91: 58-64.
- [15] Yang X, van der Kraan PM, Bian Z, Fan M, Walboomers XF and Jansen JA. Mineralized tissue formation by BMP2-transfected pulp stem cells. *J Dent Res* 2009; 88: 1020-1025.
- [16] Nakashima M and Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. *J Endod* 2005; 31: 711-718.
- [17] Gronthos S, Mankani M, Brahimi J, Robey PG and Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000; 97: 13625-13630.
- [18] Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG and Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 2003; 100: 5807-5812.
- [19] Wang Y, Li L, Zheng Y, Yuan G, Yang G, He F and Chen Y. BMP activity is required for tooth development from the lamina to bud stage. *J Dent Res* 2012; 91: 690-695.
- [20] Mangano C, Paino F, d'Aquino R, De Rosa A, Iezzi G, Piattelli A, Laino L, Mitsiadis T, Desiderio V, Mangano F, Papaccio G and Tirino V. Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex. *PLoS One* 2011; 6: e18721.
- [21] Chen S, Rani S, Wu Y, Unterbrink A, Gu TT, Gluhak-Heinrich J, Chuang HH and MacDougall M. Differential regulation of dentin sialophosphoprotein expression by Runx2 during odontoblast cytodifferentiation. *J Biol Chem* 2005; 280: 29717-29727.
- [22] Feng JQ, Luan X, Wallace J, Jing D, Ohshima T, Kulkarni AB, D'Souza RN, Kozak CA and MacDougall M. Genomic organization, chromosomal mapping, and promoter analysis of the mouse dentin sialophosphoprotein (Dspp) gene, which codes for both dentin sialoprotein and dentin phosphoprotein. *J Biol Chem* 1998; 273: 9457-9464.
- [23] MacDougall M, Gu TT and Simmons D. Dentin matrix protein-1, a candidate gene for dentinogenesis imperfecta. *Connect Tissue Res* 1996; 35: 267-272.
- [24] Suzuki S, Sreenath T, Haruyama N, Honeycutt C, Terse A, Cho A, Kohler T, Muller R, Goldberg M and Kulkarni AB. Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization. *Matrix Biol* 2009; 28: 221-229.