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TGF-β2 and TGF-β1 differentially regulate the odontogenic and osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

Objective: To explore the effects of transforming growth factor-β2 (TGF-β2) and TGF-β1 on the odontogenic and osteogenic differentiation of mesenchymal stem cells (MSCs).
Design: We used lentiviral transduction to knock down TGF-β1 or TGF-β2 in stem cells from dental apical papilla (SCAPs), and to generate bone marrow mesenchymal stem cells (BMSCs) with overexpression of TGF-β1 or TGF-β2. We investigated the odontogenic and osteogenic differentiation abilities of these transductants *in vitro* and *in vivo*.
Results: In vitro, TGF-β2 knockdown in SCAPs reduced the expression of odontoblast-related markers DSPP and DMP-1, and increased the expression of osteoblast-related markers OCN and RUNX-2. Conversely, TGF-β1 knockdown had the opposite effects. TGF-β2 overexpression promoted expression of odontoblast-related markers in BMSCs at early differentiation, but inhibited the expression of odontoblast-related markers at later stages. TGF-β2 overexpression attenuated expression of osteogenic-related markers in BMSCs, while TGF-β1 overexpression enhanced odontoblast-related and osteoblast-related markers. SCAP or BMSC transductants were transplanted underneath kidneys *in vivo*. Masson staining showed that knockdown of TGF-β1, but not TGF-β2 promoted the expression of type I collagen in SCAPs. Immunohistochemical staining showed that TGF-β2 knockdown inhibited DSPP expression in SCAPs, but TGF-β1 knockdown had no obvious effect on DSPP expression. *In vivo*, TGF-β1 overexpression and TGF-β2 overexpression had no effect on the expression of type I collagen and DSPP in BMSCs.
Conclusions: TGF-β2 promotes odontogenic differentiation of SCAPs and attenuates osteogenic differentiation of SCAPs and BMSCs. TGF-β1 promotes osteogenic differentiation of BMSCs and plays a complex role in regulating odontogenic differentiation of MSCs.

1. Introduction

Regenerative endodontics, which involves attempting to fill a root canal with vital dentin-pulp tissues instead of artificial materials, has gained the attention of dental professionals in the past decade. There are two primary approaches to regenerate dentin-pulp: revascularization/revitalization and tissue engineering. Revascularization/revitalization is a novel therapy for the treatment of immature permanent teeth with apical periodontitis. During this procedure, it is thought that stem cells from dental apical papilla (SCAPs) migrate into the root canal with the influx of bleeding, and regenerate dentin-pulp tissue. However, in some cases, when SCAPs are destroyed by the inflammation, stem cells from alveolar bone may enter into the root canal and initiate mineral deposition, similar to osteoid tissues (Cao et al., 2015; Saoud et al., 2015). Tissue engineering involves three materials: stem cells, scaffolds, and growth factors. Dentin-pulp tissue engineering is undergoing clinical trials in humans (Nakashima et al., 2017; Xuan et al., 2018). During tissue engineering, mesenchymal stem cells (MSCs) are differentiated

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into odontoblasts and produce dentinal tubules. The regeneration of dentin-pulp ideally will result in vascularized pulp with well-organized tubular dentin. However, sometimes amorphous mineral deposits are formed, instead of tubular dentin, and mineral deposition may not always occur (Huang et al., 2020). To successfully regenerate dentinal tissue, it is necessary to find the key factors to precisely regulate the differentiation of MSCs into odontoblasts, and prevent osteoblast differentiation.

MSCs from different dentoalveolar tissues are unique and retain identity from their primary tissue source. SCAPs were found in the root apex of immature permanent teeth in 2006 (Sonoyama et al., 2006). These cells appear to be the source of primary odontoblasts. As dental stem cells, SCAPs have a natural advantage in odontogenic differentiation (Huang et al., 2008; Pelissari, Paris, Mantesso, & Trierveiler, 2018; Sonoyama et al., 2008). Huang et al. inoculated SCAPs or dental pulp stem cells (DPSCs) with poly lactic-co-glycolic acid (PLGA) synthetic scaffolds into emptied root canals. Three months later, dentin-like structures with blood vessels were found in the SCAP group. In contrast, the thickness and continuity of dentin in the DPSC group were significantly lower than in the SCAP group, indicating that SCAPs have a greater ability to regenerate dentin than DPSCs (Huang et al., 2010).

Bone has a similar composition to dentin, but a different structure. During bone formation, bone marrow mesenchymal stem cells (BMSCs) are the source of osteoblast cells, which form bone tissue. A large number of studies have demonstrated the osteogenic potential of BMSCs, and BMSCs are considered to be potent seed cells for bone regeneration (Krause et al., 2001; Pittenger et al., 1999; Zhang, Walboomers, van Osch, van den Dolder, & Jansen, 2008).

In a previous study, we compared the secretomes of SCAPs and BMSCs after inducing odonto/osteogenic differentiation *in vitro*. We reported that transforming growth factor-β2 (TGF-β2) might be a key molecule in regulating the differentiation of SCAPs and BMSCs (Yu, Li, Zhao, Li, & Ge, 2020). SCAPs secrete more TGF-β2 than BMSCs, and TGF-β2 was significantly upregulated during the odonto/osteogenic differentiation of SCAPs, but was significantly downregulated during the odonto/osteogenic differentiation of BMSCs (Yu et al., 2020).

TGF-βs play important roles in cell growth, differentiation, and apoptosis. Three mammalian isoforms (TGF-β1, 2 and 3) have been identified in bone and dentin, and TGF-β1 the predominant isoform (Cassidy, Fahey, Prime, & Smith, 1997). During tooth development, TGF-β1 is widely expressed in the epithelium and mesenchyme in the bud and cap stages (Huang & Chai, 2010). TGF-β2 is expressed in dental papilla and odontoblasts, but not in dental epithelial cells (Huang & Chai, 2010). TGF-β3 expression is found in the stellate reticulum (Huang & Chai, 2010). In pulp cells, collagen synthesis is stimulated by TGF-β1and TGF-β2, but not by TGF-β3 (Chan et al., 2005).

To verify the effects of TGF-β2 on MSCs in odontogenic and osteogenic differentiation and to optimize preparation of MSCs appropriate for dentin regeneration, we used lentiviral transduction to generate SCAPs with TGF-β2 knockdown and BMSCs with TGF-β2 overexpression. We investigated the effects of TGF-β2 knockdown or overexpression on the odontogenic and osteogenic differentiation abilities of SCAPs and BMSCs *in vitro* and *in vivo*, in comparison with TGF-β1.

