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Blood vessel formation in the tissue-engineered bone with the constitutively active form of HIF-1α mediated BMSCs

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ABSTRACT

The successful clinical outcome of the implanted tissue-engineered bone is dependent on the establishment of a functional vascular network. A gene-enhanced tissue engineering represents a promising approach for vascularization. Our previous study indicated that hypoxia-inducible factor-1α (HIF-1α) can up-regulate the expression of vascular endothelial growth factor (VEGF) and stromal-derived factor 1 (SDF-1) in bone mesenchymal stem cells (BMSCs). The angiogenesis is a co-ordinated process that requires the participation of multiple angiogenic factors. To further explore the angiogenic effect of HIF-1α mediated stem cells, in this study, we systematically evaluated the function of HIF-1α in enhancing BMSCs angiogenesis *in vitro* and *in vivo*. A constitutively active form of HIF-1α (CA5) was inserted into a lentivirus vector and transduced into BMSCs, and its effect on vascularization and vascular remodeling was further evaluated in a rat critical-sized calvarial defects model with a gelatin sponge (GS) scaffold. The expression of the key angiogenic factors including VEGF, SDF-1, basic fibroblast growth factor (bFGF), placental growth factor (PLGF), angiopoietin 1 (ANGPT1), and stem cell factor (SCF) at both mRNAs and proteins levels in BMSCs were significantly enhanced by HIF-1α overexpression compared to the *in vitro* control group. In addition, HIF-1α-over expressing BMSCs showed dramatically improved blood vessel formation in the tissue-engineered bone as analyzed by photography of specimen, micro-CT, and histology. These data confirm the important role of HIF-1α in angiogenesis in tissue-engineered bone. Improved understanding of the mechanisms of angiogenesis may offer exciting therapeutic opportunities for vascularization, vascular remodeling, and bone defect repair using tissue engineering strategies in the future.

1. Introduction

The possible risks of infection, rejection, the limited supply, and cost are serious limitations faced by autologous and allogeneic bone used to repair bone defects [1,2]. The tissue-engineered bone offers new therapeutic strategies to repair tissue defects. When bone defects are repaired using tissue engineering technology, particularly larger bone defects, angiogenesis is a prerequisite step to achieve the successes of bone regeneration [3]. However, unlike organ transplants, where there is a pre-existing vascular supply, the tissue-engineered bone usually is devoid of pre-existing vasculature [4]. Therefore, how to promote the angiogenesis and vascularization of these bone constructs remains a big challenge in the clinic.

The importance of blood vessels in the formation of the skeleton and in bone repair was documented as early as the 1700s [5,6]. The vasculature transports oxygen, nutrients, soluble factors, and numerous types of cells to the tissues of the body. Many of the factors that lead to the normal development of embryonic

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vasculature are recapitulated during neoangiogenesis in adults [7]. Factors involved in neoangiogenesis include VEGF, basic fibroblast growth factor (bFGF), various members of the transforming growth factor beta (TGF-β) family, Ang-1, placental growth factor (PLGF), angiopoietin 1 (ANGPT1), PDGF-BB, IGF-1, IGF-2, and SDF-1 [8]. Among these factors, VEGF, which is activated by hypoxia, plays a critical role in angiogenesis during the development of most tissues including bone [9]. It has been demonstrated that some growth factors can accelerate angiogenesis in the tissue-engineered bone, such as VEGF [10,11]. However, the formation of blood vessels is a complex process that requires the coordination of multiple angiogenic factors, communication of cells with one another, and with their surrounding extracellular matrix (ECM) [12]. Besides VEGF, angiogenesis is also modulated by other growth factors, such as bFGF, PDGF, and SCF [13]. More recently, VEGF has been reported to be insufficient as a single agent to create the complex structures required for a functional vasculature. VEGF-induced vessels are often leaky and improperly connected to the existing vasculature [14]. To overcome these problems, several studies have used two angiogenic factors in combination to remodel and repair vasculature, such as Ang-1 and VEGF [15].

Hypoxia inducible factor-1 (HIF-1), which activates the transcription of hundreds of target genes in response to reduced O_2 availability, is the master regulator of oxygen homeostasis in metazoans [16–18]. One of the main targets of HIF-1α-mediated gene induction is VEGF. As an upstream regulator, HIF-1α is known to activate the transcription of many other angiogenic genes, including TGF-β, Ang-1, PLGF, ANGPT1, PDGF-BB, stem cell factor (SCF), and SDF-1 [8,19]. Therefore, HIF-1α should have more advantages in promoting angiogenesis compared with the above single agent gene therapies. Under normoxic conditions, HIF-1α is subject to degradation regulated by von Hippel-Lindau protein, which recruits an ubiquitin-protein ligase. A lack of oxygen, certain metabolic intermediates, or iron can inhibit prolyl-4-hydroxylase domain proteins (PHD) activity and stabilize HIF-1α [20,21]. When stabilized, HIF-1α forms a heterodimer with HIF-1β and binds to the hypoxia response element to transactivate the expression of many downstream genes that are involved in angiogenesis.

