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TRANSCRIPTION FACTOR AND BONE MARROW STROMAL CELLS IN OSSEOINTEGRATION OF DENTAL IMPLANTS

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Abstract

Titanium implants are widely used in dental clinics and orthopaedic surgery. However, bone formation surrounding the implant is relatively slow after inserting the implant. The current study assessed the effects of bone marrow stromal cells (BMSCs) with forced expression of special AT-rich sequence-binding protein 2 (SATB2) on the osseointegration of titanium implants. To determine whether SATB2 overexpression in BMSCs can enhance the osseointegration of implants, BMSCs were infected with the retrovirus encoding *Satb2* (pBABE-*Satb2*) and were locally applied to bone defects before implanting the titanium implants in the mouse femur. Seven and twenty-one days after implantation, the femora were isolated for immunohistochemical (IHC) staining, haematoxylin eosin (H&E) staining, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and micro-computed tomography (μCT) analysis. IHC staining analysis revealed that SATB2-overexpressing BMSCs were intensely distributed in the bone tissue surrounding the implant. Histological analysis showed that SATB2-overexpressing BMSCs significantly enhanced new bone formation and bone-to-implant contact 3 weeks after implantation. Real-time qRT-PCR results showed that the local delivery of SATB2-overexpressing BMSCs enhanced expression levels of potent osteogenic transcription factors and bone matrix proteins in the implantation sites. μCT analysis demonstrated that SATB2-overexpressing BMSCs significantly increased the density of the newly formed bone surrounding the implant 3 weeks post-operatively. These results conclude that local delivery of SATB2-overexpressing BMSCs significantly accelerates osseointegration of titanium implants. These results provide support for future pharmacological and clinical applications of SATB2, which accelerates bone regeneration around titanium implants.

Keywords: Implant; special AT-rich sequence-binding protein 2 (SATB2); osseointegration; bone marrow stromal cells (BMSCs).

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Introduction

Osseointegration, also known as direct bone-to-implant contact (BIC), plays an important role in the long-term success of established titanium implants (Branemark, 1983; Linder *et al.*, 1983). In order to improve osseointegration, various studies on surface properties of the titanium implants and bone regeneration surrounding the implants have been undertaken (Rupp *et al.*, 2006; Sisti *et al.*, 2006; Traini *et al*., 2008; Faeda *et al.*, 2009). Stem cells, specifically dental pulp stem cells can hook into Biocoral scaffold (Mangano *et al.*, 2011), differentiate into osteoblasts, and form bone on different titanium surface textures (Mangano *et al.*, 2010). Osseointegration of implants is achieved by the activity of osteoblasts (Schneider *et al.*, 2004). Bone marrow stromal cells (BMSCs) contain a subset of stem cells, mesenchymal stem cells that possess multipotential and differentiation features. BMSCs are capable of self-renewal and can differentiate into several phenotypes including osteoblasts, chondroblasts, and adipocytes (Nussenbaum and Krebsbach, 2006; Robey and Bianco, 2006). Osteoblast progenitors are recruited from bone marrow and are involved in bone regeneration from peripheral circulation (Li *et al.*, 2008). Transplanted BMSCs can be recruited from peripheral circulation to implantation sites and participate in the osseointegration of the titanium implants (Xu *et al.*, 2009). However, there is only a small population of mesenchymal stem cells with osteogenic potential in the implant region, which explains the slow bone regeneration around implants after surgery.

Special AT-rich sequence-binding protein 2 (SATB2) is a nuclear matrix protein that plays pivotal role in osteoblast differentiation and craniofacial development (Dobreva *et al.*, 2006). SATB2 regulates gene transcription by binding to the nuclear matrix-attachment regions. *Satb2* gene knockout mice show multiple craniofacial defects including significant mandible truncation, shortened oral maxillofacial bones, and hyoid bone malformations. It is suggested that in craniofacial reconstruction, SATB2 can be a robust osteoinductive molecule recruiting other transcription factors to form a platform or a molecular node for a transcriptional network. It can synergise, amplify, and thus exponentially augment the activity of multiple osteogenic factors including runt-related transcription factor 2 (Runx2), Osterix (Osx), and activating transcription factor 4 (Atf4) regulating skeletal development and osteoblast differentiation (Dobreva *et al.*, 2006). However, we recently found that SATB2 upregulates Osx expression independent of Runx2, but synergistically enhanced the regulatory effect of Runx2 on Osx promoter (Zhang *et al.*, 2011). Additionally, SATB2 plays an important role in embryonic stem (ES) cell pluripotency (Savarese *et al.*, 2009). It has also been reported that Osterix (Osx) is an upstream regulator of SATB2 and can activate the SATB2 promoter reporter in a dose-dependent manner during bone formation (Tang *et al.*, 2011). Our previous study demonstrated that local delivery of SATB2 enhanced implant osseointegration (Yan *et al.*, 2011).

To determine the prominent properties of *Satb2* in bone regeneration, green fluorescent protein (GFP) labelled SATB2-overexpressing BMSCs were locally administrated before implantation. The function of SATB2-overexpressing BMSCs in bone formation around the implant was explored.

