Calvarial defect healing by recruitment of autogenous osteogenic stem cells using locally applied simvastatin

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Local statins implant has been shown to promote bone healing, the underlying mechanisms are unclear. The purpose of this study was to test the effect of local simvastatin implant on bone defect healing; to evaluate the mobilization, migration, and homing of bone marrow-derived mesenchymal stem cells (BMSCs) and endothelial progenitor cells (EPCs) induced by simvastatin. We found that local simvastatin implant increased bone formation by 51.8% (week 6) and 64.8% (week 12) compared with polyglycolic acid controls \( (P < 0.01) \), as verified by X-ray, CT, and histology. Simvastatin increased migration capacity of BMSCs and EPCs in vitro \( (P < 0.05) \). Local simvastatin implant increased mobilization of EPCs to the peripheral blood by 127% revealed by FACS analysis \( (P < 0.01) \), and increased osteogenic BMSCs to the peripheral blood dramatically revealed by Alizarin Red-S staining for mineralized nodules formation. Pre-transplanted GFP-transfected BMSCs as a tracing cell and bioluminescence imaging revealed that local simvastatin implant recruited GFP-labeled BMSC. Also, local simvastatin implant induced the HIF-1a and BMP-2 expression. In conclusion, local simvastatin implantation promotes bone defect healing, where the underlying mechanism appears to involve the higher expression of HIF-1a and BMP-2, thus recruit autogenous osteogenic and angiogenetic stem cells to the bone defect area implanted with simvastatin.

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1. Introduction

The healing of bone defects remains a significant clinical orthopedic challenge. Autologous bone grafts are considered as the gold standard. However, these procedures may cause donor-site morbidity, such as hemorrhage, infection, and chronic pain [1]. Bone tissue-engineering methods use bone marrow-derived mesenchymal stem cells (BMSCs) or endothelial progenitor cells (EPCs) to enhance repair [2,3]. However, these approaches are restricted by the limited availability of stem cell sources, the potential immune response, and the complexity of the procedures.

Bone marrow harbors an abundant source of stem/progenitor cells, which participate in the regeneration of a variety of tissues following injury. Generation of multilineage stem/progenitor cells from the bone marrow is one major response during tissue repair [4,5]. Therefore, an alternative strategy for bone defect healing is to stimulate endogenous stem cell populations from the mature body and actively attract them to the sites of injury. This strategy, known as endogenous cell mobilization, has the potential to provide new therapeutic options for in situ bone regeneration [6].

Statins, such as simvastatin, inhibitors of 3-hydroxy-3-methyl glutaryl-coenzyme A (HMG-CoA) reductase, were widely used in the prevention of cardiovascular disease, in addition to their cholesterol-lowering activities. Although the original discovery that statins can effectively and significantly stimulate bone formation [7] has been confirmed by further studies [8–10], controversies remain [11,12]. Discrepancies in simvastatin effectiveness on bone formation might be due to low bioavailability of orally administered statins to the skeleton [13]. After the first-pass hepatic metabolism, only approximately 5% of the active compound remains in the circulation [14], even less at the defect site. Increasing the dosage is not an option, as high-dose administration of statins may cause adverse side effects. An alternate route of
statin administration may be the best solution. Locally applied statins have been shown to promote bone fracture healing [15,16] and defect repair [17,18], but the underlying mechanism is still not well understood.

The bone defect cannot heal without osteogenic cells. Unlike bone tissue-engineering methods, no allogeneic stem “seeding” cells were utilized in these locally applied statin therapies. A number of important questions have been raised following those observations, including as to be it possible that locally applied statins induced osteogenic cells “homing” to the bone defect? Statins have been reported to mobilize EPCs to peripheral blood [19], and have a synergetic effect with transplanted BMSCs on bone repair [20,21] and spinal cord injury [22]. As endogenous stem cells are abundant in adults and can be recruited to injury sites to participate in tissue regeneration, we hypothesized that the local implantation of scaffolds loaded with simvastatin would induce the mobilization of endogenous stem cells, enhance their migration, and promote homing to the bone defect, thereby contributing to bone repair. To test this hypothesis, we determined the effects of locally implanted simvastatin on 1) the repair of calvarial defects in an animal model, 2) the mobilization of BMSCs and EPCs in vivo, 3) the migration capacities of BMSCs and EPCs in vitro, 4) the recruitment and homing of BMSCs to the bone defect, and 5) the induction of bone morphogenetic protein 2 (BMP-2) and hypoxia-inducible transcription factor 1 α (HIF-1α) expression at the defect site.

