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From Cell Biology to Tissue Engineering

Depletion of *SHANK2* inhibited the osteo/dentinogenic differentiation potentials of stem cells from apical papilla

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Summary. The aim of this study was to investigate the biological function of *SHANK2* on the osteo/dentinogenic differentiation potentials of human stem cells from apical papilla (SCAPs). Real-time RT-PCR was used to detect the expression of *SHANK2* in human mesenchymal stem cells (MSCs). Small hairpin RNA (shRNA) was used to knockdown the *SHANK2* in SCAPs. The knockdown efficiency was determined by real-time RT-PCR and Western Blot. The *in vitro* osteo/dentinogenic differentiation potentials of SCAPs were investigated using ALP staining, ALP activity, alizarin red staining, quantitative calcium, the expression levels of *DSPP, DMP1, RUNX2* and *OSX*. *In vivo* transplantation experiments in immunocompromised mice were used to evaluate the capacity of SCAPs to form bone/dentine-like structure. *SHANK2* was highly expressed in dental tissue-derived MSCs compared with cells of other origins. Silencing of *SHANK2* inhibited the ALP activity, mineralization, and the expressions of *DSPP, DMP1, RUNX2* and *OSX* in SCAPs. Furthermore, *in vivo* transplantation experiments indicated that knockdown of *SHANK2* in SCAPs generated less bone/dentin-like mineralized tissue compared with the control group. The present study demonstrated that depletion of *SHANK2* inhibited the osteo/dentinogenic differentiation potentials in SCAPs, explored the new function of *SHANK2*, and provided useful information to elucidate the molecular mechanism underlying directed differentiation in dental tissue-derived MSCs.

Key words: *SHANK2*, Osteo/dentinogenic differentiation, Stem Cells, Apical Papilla (SCAPs)

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Introduction

Stem cells are clonogenic cells characterized by the capacity of self-renewal and give rise to many cell types. Mesenchymal stem cells (MSCs), a type of somatic stem cell that were initially identified in bone marrow (Friedenstein et al., 1968) have generated a large amount of attention in regenerative medicine owing to their promising potential to repair or regenerate damaged tissues. Over the past decades, MSCs have been discovered in various tissues such as peripheral blood, adipose tissue, umbilical cord, liver, placenta, and also teeth (Pittenger et al., 1999). MSCs isolated from dental tissues, including dental pulp stem cells (DPSCs), dental follicle stem cells (DFSCs) (Gronthos et al., 2000), stem cells from exfoliated deciduous teeth (SHED) (Miura et al., 2003), periodontal ligament stem cells (PDLSCs) (Seo et al., 2004), and stem cells from apical papilla

(SCAPs) (Sonoyama et al., 2008) are considered as potential cells suitable for tissue engineering applications, including dental tissues, bone and nerve regeneration (Huang et al., 2009). When transplanted into mice, rat, swine or human, MSCs generated bone/dentin-like mineralized tissue and were capable of repairing tooth and mandible defects (Laino et al., 2005; Chang et al., 2007; D'Aquino et al., 2007, 2009; Liu et al., 2008; Ding et al., 2010). MSCs represent a reliable resource for tissue regeneration. Among these dental stem cells, SCAPs are a unique population of postnatal stem cells and represent a novel population of multipotent stem cells. SCAPs have demonstrated their capacity to develop into odontoblast-like cells *in vitro*. This population of cells was also found to express high levels of survivin and telomerase, which are both important molecules in mediating cell proliferation. SCAPs showed a superior tissue regeneration potential that of DPSCs-mediated tissue regeneration and may offer a promising cell-based therapy for root regeneration (Sonoyama et al., 2006).

The therapeutic utility of MSCs relies on the understanding of the molecular mechanisms controlling cell fate decisions. Numerous extrinsic and intrinsic signals are suggested to regulate the differentiation of MSCs (Iglesias-Bartolome and Gutkind, 2011). Our previous study compared the gene expression patterns of PDLSCs with stem cells derived from Wharton's Jelly of the umbilical cord (WJCMSCs) which were regarded as a potential source of MSCs for clinical therapy. Our results showed that the gene expression profiles were significantly different between these two cell types which may contribute to their distinct dentinogenic differentiation capacity (Yu et al., 2013). The origin of dental tissue-derived MSCs may be associated with neural crest cells, suggesting that the genes which play the important role in the nerve system might also be involved in regulation of dental tissue-derived MSCs. Among the up-regulated genes in PDLSCs compared with those in WJCMSCs, *SHANK2* aroused our interest for its role in the nerve system. *SHANK2*, one member of *SHANK* (SH3 domain and multiple ankyrin repeats) scaffold protein family, was originally identified as a major component of the postsynaptic density (PSD) (Boeckers et al., 1999; Naisbitt et al., 1999; Yao et al., 1999). *SHANK2* could mediate signal transduction in multiple cell biological contexts as an organizer of cytoskeletal/signaling complexes (Sheng and Kim, 2000). Previous knowledge about the effects of *SHANK2* on embryonic development and neurodevelopmental disorders (Berkel et al., 2010; Gessert et al., 2011; Leblond et al., 2012), the role of *SHANK2* in determining the fate of dental MSCs is unclear.