2. Materials and methods

2.1. Sample collection and cell culture

Immature normal human impacted third molars and normal human bone marrow from the mandibular alveolar bone (n = 5) were collected from healthy patients (aged 16–30 years). Approval for sample collection was provided by the Ethics Committee of the Health Science Center of Peking University (Beijing, China; PKUSSIRB-201734036). We isolated SCAPs from dental apical papilla tissue and BMSCs from bone marrow. The MSCs were maintained in α-modified Eagle's minimum essential medium (α-MEM; Gibco, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) in 5% CO_2 at 37 °C. Our experiments used cells that had been passaged three to five times. Flow cytometry with antibodies against CD73, CD90, CD105, CD146, and CD34 (BD Biosciences, United States) was performed to characterize SCAPs and BMSCs, as previously described (Yu, Zhao, Ma, & Ge, 2016).

In addition, the differentiation of SCAPs and BMSCs into osteogenic/odontogenic, adipogenic and chondrogenic lineages was performed as previously described (Yu et al., 2013). Adipogenic or chondrogenic differentiation was induced by the STEMPRO Adipogenesis or Chondrogenesis differentiation Kit (Invitrogen) for three weeks, respectively. To determine the differentiation of adipocytes, lipid droplets were stained with 60% Oil Red solution. Alcian Blue staining or immunochemistry was performed to assess the differentiation of chondrocytes. For immunochemistry, cells were fixed, embedded, deparaffinized, blocked, incubated with a primary antibody (collagen II, Origene, China), and incubated with secondary antibody.

2.2. Lentiviral knockdown and overexpression of TGF-β1 and TGF-β2a in SCAPs and BMSCs

TGF-β1 knockdown lentivirus (TGFβ1-sh), TGF-β2 knockdown lentivirus (TGFβ2-sh), TGF-β1 overexpression lentivirus, TGF-β2 overexpression lentivirus, and the corresponding control vector viruses were purchased and stored at −80 °C (Xibei Hongcheng, China). SCAPs and BMSCs at passage two or three were seeded in 24-well plates. When the confluency of the cells reached 60–70%, the normal growth medium was replaced by media containing lentivirus (MOIs: 50, 70, and 100). After 24–48 h of transfection, the media was replaced with normal medium. Successful transduction was observed under a fluorescence microscope 48–72 h after lentivirus treatment. Medium containing 3 μg/ml puromycin was used to screen for transfected cells until few cells died 4–5 days after transfection. Cells were kept under selection with 1.5 μg/ml puromycin for further culture and experiments.

After selection, TGFβ1sh-SCAPs, TGFβ2sh-SCAPs, and Contsh-SCAPs (empty virus-transfected SCAPs) were obtained, and TGFβ1-BMSCs, TGFβ2-BMSCs, and Vector-BMSCs (empty virus-transfected BMSCs) were obtained.

2.3. Osteogenic/odontogenic differentiation

Cells were seeded in 6-well plates at a density of 1×10^5 cells per well. When the confluency of the cells reached approximately 70–80%, mineralization-inducing medium composed of α-MEM supplemented with 10% FBS, 50 mM ascorbate-2-phosphate (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich, United States), and 100 nM dexamethasone (Sigma-Aldrich) was used. The media was changed every 3 days, and cell growth and morphological changes were observed under a microscope daily.

After three weeks, the cells were fixed with 4% paraformaldehyde and stained with 2% alizarin red (Sigma-Aldrich). After photos were taken, 1 ml of 10% cetylpyridinium chloride (Sigma-Aldrich) was added to each well and incubated at room temperature for 30 min, and the supernatant was aspirated. The absorbance at 570 nm was determined using an enzyme-linked immunosorbent assay reader.

2.4. Real-time quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, United States), according to the manufacturer's instructions. cDNA was synthesized from oligo (dT) primers using a reverse transcriptase kit (Promega, United States). The primer sequences were designed by a primer bank, shown in Supplementary Table. We performed reverse transcription-polymerase chain reaction (RT-PCR) in triplicate in 96-well plates using a 7900HT Fast Real-Time System (Applied Biosystems, United States). We used the comparative cycle threshold (2^-

$^{\Delta\Delta CT}$) method to calculate the relative expression levels of the target genes.

2.5. Western blot analysis

After harvesting protein from the cells, we used 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate thirty micrograms of protein. Proteins were transferred onto a polyvinylidene fluoride membrane. The membrane was then blocked with 5% (w/v) nonfat dried milk, incubated overnight at 4 °C with primary antibodies (TGF-β1, Abcam ab179695, rabbit monoclonal; TGF-β2, Abcam ab36495, mouse monoclonal; runt-related transcription factor 2, RUNX2, Abcam ab133612, rabbit monoclonal; dentin sialophosphoprotein, DSPP, Santa Cruz sc-73632, mouse monoclonal; dentin matrix protein 1, DMP-1, Bioss bs12359R, rabbit polyclonal; osteocalcin, OCN, Bioss bs0470R, rabbit polyclonal; β-actin, Abcam ab8227, rabbit polyclonal), then incubated with horseradish peroxidase-conjugated secondary antibodies (Origene, China). Immunoreactive bands were visualized using enhanced chemiluminescence (Cwbiotech, China) at room temperature. Images were digitized using a Fusion FX image analyzer (Viber Loumat, Germany).

2.6. In vivo transplantation

All animal experiments were approved by the Animal Care Committee of Peking University (Reference No. LA2019311) and conducted according to the accepted standards of humane animal care. Approximately 1×10^6 cells at passage 2 were lifted with trypsin/EDTA for 5 min, spun down into cell pellets, maintained in α-MEM with 10% FBS, and incubated for 3 h in tubes to ensure that the cells were well aggregated. Cell pellets from the six different transductants were seeded onto absorbable gelatin sponges (AGS) and transplanted into the renal capsules of BALB/C nude mice. Untreated cell pellets on AGS materials served as controls. Each mouse was implanted with one AGS, which contained one transductant. Six weeks following transplantation, the mice were euthanized by anesthesia overdose and the 21 pellets (3 from each treated group and 3 from the untreated group) were retrieved. The implants were then fixed in 4% polyoxymethylene, decalcified, and processed for Masson's trichrome staining and immunocytochemical analysis.

2.7. Masson's trichrome staining

Briefly, 5 μm tissue sections from representative paraffin blocks were deparaffinized in xylene and rehydrated through graded ethanol solutions. Sections were then stained with Masson's Trichrome Stain Kit (G1281, Solarbio) to evaluate collagen distribution.

2.8. Immunohistochemistry

Immunohistochemical analyses of recovered implants was performed using the streptavidin-biotin complex method, in accordance with the manufacturer's recommended protocol. First, 3% hydrogen peroxide was used to block endogenous peroxidases. The sections were then processed by conventional microwave heating in 0.01 M sodium citrate retrieval buffer (0.01 M sodium citrate and 0.01 M citric acid, pH 6.0) for 5–10 min for antigen retrieval. Then, sections were blocked with 5% normal swine serum for 20 min and incubated with a primary antibody (DSPP, 1:100; human nuclei, Gene Tex GTX82624, mouse monoclonal, 1:100) overnight at 4 °C. Incubation with PBS served as a negative control. Finally, the sections were incubated with secondary antibody for 45 min at room temperature. The reaction products were developed by 3,3′-diaminobenzidine solution with hydrogen peroxide and counterstained with hematoxylin.