We previously reported that a truncation mutant of HIF-1α (CA5), which contains a deletion (amino acids 392–520) and 2 substitutions (Pro567Thr and Pro658Gln), could effectively maintain the stability and activity of HIF-1α under normoxic conditions [22]. The expression of VEGF and SDF-1 in BMSCs was found up-regulated by HIF-1α overexpression [23]. However, whether HIF-1α could regulate angiogenesis in BMSCs through additional pathways and its *in vivo* role played in blood vessels formation in the tissue-engineered bone are largely unknown. In this study, we systematically explore the angiogenesis of HIF-1α mediated stem cells *in vitro* and evaluate the angiogenic function of CA5 gene inducing BMSCs in tissue-engineered bone in a rat model of critical-sized calvarial defects (CSD).

2. Materials and methods

2.1. Culture of rat BMSCs

Male F344 rats were obtained from the Ninth People's Hospital Animal Center (Shanghai, China), and all procedures were approved by the Animal Research Committee of the Shanghai Ninth People's Hospital. Rat bone marrow was extracted from the femurs and tibias of 4-week-old male F344 rats, as described previously [22], and the BMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2 for 5–7 d. After reaching 80%–90% confluency, the BMSCs were detached with 0.25% (w/v) trypsin/1 mM EDTA solution (1:1, v/v) and transferred to 10 cm dishes at a concentration of 1.0×10^5 cells/mL in 10 mL of medium. The culture medium was changed twice a week, and the BMSCs were used for *in vitro* or *in vivo* experiments at passages 2–4.

2.2. Lentiviral vector construction and transduction

A replication-defective lentivirus that encodes for enhanced green fluorescent protein (EGFP) was used as the vector for the control (only GFP protein, Lenti-GFP) and target gene groups (Lenti-WT, wild-type HIF-1α and Lenti-CA5, a constitutively active form of HIF-1α). Lenti-WT, Lenti-CA5, and Lenti-GFP were constructed as previously described [22]. The CA5 construct is a truncation mutant of HIF-1α that contains a deletion (amino acids 392–520) and two substitutions (Pro567Thr and Pro658Gln). Large-scale lentiviral production was done by Shanghai R&S Biotechnology Co., Ltd. (China). The efficiency of lentiviral gene transfer in BMSCs was greatest when the multiplicity of infection (MOI) was 15. The transduction efficiency was assessed by counting the number of GFP-positive cells using flow cytometry after 4 d of culture.

2.3. Quantitative real-time reverse transcription-PCR analysis

Total RNA was extracted from BMSCs transduced with Lenti-WT, Lenti-CA5, or Lenti-GFP on days 0, 1, 4, 7, 14, and 21. The extracted RNA was DNase treated and purified using an RNeasy Mini kit (Qiagen, Germany). RT-qPCR was performed with a thermal cycler (iCycler, Bio-Rad, USA) using 39 cycles of 95 °C for 15 s and at 48 °C–58 °C for 30 s depending on the melting temperatures of the primers. A 1 μg aliquot of the total RNA in a final volume of 20 μL was used as the template for cDNA synthesis using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). The △Ct method was used to calculate the relative expression of each angiogenic growth factor relative to the amount of 18S rRNA detected [24]. The RNA primers were commercially synthesized by Shengong Co., Ltd., Shanghai (China). The gene names, accession numbers, primer sequences, and amplicon sizes are shown in Table 1.

2.4. Western blot analysis

To determine the protein level of the angiogenic factors, whole cell extracts were prepared from BMSCs on days 0, 1, 4, 7, 14, and 21 following gene transduction. Briefly, after washing with ice-cold phosphate-buffered saline, the cells were lysed using a protein extraction regent (Kangchen Bio-tech, Shanghai, China). Total protein extracts were quantified with the DC protein assay kit (Invitrogen, Carlsbad,

Table 1
Nucleotide sequences for real-time RT-PCR primers.

Genes	Primer sequence (5′–3′) (forward/reverse)	Product size (bp)	Annealing temperature (°C)	Accession number
HIF-1α	CCCTACTATGTCGCTTTCTTGG GTTTCTGCTGCCTTGTATGGG	199	60	NM_001530.3
VEGF	GGCTCTGAAACCATGAACTTTCT GCAGTAGCTGCGCTGGTAGAC	165	60	NM_031836.2
SDF1	ACCTCGGTGTCCTCTTGCTG GATGTTTGACGTTGGCTCTGG	172	60	NM_001033883.1
bFGF	AAATCGCTATCTTGCTATGAAGGA GTTCGTTTCAGTGCCACATACC	153	60	NC_005101.2
ANGPT2	CCAATGGCATCTATACGCTAACA CCAGAAGGGTTCCCAAATCC	172	60	NC_005115.2
PLGF	GGGAACAACTCAACAGAAATGG CACTACAGCGACTCAGAAGGACA	158	60	NM_053595.2
SCF	AACCCTCAACTATGTCGCCG ATGCCACGAGGTCATCCACTA	172	60	M59966.1
GAPDH	ATGTTTGTGATGGGCGTGAAGGTCT TCTGGGTGGCAGTGAT	174	60	NM_017008.3