In the present study, we investigated the function of *SHANK2* on osteo/dentinogenic differentiation potentials of SCAPs, and found that the knockdown of *SHANK2* impaired osteo/dentinogenic commitment of SCAPs *in vitro* and *in vivo*, and explored the novel role of *SHANK2* in MSCs fate determination.

Materials and methods

Cell cultures

Tooth tissues were obtained under approved guidelines set by Beijing Stomatological Hospital, Capital Medical University with informed patient consent. Wisdom teeth were first disinfected with 75% ethanol and then washed with PBS. SCAPs were isolaed, cultured and identified as previously described (Gronthos et al., 2000; Miura et al., 2003; Sonoyama et al., 2006; Iwata et al., 2010). SCAPs were gently separated from the apical papilla of the root and then digested in a solution of 3 mg/mL collagenase type I (Worthington Biochem, USA) and 4 mg/mL dispase (Roche, Germany) for 1 hr at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon, BD Labware, USA). SCAPs were grown in a humidified 5% CO_2 incubator at 37°C in MEM alpha modified Eagle's medium (Invitrogen, USA) supplemented with 15% fetal bovine serum (FBS; Invitrogen), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg /mL streptomycin (Invitrogen). The culture medium was changed every 3 days. 293T cells were maintained in complete DMEM medium with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 100 μg /mL streptomycin (Invitrogen).

Plasmid construct and viral infection

The plasmids were constructed by standard methods; all structures were verified by appropriate restriction digest and/or sequencing. The shRNA of target gene was subcloned into pLKO.1 lentiviral vector (Addgene, USA). Viral packaging was prepared as described previously (Sonoyama et al., 2006). 3×10^6 293T cells were incubated in a 10 cm tissue culture plate in 10 ml DMEM + 10% FBS without antibiotics overnight. 2 μg Virus plasmid, 2 μg packaging plasmid and 2μg envelope plasmid with 18 ulFuGENE® 6 transfection reagent (promega, USA) in 600 μl serum-free OPTI-MEM (Invitrogen, USA) was incubated at room temperature for 30 minutes, and then gently added DNA: FuGENE® mix dropwise to 293T cells. After 12-15 hr, 10 ml DMEM + 10% FBS with 100 U/mL penicillin, and 100 μg /mL streptomycin was used to remove the transfection reagent. 48 hr after transfection, media was harvested, centrifuged at 1,250 rpm and filtered through a 0.45 μm filter to remove the cells. Virus would be frozen at -80°C for long-term storage. For viral infection, MSCs were plated overnight and then infected with lentiviruses in the presence of polybrene (6 μg/ml, Sigma-Aldrich, USA) for 6 hr, and then after 48 hr, selected by antibiotics. Scramble shRNA was purchased from Addgene (USA).

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) and Real-time RT-PCR

Total RNA was isolated from SCAPs using

Trizolreagents (Invitrogen, USA). For the real-time RT-PCR, two-μg aliquots of RNAs were synthesized using random hexamers or Oligo (dT) and reverse transcriptase according to the manufacturer's protocol (Invitrogen, USA). The real-time PCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiangen, Germany) and IcycleriQ Multi-color Real-time PCR Detection System.

Western blot analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCL, 1 mM EDTA, 1% sodium dodecylsulfate (SDS), 1% Nonidet P-40, 1: 100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, 50 mM sodium fluoride). The samples were separated on a 10% SDS poly-acrylamidegel and transferred to PVDF membrane by a semi-dry transfer apparatus (Bio-Rad). The membranes were blotted with 5% milk for 2 hr and then incubated with primary antibodies overnight. The immune complexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, WI, USA) and visualized with Super Signal reagents (Pierce, IL, USA). Primary antibodies were purchased from the following commercial sources: monoclonal antibodies against *SHANK2* (Clone No. S23B-49 Cat No. ab94575, Abcam, USA) and monoclonal antibodies against beta Actin (Clone No. mAbcam 8226, Cat No. ab8226, Abcam, USA).

