INTRODUCTION

BMP-7, which regulates the differentiation of pulp cells into odontoblasts and stimulates the formation of reparative dentin, is a potential bioactive pulp-capping agent\(^1\)-\(^4\). However, the requirements for large doses of bone morphogenetic proteins as well as the short half-life and the rapid degradation of BMPs have suggested the need for an optimized delivery system\(^5\). Gene therapy abolishes the need for external growth factors or hormones such as dexamethasone.

Transfection may be a new strategy to enhance the efficacy of DPC application\(^6\). An adenoviral-mediated human BMP-7 gene has been transfected into DPCs, promoting differentiation into the odontoblast phenotype, and the production of mineralized tissues in vitro\(^7\). However, our preliminary study in our laboratory showed that the teeth of immunodeficient mice that were directly infected in vivo with adenoviral-mediated BMP-7 invariably failed to produce reparative dentin; this negative result in vivo may be due to the limited duration of the expression of the transient transgene with adenoviruses.

Gene therapy products for the treatment of genetic diseases are currently in clinical trials\(^8\)-\(^11\), and one of these, an adeno-associated viral (AAV) product, has recently been licensed in Europe. AAV vectors exhibit the unique ability to integrate into the genome of cells enables their relatively long and stable transgene expression with very little accompanying toxicity\(^12\). AAV vectors have been used to transduce cells in the central nervous system\(^13\), the liver\(^10\), the retina, and skeletal\(^14\) and cardiac muscle\(^15\). In this study, the differentiation characteristics of human dental pulp cells (DPCs) infected with generated adeno-associated virus serotype 2 vector-mediated BMP-7 (AAV2-BMP-7) were evaluated. We hypothesized that AAV2-BMP-7-transfected cells have greater odontoblastic differentiation potential than non-transfected cells, even when cultured in medium without dexamethasone or other stimuli.

MATERIALS AND METHODS

Cell culture

DPCs were isolated and characterized as described previously\(^16\),\(^17\). Dental pulp tissues were obtained from the explants of clinically healthy dental pulp from human adult third molars that were removed from individuals who underwent tooth extractions for orthodontic treatment. All of the patients gave informed consent before the collection of samples, and the ethics committee of the Guanghua School of Stomatology at Sun Yat-sen University approved the experimental protocols. DPCs were cultured in alpha modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) that was supplemented with 10% fetal calf serum (Invitrogen) at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Cell cultures between the fourth and sixth passages were used in this study.

HEK293 and BHK21 cells (all from ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), which contained 10% fetal bovine serum (FBS) and penicillin/streptomycin.
Plasmid construction and AAV2 viral vector production
pDC316-BMP-7-IRES-EGFP and pSNAV2.0-LacZa were purchased from Vector Gene Technology Company Ltd. (VGTG, Beijing, China). The BMP-7 coding sequence was excised from pDC316-BMP7-IRES-EGFP and was subcloned into pSNAV2.0-LacZa using Age I and Nhe I restriction sites. After sequencing and RT-PCR, the pSNAV-BMP-7 expression vector that contained the coding sequence was constructed. The primer sequences for BMP-7 were 5'-GTTGGACCTCATGGCCTACATG-3' (sense) and 5'-TCAGCAACTGAGGGCCTCTC-3' (antisense). AAV2 vectors were prepared as previously described\(^{18}\). Briefly, BHK-21 cells were transfected with the pSNAV-BMP-7 vector using Metafectene and were selected by G418. AAV2-BMP-7 viruses were rescued and produced by infecting the G418-resistant BHK-21 colonies that contained the BMP-7 gene with recombinant HSV1-rec/AUL2 helper viruses. The crude viral lysate was purified by CsCl gradients.