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California, USA). The proteins were separated by electrophoresis on a 6% polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ). The membranes were probed with an HIF-1α mouse monoclonal antibody (1:600 dilution, Abcam, Inc., Cambridge, UK) overnight at 4 °C. On the second day, the membranes were washed three times using TBS containing 0.1% Tween-20 detergent, and the secondary antibody was added to the membranes for 2 h. The protein bands were visualized with Kodak XOMAT film (Rochester, New York, USA). This same procedure was used for antibodies against other proteins, including VEGF, SDF-1, bFGF, PLGF, ANGPT1 and SCF (Abcam, Inc., Cambridge, UK). Each experiment was repeated at least twice with different cellular extracts.

2.5. Preparation of the BMSCs/GS constructs

Gelatin sponge (GS) scaffolds (Gelfoam; Upjohn, Kalamazoo, MI, USA) were molded into cylinders (Φ 5 mm × 2 mm) with a pinhole plotter. BMSCs (2.0×10^5 cells/mL) were slowly added to the GS cylinder until saturated. To allow for cell attachment, the cells were incubated with the GS for at least 4 h. These BMSC scaffolds constructs were used for the following animal studies. A parallel experiment was carried out to determine the characteristics of the GS cylinders. The GS cylinders were seeded with BMSCs at an identical cell density. After 4 and 24 h, the constructs were fixed in 2% glutaric dialdehyde for 2 h and observed with scanning electron microscopy (Philips SEM XL-30, Amsterdam, Netherlands).

2.6. Animal experiments

BMSCs 4 days after gene transduction were used for the *in vivo* experiments. As described previously [22], twelve-week-old male Fisher 344 rats were the subjects of the *in vivo* experiments. Briefly, the animals were anesthetized by intramuscular injection of xylazine (6 mg/kg of body weight) and ketamine (70 mg/kg of body weight). After aseptic preparation, a 1.0- to 1.5-cm semilunar incision was made through the skin and the muscle down to the cranial vertex. After exposing the calvarium, two 5 mm diameter CSDs were created using a 5-mm-diameter trephine bur (Fine Science Tools, Foster City, CA, USA). Twenty-four rats with two defects were generated and randomly allocated into the following graft study groups: (1) GS ($n = 6$), (2) GS/BMSCs/Lenti-GFP ($n = 6$), (3) GS/BMSCs/Lenti-WT ($n = 6$), and (4) GS/BMSCs/Lenti-CA5 ($n = 6$). Following surgery, the soft tissues were repositioned and sutured to achieve primary closure with 4-0 silk sutures. Each animal received an intramuscular injection of antibiotics post-surgically.

2.7. Microfil perfusion

To evaluate blood vessel formation, all 8-week rats were perfused with Microfil (Flowtech, Carver, MA, USA) after euthanasia. As previously described [25], after the hair of the chest was shaved, a long incision was made in the chest and abdomen (from the front limbs down to the xyphoid process). One side of the sternum was cut with scissors, and the rib cage was retracted laterally. The left ventricle was penetrated with an angiocatheter after the descending aorta was clamped. After the inferior vena cava was incised, 20 mL of heparinized saline was perfused. Next, 20 mL of Microfil was perfused at 2 mL/min following perfusion with saline. Finally, the samples were set overnight at 4 °C.

2.8. In vitro observation of gross specimen blood vessels

In order to detect blood vessel formation, the samples (half of the specimens for each group, $n = 6$) were processed using an alcohol-methyl salicylate clearing sequence. Briefly, samples from days 1, 2, 3, 4, and 5 were dehydrated in ascending solutions of ethyl alcohol from 25% to 100%. The samples were then immersed for 12–24 h in methyl salicylate. Digital pictures were acquired (Nikon digital SLR, D5100, Japan). The areas of newly formed blood vessels in the bone defect areas were measured using ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA).

2.9. Micro-computed tomography

The cranial samples were imaged with micro-CT (GE eXplore Locus SP Micro-CT, USA) to determine the formation of blood vessels. The decalcified samples were scanned at 1600 angular positions with settings of 45 Kv and 215 μA and with the number of frames set to 16. After scanning, the initial data were collected and reconstructed using Next Generation Imaging (NGI) software package version 1.4.59 (X-TEK Systems Ltd), with an average voxel size of 14 μm and the radiopacity represented by a 16-bit grayscale value. The 3-D images were reconstructed using GEHC MicroView software (GE Healthcare BioSciences, Chalfont St. Giles, UK). A region corresponding to a vessel perfused with Microfil was selected, and the binarization threshold for that region was determined (Fig. 4A). The binarization threshold and scanning parameters were kept identical for all samples. The blood vessel area and the vessel number were analyzed by micro-CT. Besides, bone volume to total bone volume (BV/TV) and local bone mineral densities (BMD) in the bone defect were also measured.