ALP and Alizarin red staining

SCAPs were grown in mineralization-inducing media containing 100 μM/ml ascorbicacid, 2 μM β-glycerophosphate and 10 μM dexamethasone. For ALP staining, after induction, cells were fixed with 4% paraformaldehyde and stained with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast red FRV by an ALP kit according to the manufacturer's protocol (Sigma-Aldrich). ALP activity assay was performed with an ALP activity kit according to the manufacturer's protocol (Sigma-Aldrich) and normalized based on protein concentrations. For detecting mineralization, cells were induced for 2-3 weeks, fixed with 70% ethonal and stained with 2% Alizarin red (Sigma-Aldrich). To quantitatively determine calcium mineral, Alizarin Red was destained with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 30 minutes at room temperature. The concentration was determined by absorbance measurement at 562 nm on a multiplate reader using a standard calcium curve in the same solution. The final calcium level in each group was normalized with the total protein concentrations prepared from a duplicate plate (Gregory et al., 2004)

Transplantation in nude mice

Approximately $4.0x10^6$ of SCAPs were mixed with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (Engineering Research Center for Biomaterials, Sichuan University, China) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (nu/nu nude mice) as previously described (Sonoyama et al., 2006). These procedures were performed in accordance with specifications of an approved animal protocol.

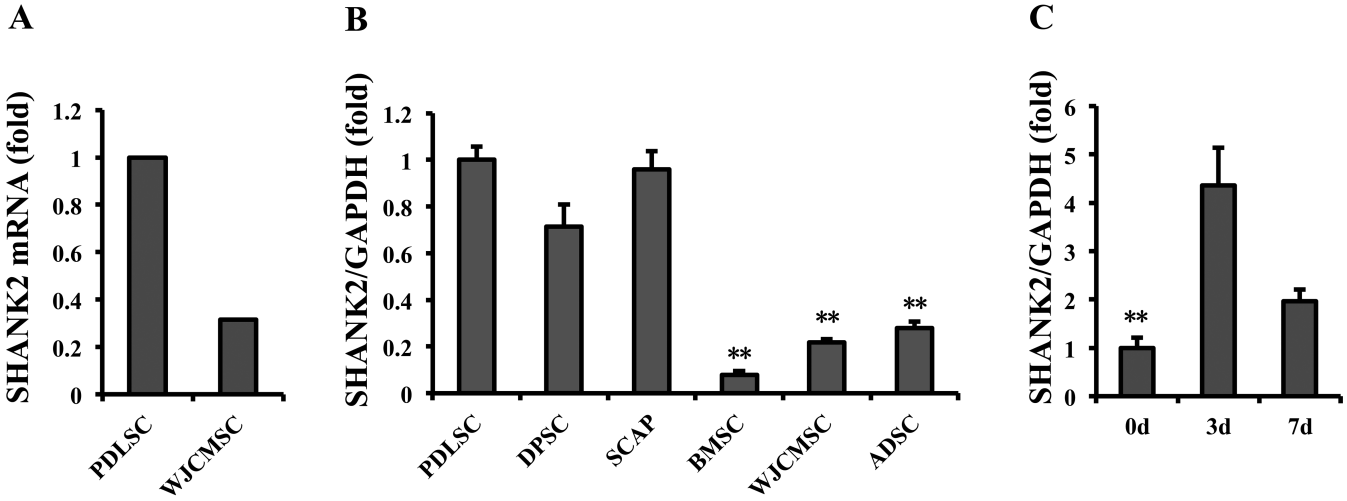

Fig. 1. SHANK2 is highly expressed in differentiated dental tissue-derived MSCs. **A.** Microarray data showed that SHANK2 mRNA expression level was much higher in PDLSCs compared with WJCMSCs. **B.** Real-time RT-PCR results showed that SHANK2 mRNA levels in PDLSCs, DPSCs and SCAPs were much higher than BMSCs, WJCMSCs and ADSCs. GAPDH was used as an internal control. **C.** The expression of SHANK2 in SCAPs was increased at 3 and 7d after osteogenic induction as detected with real-time RT-PCR. GAPDH was used as an internal control. One way ANOVA was performed to determine statistical significance. All error bars represent s.d. (n=3). *P≤0.05. **P≤0.01.

Histological staining

Eight weeks after transplantation, the transplants were harvested, fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) overnight at 4°C, and decalcified with 10% EDTA in PBS (pH 7.4) at 4°C. The decalcified samples were then dehydrated and embedded in paraffin. The sections were deparaffinized, rehydrated, and stained with H&E.

Statistics

All statistical calculations were performed using SPSS10 statistical software. Student's t test or one-way ANOVA were performed to determine statistical significance and are indicated on figures as follow: *p≤0.05, **p≤0.01.