2. Materials and methods

2.1. Preparation of PLA—simvastatin compound implant pellets

To prepare the implants for implantation in rabbits, 200 mg polyactic acid (PLA, Mw 20,000 Dalton, Shandong Institute of Medical Instrument, Jinan, China) with or without 50 mg simvastatin was dissolved in 2 mL acetone and injected into a round glass mold (15 mm diameter, 2 mm depth). The acetone was then evaporated in a laminar flow hood to form an implant pie (Fig. 1A, B). Similarly, the implants for rats (5 mm diameter, 2 mm depth) were prepared by dissolving 20 mg PLA in 200-μL acetone with or without 5 mg simvastatin. The surface morphology of PLA (Fig. 1C) and PLA—simvastatin scaffold (Fig. 1D) was observed by scanning electron microscopy (SEM, JEOL JSM-5000LV, Japan) after the samples were coated with platinum.

2.2. Animals and preparations

All animal experimental protocols were approved by the Ethical Committee of Peking University Third Hospital. Sixteen New Zealand White rabbits (5 months old; 3.5–4.0 kg in weight) were anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg). Rabbit critical-sized full-thickness calvarial bone defects were made using a trephine (Ø 15 mm) under low speed drilling with continuous cool saline irrigation. PLA scaffolds (15 mm diameter, 2 mm thickness) with or without simvastatin were implanted in the defects (Fig. 1E).

To investigate the effect of locally applied simvastatin on recruiting the autogenous BMSCs homing to the defect, we used GFP-labeled BMSCs. As the only available instruments for GFP signal detection were for small animals and the rabbit BMSCs were utilized in these locally applied statin therapies. A method for GFP signal detection was developed for rabbit stem cells. The only method for GFP signal detection was for small animals and the rabbit BMSCs were utilized in these locally applied statin therapies. A method for GFP signal detection was developed for rabbit stem cells. To investigate the possible molecular mechanisms of stem cell homing, the expression of bone morphogenetic protein 2 (BMP-2) and hypoxia-inducible transcription factor 1α (HIF-1α) in the defect region was assessed by immunohistochemistry (IHC). IHC staining was performed using rabbit anti-BMP-2 and HIF-1α antibodies (Santa Cruz Biotechnology) at 1:200.

2.3. X-Ray and CT analyses

Six and twelve weeks after implantation, randomly selected rabbits (n = 4 for each group at both time points) were euthanized by an overdose of anesthetic and calvarial specimens were collected. New bone formation at the defect site was evaluated by CT (GE Medical System Light Speed 16 scanner, 120 kV and 256 mA), with a slice thickness of 0.625 mm and a pixel size of 0.215 mm, and three-dimensional images were reconstructed. A digital radiographic system (Hitachi, Japan, 40 kV and 1.6 mA) was used to image the rabbit calvarial bone at a distance of 50 cm, and the radio-opacity within the defects was thresholded and measured manually twice by two independent examiners who were blinded to experimental treatment, using Image-Pro plus 6.0 (Media Cybernetics, USA). The percentage of new bone formed per total defect area was calculated.

2.4. Histology and immunohistochemical staining of rabbit calvarial defects

Rabbit calvarial bones were fixed in 4% paraformaldehyde overnight, decalcified in 10% ethylenediamine tetra-acetic acid, and embedded in paraffin. Bone sections of 5-μm thickness were stained with hematoxylin and eosin (H&E).

To explore the possible molecular mechanisms of stem cell homing, the expression of bone morphogenetic protein 2 (BMP-2) and hypoxia-inducible transcription factor 1α (HIF-1α) in the defect region was assessed by immunohistochemistry (IHC). IHC staining was performed using rabbit anti-BMP-2 and HIF-1α antibodies (Santa Cruz Biotechnology) at 1:200.