2.10. Histological observation

Four groups of twelve animals were sacrificed with xylazine and ketamine at 8 weeks post-surgery. Sections of undecalcified specimens were processed by dehydrating the skulls, which were treated with alcohol-methyl salicylate as described above, in ascending concentrations of alcohol from 75% to 100%. After dehydration, the calvaria were embedded in polymethylmethacrylate (PMMA), and 150-μm-thick sections in the orientation of the sagittal surface were obtained using a microtome (Leica, Hamburg, Germany). Three sections in the central area of each defect were used for the histometric analysis. The sagittal sections were polished to a final thickness of approximately 40 μm and stained with van Gieson's picrofuchsin to identify the formation of new blood vessels. Blue spots from Microfil perfusion indicate a blood vessel, and red indicates new bone formation. The surface areas, the numbers of newly formed blood vessels, and areas of newly formed bone were measured using Image Pro 5.0 (Media Cybernetics, USA) based on the entire bone defect area.

2.11. Immunohistochemical analysis

The other half of each group of samples ($n = 6$) was fixed in 4% paraformaldehyde in PBS for 24 h and decalcified in 15% EDTA for 2 weeks at 4 °C. After the decalcified samples were embedded in paraffin, 5 mm serial coronal cross sections were made using a microtome. Immunohistochemistry was performed as previously described [22]. Briefly, the sections were deparaffinized and incubated with primary antibodies against GFP or HIF-1α (1:100 dilution; Abcam, Inc., Cambridge, UK). ABC complex (Vector Laboratories, Inc., Peterborough, UK) was applied to the sections following incubation with a biotinylated secondary antibody (Dako Corporation, Carpinteria, CA), and DAB substrate (DAKO, Cambridge, UK) was used to stain the slides. The sections were then treated with hematoxylin and mounted.

2.12. Statistical analysis

The values are given as the mean ± SD. The statistical significance was assessed by ANOVA with Tukey's post-hoc test, where $P < 0.05$ was considered statistically significant. The following notation is used throughout this study: *, $P < 0.05$, and **, $P < 0.01$, when the target gene groups were compared to the control group; #, $P < 0.05$, and ##, $P < 0.01$, when the CA5 group was compared to the WT group.

3. Results

3.1. Cell culture and gene transduction

F344 rat BMSCs had characteristics of stem cells, including high expression of CD90 and CD105. Our previous data have showed that CD90 and CD105 were overexpression, whereas CD31 and CD34 are rarely detected in BMSCs of F344 rat [22]. After preliminary experiments to test for the correct dose of lentivirus, an MOI of 15 plaque forming units (pfu)/cell was determined to have the optimal effect on transfer efficiency *in vitro*. Four days after transduction with 15 pfu/cell Lenti-GFP, Lenti-WT, or Lenti-CA5, inverted fluorescence microscopy and flow cytometry indicated that the efficiency of target gene transfer in the BMSCs was greater than 90% (Supporting Information, Fig. S1). Analysis of the HIF-1α mRNA and protein expression levels was performed in Lenti-GFP-, Lenti-WT-, and Lenti-CA5-transduced BMSCs. HIF-1α overexpression was noted upon transduction with WT and CA5 on days 4, 7, 14, 21, and the expression showed a continual increase from day 4 to day 21. However, HIF-1α remained poorly expressed in the Lenti-GFP group, as shown in our previous data [22].

3.2. mRNA expression of angiogenic factors in vitro in BMSCs transduced with the target gene

The mRNA levels of key angiogenic factors were detected on days 0, 1, 4, 7, 14, and 21. In HIF-1α-transduced BMSCs, the expression of VEGF and SDF-1, which believed to be important factors for inducing vessel formation, was markedly increased on day 1 and continuously increased between day 4 and day 21 (Fig. 1A and B). SDF-1 displayed a remarkable increase in the WT- and CA5-transduced groups. Other pivotal angiogenic genes, such as bFGF, ANGPT1, PLGF, and SCF, showed the same pattern as VEGF and SDF-

