Int J Clin Exp Med 2015;8(7):10459-10470
www.ijcem.com /ISSN:1940-5901/IJCEM0009931

Original Article

Homeobox B7 promotes the osteogenic differentiation potential of mesenchymal stem cells by activating *RUNX2* and transcript of *BSP*

Run-Tao Gao[1], Li-Ping Zhan[1], Cen Meng[1], Ning Zhang[1], Shi-Min Chang[1], Rui Yao[3], Chong Li[2]

[1]*Department of Stomatology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China;* [2]*Institute of Biophysics, CAS Key Laboratory of Infection and Immunity, Chinese Academy of Sciences (CAS), Beijing 100101, China;* [3]*Department of Pediatrics, Stomatological Hospita lof Nankai University, Tianjin 300041, China*

Received May 5, 2015; Accepted July 11, 2015; Epub July 15, 2015; Published July 30, 2015

Abstract: Mesenchymal stem cells (MSCs) are a reliable cell source for tissue regeneration. However, the molecular mechanisms underlying the directed differentiation of MSCs remain unclear; thus, their use is limited. Here, we investigate *HOXB7* function in the osteogenic differentiation potentials of MSCs using stem cells from apical papilla (SCAPs) and bone marrow stem cells (BMSCs). The *HOXB7* gene is highly expressed in BMSCs compared with dental tissue-derived MSCs. We found that, *in vitro*, over-expression of *HOXB7* in SCAPs enhanced alkaline phosphatase (ALP) activity and mineralization. *HOXB7* over-expression affected the mRNA expression of osteonectin (*ON*), collagen alpha-2(I) chain (*COL1A2*), bone sialoprotein (*BSP*), and osteocalcin (*OCN*), led to the expression of the key transcription factor, runt-related transcription factor 2 (*RUNX2*), and promoted SCAP osteogenic differentiation *in vitro*. The knock-down of *HOXB7* inhibited ALP activity, mineralization, and the expression of *ON*, *BSP*, *COL1A2*, *OCN*, and *RUNX2* in BMSCs *in vitro*. In addition, transplant experiments in nude mice confirmed that SCAP osteogenesis was triggered when HOXB7 was activated. Furthermore, Over-expression of *HOXB7* significantly increased the levels of *HOXB7* associated with the *BSP* promoter by ChIP assays. Taken together, these results indicate that HOXB7 enhances SCAP osteogenic differentiation by up-regulating *RUNX2* and directly activating transcript of *BSP*. Thus, the activation of HOXB7 signaling might improve tissue regeneration mediated by MSCs. These results provide insight into the mechanism underlying the directed differentiation of MSCs.

Keywords: Homeobox B7 (*HOXB7*), osteogenic, differentiation, mesenchymal stem cells (MSCs), bone sialoprotein (*BSP*)

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that were originally isolated from bone marrow. They can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes. Increasing evidence indicates that MSCs are also present in non-bone marrow tissues [1-3]. Recently, a new population of MSCs was isolated from dental and craniofacial tissues on the basis of their stem cell properties. This population included stem cells from the periodontal ligament (PDLSCs), from dental pulp (DPSCs), from apical papilla (SCAPs), and from human exfoliated deciduous teeth (SHEDs) [4-8]. These isolated cells were multipotent, exhibited osteo/dentinogenic differentiation, and could self-renew. When transplanted into mice or miniature pigs, they generated bone/dentin-like mineralized tissue, and they were capable of repairing tooth defects [8-10]. MSCs are a reliable resource for tissue regeneration, but the molecular mechanisms underlying their directed differentiation remain unclear; thus, their use is limited.

Homeobox genes (*HOX)* are characterized by a conserved 180 bp DNA sequence coding for a 60aa DNA-binding homeodomain, and the master regulators in determining patterning, specification, and differentiation during embryonic development [11-13]. Homeodomain proteins are transcription factors that can activate or inhibit transcription of downstream genes

through the DNA binding. Recent studies show that homeobox genes play crucially regulatory roles in the process of maxillofacial and dental development. In *Pbx1*-deficient mice, absence of pre-B-cell leukemia homeobox proteins 1 (*Pbx1*) caused precocious endochondral ossification and abnormal bone formation by perturbing chondrocyte proliferation and differentiation [14]. HOXA10 could mediate chromatin hyperacetylation and trimethyl histone K4 (H3K4) methylation, induce expression of osteogenic genes through activation of *RUNX2*, or directly regulate other osteoblastic phenotypic genes, and contribute to the onset of osteogenesis and subsequent bone formation [15]. More importantly, recent studies have showed that homeobox gene, such as *DLX2, DLX5* and *HOXC6* as key regulators in the osteogenic differentiation of MSCs [16-18], indicating that homeobox genes play essential roles in the differentiation of stem cells. Among these homeobox genes, HOXB7 was attracted, since its downregulation in aged MSC was preliminarily observed [19]. HOXB7 regulates several genes playing a significant role in cell proliferation and differentiation [20-23]. Some researchers found that *HOXB7* s highly expressed in non-dental tissue-derived MSCs [24], a forced HOXB7 expression was associated with an improved cell growth, a reduction of senescence and an improved osteogenesis [25]. However, at present, functional role of HOXB7 in differentiation of human MSCs, especially, the dental tissue derived MSCs, haven't been reported.

Based on those previous findings, we hypothesized that HOXB7 might have an effect on human MSCs. In the present study, we studied human SCAPs and bone marrow stem cells (BMSCs) to investigate the effects of HOXB7 and the mechanisms underlying these effects. Our results indicated that HOXB7 could enhance the osteogenic differentiation potential in SCAPs and BMSCs by up-regulating the expression of runt-related transcription factor 2 (*RUNX2*) and directly activating transcript of bone sialoprotein (*BSP*). This finding may have practical implications in terms of enhancing the directed differentiation of MSCs for tissue regeneration applications.

Materials and methods

Cell cultures

Tooth tissues were obtained according to approved guidelines set by Beijing Friendship Hospital, Capital Medical University. Informed patient consent was obtained.