Materials and Methods

Plasmids

The mouse *Satb2* cDNA was released from pBs-SK-*Satb2* (a gift from Dr. Grosschedl, Gene Centre and Institute of Biochemistry, University of Munich, Munich, Germany), and was ligated into the *BamH*I/*EcoR*I sites of a retroviral vector, pBABE-hygro (ID: 1765, Addgene, Cambridge, MA, USA), creating the plasmid pBABE-*Satb2* (Yan *et al.*, 2011; Zhang *et al.*, 2011).

Production of high-titre pBABE viral stocks

The retroviral vectors, pBABE-*Satb2* and pBABE-hygro (Zhang *et al.*, 2011), were transfected into HEK-293T (ATCC, Manassas, VA, USA, CRL-11268™) cells using Lipofectamine transfection reagent (Life Technologies, Grand Island, NY, USA). Forty-eight hours after the transfection, the supernatant was collected, filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA), and mixed with 40 % PEG-8000 in phosphate buffered solution (PBS) to reach a final concentration of 12 % (Sigma-Aldrich, St. Louis, MO, USA). The virus-PEG8000 mixtures were incubated on ice for 12 h, and then centrifuged at 4000 rpm (4 °C) for 10 min. The pellets were dissolved in Dulbecco's Modified Eagle Medium (DMEM) to achieve a viral titre of 10^8 cfu/mL.

BMSC culture and gene transduction

BSP-Luc/ACTB-EGFP mice were genetically double labelled with a luciferase reporter gene driven by a bone sialoprotein (BSP) promoter and an enhanced green fluorescent protein (EGFP) driven by a beta-actin promoter (Li *et al.*, 2008). The BMSCs were obtained and cultured as described previously (Wu *et al.*, 2003; Valverde *et al.*, 2005). Briefly, BMSCs from 8-week-old BSP-Luc/ACTB-EGFP mice were obtained and cultured under non-differentiating conditions (DMEM, with 20 % foetal bovine serum (FBS), 100 mg/mL penicillin and 100 mg/mL streptomycin). Afterward, the IVIS 200 imaging system (Xenogen, Alameda, CA, USA) determined GFP expression in the BMSCs.

The BMSCs were infected by the viral supernatant with polybrene at a final concentration of 8 μg/mL for 6 h. BMSCs overexpressing SATB2 were then cultured under non-differentiating conditions (DMEM with 20 % FBS, 100 mg/mL penicillin and 100 mg/mL streptomycin). Empty vector infected BMSCs served as a control. Two days after viral infection, $1x10^6$ SATB2 overexpressing BMSCs or $1x10^6$ empty vector infected BMSCs were resuspended in 100 μL of cultured medium and prepared for use. We detected the Osx and Runx2 expression levels to evaluate the BMSC differentiation potential after transduction as previously described (Tu *et al.*, 2006; Zhang *et al.*, 2011).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of the infected BMSCs Infected BMSCs (pBABE-*Satb2* group and control group) were collected at 2, 9 or 23 days (the time points of the implantation and animals sacrifice) after infection. Total RNA was extracted from the infected BMSCs with TRIzol reagent (Life Technologies), and the first strand cDNA was generated with SuperScript III reverse transcriptase (Life Technologies) and oligo $(dT)_{20}$ primer (Life Technologies). Real-time qRT-PCR analysis was performed using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a Bio-Rad iQ5 thermal cycler (Bio-Rad). In Table 1, the sequences of the primers for amplification of mouse Osx, Runx2, BSP, Osteocalcin (OC), COLI, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed.

Animal surgery and BMSC application

The animals were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985). The Institutional Animal Use and Care Committee at the Tufts Medical Centre (Boston, MA, USA) approved the animal protocol.

The titanium implants (1.05 mm in diameter and 2 mm in length, Institute Straumann AG, Basel, Switzerland) were SLA (sand-blasting and acid etching) surfaced and the surgery was performed as previously described (Xu *et al.*, 2009). Briefly, two implantation sites, one in each femur, were prepared on the anterior-distal surface of the

Table 1. The sequences of the primers for qRT-PCR in the article.

Primer	Sequence
SATB2	forward : 5'-GAGATGAGTTGAAGAGGGCTAGTG--3'
	reverse : 5'-CCCTGTGTGCGGTTGAAT -3
OSX	forward: 5'-ATGGCGTCCTCTCTGCTTG-3'
	reverse: 5'-TGAAAGGTCAGCGTATGGCTT-3'
RUNX2	forward : 5'-CCCAGCCACCTTTACCTACA--3'
	reverse : 5'-TATGGAGTGCTGCTGGTCTG -3'
BSP	forward: 5'-CAGGGAGGCAGTGACTCTTC-3'
	reverse: 5'-AGTGTGGAAAGTGTGGCGTT-3'
COLI	forward: 5'-TGACTGGAAGAGCGGAGAGT-3'
	reverse: 5'-GTTCGGGCTGATGTACCAGT-3'
OCN	forward : 5'-GCGCTCTGTCTCTCTGACCT-3'
	reverse : 5'-GCCGGAGTCTGTTCACTACC-3'
GAPDH	forward: 5'-AGGTCGGTGTGAACGGATTTG-3'
	reverse: 5'-TGTAGACCATGTAGTTGAGGTCA--3'