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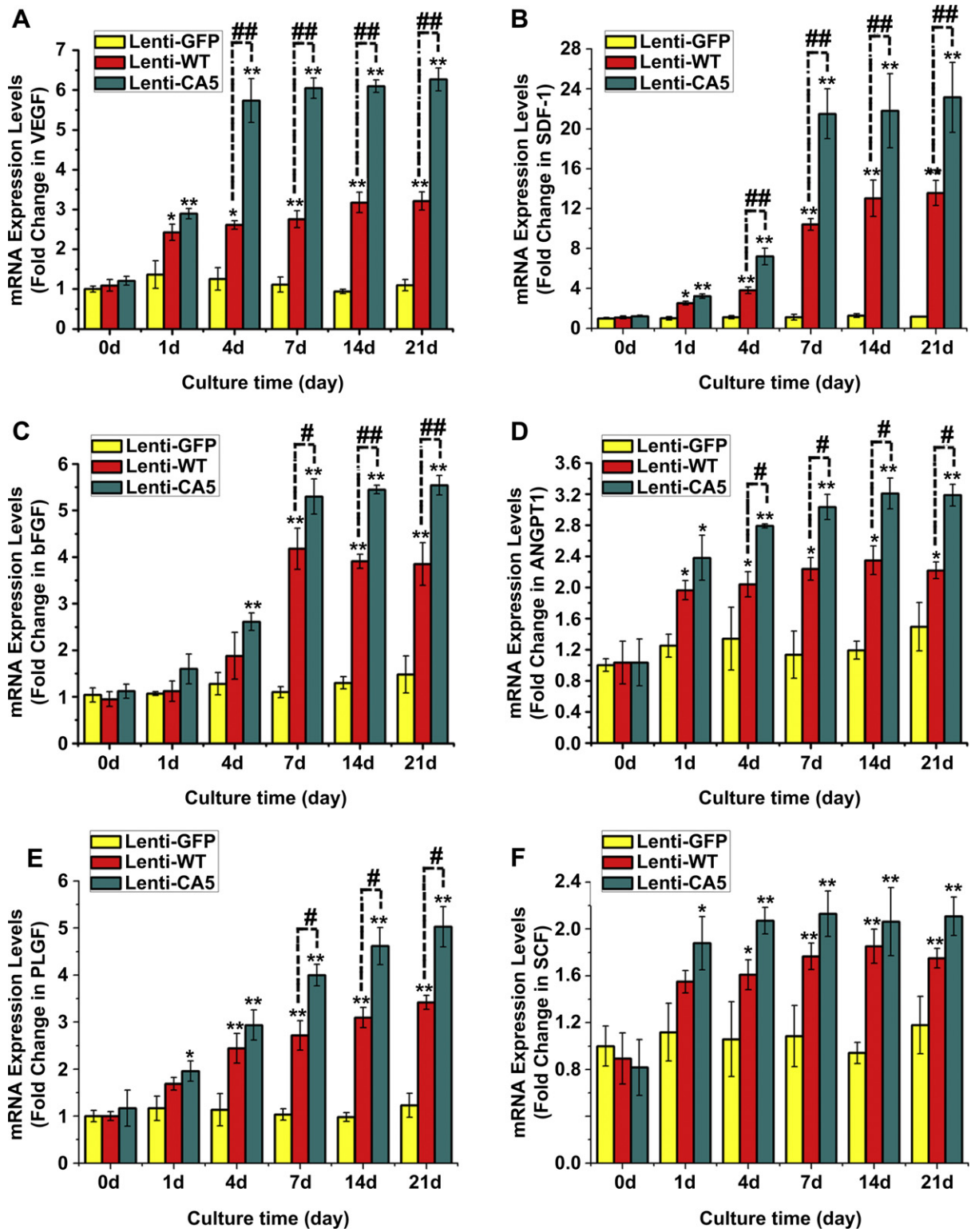

Fig. 1. Detection of mRNA from selected angiogenic markers in BMSCs. The mRNA levels of VEGF (A), SDF-1 (B), bFGF (C), ANGPT1 (D), PLGF (E), and SCF (F) were quantified (*, $P < 0.05$ and **, $P < 0.01$, the target gene groups compared to the GFP group; #, $P < 0.05$ and ##, $P < 0.01$, the CA5 group compared to the WT group).

1 (Fig. 1C, D, E and F). The mRNA expression of angiogenic factors in the CA5 group was higher than that in the WT group. However, the transcripts of angiogenic markers in the GFP group remained at low levels from day 0 to day 21. Taken together, these data support an angio-inductive effect of transduction with CA5.

3.3. Western blot analysis of angiogenic markers in vitro after gene transduction

To further detect angiogenesis, the protein expression of angiogenic factors was analyzed in the gene-modified BMSCs using

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samples from days 0, 1, 4, 7, 14, and 21. The protein levels detected were consistent with the qPCR data (Fig. 2A). Following gene transduction, VEGF levels were detected on days 0, 1, 4, 7, 14, and 21 in the Lenti-GFP-, Lenti-WT-, and Lenti-CA5-transduced groups. Quantitative analysis revealed a six- to seven-fold increase in VEGF in the Lenti-WT- and Lenti-CA5- transduced groups on day 21 (Fig. 2B-a). The expression of SDF-1 in the WT and CA5-transduced groups was enhanced seven- to eleven-fold compared to the GFP group (Fig. 2B-b). In addition, we investigated whether bFGF, ANGPT1, PLGF, and SCF were also up-regulated via the HIF-1 pathway. The results showed that the expression of bFGF was increased two- to three-fold in the target gene-transduced group compared to the GFP group (Fig. 2B-c). Similar results were observed for PLGF and SCF (Fig. 2B-d, e, and f). CA5 showed a significantly higher angiogenic effect in BMSCs compared to WT cells. Collectively, these data indicate that CA5 can enhance the expression of angiogenic proteins in BMSCs.