Wisdom teeth were disinfected with 75% ethanol and washed with phosphate buffered saline (PBS). SCAPs were gently separated from the apical papilla of the root, PDLSCs were separated from the periodontal ligament in the middle one-third of the root, while DPSCs were separated from crown pulp. Subsequently, the MSCs were digested in a solution of 3 mg/mL collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) and 4 mg/mL dispase (Roche Diagnostics Corp., Indianapolis, IN, USA) for 1 h at 37°C. The digested tissues were passed through a 70-μm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA) to obtain isolated cell suspensions. Human BMSCs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). MSCs were grown in a humidified incubator under 5% CO_2 at 37°C in DMEM alpha modified Eagle's medium (Invitrogen, Carlsbad, CA, 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. Cells from passages 2-4 were used in further experiments.

Human embryonic kidney 293T (HEK 293T) (American Type Culture Collection, Manassas, VA, USA) cells were maintained in complete DMEM medium with 10% FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). These cells were utilized for packaging viral constructs.

Plasmid construction and viral infection

The plasmids were constructed according to standard methods, and all constructs were verified with the appropriate restriction digestion analysis and/or sequencing. Human full-length *HOXB7* cDNA was obtained by isolating total RNA from cultured BMSCs, then performing reverse-transcription and PCR amplification (RT-PCR). Next, the *HOXB7* cDNA sequence was fused to a Flag-tag sequence (Flag-*HOXB7*), with AgeI and EcoR1 restriction sites, and subcloned into the T-easy vector (Promega, Madison, WI, USA). The final sequence was confirmed with DNA sequencing. Next, the Flag-*HOXB7* was subcloned into the pQCXIN retroviral vector (BD Clontech, Mountain View, CA, USA) with the AgeI and EcoR1 restriction sites,

and that sequence was also confirmed by DNA sequencing.

Short hairpin RNAs (shRNA) with sequences complementary to those of the target genes were subcloned into the pLKO.1 lentiviral vector (Addgene, Cambridge, MA, USA). Viral packaging was performed, according to the manufacturer's protocol, with HEK 293T cells (BD Clontech). For viral infections, SCAPs were plated overnight, and then, infected with retroviruses or lentiviruses in the presence of polybrene (6 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 6 h. After 48 h, infected cells were selected with the appropriate antibiotics. BMSCs were infected with lentiviruses expressing *SMAD4* shRNA (*SMAD4sh*), expressed *HOXB7* shRNA (*HOXB7sh*) or *Scramsh*, and selected with 2 μg/mL puromycin for 7 days. SCAPs were infected with a retrovirus that carried the wild type *HOXB7* fused to a Flag-tag or an empty vector, and selected with 600 μg/mL G418 for 10 days. The target sequences for the shRNAs were as follows: *HOXB7*shRNA (*HOXB7sh*), 5'-gaggaagagacatgagaaa-3'. A scrambled shRNA (*Scramsh*) was used as a control (Addgene). The PCR primers used for cloning the *HOXB7* gene were as follows: *HOXB7* Forward, 5'-tcgaaccggtatggattacaaggacgacgatgacaagatgagttcattgtattatgc-3'; and *HOXB7* Reverse, 5'-acgtgaattctcactcttcctcttcctcct-3'.

Western blot analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCL, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% NP-40, 1:100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, 50 mM sodium fluoride). The samples were separated on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes with a semi-dry transfer apparatus (BioRad, Hercules, CA, USA). The membranes were blotted with 5% dehydrated milk for 2 h and then incubated with primary antibodies overnight. Next, membranes were incubated with anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (Promega), and the immune complexes were visualized with SuperSignal reagents (Pierce, Rockford, IL, USA). The primary antibodies were: monoclonal anti-Flag M2 (Clone No.9A3, Cat No.8146, Cell Signaling Technology, Beverly, MA, USA), and a monoclonal antibody specific for the housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Clone No.GAPDH-71.1, Cat No.G8795, Sigma-Aldrich).

Alkaline phosphatase and Alizarin Red staining

MSCs were grown in osteogenic-inducing medium that contained 100 μM/mL ascorbic acid, 2 mM β-glycerophosphate, 1.8 mM KH_2PO_4, and 10 nM dexamethasone. After induction, for ALP staining, the cells were fixed with 4% paraformaldehyde. Cells were stained with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Red (in an ALP kit), according to the manufacturer's protocol (Sigma-Aldrich). ALP activity was assayed with an ALP activity kit, according to the manufacturer's protocol (Sigma-Aldrich). Signals were normalized based on protein concentration. To detect mineralization, cells were induced for 3 weeks, fixed with 70% ethanol, and stained with 2% Alizarin Red (Sigma-Aldrich). To quantitatively determine calcium content, cells stained with Alizarin Red were destained with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 30 min at room temperature. The calcium concentration was determined by measuring absorbance at 562 nm on a multiplate reader and comparing the reading to a standard calcium curve, constructed with calcium diluted in the same solution. The final calcium level in each group was normalized to the total protein concentration detected in a duplicate plate [26].

Real time, reverse transcriptase-PCR

Total RNA was isolated from MSCs with Trizol reagent (Invitrogen). We synthesized cDNA from 2-μg aliquots of RNA with random hexamers or oligo (dT) and reverse transcriptase, according to the manufacturer's protocol (Invitrogen). Real-time PCR reactions were performed with the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) and an Icycler iQ Multi-color Real-time PCR Detection System. The expression of genes changes was calculated by the method of $2^{-\Delta\Delta CT}$. The primers used for specific genes are shown in Table S1.