femora in 20 10-week-old B6D2F1 mice (Jax #100006, Jackson Laboratory, Bar Harbor, ME, USA). The mice were divided randomly into two equal groups. After sequential drilling under cooled sterile saline irrigation with 0.4, 0.5, 0.7 and 1.0 mm surgical stainless steel twist drills, 3 µL of resuspended BMSCs in cultured medium were injected into the drilled holes for implant installation, and the SLA-surfaced implants were press-fitted into the undersized holes. The muscles were carefully sutured over the implant site to cover and stabilise the implants, and the mice were sacrificed 1 and 3 weeks after surgery.

Immunohistochemical (IHC) staining

After euthanasia, the femora with the implants were isolated, and fixed in 10 % neutral-buffered formalin solution. After decalcification, the implants were gently removed, and the femoral tissues were dehydrated, cleared and embedded in paraffin. Tissue sections, 6 µm in thickness, were mounted on glass slides and subjected to IHC and haematoxylin-eosin (H&E) staining. IHC staining was performed to detect the expression of GFP using the *Histostain*-SP*Kit* (AEC, Broad Spectrum, Life Technologies). The primary antibody for GFP (Clontech, Palo Alto, CA, USA) was used at a 1:50 dilution following the protocol. The slides were observed under the Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan).

Histomorphometric analysis

H&E staining was performed and images were taken under a Nikon Eclipse E600 microscope, and the newly formed bone area, restricted to the 0.5 mm area surrounding the implant, was measured with Spot Advanced Software (Diagnostic Instruments, Sterling Heights, MI, USA). The percentage of new bone edges in direct contact with the implant surface was also determined (Xu *et al.*, 2009).

Real-time RT-PCR analysis

The soft tissue and the femoral bone tissues bordering the implant (1 mm mesial and distal to the implantation site) were carefully dissected, snap-frozen in liquid nitrogen, and the implant was carefully removed. Total RNA was extracted from the bone tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and Real-time RT-PCR analysis was performed as described previously (Yan *et al.*, 2011).

Micro-computed tomography (µCT) analysis

After euthanasia, the femora with the implants were separated, fixed in 10 % neutral-buffered formalin solution overnight, kept in 70 % ethanol, and scanned with a high-resolution µCT (CT40; Scanco Medical, Basserdorf, Switzerland). According to the histomorphometric measurements on the H&E stained sections, the newly formed bone was restricted to a 0.5 mm area surrounding the implant. Thus, at a 3D level the Hounsfield Unit (HU) of this newly formed bone area was determined using eFilm Workstation 2.12 (Merge Technologies Inc., Milwaukee, USA) as we reported previously (Xu *et al.*, 2009).

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM) of 3 or more independent experiments. One-way ANOVA was used to test significance using the software package Origin 8 (Origin lab, Northampton, MA, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Expression of potent osteogenic transcription factors and bone matrix proteins in infected BMSCs

Real-time qRT-PCR revealed 2 days after infection the mRNA expression levels of SATB2, Osx, and Runx2 were all significantly higher in the pBABE-*Satb2* group than in the pBABE-hygro group, however there was no significant difference in the BSP, COLI, and OC mRNA levels between the two groups (Fig. 1a). The expression levels of SATB2, Osx, Runx2, BSP, COLI, and OC were all significantly higher in the pBABE-*Satb2* group than in the pBABE-hygro group 9 and 23 days after infection (Fig. 1b,c).

Tracing of exogenous BMSCs

One week after implantation, IHC staining showed the exogenous BMSCs were randomly distributed and mostly

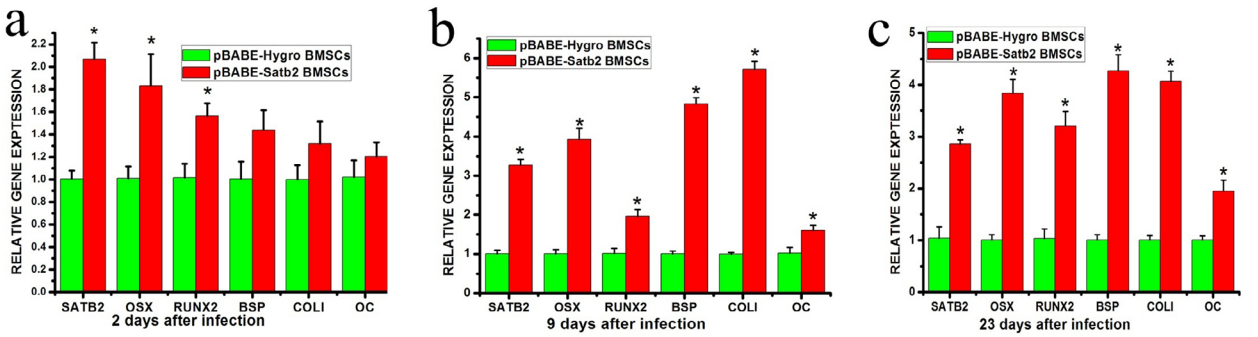

Fig. 1. Bone matrix protein and osteogenic transcription factor expression levels in pBABE-Hygro BMSCs and pBABE-Satb2 BMSCs at (**a**) 2 days, (**b**) 9 days, and (**c**) 23 days after transfection. Data were expressed as mean ± SEM (n = 6-8). *$p < 0.05$, pBABE-hygro BMSCs *vs.* pBABE-*Satb2* BMSCs.