Results

SHANK2 is highly expressed differentiated dental tissue-derived MSCs

Our previous microarray analysis showed that the expression of *SHANK2* was higher in PDLSCs compared with WJCMSCs (Yu et al., 2013) (Fig. 1A). Then real-

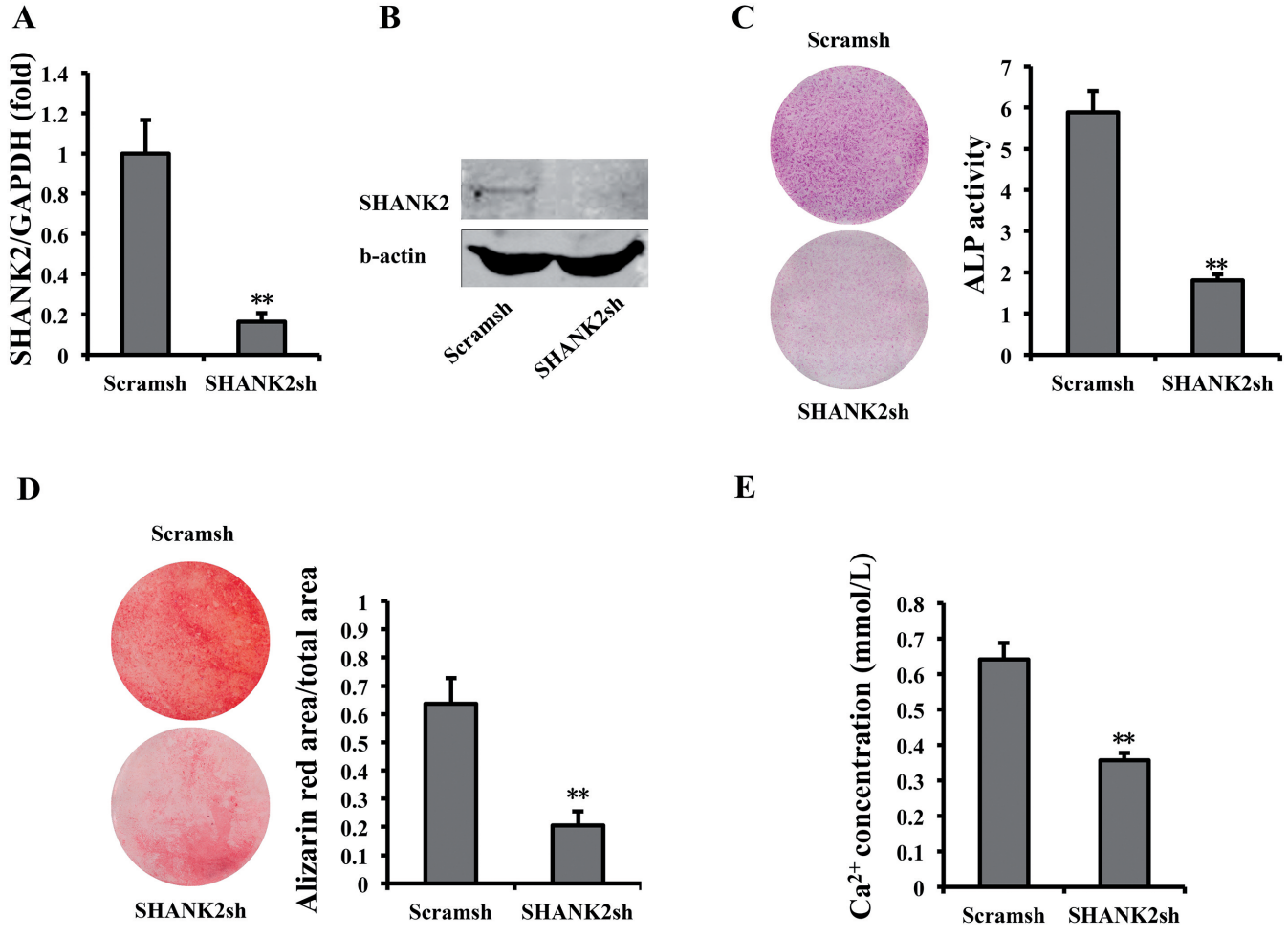

Fig 2. Osteo/dentinogenic ability was impaired in SCAPs after knock-down of SHANK2. **A.** SCAPs were infected with lentiviruses expressing SHANK2shRNA (SHANK2sh) or Scramble shRNA (Scramsh). The expression of SHANK2 was determined by real-time RT-PCR. SHANK2 can be knocked down efficiently compared with Scramsh group. GAPDH was used as an internal control. **B.** Western Blot results also confirmed that SHANK2 can be depleted efficiently by SHANK2shRNA. Beta-actin was used as an internal control. **C**. The knock-down of SHANK2 inhibited ALP activity in SCAPs. SCAPs were induced to differentiate for the indicated time periods. After induction, ALP activity was measured with an ALP assay kit and Fast Red ALP staining Kit **(C)** according to the manufacturer's protocol (Sigma-Aldrich). **D, E.** The knock-down of SHANK2 inhibited mineralization in SCAPs. SCAPs were induced to differentiate for the indicated time periods. After induction, calcium mineral of cells was stained with 2% Alizarin red **(D)** and was quantitatively determined **(E)**. Student's t test was performed to determine statistical significance. All error bars represent s.d. (n=3). **P≤0.01.

time RT–PCR results showed that the mRNA levels of *SHANK2* in dental tissue-derived MSCs such as PDLSCs, DPSCs and SCAPs were much higher than other origin MSCs, such as BMSCs, WJCMSCs and adipose-derived mesenchymal stem cells (ADSCs) (Fig. 1B). Subsequently, we cultured SCAPs with osteogenic-inducing medium, and interestingly, we found that *SHANK2* expression was increased at 3 and 7 days after induction (Fig. 1C). These results suggest that *SHANK2* may play an important role in osteo/dentinogenic differentiation in dental tissue-derived MSCs.