CHIP assays

We used a ChIP assay kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol. Briefly, cells were incubated with 1% formaldehyde for 10 min at 37°C. Each

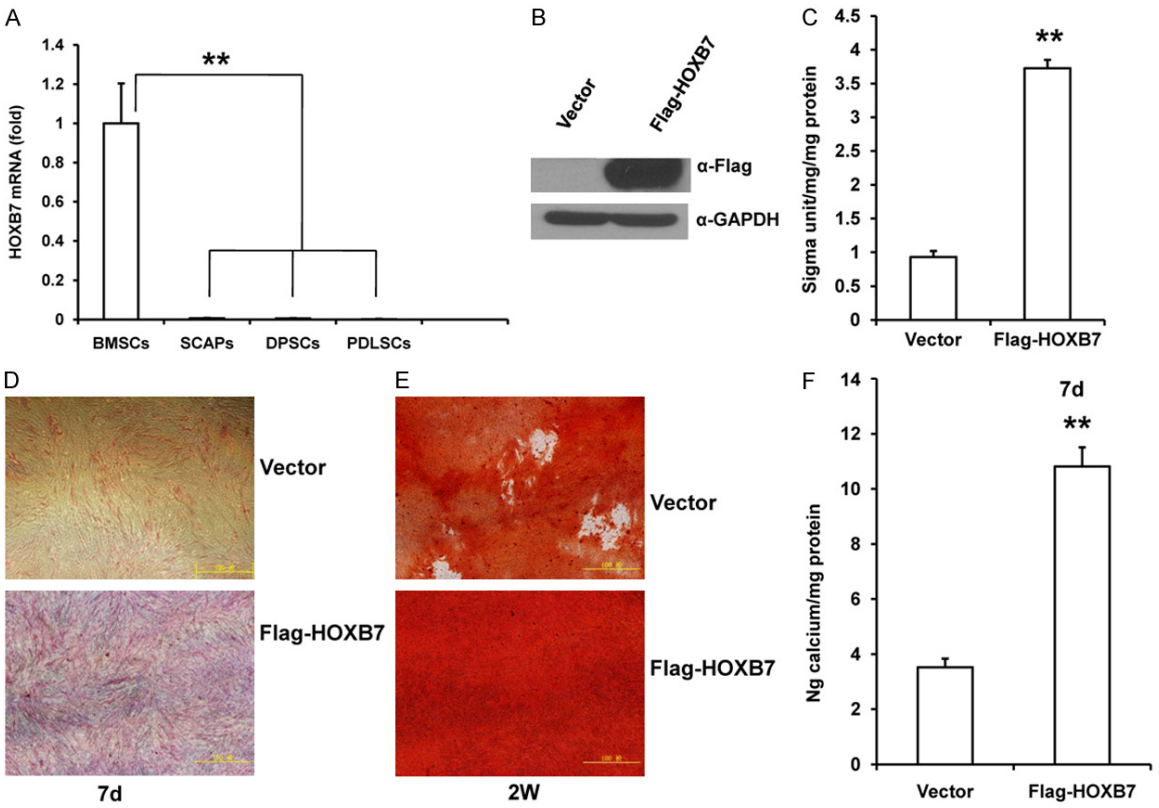

Figure 1. Wild type *HOXB7* over-expression enhances osteogenic differentiation of SCAPs. (A) *HOXB7* expression in BMSCs, SCAPs, DPSCs, and PDLSCs according to the results of real-time RT-PCR. *GAPDH* was used as an internal control. The one-way ANOVA was performed to determine statistical significance. (B) Western blot analysis showed that HOXB7 over-expression in SCAPs. GAPDH was used as an internal control. (C, D) ALP activity assay (C) and ALP (D) staining results show that *HOXB7* over-expression increased ALP activity in SCAPs. Bar: 100 μm. (E, F) Alizarin Red staining (E) and quantitative calcium analysis results (F) show that *HOXB7* over-expression enhanced mineralization in SCAPs. Bar: 100 μm. The student's t test was performed to determine statistical significance. All error bars represent s.d. (n = 3). $^{**}P \leq 0.01$.

ChIP reaction was performed using 2.0 × 10^6 cells. For DNA precipitation, we added 2 μg monoclonal anti-Flag M2 (Clone No.9A3, Cat No.8146, Cell Signaling Technology, Beverly, MA, USA). The precipitated DNA samples were quantified by real-time PCR with primers targeting the *HOXB7*-binding region of the *BSP* promoter (*HOXB7* binding site): forward, 5'-gcaagctttcctttctttcg-3' and reverse, 5'-gaaaaccccaaacttcaac-3'; and primers targeting the open reading frame (ORF) region of the *BSP* (Negative control): forward, 5'-gaaagtgtcactggagccaa-3' and reverse, 5'-tggagaggttgttgtcttcg-3'. Quantification data are expressed as the percentage of input DNA.

Transplantation into nude mice

The present study was approved by an animal care and use committee at the Beijing Friendship Hospital, Capital Medical University. Our care and use of animals followed the guidelines of the Experimental Animal Management Ordinance. We used 10-week old, immunocompromised beige mice (*nu/nu*) female nude mice. All animals were purchased from the Institute of Animal Science of the Vital River Co., Ltd. The animals had not received any drugs or previous procedures.

Approximately 4.0 × 10^6 SCAPs were mixed with 40 mg of ceramic particles made of hydroxyapatite/tricalcium phosphate (HA/TCP; Engineering Research Center for Biomaterials, Sichuan University, Chengdu, China). The mixture was transplanted subcutaneously into the dorsal surface of immunocompromised beige mice. All procedures were performed in accordance with the specifications of an approved animal protocol. Eight weeks after transplantation, the transplanted cells were harvested, fixed in 10% formalin, decalcified with buffered