2.9. Statistical analysis

Statistical analysis was performed using SPSS version 19.0 (SPSS, United States). All continuous variables were tested for normal distribution by shapiro-wilk test, and the data consistent with normal distribution were expressed as means ± standard deviation (SD). Then, we used Student's t-test to assess the significance of differences between two groups, and one-way ANOVA for differences between multiple groups. Differences with a p-value of $p < 0.05$ were considered to be statistically significant. All experiments were repeated three times ($n = 3$).

3. Results

3.1. MSCs identification

SCAPs and BMSCs showed typical fibroblast-like morphology (Fig. 1). Flow cytometry showed that SCAPs and BMSCs were positive for CD73, CD146, CD90, CD105 and negative for CD34 (Yu, Zhao, Ma, & Ge, 2016) (Supplementary Fig. 1A). In addition, the identify of SCAPs or BMSCs was confirmed by differentiation into osteogenic/odontogenic, adipogenic or chondrogenic cells (Supplementary Fig. 1B).

3.2. Generation and verification of the SCAPs and BMSCs transductants

Forty-eight to 72 h after viral transfection (MOI values of 50, 70, and 100), transfection efficiency was observed under a fluorescence microscope. The transfection efficiencies at the three MOI values were more than 70%. The transfection efficiency at an MOI of 50 was slightly lower than that of the other two. We used an MOI value of 70 for subsequent viral transfections.

We transfected SCAPs with the TGF-β1 knockdown or TGF-β2 knockdown lentivirus. The transfection efficiency of the two knockdown viruses was more than 80% (Fig. 1A–D). The expression of TGF-β1 and TGF-β2 was detected at the mRNA and protein levels. Real-time PCR and western blot analysis showed that the expression of TGF-β1 in the TGFβ1sh-SCAPs group was significantly lower than in the Contsh-SCAPs group (Fig. 1E, F), and the expression of TGF-β2 in the TGFβ2sh-SCAPs group was significantly lower than in the Contsh-SCAPs group (Fig. 1G, H). Additionally, we transfected BMSCs with the TGF-β1 or TGF-β2 overexpression lentivirus (Fig. 1I–L). Real-time PCR and western blot analysis showed that the expression of TGF-β1 in the TGF-β1-BMSCs group was significantly higher than in the Vector-BMSCs group (Fig. 1M, N), and the expression of TGF-β2 in the TGFβ2-BMSCs group was significantly higher than in the Vector-BMSCs group (Fig. 1O, P).

3.3. Impacts of TGF-β1 and TGF-β2 knockdown on the osteo/odontogenic differentiation of SCAPs

We performed real-time RT-PCR, western blot, and alizarin red staining to investigate the effects of TGF-β1 and TGF-β2 knockdown on the osteo/odontogenic differentiation of SCAPs. Experiments were performed using the TGFβ1sh-SCAPs, the TGFβ2sh-SCAPs, and the Contsh-SCAPs as controls.

Alizarin red staining and calcium quantification after 2 weeks revealed that SCAP mineralization was significantly higher in the TGFβ2sh-SCAPs group, but significantly lower in the TGFβ1sh-SCAPs group than in the Contsh-SCAPs group (Fig. 2A, B). We also examined the expression of osteoblast-related markers, OCN and RUNX2, in each group. RUNX-2 and OCN mRNA and protein levels on days 7 and 14 in the TGFβ2sh-SCAPs were significantly higher than in the Contsh-SCAPs. In contrast, western blot analysis revealed that RUNX-2 levels were significantly lower on days 7 and 14 in the TGFβ1sh-SCAPs than in the Contsh-SCAPs. Moreover, the OCN mRNA levels on days 7 and 14 in the TGFβ1sh-SCAPs were significantly lower than in the Contsh-SCAPs, while there were no significant differences at the protein level.