3.4. Cellular adhesion to the scaffold and animal experiments

The scanning electron microscope results revealed that the GS scaffold had a porous structure (Supporting Information, Fig. S2A). Twenty-four hours after the BMSCs were cultured on the GS, the cells had adhered to the scaffold surface *in vitro* (Supporting Information, Fig. S2B). There were nominal differences in cellular adhesion and proliferation between BMSCs transduced with GFP, WT, or CA5 and untransduced BMSCs. After the scaffolds were seeded with BMSCs and incubated at 37 °C in 5% CO_2 for 4 h, the compounds (GS; GS with BMSCs/Lenti-GFP; GS with BMSCs/Lenti-WT; and GS with BMSCs/Lenti-CA5) were implanted into the CSDs of the rat calvarial model (Supporting Information, Fig. S2C and D).

3.5. Specimen observation

After the skulls were treated with alcohol-methyl salicylate, photography showed that the amount of blood vessel growth in the target group was markedly greater than that in the GFP or GS groups. Furthermore, the CA5 group showed the greatest amount of blood vessel growth of the four groups (CA5, WT, GFP, and GS) (Fig. S3A–D). Analysis of the images using Image Pro 5.0 confirmed the results from the pictures (Fig. 3A a–d). The areas of newly formed blood vessels in the WT and CA5 groups were significantly larger than the areas in the GFP and GS groups, and the CA5 group showed the largest area of new growth (Fig. 3B) (**, $P < 0.01$; #, $P < 0.05$).

3.6. Micro-CT measurement

The effects of HIF-1α gene therapy on BMSCs vascularization of tissue-engineered bone 8 weeks after implantation were studied by perfusing the vessels with microfil and micro-CT imaging them at a 14 mm resolution. The reconstructions of the three-dimensional micro-CT images revealed blood vessel formation directly in the bone defects (Fig. 4B). The vascular network in the target gene groups appeared denser compared to the GFP or GS groups. Quantification of the newly formed vessels in the defect sites was conducted using morphometrical analysis. A significantly larger vascular network area was observed in the Lenti-WT- and Lenti-CA5-transduced BMSCs groups (Fig. 4C). The number of blood vessels in the Lenti-WT and Lenti-CA5 groups was also higher than in the other two groups (Fig. 4D). BV/TV and BMD demonstrated the same tendencies with the results of blood vessels (Fig. 4E and F). The data revealed that the CA5 group had the most significant increase in the total vessel volume inside the composites (**, $P < 0.01$; ##, $P < 0.01$).

3.7. Histological analysis and immunohistochemistry

The gross observations of the samples and the micro-CT findings were further supported by histological analysis (Fig. 5A). Under light microscopy, the number of blood vessels was 12 ± 1 blood vessels per 100× field in the Lenti-CA5 group, 8 ± 1 in the Lenti-WT