Depletion of SHANK2 inhibited the in vitro osteo/dentinogenic differentiation potentials in SCAPs

In order to understand the function of *SHANK2* in dental tissue-derived MSCs, we designed a short hairpin RNA (shRNA) that can target *SHANK2* and introduced it into SCAPs by lentiviral infection. After 2 μg /ml puromycin selection, the knockdown efficiency (80%) was verified by real-time RT-PCR and Western Blot (Fig. 2A,B). Then, we examined if *SHANK2* affected the osteo/dentinogenic differentiation potentials in SCAPs. SCAPs were cultured with osteogenic-inducing medium. Four days after induction, alkaline phosphatase (ALP) staining and ALP activity assay showed that the ALP activity was repressed in SCAPs-*SHANK2sh* compared with Scramsh (Fig. 2C). Two weeks after induction, Alizarin Red staining and quantitative calcium also revealed that mineralization was significantly decreased in SCAPs-*SHANK2sh* compared to SCAPs-Scramsh (Fig. 2D,E).

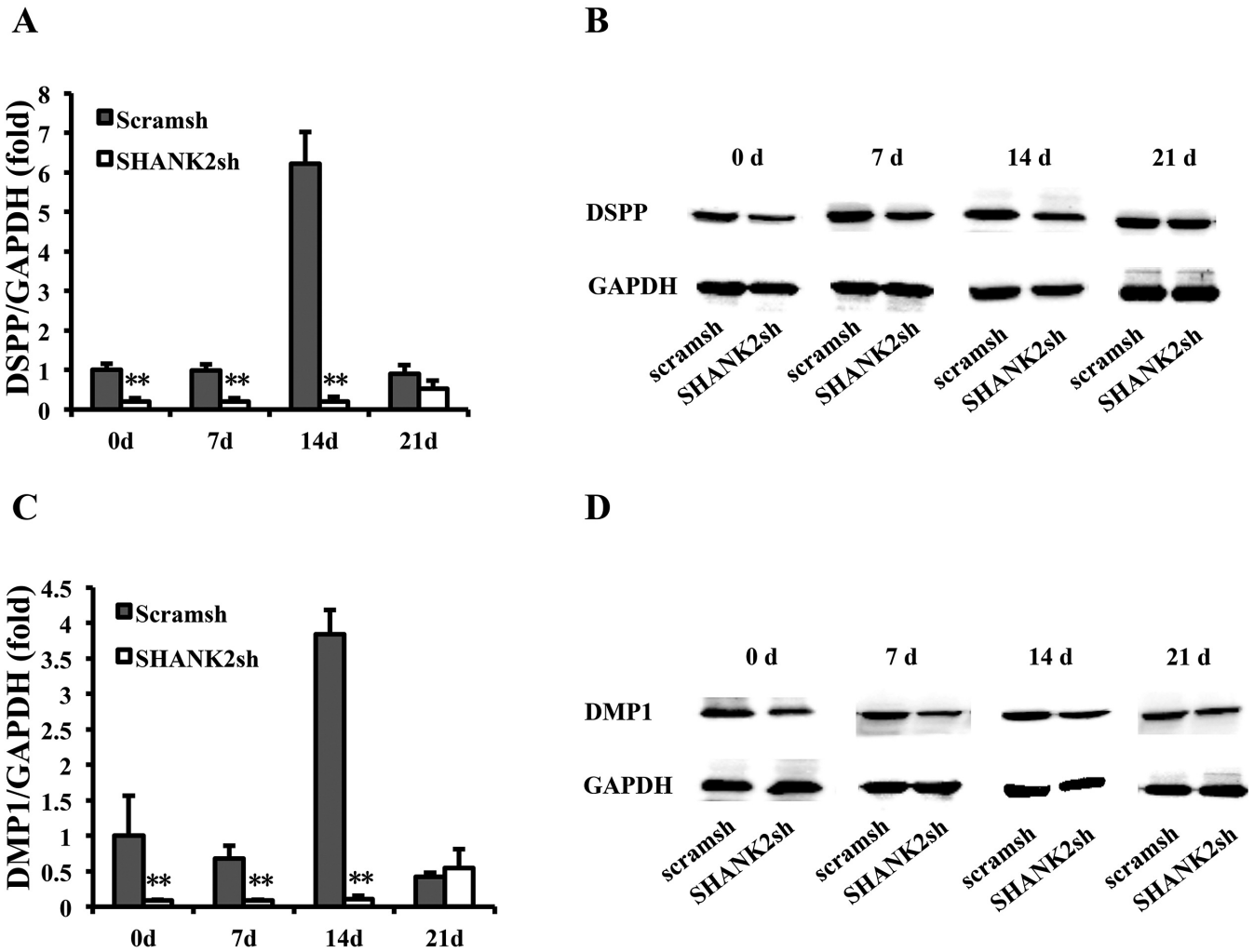

Fig 3. The knock-down of SHANK2sh decreased the expressions of DSPP and DMP1. **A-D**. The knock-down of SHANK2sh inhibited the expressions of DSPP **(A, B)** and DMP1 **(C, D)** in SCAPs after osteogenic induction. SCAPs were induced to differentiate for the indicated time periods. After induction, the RNA and protein was isolated and the expressions of DSPP and DMP1 were examined by real-time RT-PCR and Western Blot. GAPDH was used as an internal control. Student's t test was performed to determine statistical significance. All error bars represent s.d. (n=3). **P≤0.01.

Next, real-time RT–PCR and Western blot results showed that the dentinogenic marker, *DSPP* (dentin sialophosphoprotein) which encodes principal proteins of the dentin extracellular matrix of the tooth, which is critical for proper mineralization of bone and dentin, was strongly inhibited in SCAPs-*SHANK2sh*, compared with control group at 0, 7 and 14 days after osteogenic induction (Fig. 3A,B). Another dentinogenic marker, DMP1 (dentin matrix acidic phosphoprotein 1) was strongly inhibited in SCAPs-*SHANK2sh* in mRNA level at 0, 7 and 14 days and at 0, 7, 14 and 21 days in protein level after osteogenic induction, compared with control group (Fig. 3C,D). Other osteo/dentinogenic markers, such as BSP (bone sialoprotein), *OPN* (osteopontin) and *OCN* (osteocalcin) showed no significant change (data not shown).