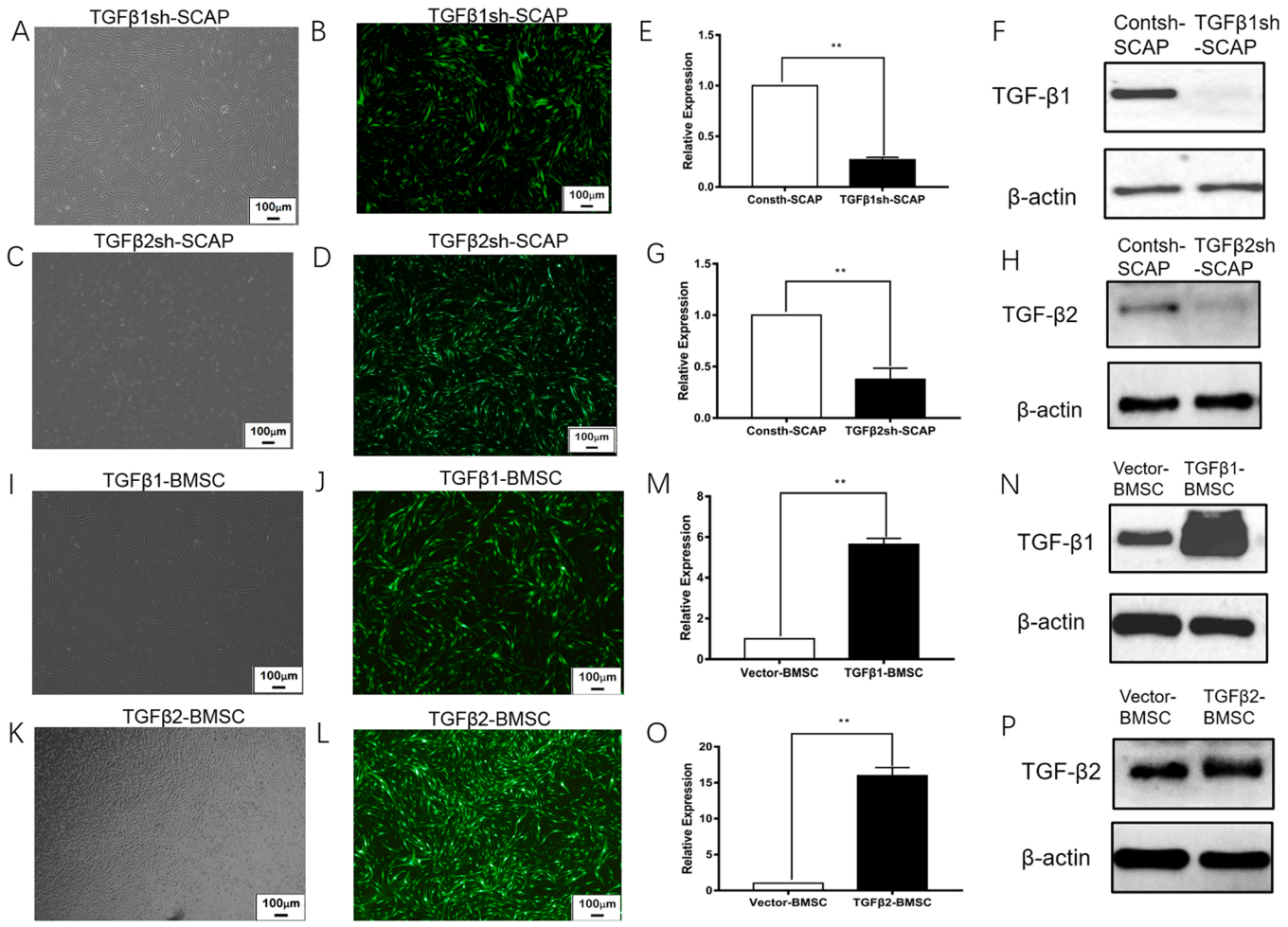

Fig. 1. Generating and verifying TGFβ1sh-SCAPs, TGFβ2sh-SCAPs, TGFβ1-BMSCs and TGFβ2-BMSCs. (A-D, I-L) Bright field and fluorescence microscopy images of the same field of view after TGF-β1/ TGF-β2 knockdown virus or TGF-β1/TGF-β2 overexpression virus transfected into MSCs. (E, G, M, O) Real-time RT-PCR showing the expression of TGF-β1 or TGF-β2 in the different groups (*$p < 0.05$, **$p < 0.01$). (F, H, N, P) Western blot showing the expression of TGF-β1 or TGF-β2 in the different groups.

The expression levels of the odontogenesis-specific markers DSPP and DMP-1 were also measured by real-time RT-PCR and western blot (Fig. 2C–F). DSPP mRNA and protein levels were significantly lower in TGFβ2sh-SCAPs than in the Contsh-SCAPs on days 7 and 14; DMP-1 mRNA and protein levels showed the same results except that the mRNA level of DMP-1 was higher in on TGFβ2sh-SCAPs than in the Contsh-SCAPs on day 14 (Fig. 2D–H). DSPP and DMP-1 mRNA and protein levels were significantly higher in the TGFβ1sh-SCAPs than in the Contsh-SCAPs on day 14.

Based on these data, we concluded that TGF-β2 knockdown may inhibit the odontogenic differentiation of SCAPs and may promote osteogenic differentiation. In addition, TGF-β1 knockdown may attenuate the osteogenic differentiation of SCAPs but may enhance odontogenic differentiation.

3.4. Impacts of TGF-β1 and TGF-β2 overexpression on the osteo/ odontogenic differentiation of BMSCs

We observed decreased mineralized nodule formation on day 14 in TGFβ2-BMSCs, and increased nodule formation in TGFβ1-BMSCs on day 14, compared with the Vector-BMSCs (Fig. 3A, B).

Real-time RT-PCR and western blot revealed significantly attenuated OCN expression on days 7 and 14 in TGFβ2-BMSCs compared with the Vector-BMSCs (Fig. 3D–H). RUNX2 expression followed a similar pattern, except that RUNX2 mRNA levels on day 7 were higher in the TGFβ2-BMSCs. In contrast, OCN and RUNX2 expression were elevated in TGFβ1-BMSCs on days 7 and 14 (Fig. 3C–H).

DSPP and DMP-1 mRNA levels were significantly higher in the TGFβ2-BMSCs than in the Vector-BMSCs on day 7, but lower on day 14 (Fig. 3C–H). In addition, the protein levels of DSPP and DMP-1 were significantly higher in the TGFβ2-BMSCs on days 7 and 14 except, for DSPP on day 14. DSPP and DMP-1 mRNA levels were significantly higher in TGFβ1-BMSCs than in Vector-BMSCs on days 7 and 14. On day 7, no differences in the DMP-1 protein levels were detected between TGFβ1-BMSCs and Vector-BMSCs, but the DSPP protein level in TGFβ1-BMSCs was higher than in the control group. However, on day 14, the protein levels of both DSPP and DMP-1 were significantly higher in the TGFβ1-BMSCs.

Based on these data, we conclude that TGF-β2 overexpression may promote the odontogenic differentiation of BMSCs and may inhibit their osteogenic differentiation at early stages. Moreover, TGF-β1 overexpression may enhance the osteogenic and odontogenic differentiation of BMSCs.

3.5. Impacts of TGF-β1 and TGF-β2 knockdown on the formation of type I collagen and DSPP expression in SCAPs in vivo

Anti-human nuclei antibody staining showed the location of implanted cells and the relation to collagen at six weeks after implantation (Supplementary Fig. 2). Human nuclei positive cells were mainly