Measurements of viral infectivity
DPCs were placed in the wells of a 24-well plate at a density of 4×10^4 cells/well. DPCs at 75% confluency were infected with AAV2-EGFP at 1×10^4, 1×10^5, or 5×10^5 v.g. (viral genome) per cell. The expression of enhanced green fluorescent protein (EGFP) in DPCs was monitored under an inverted microscope (Zeiss, Jena, Germany). Two days after the infection, the cells were harvested, and the frequency of infected cells was characterized by the fluorescence-activated cell sorter (FACS) analysis of the expression of EGFP by a FACS Aria machine (BD, Franklin Lakes, NJ, USA) to determine the efficiency of the infection.

MTT assay
DPCs at 1×10^4 cells/well in 96-well plates were infected with AAV2-EGFP and were then cultured for different periods. The cells were treated with 50 µg/mL methylthiazolyl diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) and were then incubated for 4, 7, 10, or 14 days. These cells were harvested and fixed in 0.1% Triton X-100 at 4°C overnight. Subsequently, p-nitrophenyl phosphate disodium was added to a final concentration of 2.5 mmol/L for 15 min at 37°C, followed by the addition of 0.1 mol/L NaOH to stop the reaction. The absorbance was determined at 410 nm using an enzyme-linked immunosorbent assay reader (Bio-Tek). The unmanipulated dental pulp cells were cultured for controls.

Von Kossa staining
Specific calcifications were detected by von Kossa staining. Briefly, DPCs at 1×10^4 cells/well in 6-well plates were infected with AAV2-BMP-7 and were maintained in DMEM supplemented with 10% FBS, 50 mg/mL ascorbic acid, 10 mmol/L sodium β-glycerophosphate, and 10 nmol/L dexamethasone. After 14 days of incubation, the cells were treated with a 5% silver nitrate solution and were exposed to ultraviolet light for 30 min. The solution was then neutralized with 5% sodium thiosulfate for 2 min, and the cells were rinsed with distilled water for 5 min. Finally, the cells were stained with nuclear fast red (Sigma, Deisenhofen, Germany) for 1 min. For each group, 8 random fields at 10× magnification were chosen, and the staining density was quantified using the Scion Image software (Scion Corp., Frederick, MD, USA).

The expression of DSPP and OCN genes
The expression levels of DSPP and OCN mRNA were determined by SYBR green real-time reverse transcription-PCR as described previously\(^{19,20}\). DPCs were infected with AAV2-BMP-7 or AAV2-EGFP and were cultured for 7, 14, or 21 days. The expression levels of DSPP and OCN mRNA were determined by SYBR green real-time reverse transcription-PCR (RT-PCR). Total RNAs were extracted using the TRIzol reagent. The real-time PCR and data collection were performed with an ABI PRISM 7500 sequence detection system. The housekeeping geneβ-actin was used as an internal control to normalize the expression levels of different genes. The data for gene expression were analyzed with the ΔΔCt method. The primers that were used for the amplification of the indicated genes are listed in Table 1.

Statistical analysis
The data represent the results of at least three independent experiments, each carried out in triplicate. The values are expressed as the mean±SD of at least three independent experiments. A one-way analysis of variance followed by Tukey’s HSD test post hoc was used to compare the experimental groups. p<0.05 was considered statistically significant.
Table 1 Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPP</td>
<td>F: 5’-GGGATGTTGGCGATGCA-3’</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CCAGCTACTTGGAGGTCCATCCT-3’</td>
<td></td>
</tr>
<tr>
<td>OCN</td>
<td>F: 5’-AGCAAGGTGCAGCTTTGT-3’</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GGGCTGGGTCTCTTCCT-3’</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5’-GCAAGGTTGAGGATCCCT-3’</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TCGTCAGTTTGACGAT-3’</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Construction of adeno-associated virus that contained BMP-7 gene
An approximately 700 bp fragment of the BMP-7 gene was PCR-amplified from AAV2-BMP-7 (Fig. 1). The target genes were confirmed to be correctly cloned in the adeno-associated virus vector by gene sequencing and were matched to the BMP-7 sequence in GenBank (Data not shown). This result demonstrated that the recombinant adeno-associated virus vector that contained the BMP-7 gene was successfully constructed. The final viral concentration was 5.1×10¹¹ v.g./mL.