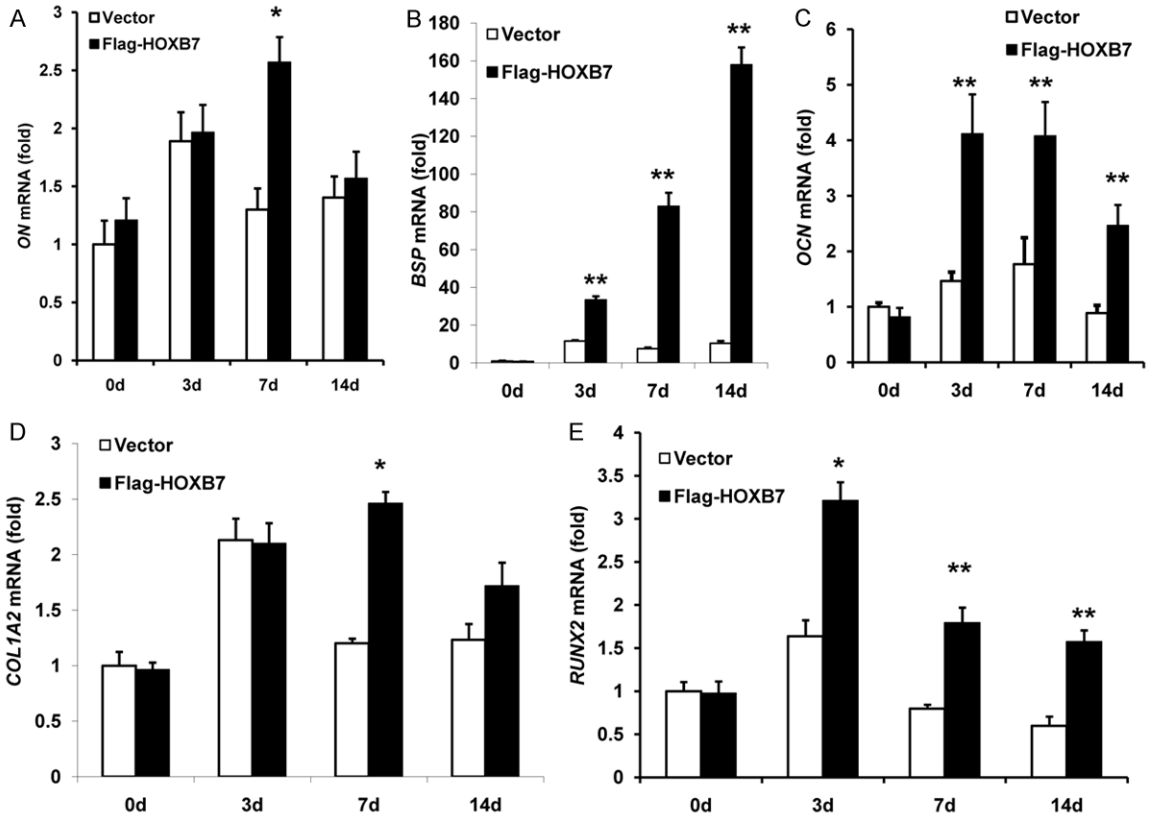

Figure 2. Wild type *HOXB7* over-expression enhances the mRNA levels of osteogenic differentiation markers in SCAPs. Real-time RT-PCR results showed that *HOXB7* over-expression up-regulated the expression of *ON* (A), *BSP* (B), *OCN* (C), *COL1* (D) and *RUNX2* (E) in SCAPs. *GAPDH* was used as an internal control. The student's t test was performed to determine statistical significance. All error bars represent s.d. (n = 3). *P ≤ 0.05. **P ≤ 0.01.

10% EDTA (pH 8.0), embedded in paraffin, and sliced in 5-μm sections. For analysis, the sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E) [26-28]. Qualitative measurements of tissue mineralization were performed with the Image-Pro Plus 6.0 program (Media Cybernetics, Rockville, MD, USA).

Statistics

All statistical calculations were performed with SPSS10 statistical software. The student's t-test or one-way ANOVA were utilized to determine statistically significant differences between groups. A *P*-value ≤ .05 was considered significant.

Results

HOXB7 over-expression enhanced osteogenic differentiation potential in SCAPs in vitro

We used real-time RT-PCR to test the *HOXB7* mRNA levels in BMSCs and dental tissue-derived MSCs. We found higher *HOXB7* expression in BMSCs than in SCAPs, DPSCs, and PDLSCs (**Figure 1A**). Then we investigated the function of *HOXB7* in SCAPs with *HOXB7* over-expression. First, we inserted the *Flag-HOXB7* sequence into a retroviral vector. This construct induced overexpression of ectopic *HOXB7* when transduced into SCAPs via retroviral infection. Ectopic *HOXB7* expression was confirmed by Western blot analysis (**Figure 1B**). Next, SCAPs transduced with the Flag-*HOXB7* or with the empty vector alone (controls) were cultured in osteogenic-inducing medium to determine the osteogenic differentiation potential. The results indicated that over-expression of *HOXB7* strongly increased ALP activity, one of the earliest markers of osteogenic differentiation in SCAPs (**Figure 1C**, **1D**). Accordingly, Alizarin Red staining and quantitative calcium measurements showed that mineralization was markedly enhanced in SCAPs infected with Flag-*HOXB7* compared to control-infected cells (**Figure 1E**, **1F**). Consistent with this finding, real-time RT-PCR results showed that the