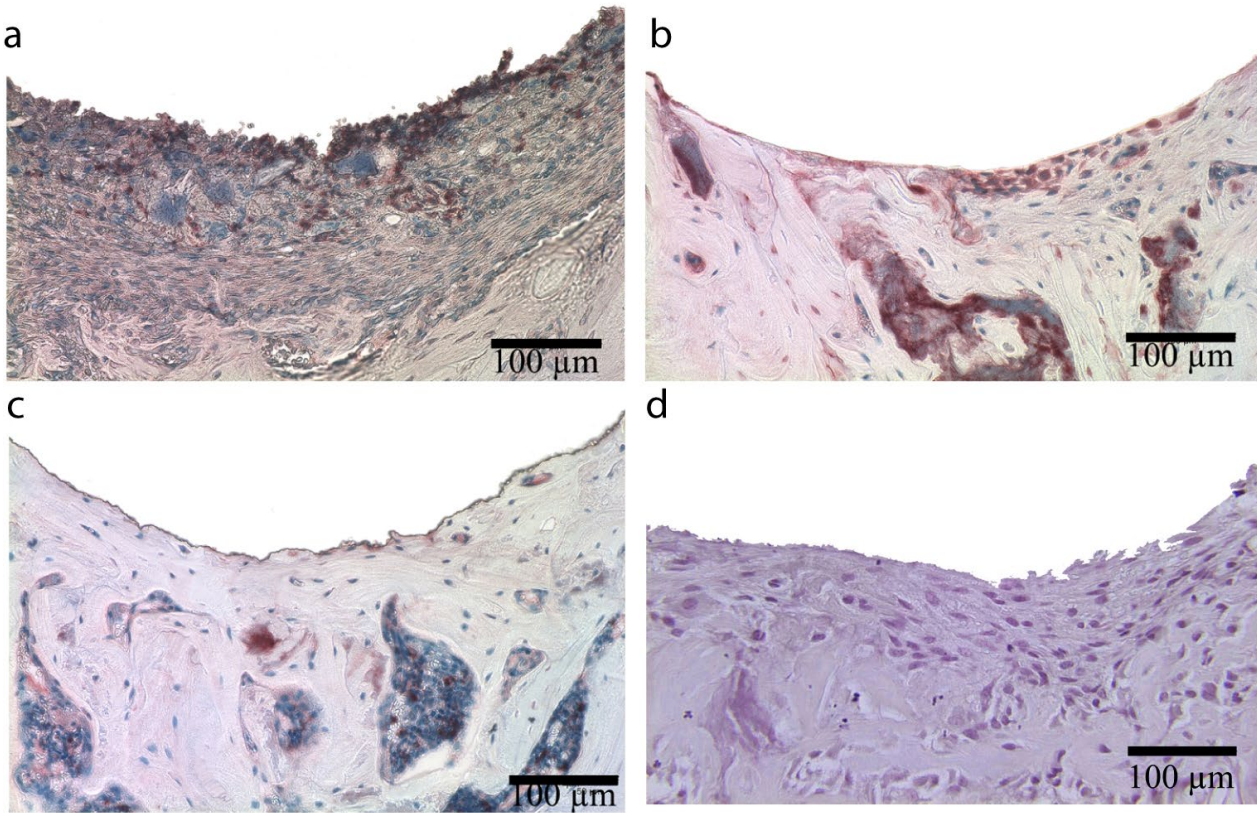

Fig. 2. Immunohistochemical staining for GFP. (**a**) 1 week after implantation, most of the exogenous BMSCs were found to distribute randomly in the tissue surrounding the implant. (**b**) 3 weeks after implantation, some of the exogenous BMSCs were embedded in the newly formed bone tissue. (**b, c**) Positive signals for GFP were found in the bone tissue surrounding the implant. (**c**) Some exogenous BMSCs could be observed in the bone marrow. (**d**) Negative control, the negative control did not show any GFP staining.

located in the tissue surrounding and adjacent to the femur implant (Fig. 2a). Three weeks after implantation, positive signals for GFP could be found in the bone tissue surrounding the femur implant (Fig. 2b,c). Specifically, the exogenous BMSCs were found embedded in the newly formed bone tissue (Fig. 2b), and in the bone marrow (Fig. 2c).