2.5. Fluorescence Activated Cell Sorting (FACS) analysis of mobilized EPCs in peripheral blood

Fourteen days after implantation, 2 mL of anti-coagulated peripheral blood was collected using a syringe containing heparin (1000 IU), and mixed with 2 mL PBS. Cells were purified from the whole blood by density gradient centrifugation using a Biocoll separating solution. FACS analysis in a FACS scanner (Becton–Dickinson, San Jose, CA) was performed on samples containing 1 × 10^7 cells in 100 mL PBS. Following standard FACS protocols, immunophenotyping was performed using Rhb-conjugated anti-CD133 and FITC-conjugated anti-CD34 rabbit monoclonal antibodies. Isotype-identical antibodies served as controls. All antibodies were purchased from Beijing Biosynthesis Biotechnology (China).

2.6. Identification of mobilized osteogenic progenitor cells in peripheral blood

Fourteen days after implantation, 2 mL of anti-coagulated peripheral blood was collected as above and mixed with 3 mL DMEM with 15% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.
mL penicillin, 100 µg/mL streptomycin; osteoinductive medium [23] was supplemented with 10 mM β-glycerophosphate sodium salt and 50 µg/mL sodium ascorbate (Sigma–Aldrich, USA). After 21 days, the formation of mineralized nodules was detected by Alizarin Red-S staining under an optical microscope (Leica, Germany) as described previously [24].

2.7. Cell migration assay

To obtain rabbit BMSCs and EPCs, bone marrow was aspirated from the ventral ilium of an additional New Zealand White rabbit using a 10 mL syringe with 16 G needle pretreated with 1000 U heparin.

BMSCs were cultured in DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin and identified by immunocytochemical staining for CD29, CD90 and CD34, as previously described [25]. EPCs were cultured in fibronectin-coated flasks and maintained in endothelial basal medium (Cell systems) supplemented with EGM (Single Quote Kit, Lonza, Switzerland) and identified by IHC staining for CD34, CD133, and VEGFR-2, as previously described [26]. All antibodies were purchased from Beijing Biosynthesis Biotechnology (China).

The effect of simvastatin on BMSCs and EPCs migration was evaluated by a modified Transwell®-based migration assay [27]. Briefly, 1 × 10^5 cells cultured for 14 day were loaded into the upper chamber of a 24-well Transwell® plate (Corning, pore size 5 µm) and 600 µL medium containing different concentrations of simvastatin was added to the lower chamber. Twenty-four hours later, the filter was gently removed and the cells from the upper surface of the membranes were removed with a cotton swab. Cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained for 10 min with 0.5% crystal violet. The number of cells that had migrated into the lower chamber was counted in five randomly selected microscopic fields per filter by blind evaluations performed twice by two independent assessors. After careful aspiration of the crystal violet solution the plates were extensively washed with de-ionized water, and air-dried prior to the solubilization of the bound dye with 100 µL of a 10% acetic acid solution incubated for 30 min. The optical density (OD) of the plates was measured at 595 nm in a µQuant microplate spectrophotometer (Varioskan Flash, Thermo Electron, Finland) [28].

2.8. Bone marrow stromal cell homing assay

GFP signals were detected using a Maestro fluorescence-based small animal imaging system (Cri Inc., Woburn, MA). After frozen sectioning at 10 µm (Leica Instruments GmbH, D-6907 Nussloch, Germany), slides with slices of rat calvarial specimen (5 slices each) were washed in PBS three times and counterstained with DAPI-Fluoromount-G® (Southern Biotech, USA) to counterstain the nuclei. The numbers of GFP-positive cells were counted manually in each specimen and images were taken using a fluorescence microscope (Leica DM3000, Germany).

2.9. Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using either unpaired, two-tailed Student’s t-tests or ANOVAs. Statistical significance was set at P < 0.05.

3. Results

3.1. Radiological and histological assessment of bone formation

X-Ray and CT images of rabbit calvarial bone defects taken at 6 and 12 weeks after implantation of the control PLA scaffolds showed no high density areas, which indicate bone formation, and the margins of the defects were smooth. In the PLA–simvastatin treated group, however, many high density spots were observed and the margins of the defects were more irregular (Fig. 2).
Quantitative analysis verified a significant increase in radiopacity in the simvastatin group compared with the control group at both 6 and 12 weeks (both $P < 0.000$) after implantation. Furthermore, new bone had filled in more of the defect at week 12 than week 6 after implantation in the simvastatin group (Table 1).

Consistent with these data, new bone formation in defects was also confirmed histologically (Fig. 3). The rabbit calvarial defect site of the control group displayed mostly fibrous tissue with little bone formation after 6 (data not shown) and 12 weeks (Fig. 3A, B) post-implantation. Conversely, defects treated with PLA–simvastatin scaffolds showed obvious new bone formation and defects were markedly filled with bone-like particles 12 weeks after implantation (Fig. 3C, D).