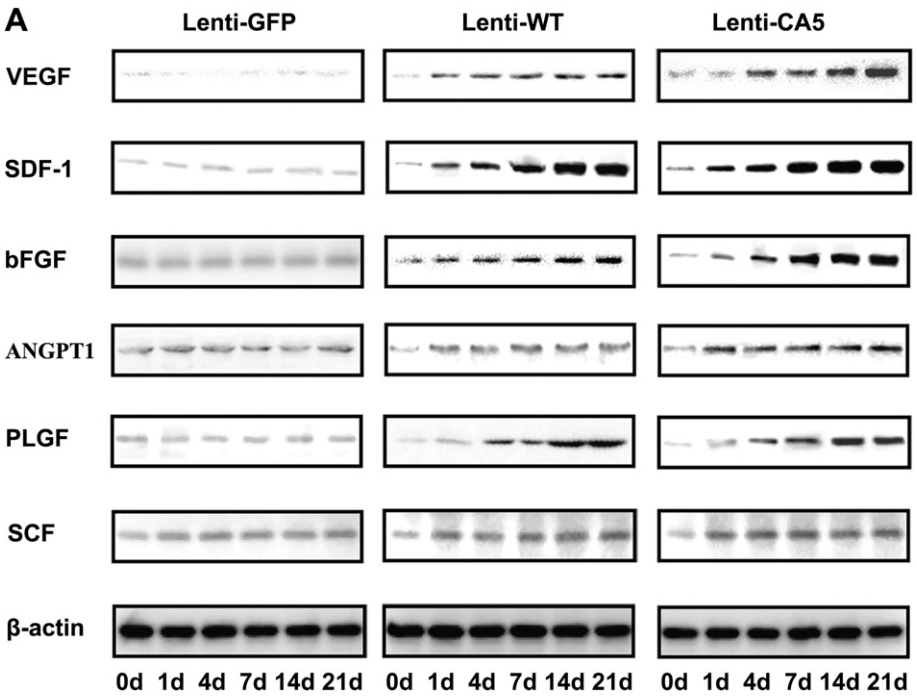

Fig. 2. Western blot to determine protein levels during angiogenesis. (A) A grayscale scan showed that VEGF, SDF-1, bFGF, ANGPT1, PLGF, and SCF proteins were expressed in BMSCs transduced with Lenti-WT, Lenti-CA5 and Lenti-GFP at 0, 1, 4, 7, 14, and 21 d after transduction. (B) Scan image software detected the gray values of the VEGF, SDF-1, bFGF, ANGPT1, PLGF, and SCF protein bands at each of the different time points. All of the values were normalized to β-actin (*, $P < 0.05$ and **, $P < 0.01$, the target gene groups compared to the GFP group; #, $P < 0.05$ and ##, $P < 0.01$, the CA5 group compared to the WT group).

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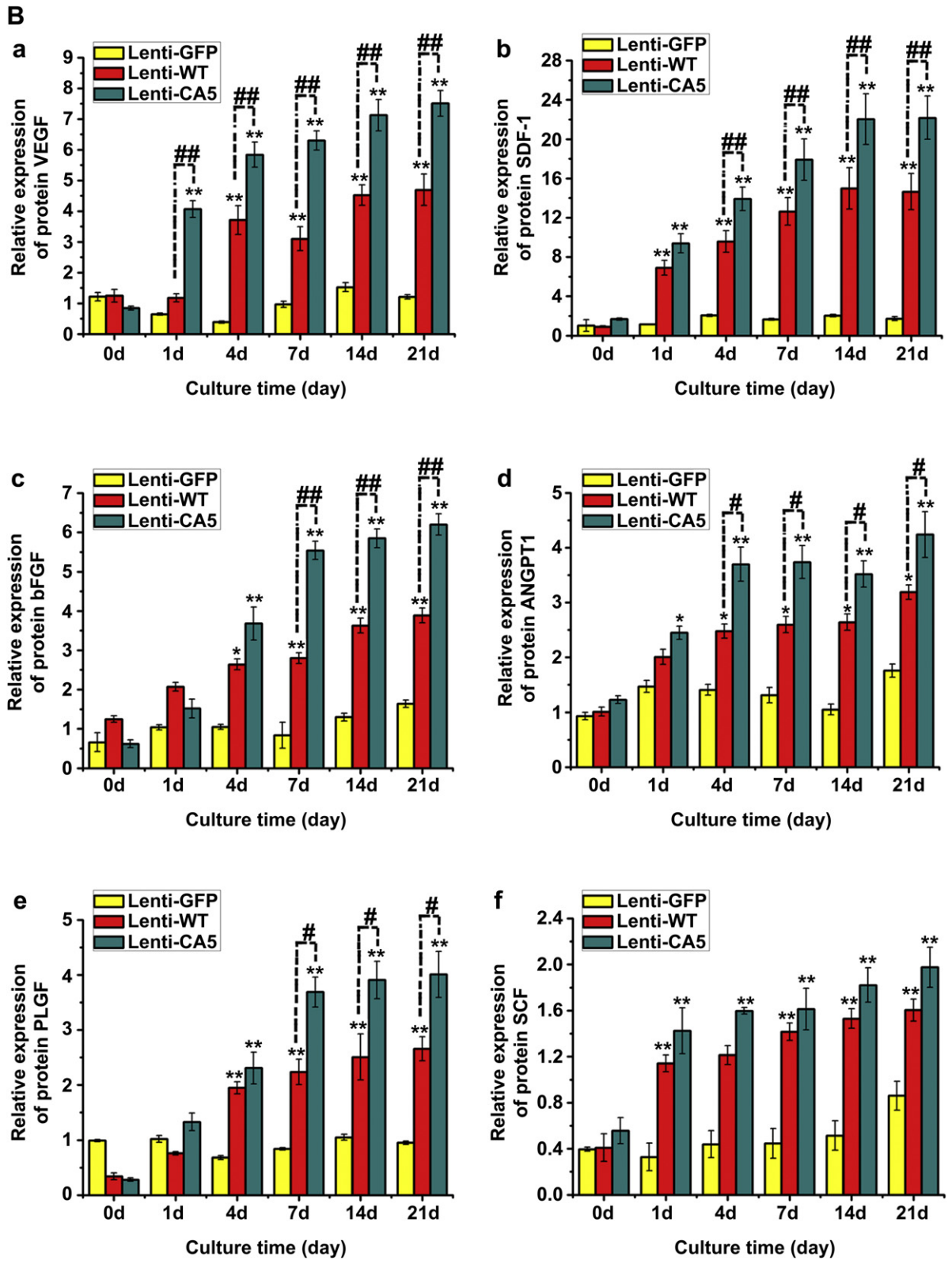

Fig. 2. (*continued*).