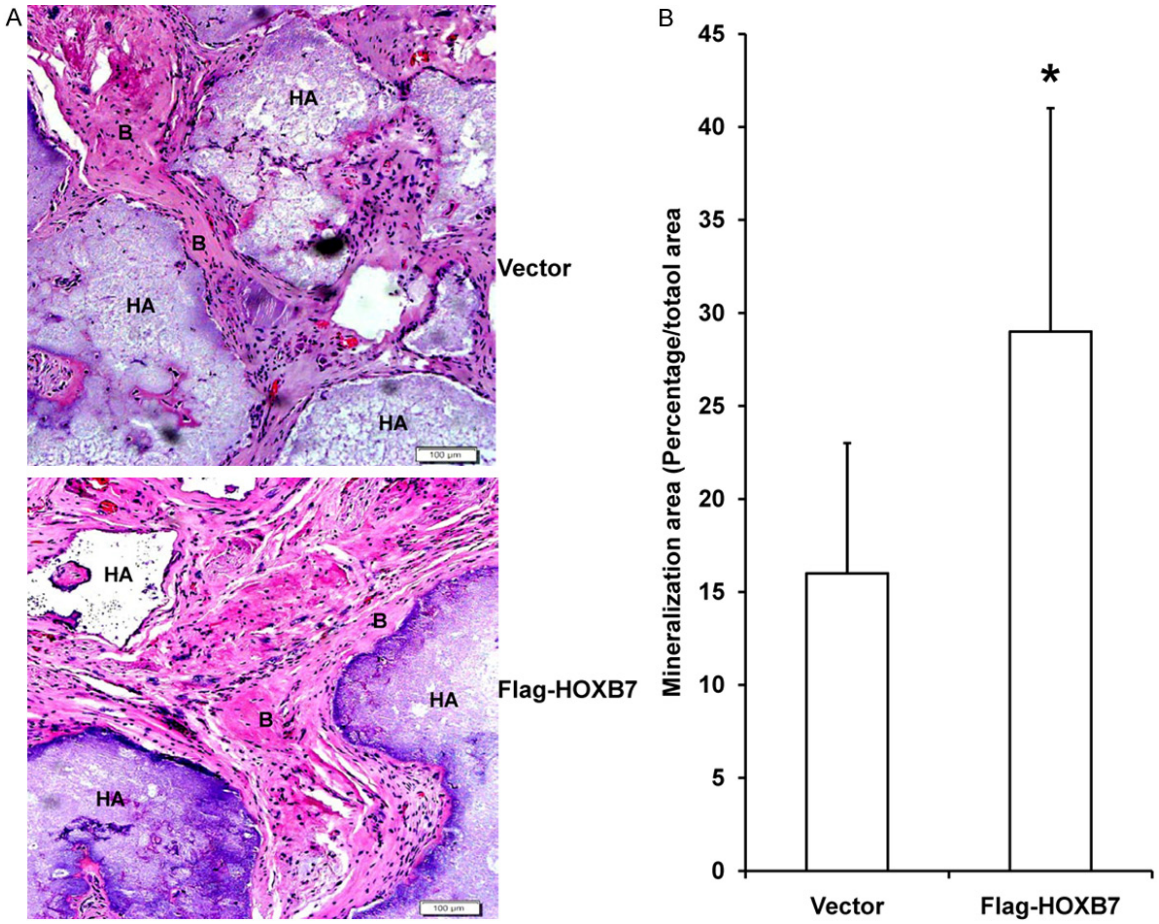

Figure 3. *HOXB7* over-expression enhances mineralized tissue formation *in vivo*. SCAPs infected with either the empty vector (control) or with Flag-HOXB7 (HOXB7 overexpression) were transplanted subcutaneously into the dorsal surfaces of 10-week-old immunocompromised beige mice. (A) Tissue sections were prepared 8 weeks after transplantation. Sections were stained with (A) H&E. (B) Qualitative measurement of mineralization in tissue samples after transplantation with SCAPs that overexpressed HOXB7 or control SCAPs. The mineralization area in the tissue sections was calculated based on the results of H&E staining. The student's t test was performed to determine statistical significance. All error bars represent s.d. (n = 5). $^{**}P \leq 0.01$. Bar: 100 μm. B, Bone-like tissues; HA, hydroxyapatite tricalcium carrier; CT, connective tissue.

osteogenic marker genes, which encodes extracellular matrix proteins in bone and dentin tissues were changed. The results showed that the overexpression of *Flag-HOXB7* in SCAPs was associated with elevated expression of osteonectin (*ON*) and collagen alpha-2(I) chain (*COL1A2*) at 7 days (**Figure 2A**, **2D**), and bone sialoprotein (*BSP*) and osteocalcin (*OCN*) at 3, 7, and 14 days after induction (**Figure 2B**, **2C**).

Next, we examined the expression of key transcription factors that regulate osteogenic differentiation, including runt-related transcription factor 2 (*RUNX2*) and osterix (*OSX*). We found that the mRNA level of *RUNX2* was significantly increased at 3, 7, and 14 days after induction in *Flag-HOXB7* overexpressed SCAPs compared to SCAP-Vector cells (**Figure 2E**). However, the expression levels of *OSX* were not significantly different between these cell groups (data not shown).

HOXB7 over-expression enhanced osteogenic differentiation potential in SCAPs in vivo

Next, we investigated whether the overexpression of *HOXB7* would affect SCAP osteogenesis *in vivo*. We transplanted control or Flag-HOXB7 SCAPs subcutaneously into nude mice. Eight weeks after transplantation, the transplanted tissues were harvested. The H&E staining revealed that more bone-like mineralized tissue was present in tissues transplanted with