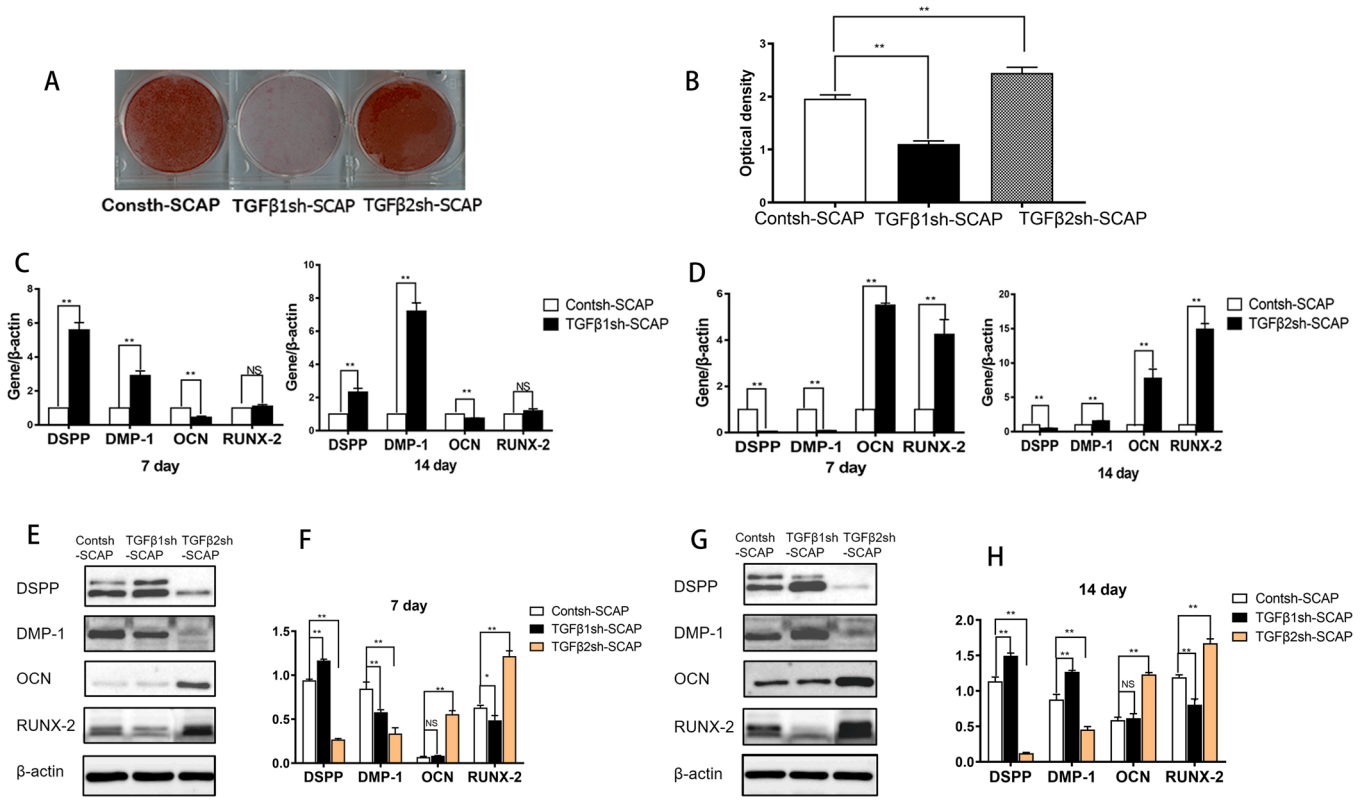

Fig. 2. Impacts of TGF-β1 and TGF-β2 knockdown on the osteo/odontogenic differentiation of SCAPs *in vitro*. (A) Osteo/odontogenic differentiation by alizarin red staining. (B) Quantitative measurement of alizarin red staining. (C) Real-time RT-PCR showing the mRNA levels of RUNX2, OCN, DSPP, and DMP-1 of TGFβ1sh-SCAPs and Contsh-SCAPs at 7 days and 14 days. (D) Real-time RT-PCR results in TGFβ2sh-SCAPs and Contsh-SCAPs. (E, F) Western blot analysis in TGFβ1sh-SCAPs, TGFβ2sh-SCAPs and Contsh-SCAPs at 7 days. (G, H) Western blot analysis at 14 days. *$p < 0.05$, **$p < 0.01$.

detected in new-formed collagen near the renal capsule. Some nuclei positive cells were migrated into the renal capsule beneath the implanted tissues.

Masson's trichrome staining showed that there were no obvious blue-stained tissues in the renal capsule membrane in the untreated negative control group (Fig. 4A1, A2). However, blue-stained tissues (yellow arrow), indicating the formation of type I collagen tissue, were seen in the Contsh-SCAPs, TGFβ1sh-SCAPs, and TGFβ2sh-SCAPs (Fig. 4B1–D1, B2–D2). The range and quantity of positively stained tissue in the TGFβ1sh-SCAPs group were higher than in the other two groups, and the type I collagen tissue formed a tight cell layer. There were no observable differences between the Contsh-SCAPs and the TGFβ2sh-SCAPs. The expression of DSPP was determined by immunohistochemical staining. No DSPP-positive tissue was found in the untreated negative control group in the renal capsule membrane (Fig. 4E1, E2). DSPP-positive tissues were detected in both the Contsh-SCAPs and TGFβ1sh-SCAPs groups (yellow arrow), and it there were no obvious differences observed between those two groups (Fig. 4F1, F2, G1, G2). In the TGFβ2sh-SCAPs group, no DSPP-positive tissue was found at the transplantation site (Fig. 4H1, H2).

These data suggest that TGF-β2 knockdown inhibits the odontogenic differentiation of SCAPs, and TGF-β1 knockdown may stimulate collagen synthesis by SCAPs *in vivo*.

3.6. Impacts of TGF-β1 and TGF-β2 overexpression on the formation of type I collagen and DSPP in BMSCs in vivo

In the untreated negative control group, there was no evidence of blue-stained tissue in the renal capsule membrane (Fig. 5A1, A2). In the Vector-BMSCs group, TGFβ1-BMSCs, and TGFβ2-BMSCs, positive tissues were observed at the transplantation sites (Fig. 5B1, B2). However, there were no major differences observed between those three groups (Fig. 5C1, C2, D1, D2).

The immunohistochemical staining results indicated that only a small amount of DSPP-positive tissue was formed at the transplantation site in the Vector-BMSCs, the TGFβ2-BMSCs, and the TGFβ1-BMSCs groups (yellow arrow), but there were no obvious differences between groups (Fig. 5F1, F2, G1, G2, H1, H2). Moreover, the amounts of DSPP-expressing tissues in the BMSCs groups were less than in the SCAPs groups.

These data indicate that TGF-β2 and TGF-β1 did not have an effect on the odontogenic differentiation of BMSCs *in vivo*.

4. Discussion

To verify the function of TGF-β2 on the odontogenesis of MSCs, we designed TGFβ2sh-SCAPs and TGFβ2-BMSCs in this study. The reasons for this design are as follows: our previous research showed that TGF-β2 secretion increased in SCAPs and decreased in BMSCs after mineralization induction, suggesting that TGF-β2 may promote the odontogenic differentiation of MSCs and inhibit the osteogenic differentiation (Yu et al., 2020). At the same time, we verified that TGF-β2 inhibit the osteogenic differentiation of SCAPs and promote the odontogenic differentiation of BMSCs (Yu et al., 2020). Therefore, we constructed TGFβ2sh-SCAPs and TGFβ2-BMSCs in this study, to observe whether it can reverse the differentiation direction of SCAPs and BMSCs, by which we can complete the reconstruction of MSCs function. The role of TGF-β1 in tooth development is different from TGF-β2. TGFβ1-overexpressing in tooth showed a significant reduction in mineralization and the expression of DSPP (Thyagarajan, Sreenath, Cho, Wright, & Kulkarni, 2001). However, TGFβ2-overexpressing mice showed an increased dentin mineral apposition (DenBesten et al., 2001).