### 3.2. Detection of EPCs and BMSCs in the peripheral blood

FACS analysis demonstrated that the number of EPCs (CD34+/CD133+) in rabbit peripheral blood two weeks post-implantation of the simvastatin group was significantly greater than the control group ($P = 0.000$) (Fig. 4A), illustrating that locally applied simvastatin can effectively mobilize EPCs to the peripheral blood. Moreover, adherent BMSCs could be obtained from the peripheral blood samples in the simvastatin group (Fig. 4B), while we failed to harvest adherent cells from the peripheral blood in the control group (data not shown). Importantly, these adherent cells could be induced to form mineralized nodules that stained positively with Alizarin Red-S (Fig. 4C), indicating that BMSCs mobilized from bone marrow by simvastatin could be induced to form bone mineral contents.

### 3.3. Migration capacity of BMSCs and EPCs in vitro

Simvastatin also had a positive effect on the migration capacity of BMSCs and EPCs. We classified CD29 and CD90 positive and CD34 negative cells from rabbits as BMSCs (Fig. 5A, upper panel), while CD34, CD133, and VEGF-2 positive cells were identified as rabbit EPCs (Fig. 5A, lower panel). Modified Transwell®-based migration assays revealed that both the BMSCs (Fig. 5B, upper panel; Table 2) and EPCs (Fig. 5B, lower panel; Table 2) in the simvastatin group displayed greater migration potential than the corresponding cell types in the control group.

### 3.4. Detection of GFP-labeled BMSCs in the bone defect region

Intense GFP signal was detected in the rat defect region 2 weeks after simvastatin implantation, while no GFP signal was seen in the control group (Fig. 6A). Frozen sections histology of the defect region confirmed the existence of GFP-BMSCs in the simvastatin group under fluorescence microscopy (Fig. 6B, C).

### 3.5. Immunohistochemical staining of BMP-2 and HIF-1α

IHC staining of the rabbit calvarial defect region demonstrated that higher expression of BMP-2 and HIF-1α (Fig. 7), which suggests that BMP-2 and HIF-1α may mediate the recruitment of stem cell homing.

### 4. Discussion

Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent cells capable of differentiating into osteoblasts and other mesenchymal cell types.
play an important role in bone regeneration [29]. Ex vivo expanded BMSCs applied to bone tissue-engineering strategies are capable of promoting bone defect healing in various animal models. However, it was still limited and has not been widely used in clinic. Even in adults, bone marrow harbors abundant stem cells to repair non-hematopoietic organs [30,31]. The identification of bone marrow-derived cells in various tissues after injury indicates that such damage can induce mobilization of these cells from the bone marrow into the peripheral blood [32,33]. Following stress, or some stimulation, a portion of the stem/progenitor cells egress from the bone marrow into peripheral blood, and contribute to tissue repairing, suggesting that promoting endogenous stem cell homing to injured tissues may be useful as a novel treatment strategy [6].
A critical-sized calvarial defect is the standard model of osseous non-union for the study of bone regeneration; a process that primarily involves bone formation [34]. Locally implanted simvastatin has been reported to be effective for bone defect healing [17,18]. However, the mechanism by which simvastatin stimulates new bone formation remained poorly understood.

We previously found that simvastatin stimulates primary BMSCs towards osteoblastic differentiation and inhibits adipogenic differentiation [35], and that it mobilizes bone marrow-derived mesenchymal stem cell migration to the injured areas in a rat spinal cord injury model [22]. Statin was also reported to induce EPCs proliferation and mobilization from the bone marrow, enhancing angiogenesis [19]. Taken together, we hypothesize that simvastatin-laden scaffolds can improve osteogenesis by promoting BMSCs and EPCs homing to a defect site. Here, we demonstrated that local simvastatin-loaded scaffold implant could repair rabbit critical-sized calvarial bone defects and evidence points to the recruitment of BMSCs to the defect site by simvastatin as the underlying mechanism.

Polyactic acid was a kind of biomaterial which demonstrated satisfactory biocompatibility and absence of significant toxicity and its intrinsic nature rendered it suitable for applications where temporally slow releases of bioactive agents in situ may be required [36]. As it had been shown to be a practically feasible packaging material in a number of researches on bone healing and already used for resorbable sutures and prosthetic devices in the human body [37].