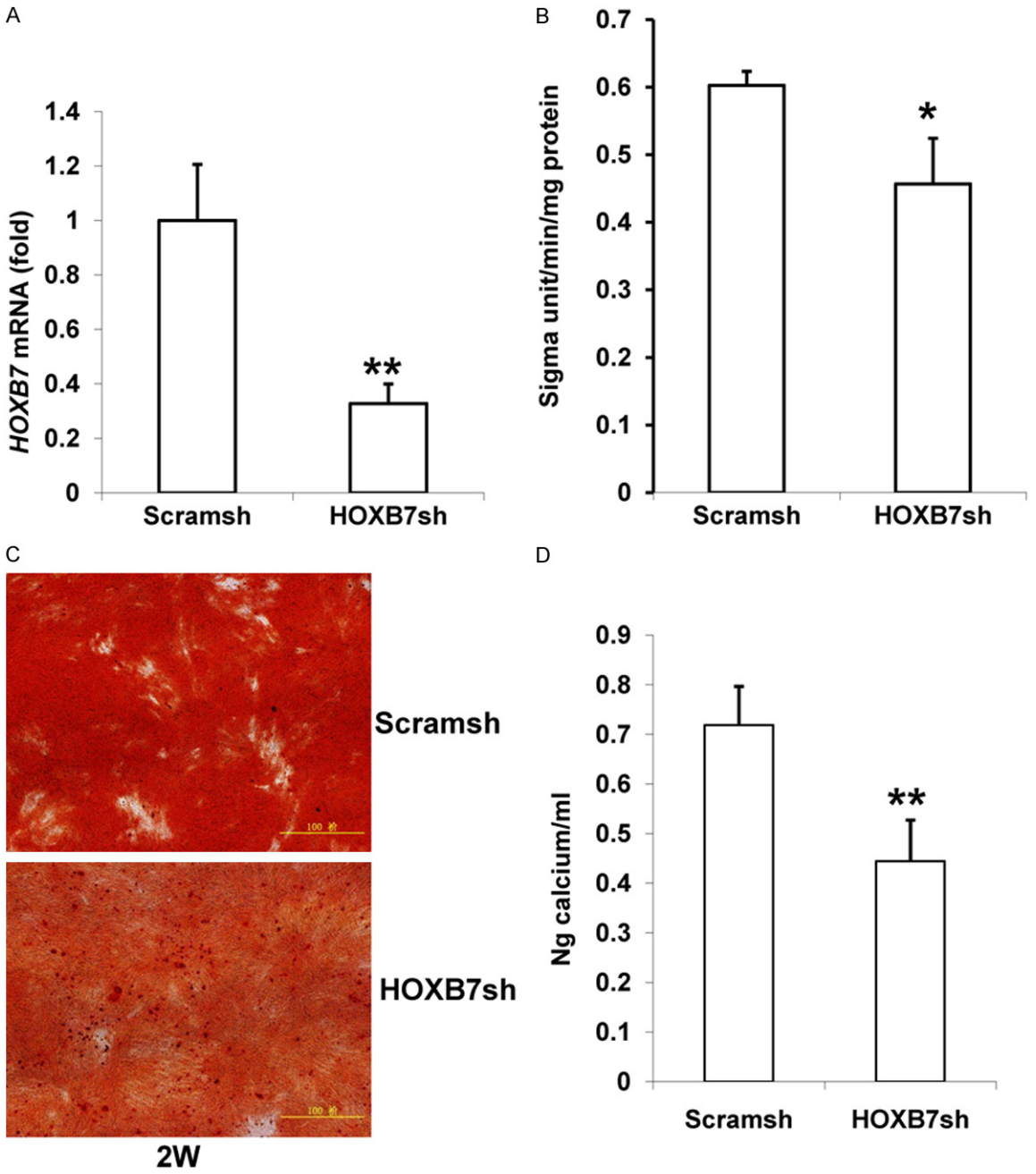

Figure 4. *HOXB7* knock-down inhibits the osteogenic potential of BMSCs. (A) The real-time RT-PCR result showed that HOXB7 knock-down in BMSCs. *GAPDH* was used as an internal control. (B) ALP activity assay result showed that, when *HOXB7* was knocked-down in BMSCs, the ALP activity was reduced. (C, D) Alizarin Red staining (C) and quantitative calcium analysis (D) showed that, when *HOXB7* was knocked-down in BMSCs, mineralization was reduced. Bar: 100 μm. The student's t test was performed to determine statistical significance. All error bars represent s.d. (n = 3). *P ≤ 0.05. **P ≤ 0.01.

Flag-HOXB7 SCAPs compared to tissues transplanted with control SCAPs (**Figure 3A**). Qualitative measurements of the mineralization showed much more mineral in tissues transplanted with Flag-HOXB7 than those transplanted with control SCAPs (**Figure 3B**). These transplantation experiments demonstrated that Flag-HOXB7 SCAPs generated more bone-like mineralized tissue than control SCAPs *in vivo*. Taken together, these results showed that *HOXB7* expression substantially triggered osteogenic differentiation in SCAPs.