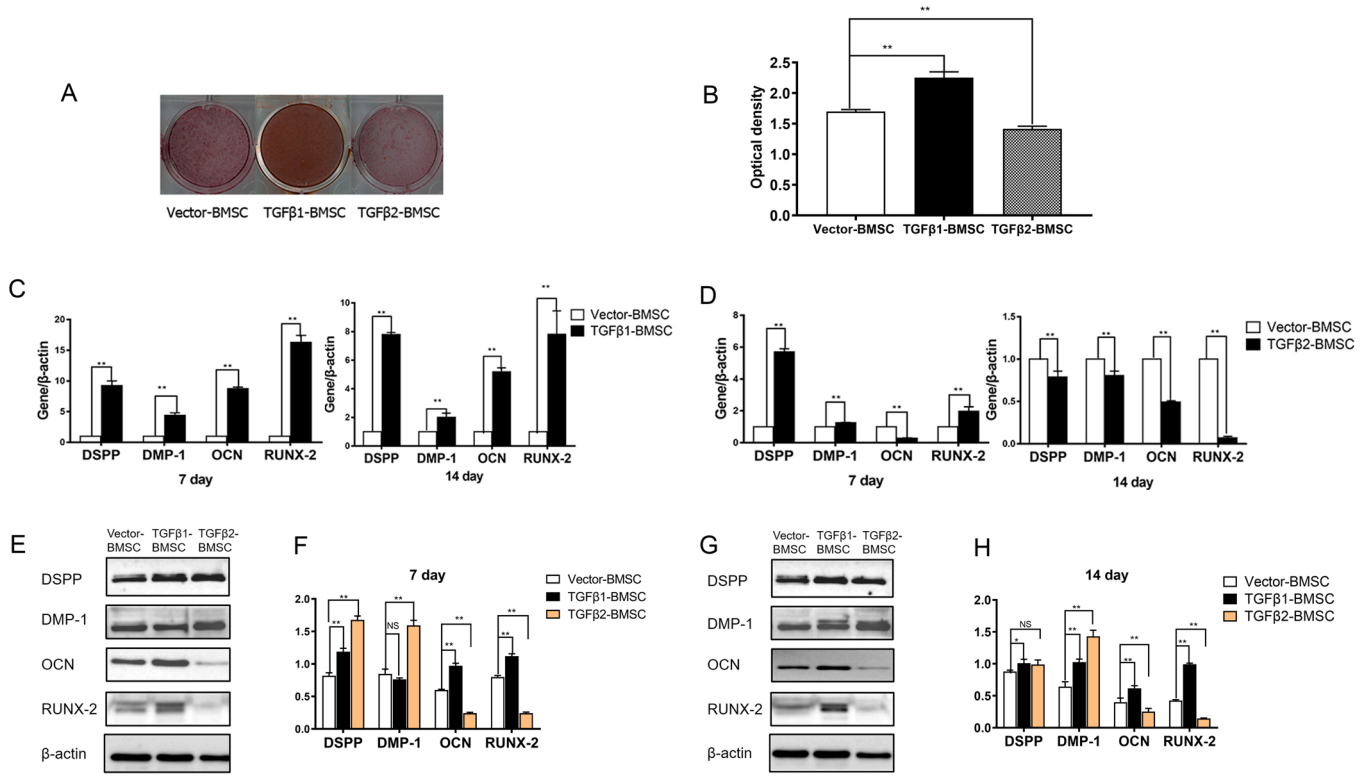

Fig. 3. Impacts of TGF-β1 and TGF-β2 overexpression on the osteo/odontogenic differentiation of BMSCs *in vitro*. (A) Osteo/odontogenic differentiation by alizarin red staining. (B) Quantitative measurement of alizarin red staining. (C) Real-time RT-PCR showing the mRNA levels of RUNX2, OCN, DSPP, and DMP-1 in TGFβ1-BMSCs and Vector-BMSCs at 7 days and 14 days. (D) Real-time RT-PCR results in TGFβ2-BMSCs and Vector-BMSCs. (E, F) Western blot analysis in TGFβ1-BMSCs, TGFβ2-BMSCs and Vector-BMSCs at 7 days. (G, H) Western blot analysis at 14 days. *p < 0.05, **p < 0.01.

To compare the function of TGF-β1 and TGF-β2, we constructed TGFβ1sh-SCAPs and TGFβ1-BMSCs in the same way.

In SCAPs, we found that TGF-β2 knockdown inhibited the expression of odontogenic markers (DSPP and DMP-1), but enhanced the expression of osteogenic markers (OCN and RUNX2) *in vitro*. DSPP is a highly specific dentin protein, and DSPP gene mutations cause dentinogenesis imperfecta, with no bone abnormalities. DMP-1 is an odontogenic-related marker, and DMP-1 expression is required for odontogenesis (Massa, Ramachandran, George, & Arana-Chavez, 2005). However, DMP-1 affects not only the mineralization of dentin, but also the mineralization of bone (Shigeki, Naoto, Fusanori, & Ashok, 2012). OCN is produced by osteoblasts, and is the most abundant non-collagenous protein in bone (Toshihisa, 2020). RUNX2 is a key transcription factor in osteogenesis, and is essential for osteoblast differentiation (Carolina et al., 2002). In our previous study, we found that recombinant human TGF-β2 enhanced odontogenic markers and inhibited osteogenic markers in MSCs, which is consistent with the present results (Yu et al., 2020). Immunohistochemical staining showed that TGF-β2 knockdown inhibited the expression of DSPPs by SCAPs *in vivo*. DSPP is a specific odontogenic marker. Therefore, TGF-β2 induces the odontogenic differentiation of SCAPs and attenuates their osteogenic differentiation.

In contrast, TGF-β1 had the opposite effect compared to TGF-β2 in regulating the odontogenic and osteogenic differentiation of SCAPs. *In vitro*, TGF-β1 knockdown inhibited the osteogenic differentiation of SCAPs and promoted their odontogenic differentiation. However, *in vivo*, immunohistochemical staining showed that TGF-β1 knockdown had no effect on the expression of DSPP in SCAPs. Our previous study proved that recombinant human TGF-β1 weakened the odontogenic differentiation of SCAPs at early stages, but enhanced odontogenic differentiation of SCAPs at later stages (Yu et al., 2020). The effect of TGF-β1 on the differentiation of SCAPs is complex. He et al. reported that TGF-β1 inhibits the expression of DSPP and OCN in SCAPs (He et al., 2014). However, Sara et al. demonstrated that TGF-β1 promotes the mineralization of SCAPs (Sara, Koyo, & Anibal, 2017). Bellamy et al. found that the expression of DSPP and DMP-1 in SCAPs is higher in scaffolds with TGF-β1 than in scaffolds without TGF-β1 (Bellamy, Shrestha, Torneck, & Kishen, 2016). The potential mechanism underlying these differences is still not well elucidated. The effects of TGF-β1 on the differentiation of MSCs is impacted by many factors. For example, low concentrations of TGF-β1 were shown to promote the osteogenic differentiation of MSCs, while high concentrations of TGF-β1 inhibited it (Chang et al., 2015; He et al., 2008; Lieb, Vogel, Milz, Dauner, & Schulz, 2004; Lin et al., 2011; Wada, Yamamoto, Nanbu, Mizuno, & Tamura, 2008). However, the thresholds for high and low concentrations in different cells and different experiments are not identical.

In BMSCs, we found significantly higher DSPP mRNA expression in the TGF-β2 group than in the control group at day 7, but significantly lower DSPP mRNA levels in the TGF-β2 group at day14. The protein level of DSPP was also significantly higher in the TGF-β2 group than in the control group at day 7, but showed no difference between these groups at day 14. The variation in expression of DMP-1 between the TGF-β2-BMSCs and Vector-BMSCs was similar to DSPP. RNA expression occurs prior to protein formation, and TGF-β2 may promote the odontogenic differentiation of BMSCs at early differentiation, but may inhibit it at later stages. In addition, TGF-β2 attenuated osteogenic-related markers in BMSCs. *In vivo*, TGF-β2 had no effect on the expression of DSPP in BMSCs. Therefore, TGF-β2 inhibited the osteogenic differentiation of BMSCs, and had complicated effects on the odontogenic differentiation of BMSCs. Our data demonstrated that TGF-β1 enhanced the mineralization, the odontogenic differentiation, and the osteogenic differentiation of BMSCs *in vitro*. Sun et al. also reported that TGFβ1-overexpressing BMSCs had significantly higher mineralization ability *in vitro,* which promoted new bone formation *in vivo* (Sun et al., 2018).