Gene transfer and cell proliferation
To quantify the infectivity of the recombinant adeno-associated virus, DPCs were harvested 48 h after the infection, and the frequency of EGFP-positive cells was characterized by FACS analysis. The frequency of EGFP-positive cells was correlated to the doses of the adeno-associated virus. The transduction efficiencies of AAV2-EGFP in DPCs were shown in Fig. 2a. In our study, we found that the cells became round at 5×10⁵ particles. Therefore, 1×10⁵ AAV2-BMP-7 particles should be optimal for the infection of dental pulp cells. We chose this dose for the infection titer in the subsequent study. Importantly, the infection of DPCs with this dose of AAV did not change the growth of cells in vitro. As shown in Fig. 2b, after the infection with AAV2-BMP-7 or AAV2-EGFP, the proliferation through this experimental period was indistinguishable from that of unmanipulated cells.

Fig. 1 The identification of AAV2-BMP-7.
Lane M: DL2000 Marker; Lane 1: negative control; Lane 2–3: AAV2-BMP-7; Lane 4: positive control (pDC316-BMP7-IRE8-EGFP).

Fig. 2 The effect of AAV2-BMP-7 on cell proliferation.
(a) DPCs were infected with AAV2-EGFP at a variable v.g. for 48 h and were then monitored under an inverted fluorescent microscope. The lower panels indicate the infection efficiency of AAV2-EGFP, as determined by FACS analysis. The data shown are representative of three independent experiments from different cell samples. *p<0.05 compared with the 1×10⁵ group. (b) DPCs were infected with AAV2-BMP-7 or AAV2-EGFP or were unmanipulated, and the dynamics of their growth was determined by MTT assays. The values represent the mean±SD of three independent experiments. There were no significant differences between AAV2-BMP-7 and the control group. Scale bar: 20 µm.
The overexpression of BMP-7 protein in AAV2-BMP-7-infected cells
DPCs were infected with AAV2-BMP-7 or AAV2-EGFP, and their expression of BMP-7 was characterized by Western blotting. Although no clear protein band could be probed by BMP-7-specific antibodies in both AAV2-EGFP-infected and unmanipulated groups, protein bands were observed in the AAV2-BMP-7-infected groups at 7, 14, and 21 days (Fig. 3), which indicated that the AAV2-BMP-7 infection had induced the expression of BMP-7 in DPCs.

Effect of AAV2-BMP-7 on ALP activity and formation of mineralized nodules
To determine the function of BMP-7 on odontoblastic differentiation, ALP activity was characterized by ALP enzymatic assays. As shown in Fig. 4a, the activity of ALP in the cells that were infected with AAV2-EGFP increased slightly with time. In contrast, the activity of ALP was significantly elevated in AAV2-BMP-7-infected DPCs. Next, the formation of mineralized nodules was investigated after 14 days of incubation; AAV2-BMP-7 increased the formation of mineralization in DPCs (Fig. 4b). These results suggested that AAV2-BMP-7 promoted odontoblastic differentiation and mineralization in DPCs in vitro.

Effect of AAV2-BMP-7 on odontoblastic differentiation
To further evaluate whether AAV2-BMP-7 could promote odontoblastic differentiation, the expression levels of DSPP and OCN mRNA were determined by real-time PCR. Messenger RNA levels of DSPP and OCN in the AAV2-BMP-7 group were 7.03-fold and 3.12-fold greater than that in the control group at 7 days, respectively (Fig. 5). Although the expression of DSPP and OCN mRNA showed downregulation with time, the expression levels were higher than that of the AAV2-EGFP group. Together, the significantly increased levels of ALP activity and DSPP and OCN mRNA in the AAV2-BMP-7 group strongly suggested that BMP-7 promoted the differentiation of DPCs to odontoblast-like cells in vitro.