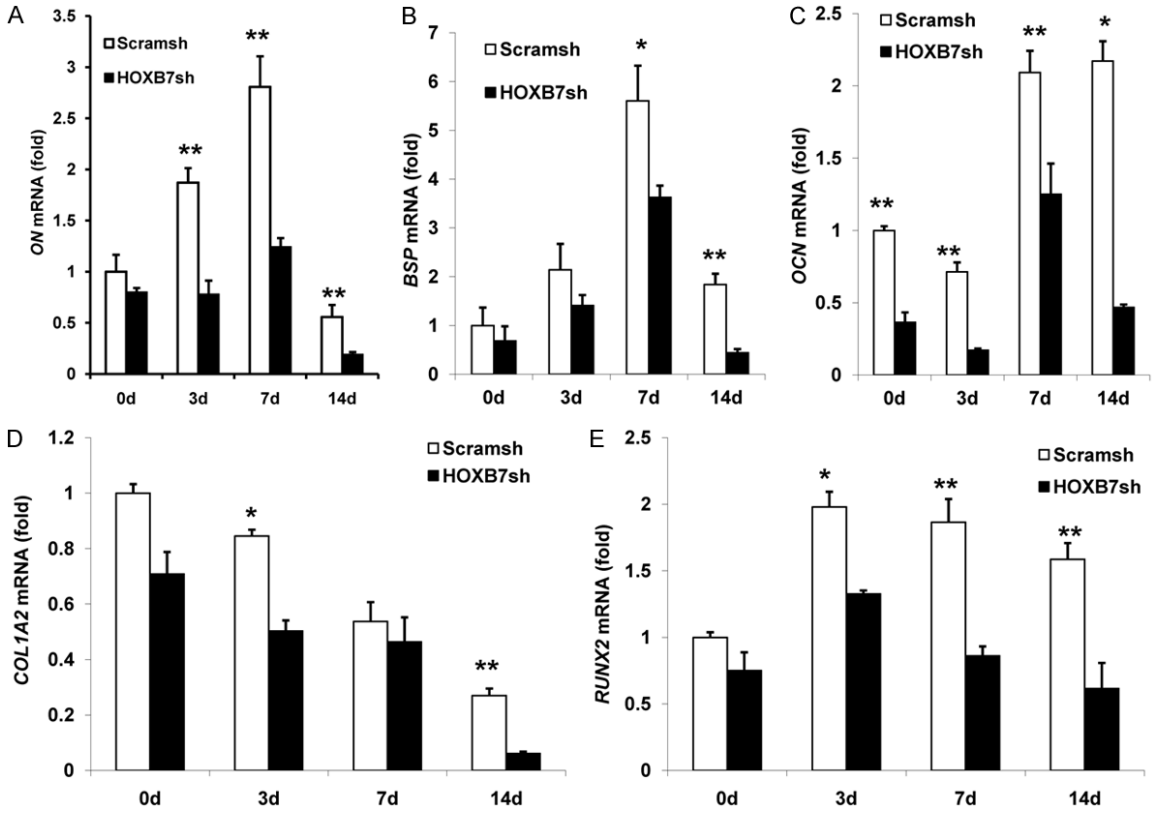

Figure 5. *HOXB7* knock-down decreases the mRNA levels of osteogenic differentiation markers in BMSCs. Real-time RT-PCR results show that *HOXB7* knock-down in BMSCs caused reduced expression of *ON* (A), *BSP* (B), *OCN* (C), *COL1* (D) and *RUNX2* (E). *GAPDH* was used as an internal control. The student's t test was performed to determine statistical significance. All error bars represent s.d. (n = 3). *P ≤ 0.05. **P ≤ 0.01.

HOXB7 depletion inhibited the osteogenic differentiation potential of BMSCs in vitro

To further elucidate the function of *HOXB7* in MSCs, we designed a shRNA to target and inhibit *HOXB7* expression. We introduced this shRNA (BMSC-HOXB7sh) or a Scramble control shRNA (BMSC-Scramsh) into BMSCs by lentiviral infection. After antibiotic selection of infected BMSCs, we determined that the knockdown efficiency in BMSC-HOXB7sh cells was 70% with real-time RT-PCR (**Figure 4A**). To determine whether *HOXB7* intrinsically affected osteogenesis in BMSCs, we cultured these transgenic BMSCs in osteogenic-inducing medium. We found that ALP activity was reduced in BMSC-HOXB7sh cells compared to BMSC-Scramsh cells (**Figure 4B**). Two weeks after induction, Alizarin Red staining and quantitative calcium measurements revealed that mineralization was also significantly lower in BMSC-HOXB7sh cells compared to BMSC-Scramsh cells (**Figure 4C**, **4D**). Consistent with this finding, real-time RT-PCR results showed that the osteogenic marker genes, *ON* was down-regulated in *HOXB7*-depleted cells at 3, 7, and 14 21 days after induction (**Figure 5A**); *BSP* was down-regulated in *HOXB7*-depleted cells at 7, and 14 days after induction (**Figure 5B**); *OCN* was strongly inhibited in *HOXB7*-depleted cells at 0, 3, 7, and 14 days after induction (**Figure 5C**); *COL1A2* was inhibited in *HOXB7*-depleted cells at 3, and 14 days after induction (**Figure 5D**). Next, we examined the expression of key transcription factor that regulate osteogenic differentiation, *RUNX2*. We found that the mRNA level of *RUNX2* was significantly lower at 3, 7, and 14 days after induction in BMSC-HOXB7sh cells compared to BMSC-Scramsh cells (**Figure 5E**).

HOXB7 over-expression enhanced BSP transcription by increasing the binding of HOXB7 in BSP promoter

Next, we investigated how HOXB7 regulated the expression of *BSP*. We analysis the promoter of *BSP*, and identified the specific sequence for

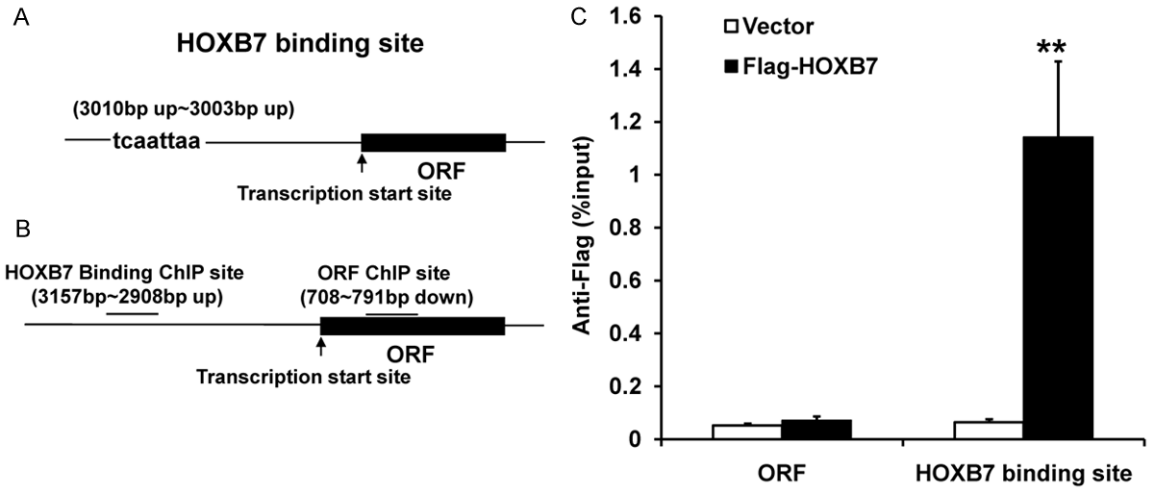

Figure 6. *HOXB7* over-expression increased the binding of HOXB7 in the *BSP* promoter. A. HOXB7 binding site in the *BSP* promoter. B. Location of ChIP assay primers targeting the *BSP* promoter at +3157 to +2908 bp upstream, and ORF at -708 to -791 bp downstream of the transcription start site (TSS). C. ChIP assay showed that HOXB7 over-expression led to changes in the binding of HOXB7 in the *BSP* promoter at 3days after cultured in osteogenic-inducing medium. Student's t test was performed to determine statistical significance. Error bars represent SD ($n = 3$). $^{**}P \leq .01$.