Furthermore, we examined the key transcription factors regulating osteo/dentinogenic differentiation, including *RUNX2* (runt related transcription factor 2) and *OSX* (osterix). We found that the mRNA and protein levels of *RUNX2* and *OSX* were significantly decreased after silence of *SHANK2* (Fig. 4).

The knock-down of SHANK2 diminished the in vivo osteo/dentinogenesis capacity

In order to validate whether *SHANK2* can affect the osteo/dentinogenic differentiation potentials of SCAPs *in vivo*, we transplanted SCAPs-*SHANK2sh* and SCAPs-*Scramsh* mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) into nude mice. The H&E staining results also demonstrated that SCAPs-*SHANK2sh* generated less bone/dentin-like mineralized tissue when compared with SCAPs-Scramsh (Fig. 5A), and qualitative measurements of the mineralization showed much less mineral in tissues transplanted with SCAPs-*SHANK2sh* than in those transplanted with SCAP-Scramsh cells (Fig. 5B). These transplantation experiments demonstrated that SCAPs-*SHANK2sh* generated less bone/dentin-like mineralized tissues than SCAP-Scramsh cells *in vivo*. Taken together, these results showed that knock-down of *SHANK2* considerably inhibited osteo/dentinogenic differentiation in SCAPs.

Discussion

In the present study, we found that *SHANK2* is more highly expressed in dental tissue-derived MSCs than other origin MSCs. Subsequently, *SHANK2* expression was increased at 3 and 7 days after osteogenic induction. These results suggest that SHANK2 may play an important role in osteo/dentinogenic differentiation in dental tissue-derived MSCs.

The *SHANK* family of proteins was initially identified as molecular scaffolds in neuronal cells, in which they coordinate membrane and cytoplasmic protein complexes in the postsynaptic density (Sheng and Kim, 2000). Among the three members of *SHANK* proteins in human (*SHANK1-3*), *SHANK2* is a large scaffolding protein originally identified as a key component of the neuronal postsynaptic density (Naisbitt et al., 1999). *SHANK2* binds proteins involved in modulating actin dynamics and endocytosis regulation including dynamin2 and cortactin (Du et al., 1998; Okamoto et al., 2001).