Bone healing constitutes a unique regenerative process that requires coordinated coupling between angiogenesis and osteogenesis [38]. EPCs are bone marrow-derived cells with the ability to differentiate into endothelial cells and participating in postnatal vascular regeneration, can also significantly enhance bone regeneration during repair of bone defect [39]. The higher number of CD133+/CD34+ (phenotype of EPCs) cells in the peripheral blood, as verified by FACS analyses, suggesting simvastatin induced EPC migration into the peripheral blood. Also, cultures of adherent peripheral blood cells from the simvastatin-treated groups were able to form mineralized nodules. This indicated BMSCs were not

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**Fig. 6.** Simvastatin recruits BMSC homing to bone defects in vivo. (A) GFP signal could be detected within the defect region in the simvastatin group while no signal was detected in the control group. (B) The presence of GFP-BMSCs in the defect region was verified with fluorescent microscopy. (C) The total number of GFP-positive cells (five slices each specimen) presented in the control and simvastatin group. (Scale bars are 50 μm). *P* < 0.01 vs. the control.
only mobilized, but could also induce towards osteogenic progenitor cells. Besides, the Transwell-based migration assays, indicate simvastatin influences the migration potential of BMSCs and EPCs. These data suggest that statin-induced promotion of EPCs and BMSCs may improve angiogenesis and osteogenesis, thus enhancing bone defects healing.

Bone fracture could mobilize BMSCs and EPCs [40,41], which usually peaked in 3 days and decreased to normal within 2 weeks [42,43]. Meanwhile, continuous statin therapy could effectively mobilize EPCs 14 days after injury [44,45]; extracorporeal shock wave treatment enhanced recruitment of mesenchymal stem cells, which peaked at 14 days [46]. In this study, PLA compound with simvastatin could mobilize BMSCs and EPCs 14 days after local implantation, yet its temporal effect on mobilization of BMSCs and EPCs needs further research.

Bioluminescence imaging has been used in bone repair researches [47]. Since stem cells are known to selectively migrate to the bone marrow compartment soon after intravenous infusion [48]. In this study, GFP-labeled BMSCs were injected before defect induction as tracing cells [49]. The detection of strong GFP signal in the simvastatin-treated defects provided substantial evidences for our hypothesis. This selective homing indicated that simvastatin could recruit GFP-BMSCs to the bone defect region to participate in osteogenesis, suggesting a similar effect on endogenous stem cells. This cellular recruitment may be a consequence of elevated expression of various growth factors and cytokines induced by simvastatin.

Circulating bone marrow-derived cells can form new blood vessels through a process of postnatal vasculogenesis, with endothelial progenitor cells selectively recruited to injure or ischemic tissue [50]. The endothelial expression of stromal cell-derived factor-1 (SDF-1) acts as a signal indicating the presence of tissue ischemia. This expression is directly regulated by HIF-1α, suggesting that hypoxia may be a fundamental requirement for progenitor cell trafficking and function [51].

BMP-2 also has the capacity to mobilize bone marrow-derived osteoblast progenitor cells to the peripheral blood and to induce migration to the BMP-2 implanted site [52]. In the current study, locally applied simvastatin induced expression of HIF-1α and BMP-2, which may be the molecular mechanism directing mobilization of autogenous BMSCs and EPCs and homing to bone defect sites implanted with simvastatin.

There were some limitations of our study. First, we use rat model instead in the stem cell homing assay and differences may exist between rabbit and rat model. We made that choice because there were only instruments for small animals to detect GFP signals so far. Second, the GFP-labeled cell lines may not behave exactly the same as primary autogenous cells. Considering the difficulty of directly labeling rat primary cells, we have to use GFP-BMSC cell lines as tracer cells as an alternative. And the GFP-labeled bone marrow stromal cells were already commonly used as an autogenous stem cell tracer [53,54].

5. Conclusions

Locally applied simvastatin can mobilize autogenous EPCs and BMSCs of the peripheral blood, and these stem cells with increased migration capacity might be recruited to the defect, thus to contribute to the beneficial effects of simvastatin in osteogenesis and promoting the calvarial bone defect healing. The underlying mechanism appears to involve the higher expression of HIF-1α and BMP-2 induced by simvastatin.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.08.060.
References