HOXB7 binding (tcaattaa) in the *BSP* promoter at +3010 bp up~+3003 bp up upstream of the transcription start site (**Figure 6A**). Then we investigated whether the HOXB7 could bind on the *BSP* promoter by a ChIP assay. However, despite repeated efforts, the available commercial anti- HOXB7 antibodies were ineffective in our ChIP assays. To overcome this problem, we constructed retroviruses to express Flag-*HOXB7* or the empty Vector in SCAPs (**Figure 1A**), and we performed ChIP assays with anti-Flag antibodies. The results indicated that, compared to the Vector group, significantly higher levels of Flag-*HOXB7* were on associated with the *BSP* promoter at 3 days after induction (**Figure 6B**, **6C**).

Discussion

MSCs can differentiate into several cell types, including osteoblasts. The molecular mechanisms underlying directed differentiation remain unclear. *HOXB7* is a critical member of homeobox genes and plays a key role in regulating cell fate. Evidence for the potential function of *HOXB7* in controlling differentiation of stem cells involved in its critical roles in osteogenesis and age of stem cells [19, 25]. Here, we present evidence to extend the functions of *HOXB7* to be a critical regulator in maintaining osteogenic differentiation of human post-natal MSCs.

In this study, we investigated the function of *HOXB7* in the differentiation of MSCs derived from human dental tissues and bone marrow. First, we found that *HOXB7* enhanced ALP activity and mineralization *in vitro.* Moreover, *HOXB7* overexpression enhanced osteogenesis *in vivo*. These findings indicated that *HOXB7* might be a key transcription factor that controls the osteogenic differentiation potential of MSCs.

The commitment of MSCs to an osteogenic lineage requires coordinated inhibition of differentiation toward other lineages. Thus, the activation of multiple transcription factors is associated with MSC differentiation [29, 30]. Two of the key transcription factors, *RUNX2* and *OSX*, are necessary for osteogenic differentiation [31-33]. *RUNX2* directs the differentiation of MSCs into an osteoblastic lineage, and inhibits them from differentiating into adipocytic and chondrocytic lineages. After differentiating into preosteoblasts, *OSX* and *RUNX2* can drive the cells to become immature osteoblasts and produce bone matrix. As a downstream gene of *RUNX2*, *OSX* is a second transcription factor that is essential for osteogenic differentiation; it is specifically expressed in all developing bones [31-34]. Previous study showed that *HOXA10* was markedly stimulated in osteoblasts after BMP2 treatment, coincident with the robust expression of *RUNX2* [35]. Also, we

showed that *HOXB7* evoked the significant up-regulation of *RUNX2*, but didn't affect *OSX* expression, indicating that regulation of *HOXB7* on osteogenesis mediated by *RUNX2*. Furthermore, *HOXB7* induced expression of the *ON*, *COL1A2*, *BSP*, and *OCN* genes, which encode extracellular matrix proteins. These results suggested that *HOXB7* is a key enhancer of osteogenic differentiation.

In addition, compared with other osteogenic marker genes, *BSP* was significantly induced by *HOXB7* after osteogenic induction, suggested *HOXB7* might regulated *BSP* transcript directly. We analysis the promoter of *BSP*, and identified the specific sequence for HOXB7 binding (tcaattaa) in the *BSP* promoter. Furthermore, Overexpression of *HOXB7* significantly increased the levels of *HOXB7* associated with the *BSP* promoter by ChIP assays at 3 days after osteogenic induction. These results indicated that *HOXB7* could directly bind with the promoter of *BSP* and promote its transcript.

In conclusion, this study found that HOXB7 was a positive effector for the osteogenic differentiation of MSCs via up-regulation of *RUNX2* and directly activating transcript of *BSP*. These results suggested that activation of HOXB7 signaling might improve tissue regeneration mediated by MSCs. These results have provided insights into the mechanism underlying directed differentiation of MSCs.

Acknowledgements

This work was supported by the National Sciences Foundation of China (Grant No. 81271164 to R Gao) and Tianjin Natural Science Foundation (12JCYBJC18400 to R Yao).

Disclosure of conflict of interest

None.

Abbreviations

MSCs, mesenchymal stem cells; SCAPs, stem cells from apical papilla; PDLSCs, periodontal ligament stem cells; DPSCs, dental pulp stem cells; BMSCs, bone marrow stromal stem cells; ALP, alkaline phosphatase; ARS, Alizarin red staining; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCN, osteocalcin; OSX, osterix; RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; OPN, osteopontin.

Address correspondence to: Dr. Run-Tao Gao, Department of Stomatology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China. Tel: +86 10 63139773; Fax; +86 10 63139773; E-mail: gaoruntao@ccmu.edu.cn; Dr. Chong Li, Institute of Biophysics, CAS Key Laboratory of Infection and Immunity, Chinese Academy of Sciences (CAS), Beijing 100101, China. Tel: +86 10 64884329; Fax; +86 10 64884329; E-mail: lichong@moon.ibp.ac.cn

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Table S1. Primers used in the Real-time RT-PCR

Gene Symbol	Primer Sequences (5'-3')
GAPDH-F	CGGACCAATACGACCAAATCCG
GAPDH-R	AGCCACATCGCTCAGACACC
BSP-F	CAGGCCACGATATTATCTTTACA
BSP-R	CTCCTCTTCTTCCTCCTCCTC
OCN-F	Agcaaaggtgcagcctttgt
OCN-R	gcgcctgggtctcttcact
RUNX2-F	TCTTAGAACAAATTCTGCCCTTT
RUNX2-R	TGCTTTGGTCTTGAAATCACA
COL1A2-F	ACAGGGCTCTAATGATGTTGA
COL1A2-R	AGGCGTGATGGCTTATTTGT
ON-F	TCCCTGTACACTGGCAGTTC
ON-R	TTGTCCAGGTCACAGGTCTC
HOXB7-F	CGGTTCAAGGAATCTCGTAAAACCG
HOXB7-R	TTCCGGCTCTCTATCTCAATGTTT