We have demonstrated that knock-down of *SHANK2* by shRNA significantly impaired the ALP activity and mineralization ability of SCAPs *in vitro*, as well as decreasing the levels of osteoblast/odontoblast-related marker mRNAs, including *DSPP, DMP1*, and the key osteo/dentinogenic transcription factors, *RUNX2* and *OSX*. In addition, the *in vivo* transplantation results further confirmed that SCAPs-*SHANK2sh* showed

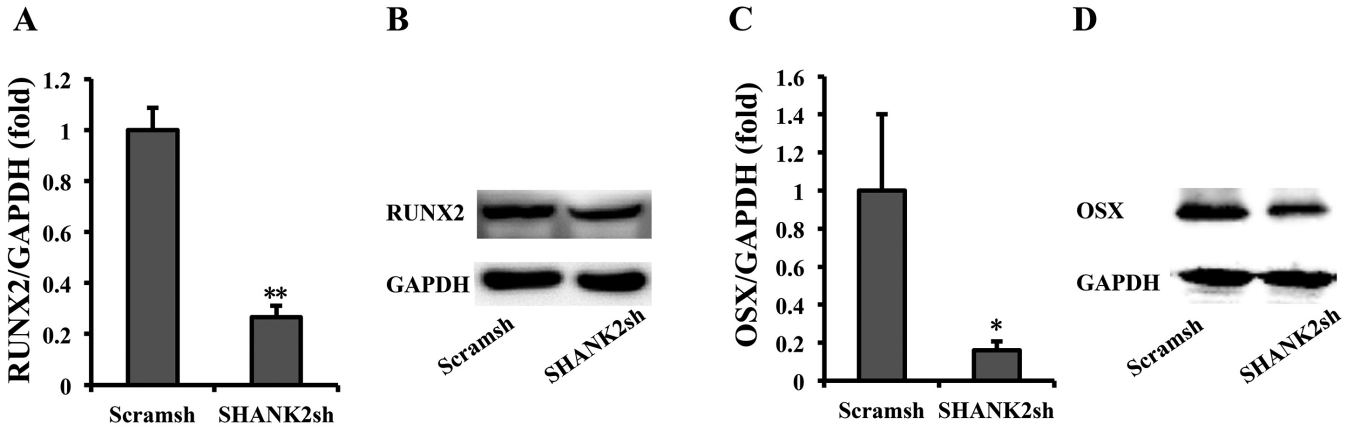

Fig 4. The expression of RUNX2 and OSX was significantly inhibited in SCAPs-SHANK2sh. **A-D.** Real-time RT-PCR and Western Blot results showed that the expression of RUNX2 **(A, B)** and OSX **(C, D)** was much lower in SCAP-Shank2sh cells compared with SCAP-Scramsh. GAPDH was used as an internal control. Student's t test was performed to determine statistical significance. All error bars represent s.d. (n=3). *P≤0.05. **P≤0.01.

dramatically decreased capability to form bone/dentin-like structure, compared with the SCAP-*Scramsh*.

DSPP and *DMP1* are critical for odontoblast differentiation and dentinogenesis (Lu et al., 2005; Chen et al., 2009). The present study revealed that disruption of endogenous *SHANK2* down-regulated the expression of *DSPP* and *DMP1* mRNA rather than osteoblast-related genes like *BSP, OPN* and *OCN*, suggesting that *SHANK2* may be involved in inducing SCAPs to differentiate, mainly along the odontoblast lineages instead of osteoblast lineages. There is evidence that tissue-specific adult stem cells exhibit significant differences in terms of gene expression, proliferation ability, differentiation potential *in vitro*, and, more importantly, their capacity to develop into distinct structures representative of the tissues from which they were derived *in vivo*, most probably due to the influence of local signals and micro-environments (Huang et al., 2009; Dangaria et al., 2011).

RUNX2 is known as a transcription factor essential for osteoblast and odontoblast differentiation and induces the commitment of mesenchymal stem cells to osteoblast. *RUNX2* null mice showed skeletal defects and impaired tooth development, progressing only to the cap/early bell stage (Otto et al., 1997; D'Souza et al., 1999). *OSX* is another osteoblast-specific transcription factor which is expressed in tooth germ mesenchymal cells and is involved in tooth root development (Nakashima et al., 2002; Cao et al., 2012). *RUNX2* and *OSX* exert their functions by regulating many bone- and tooth-related gene expressions. It is known that *RUNX2* regulates *DSPP* transcriptional expression in both a positive and negative manner, depending on stages of odontoblast differentiation. Indeed, *RUNX2* activates *DSPP* expression in preodontoblast cells and inhibits this gene during the maturation stages of cell differentiation (Chen et al., 2005). Distinct effects of *RUNX2* on *DMP1* expression have also been reported (Feng et al., 2002). In *RUNX2*-deficient mice, *DMP1* expression vanished in developing bones, whereas *DMP1* expression in developing teeth was unaffected. In mouse odontoblast-like cells, *OSX* overexpression could enhance *DSPP* transcription (Chen et al., 2009). Our data revealed that the expressions of *RUNX2, OSX, DSPP* and *DMP1* were obviously down-regulated in SCAP-*SHANK2sh* compared with the control cells.