Histological analysis of bone regeneration

In both pBABE-hygro and pBABE-*Satb2* BMSCs, newly formed bone was observed 1 week after implantation (Fig. 3a,b). There was no difference detected in the percentage of newly formed bone area and bone-to-implant contact between these two groups (Fig. 3e). Three weeks after implantation, the implants successfully integrated with the host bone and organised lamellar bone formed in both the pBABE-S*atb2* and the pBABE-hygro BMSCs (Fig. 3c,d). Histomorphometric analysis showed that both the percentage of newly formed bone area and bone-to-implant contact increased by about 20 % in the pBABE-*Satb2* BMSCs when compared with those in the pBABE-hygro BMSCs (Fig. 3f).

Expression of potent osteogenic transcription factors and bone matrix proteins in bone tissue

One week after implantation, expression levels of SATB2, Osx, Runx2, BSP, and COLI were all significantly higher in the pBABE-*Satb2* group than in the pBABE-hygro group

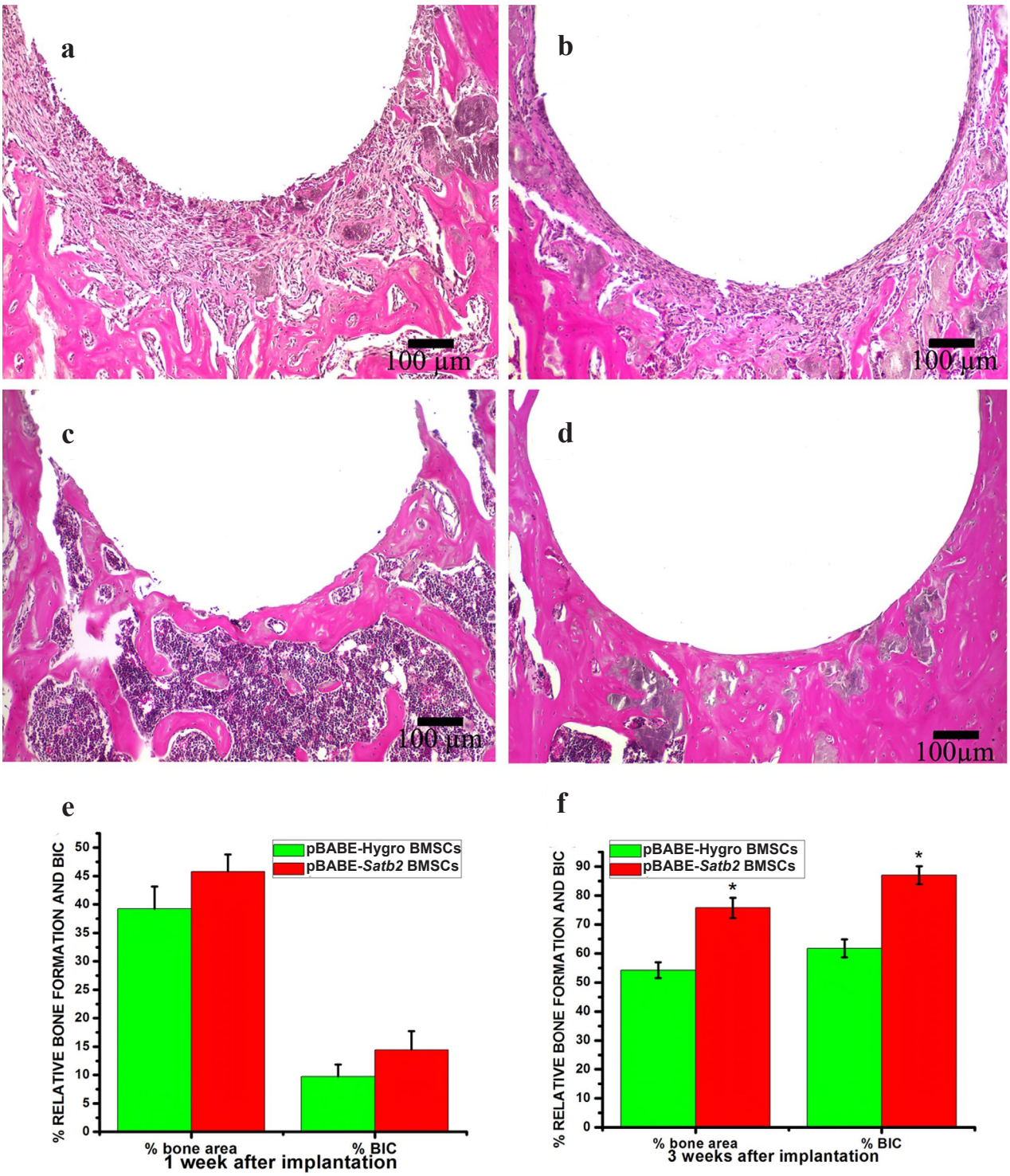

Fig. 3. Histomorphometric analysis. H&E staining 1 week after implantation showed newly formed bone in (**a**) pBABE-hygro BMSCs and (**b**) pBABE-*Satb2* BMSCs. No significant difference was detected in the percentage of newly formed bone area and bone-to-implant contact between these two groups (**e**). H&E staining 3 weeks after the implantation showed successful integration of the implants with the host bone and organised lamellar bone in (**c**) pBABE-hygro BMSCs and the (**d**) pBABE-*Satb2* BMSCs. (**f**) Histomorphometric analysis showed that both the percentage of newly formed bone area and bone-to-implant contact increased by about 20 % in pBABE-*Satb2* BMSCs compared with pBABE-hygro BMSCs. Data were expressed as mean ± SEM (n = 6-8). *$p < 0.05$, pBABE-hygro BMSCs *vs.* pBABE-*Satb2* BMSCs.