DISCUSSION
Although our previous study proved that Ad-BMP-7 could promote the odontoblastic differentiation of DPCs\(^7\)
Fig. 5  The expression of dentin-related genes in DPCs that were infected with AAV2-BMP-7. (a and b) DPCs were infected with AAV2-BMP-7 for the indicated periods, and the mRNA expressions of odontogenic marker genes were determined by real-time PCR. β-actin was used as an internal control. The data shown are representative of three independent experiments from different cell samples. *p<0.05 compared with the AAV2-EGFP group.

and transient expression methods could avoid genomic alteration of DPCs, BMP-7 overexpression in vivo by Ad-mediated gene therapy was ineffective at inducing reparative dentin. Thus, we need a new virus vector that has been employed to deliver genes to cells to provide permanent transgene expression. Adeno-associated viral (AAV) vectors are derived from a non-pathogenic human virus that efficiently provide stable transgene expression. So far, no research group has reported whether AAV2 can work as a BMP-7 gene delivery tool and induce the differentiation of DPCs.

To examine the efficiency of the AAV2-BMP-7 infection on human dental pulp cells, we successfully generated AAV2-BMP-7, which is a human BMP-7-expressing adeno-associated virus vehicle, and the control, AAV2-EGFP. The infection with 1×10^5 AAV2-EGFP particles induced a high frequency of cells that expressed the target protein, and the infection with AAV2-BMP-7 promoted the long-term expression of BMP-7 in DPCs, which is consistent with the notion that the dental cells are quite sensitive to adeno-associated virus infections. Furthermore, this induction of BMP-7 did not alter the dynamics of the proliferation and viability of infected cells, which suggests that the generated AAV2-BMP-7 has a high infectivity and a low cytotoxicity.

ALP activity has been considered to be an early marker of odontoblastic cell differentiation because ALP activity is higher than undifferentiated dental mesenchymal cells and is closely related to dentine formation. In our current study, significantly increased levels of ALP activities were observed in AAV2-BMP-7-infected DPCs compared with AAV2-EGFP-infected cells. Taken together, the higher level of ALP activity and the enhanced expression of DSPP and OCN indicated that AAV2-BMP-7 could promote the odontoblastic differentiation of human periodontal ligament stem cells. The strong odontogenic effects that we observed in our study are consistent with previous reports that used adeno-associated viral vectors in a variety of cell types.

Additionally, DSPP and OCN have been used as indicators of odontoblastic differentiation. DSPP, which is a noncollagenous extracellular matrix, traditionally bone- and dentine-specific gene, is believed to play a significant role in dentine mineralization and tooth development. DSPP was originally considered dentine-specific. However, several studies have demonstrated the expression of DSPP in some nonmineralized tissues, for example, cementum and bone. Although the amount of DSPP in nondental tissues is lower than in dentine. In spite of its expression in nondental tissues, DSPP remains a significant marker for odontoblastic differentiation. OCN, as a later marker of cell differentiation, is related to matrix deposition and mineralization. Wang et al. demonstrated that DSPP and OCN played a crucial role in the differentiation of cells with odontogenic and osteogenic phenotypes, respectively. In the present study, AAV2-BMP-7 stimulated the increased expression of DSPP and OCN for approximately 3 weeks in DPCs compared with the AAV2-EGFP group. Similar effects were also observed when a plasmid containing BMP-7 was introduced into human dental pulp stem cells. Taken together, the higher level of ALP activity and the enhanced expression of DSPP and OCN indicated that AAV2-BMP-7 could promote the odontoblastic differentiation of DPCs.

In conclusion, human dental pulp cells were successfully transfected with an adeno-associated virus vector that contained the BMP-7 gene. Transfected cells underwent more effective induction toward the odontoblast phenotype than non-transfected cells. In support of using a gene therapy approach in dental clinical applications, we noted that the teeth of the exposed pulp that had been directly infected in vivo with AAV2-BMP-7 were instrumental in producing...
the dentin-pulp complex. Despite some advantages, the two main hurdles in using gene therapy for clinical applications are safety concerns and the unknown duration of the curative effect that results from the transfected treatment. Therefore, further studies are needed to investigate the long-term effects of the in vivo exposure of dental pulp cells to AAV2-BMP-7.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Sciences Foundation of China (No. 81070829).

REFERENCES