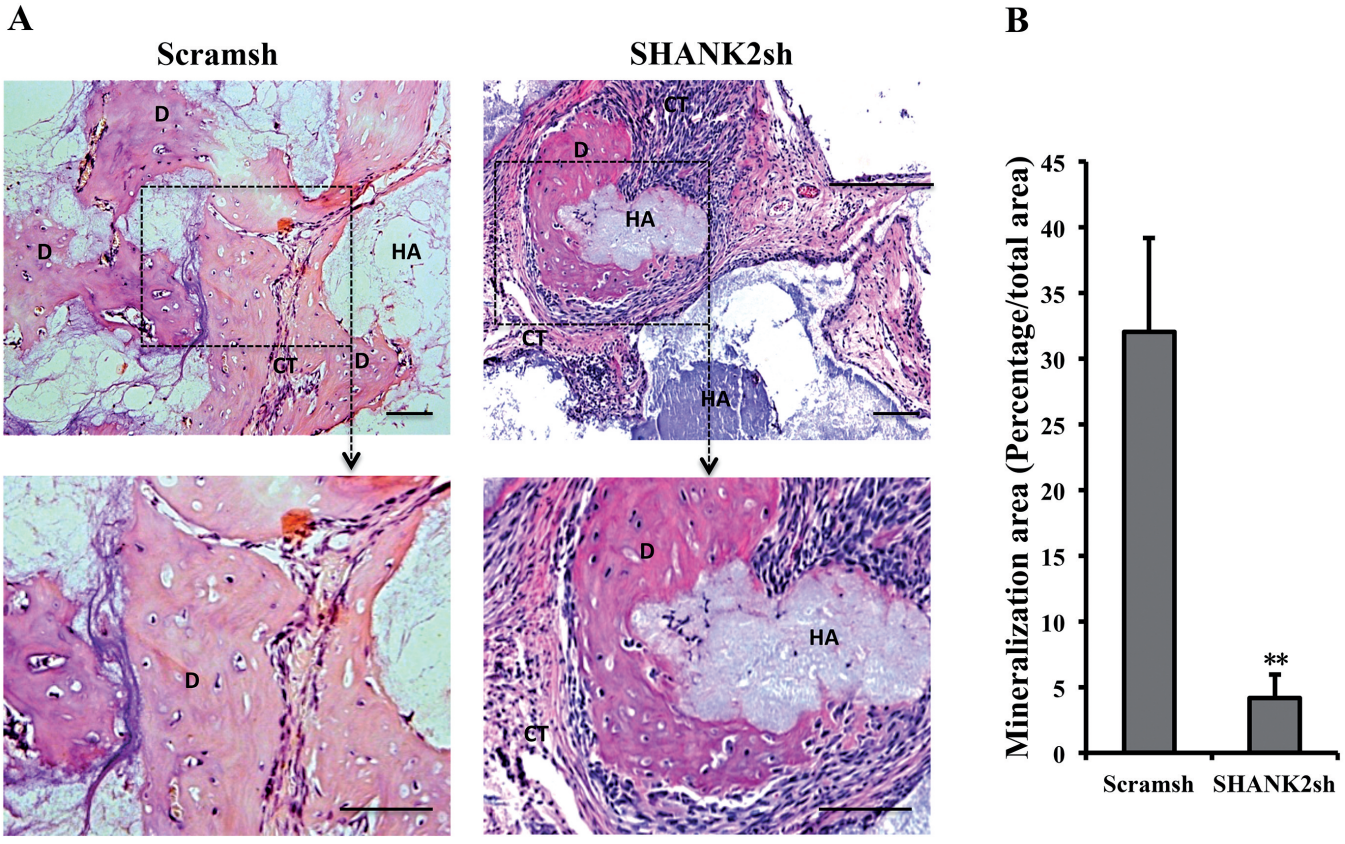

Fig 5. Depletion of SHANK2 diminished the ability of mineralized tissue formation *in vivo* in SCAPs. **A**. SCAP-Scramsh and SCAP-SHANK2sh mixed with HA/TCP were transplanted in Nude mice over a period of 8 weeks. The transplants were examined by H&E staining. D, bone/dentin-like tissues; HA, hydroxyapatite tricalcium carrier; CT, connective tissues. **B**. The mineralization area was calculated according the results of H&E staining. Student's t test was performed to determine statistical significance. All error bars represent s.d. (n=5). **P≤0.01. Bar: 100 μm.

Whether *DSPP* and *DMP1* transcription repression is in response to *RUNX2* and *OSX* needs further investigation.

In conclusion, our present findings showed the potential of *SHANK2* to determine the commitment of SCAPs, explored the new function of *SHANK2*, and provideduseful information to elucidate the molecular mechanism underlying directed differentiation in dental tissue-derived MSCs. Our findings indicate that *SHANK2* may be used as a potential candidate for tooth engineering applications.

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Disclosure of potential conflicts of interest. The authors declare no potential conflicts of interest.

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