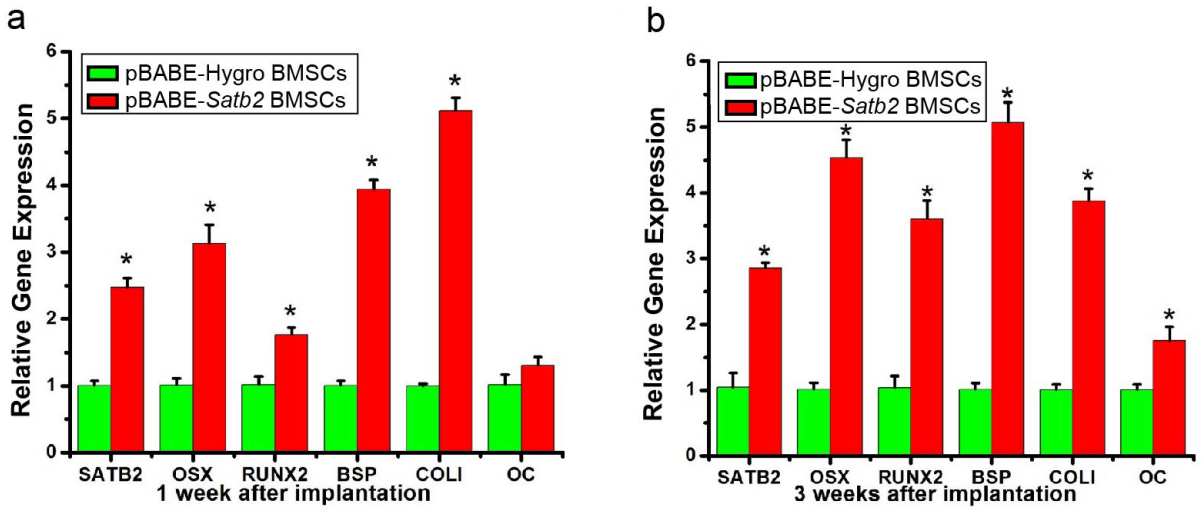

Fig. 4. In newly formed bone tissues, the SATB2 overexpressing BMSCs showed enhanced expression levels of potent osteogenic transcription factors and bone matrix proteins. To evaluate the function of SATB2 overexpressing BMSCs in osteogenic differentiation, qRT-PCR analysis was performed to detect the expression levels of SATB2, Osx, Runx2, BSP, COLI, and OC in the bone tissues surrounding the implant (**a**) 1 week and (**b**) 3 weeks after implantation in pBABE-hygro BMSCs and pBABE-*Satb2* BMSCs. Data were expressed as mean ± SEM ($n = 3$). *$p < 0.05$.