TGFβ2-overexpressing mice show increased mineral apposition

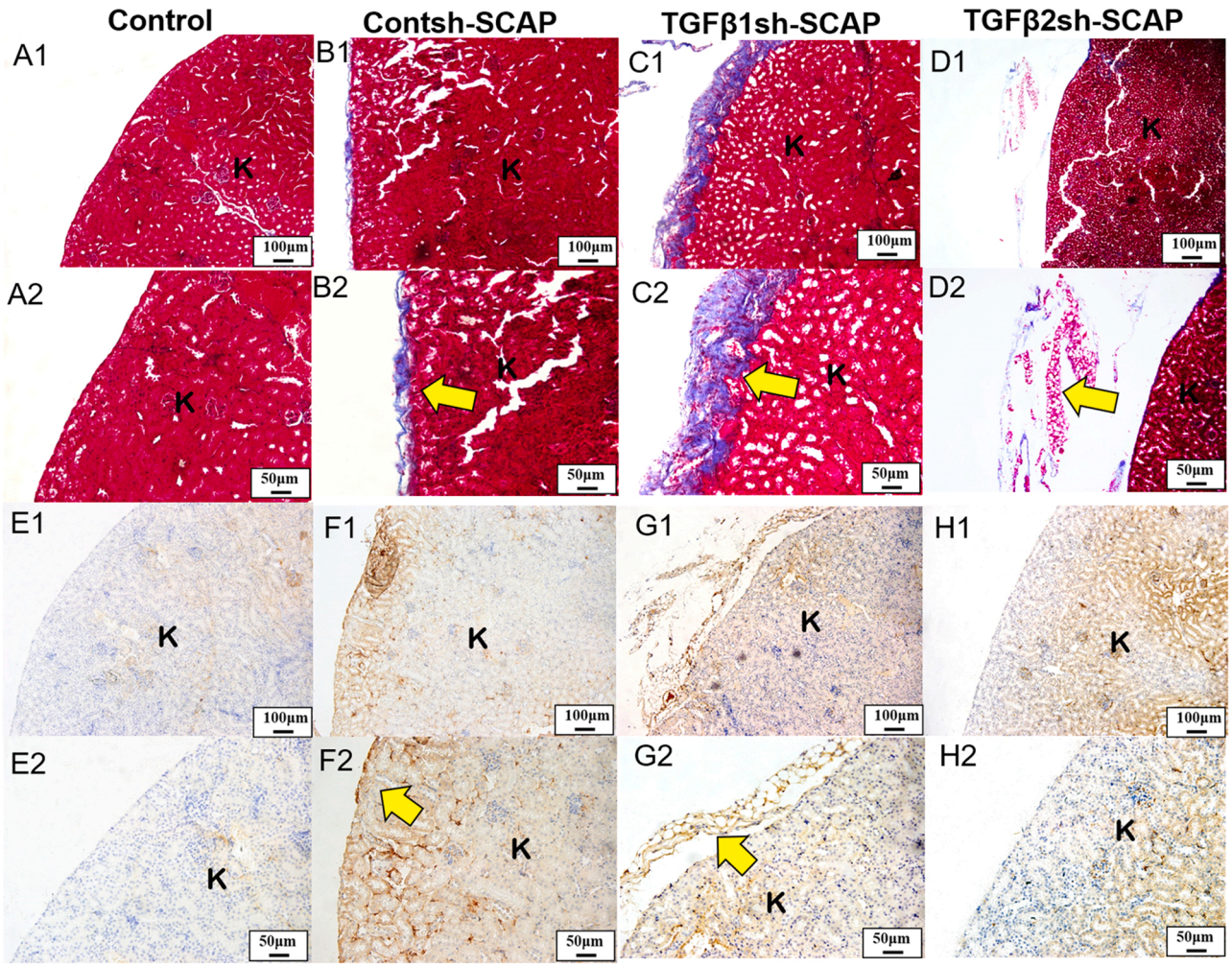

Fig. 4. Impacts of TGF-β1 and TGF-β2 knockdown on the formation of type I collagen and DSPP in SCAPs *in vivo*. (A1–D2) Masson staining (E1–H2) DSPP staining. The yellow arrow represents positive tissue. K represents the renal tissue. "Control group" means the untreated group.

rates, with no significant change in dentin microhardness and a more porous, osteoporotic phenotype in bone (DenBesten et al., 2001). These data indicate that TGF-β2 plays distinct roles in dentin and bone development. Erlebacher et al. constructed transgenic mice and reported bone loss in mice with TGF-β2 overexpression (Erlebacher & Derynck, 1996). The expression of TGF-β2 in inflammatory bone was significantly increased, and bone healing was inhibited, suggesting that TGF-β2 might inhibit the process of bone healing. Soyoun et al. demonstrated that inflamed dental follicle stem cells (DFSCs) display low levels of TGF-β1 and high levels of TGF-β2 (Um, Lee, & Seo, 2018). The mineralization and the expression of OCN and TGF-β1 is elevated after the inhibition of TGF-β2, suggesting that TGF-β2 inhibits the osteogenic differentiation of DFSCs.

There are no obvious functional redundancies between TGF-β1 and TGF-β2, although their structures are similar. The reason for this difference may be related to the differences in receptor binding and expression in cells. TGF-β proteins phosphorylate TβRII receptors and reactivate TβRI receptors to transmit information *via* Smad or non-Smad pathways. However, the transfer process also requires the participation of coreceptors.

Coreceptors are located on the cell surface and can regulate and/or mediate the signal transduction intensity, duration, specificity, and diversity of TGF-β family receptors (Nickel, Ten Dijke, & Mueller, 2018). The coreceptors of TGF-β are mainly endoglin and β-glycan. The former binds to TGF-β1 and TGF-β3, which are mainly distributed in endothelial cells, while the latter binds to three subtypes and has a wider distribution. TGF-β2 has a low affinity for "captured" TβRII receptors and needs to be recruited and presented by β-glycan, indicating that β-glycan functions as a TGFβ2-specific coreceptor, therefore, β-glycan is also called TβRIII. This suggests that cells without the TβRIII receptor will not be able to respond to TGF-β2. Furthermore, β-glycan also affects TGF-β1 and TGF-β3, but these effects do not require ligand presentation, indicating that β-glycan does not only have ligand-presenting activity (Bilandzic & Stenvers, 2011; Zuniga et al., 2005).