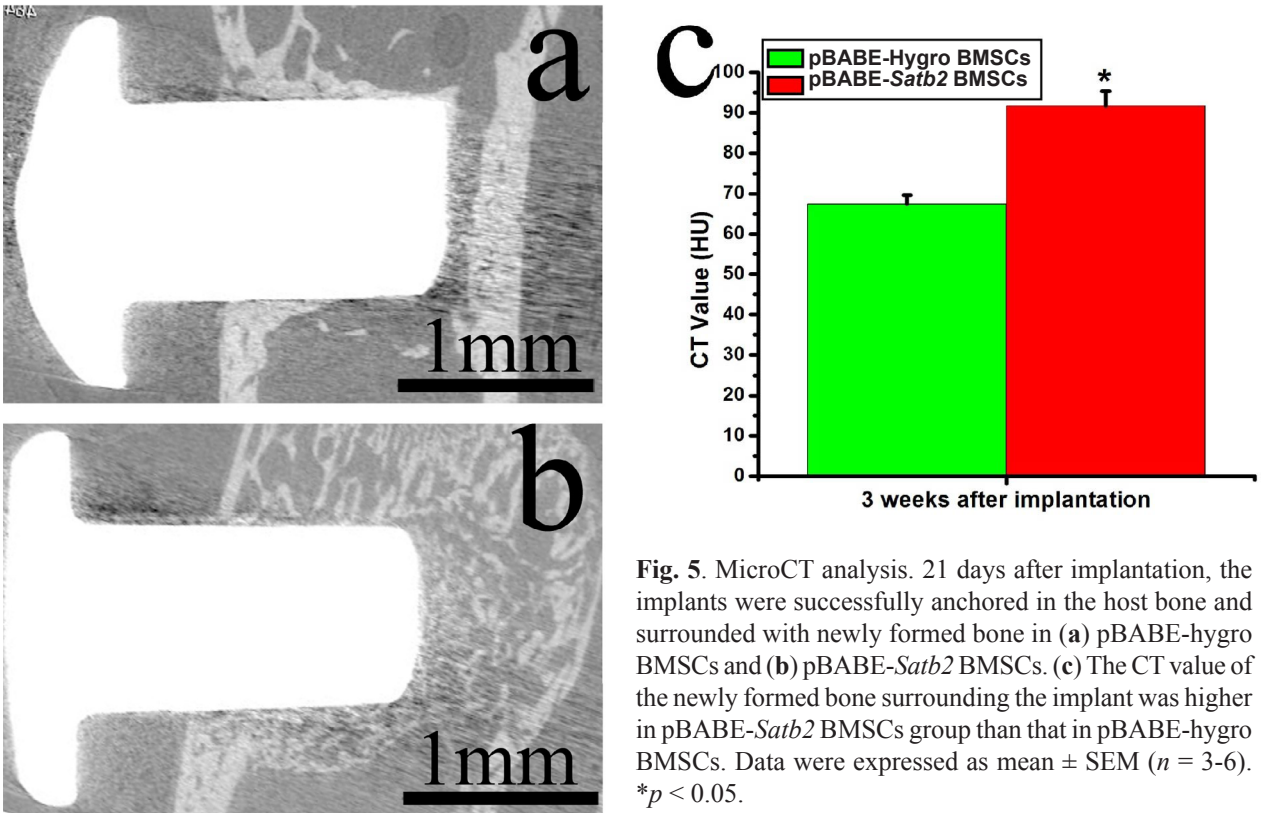

Fig. 5. MicroCT analysis. 21 days after implantation, the implants were successfully anchored in the host bone and surrounded with newly formed bone in (**a**) pBABE-hygro BMSCs and (**b**) pBABE-*Satb2* BMSCs. (**c**) The CT value of the newly formed bone surrounding the implant was higher in pBABE-*Satb2* BMSCs group than that in pBABE-hygro BMSCs. Data were expressed as mean ± SEM ($n = 3$-6). *$p < 0.05$.

in the newly formed bone tissues, however there was no significant difference in the OC mRNA level between the two groups (Fig. 4a). Three weeks after surgery, the expression levels of SATB2, Osx, Runx2, BSP, COLI, and OC were still significantly higher in the pBABE-*Satb2* group than those in the pBABE-hygro group (Fig. 5b).

µCT analysis

Twenty-one days after implantation, µCT analysis (Fig. 5) showed that the femur implants were surrounded by newly formed bone and successfully anchored with the host bone in pBABE-*Satb2* BMSCs (Fig. 5a), and pBABE-hygro BMSCs (Fig. 5b). SATB2 overexpressing BMSCs markedly enhanced the density of the newly formed bone around the implants (Fig. 5c).

Discussion

Satb2 plays an important role in craniofacial patterning and bone development, making it a plausible candidate gene for bone tissue engineering techniques. SATB2

interacts with Runx2 and ATF4, which play essential roles in osteogenic differentiation (Dobreva *et al.*, 2006). This study explored the role of SATB2 overexpressing BMSCs in accelerating implant osseointegration by introducing SATB2 overexpressing BMSCs to the implantation sites and observing the bone-to-implant contact. Though much effort has been made to increase the bone-to-implant contact, bone-to-implant integration is still a challenge. It is generally accepted that the bone forming cells at the titanium implant surface are from the neighbouring host bone. It is also known that the population of mesenchymal stem cells with osteogenic potential is limited to the vicinity of the implant. This can explain why bone regeneration around the implants is relatively slow after placement. The scarceness of bone forming cells in the implant site also poses the difficulty in early implant placement and immediate loading and thus limits the use of titanium implants in certain patients. Our previous study demonstrated that transplanted BMSCs can be recruited from circulation to the implantation sites and participate in implant osseointegration (Xu *et al.*, 2009). Based on this study, BMSCs were labelled with GFP prior to transplantation into the implant sites and GFP staining was performed to determine the BMSC fate.

To investigate the *in vitro* function of SATB2 in osteogenic differentiation of BMSCs, real-time qRT-PCR analysis was performed. Consistent with previous findings (Zhang *et al.*, 2011), the expression levels of potent osteogenic transcription factors and bone matrix proteins were upregulated by the forced expression of *Satb2*. This study focused on the function of SATB2 overexpressing BMSCs in early implant osseointegration. It was found that the exogenous SATB2 overexpressing BMSCs were distributed in the tissue surrounding the implant 1 week after the implantation. Most of the exogenous BMSCs were located in the tissue adjacent to the implant. Three weeks after implantation, the exogenous SATB2 overexpressing BMSCs were observed in the newly formed bone tissue. Some of them were embedded in the newly formed bone to form bone cells, and some could be observed in the bone marrow around the implant. Therefore, we conjecture that SATB2 overexpressing BMSCs enhanced bone formation around the implant than compared to the control group.

The expression levels of bone matrix proteins and osteogenic transcription factors were all significantly elevated in bone tissue surrounding the implant 1 and 3 weeks after implantation, which is consistent with *in vitro* data. Local administration of SATB2-overexpressing BMSCs resulted in increased new bone formation and enhanced implant osseointegration 3 weeks after implantation. These results lay a foundation for future clinical studies to develop novel pharmacological approaches that accelerates osseointegration and bone regeneration surrounding implants. This study explores if exogenous SATB2-overexpressing BMSCs can accelerate the bone formation and implant osseointegration. Future studies could investigate SATB2 as a candidate transcription factor for bone regeneration and early anchor of titanium implantation and study the long-term role of exogenous BMSCs in implant osseointegration.

Conclusion

SATB2-overexpressing BMSCs significantly enhanced expression levels of osteogenic transcription factors and bone matrix proteins, and accelerated new bone formation and enhanced implant osseointegration. This study provides evidence that BMSCs can contribute to implant osseointegration and SATB2 can be used as a candidate transcription factor for bone tissue engineering and regeneration.

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Discussion with Reviewers

L. Jimenez-Rojo: Please specify the novelties contained in this article with respect to your previous study (Yan *et al*., 2011, text reference).

Authors: In our previous study, the RCAS viral system was found to function in a relatively shorter time period. The RCAS-*Satb2* group showed dramatic increases in mRNA levels of SATB2, Osx, Runx2, BSP, COLI, and OC at 1 week after implantation when compared with the RCAS group. In contrast, only moderate changes in the mRNA levels of these genes were observed between the pBABE-*Satb2* group and the pBABE-hygro group. Three weeks after surgery, the expression levels of SATB2, Osx, Runx2, BSP, COLI, and OC were still significantly higher in the pBABE-*Satb2* group than those in the pBABE-hygro group, while there was no significant difference between RCAS-*Satb2* and RCAS group. pBABE-*Satb2* has a longer effect on the targeted gene expression compared with RCAS-*Satb2*. When RCAS-*Satb2* was applied, only BSP-expressing cells, which had already committed to the osteoblastic lineage were infected and overexpressed SATB2. In contrast, pBABE-*Satb2* could infect all local cells including adult stem cells with the ability of self-renewal and multi-potential of differentiation, which may prolong and enlarge the effect of the original SATB2 infection until a later time point. Based on our previous study, we hypothesised that exogenous bone forming cells with forced expression of SATB2 might elevate bone forming around the implant dramatically. So, this study was carried out and the functions of BMSCs with forced expression of SATB2 were detected *in vitro* and *in vivo*, and it was found that BMSCs can contribute to osseointegration of dental implants and SATB2 can be used as a candidate transcription factor for bone tissue engineering and regeneration.

G. Papaccio: The aim of this work is to describe a new system for improving osseointegration of dental implants. However, authors implant them in the femur instead of doing it in the alveolar bone. This may not be a good strategy, since these bones have different characteristics and the attachment of the tooth depends on more elements (periodontal ligament) that cannot be assessed if the implantation is done in the femur.

Authors: Most of the authors are from the dental school and focus on the early anchor of dental implants. In this study, we focused on the function of SATB2-overexpressing BMSCs in titanium implant osseointegration. This femur model characterised an *in vivo* dental implant experimental model to study implant osseointegration, as others have reported (Rahal *et al*., 1993; Nociti *et al*., 1997; Mushahary *et al*., 2013, additional references). Additionally, the periodontal ligament is not involved in the process of implant osseointegration.

G. Papaccio: BMSCs significantly enhanced expression levels of osteogenic transcription factors and bone matrix proteins around the implantation site? The authors did not demonstrate if the BMSCs are inducing the expression of these factors in the endogenous cells. The increase in these factors may be true only for the Satb2-transfected cells that actually may be already differentiated osteoblasts at the moment of the implantation. Please clarify this issue!

Authors: SATB2 is a nuclear matrix protein and regulates gene transcription by binding to the nuclear matrix-

attachment regions. In this study, BMSCs transfected with pBABE-hygro virus served as the control. The difference between the two groups is whether the BMSCs were forced to express SATB2. We did not detect if the endogenous cells around the implant site was involved in inducing the expression of the factors mentioned in the current experiment.

G. Papaccio: Are the BMSCs already differentiated to osteoblasts at the moment of the implantations, Should you not have included TRAP staining?
Authors: This should certainly have been done. However, we focused this experiment on bone forming and osseointegration around the implants, therefore TRAP staining was not included.

Additional References

Mushahary D, Sravanthi R, Li Y, Kumar MJ, Harishankar N, Hodgson PD, Wen C, Pande G (2013) Zirconium, calcium, and strontium contents in magnesium based biodegradable alloys modulate the efficiency of implant-induced osseointegration. Int J Nanomed **8**: 2887-2902.

Nociti Jr FH, Sallum AW, Sallum EA, Bozzo L (1997) Titanium implants in rabbit femur: a histologic evaluation. Braz Dent J **8**: 105-111.

Rahal MD, Branemark PI, Osmond DG (1993) Response of bone marrow to titanium implants: osseointegration and the establishment of a bone marrow-titanium interface in mice. Int J Oral Maxillofac Implants **8**: 573-579.