Cook et al. constructed β-glycan knockdown MSCs, and found a significant increase in TGF-β1 and TβRII expression, but a significant reduction in TGF-β2 expression (Cook et al., 2019). Furthermore, the loss of β-glycan in MSCs provoked a 63-fold increase in the expression of Wnt5a, and blockade of TGF-β signaling significantly reduced Wnt5a expression (Cook et al., 2019). Wnt5a is a representative noncanonical Wnt ligand. Wnt5a promotes the mineralization of dental papilla cells, and enhances the expression of osteogenic-related markers-BSP and OCN in dental papilla cell (Peng et al., 2010). In addition, Wnt5a attenuates expression of Wnt3a (a representative canonical Wnt ligand) in dental follicle cells (Sakisaka et al., 2015). Wnt3a was shown to increase the number of preodontoblasts and odontoblasts, and to enhance the expression of DMP-1 and DSPP (Vijaykumar, Root, & Mina, 2021). TGF-β2 may bind to β-glycan to regulate TGF-β and Wnt signaling networks, thereby controlling the odontogenic and osteogenic differentiation of MSCs.

In addition, Diana et al. found that another splicing variant of the TβRII receptor, TβRII-b, is a TGFβ2-binding receptor that can mediate

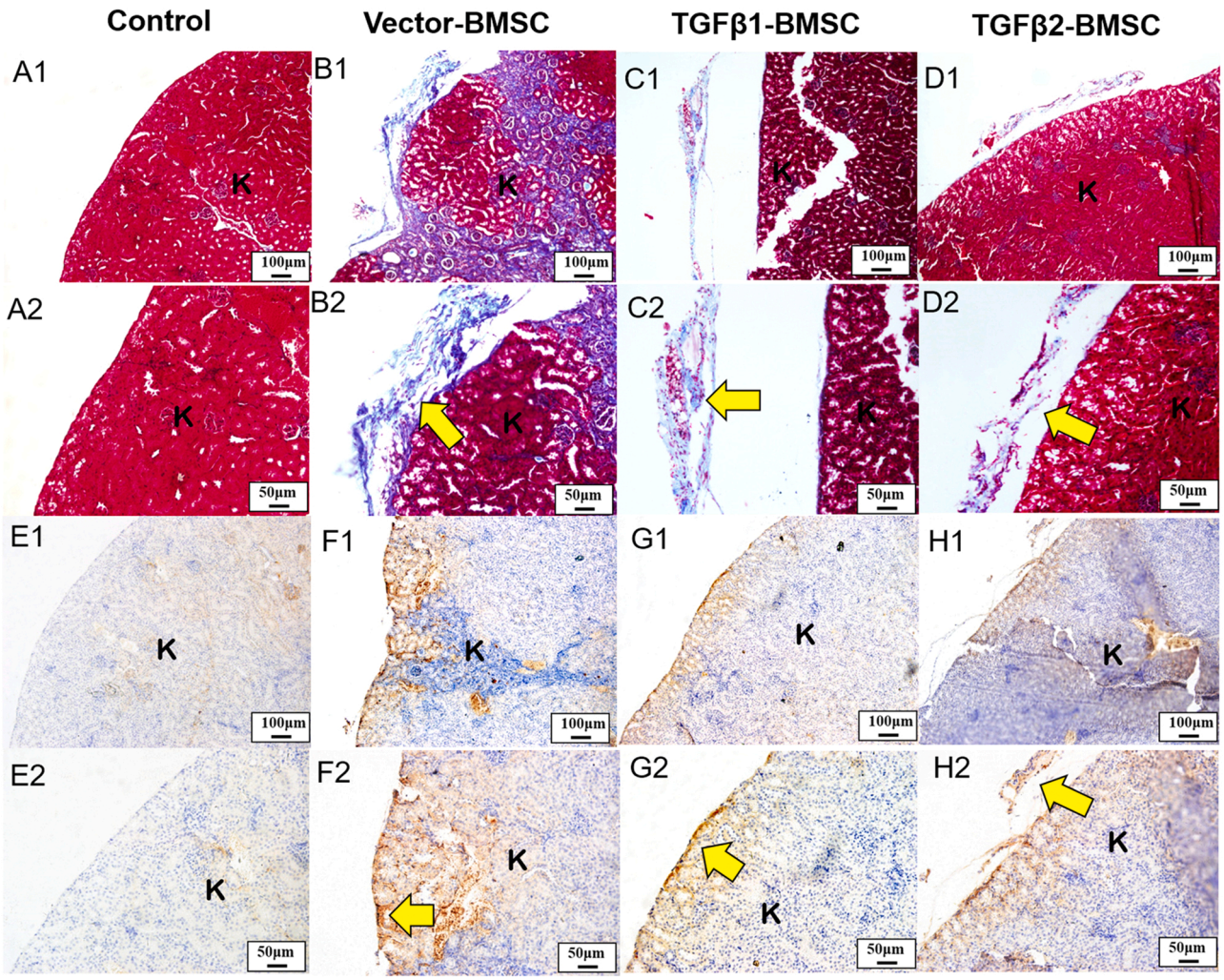

Fig. 5. Impacts of TGF-β1 and TGF-β2 overexpression on the formation of type I collagen and DSPP in BMSCs *in vivo*. (A1-D2) Masson staining (E1-H2) DSPP staining. The yellow arrow represents positive tissue. K represents the renal tissue. "Control group" means the untreated group.

signal transduction through the Smad pathway without the TβRIII receptor. TβRII-b is expressed in some tissues, including bone tissue (Poniatowski, Wojdasiewicz, Gasik, & Szukiewicz, 2015; Rotzer et al., 2001). Based on this and our data, we suggest that the differential effects of TGF-β1 and TGF-β2 may be related to their diverse actions on signal transduction processes, and the specific mechanisms need to be further explored.

In conclusion, we evaluated the effects of TGF-β1 and TGF-β2 on the osteo/odontogenic differentiation of SCAPs and BMSCs *in vitro* and *in vivo*. We report that TGF-β2 enhances the odontogenic differentiation and suppresses the osteogenic differentiation of SCAPs. In BMSCs, TGF-β2 promoted odontogenic differentiation at early differentiation, but inhibited it at later stages of differentiation. In addition, TGF-β2 inhibited osteogenic differentiation of BMSCs. TGF-β1 plays a more complex role in regulating the differentiation of SCAPs and BMSCs than TGF-β2. TGF-β1 promotes the osteogenic differentiation of BMSCs. This study provides theoretical support for the application of TGF-β1 and TGF-β2 as target genes to regulate the functional remodeling of stem cells in reconstructive dental applications.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Health Science Center of Peking University (Beijing, China; IRB00001052–11060 and PKUSSIRB-201734036). All animal experiments were approved by the Animal Care Committee of Peking University (Reference No. LA2019311).

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CRediT authorship contribution statement

SY contributed the conception, design of the study, and revise the manuscript critically for intellectual content. JL organized the database, performed the statistical analysis, and draft the manuscript. YZ and LG contributed the supervision. All authors contributed to manuscript revision. JY and JL contributed the MSCs identification.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All datasets generated for this study are included in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.archoralbio.2022.105357.

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