group, 4 ± 1 in the Lenti-GFP group, and 3 ± 1 in the GS group (Fig. 5B). The number of blood vessels in these sections was confirmed by the results of the vessel network area analysis (Fig. 5C). The percentage of new vessel area after 8 weeks was 1.23% ± 0.21% of the total area per 100× field in the GS group, 1.36% ± 0.25% of the total area per 100× field in the GFP group, 6.64% ± 0.33% of the total area per 100× field in the WT group, and 9.6% ± 0.27% of the total area per 100× field in the CA5 group. The

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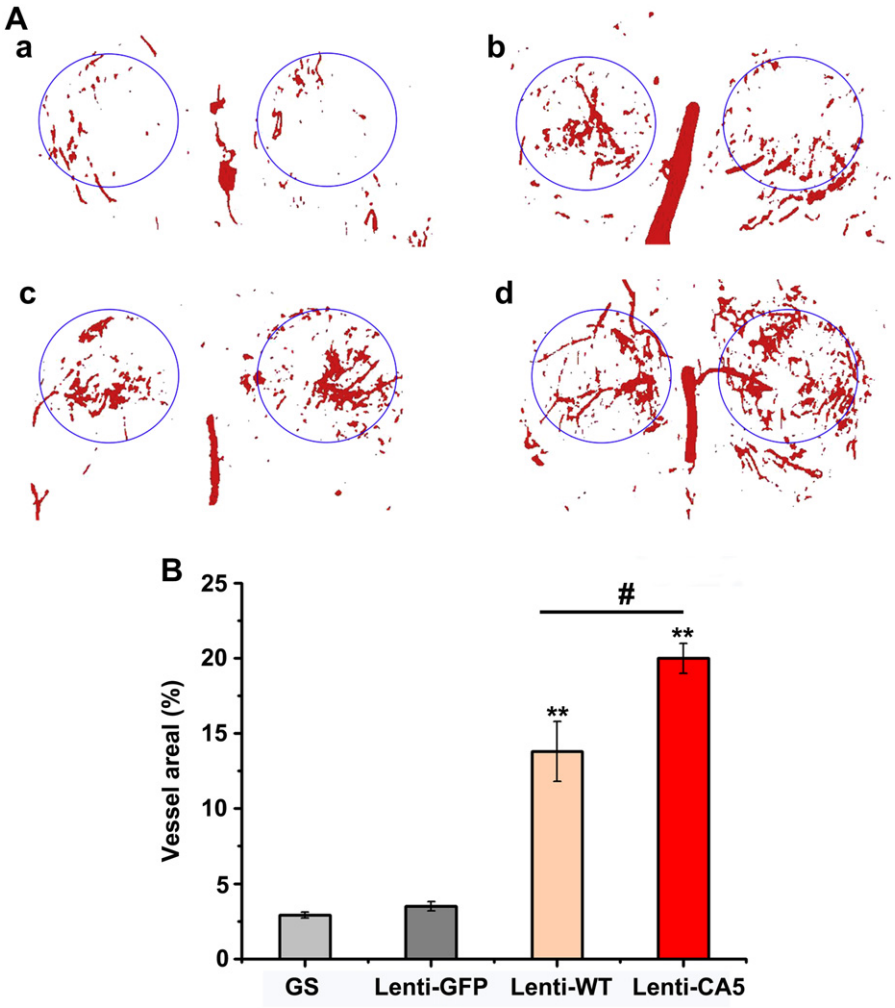

Fig. 3. Observation of blood vessels in the gross specimen. (A) The photographs were analyzed with ImageJ software: The GS group (a), the Lenti-GFP group, the Lenti-WT group, and the Lenti-CA5 group. (B) Analysis of the local vessel area in the CSD (*, $P < 0.05$ and **, $P < 0.01$, the target gene groups compared to the GFP group or the GS group; #, $P < 0.05$ and ##, $P < 0.01$, the CA5 group compared to the WT group).

newly formed bone area was, 57.43 ± 0.21% of total area per 100× field in the CA5 group, 40.82 ± 0.57% of total area per 100× field in the WT group, 14.67 ± 0.52% of total area per 100× field in the GFP group, and 2.12 ± 0.36% of total area per 100× field in the GS (Fig. 5D).

To detect the BMSCs that were implanted in the defect sites, immunohistochemistry of GFP was used. GFP (Lenti-GFP, Lenti-WT and Lenti-CA5) were apparent in the new bone matrix or fibrous tissue in the WT-, CA5- and GFP-transduced BMSCs groups (Fig. 6B–D) 8 weeks post-surgery, but negative staining was found in the GS group (Fig. 6A). There was intensely increased HIF-1α expression in the Lenti-WT and Lenti-CA5 groups in both the bone matrix and the surrounding fibroblastic-like tissue (Fig. 6G and H). However, in the Lenti-GFP and the GS groups there was no obvious positive staining for endogenous HIF-1α (Fig. 6E and F).

4. Discussion

The development of new therapeutic approaches that can enhance vascularized tissue growth has become one of the most active areas of tissue engineering. Due to the close association between angiogenesis and osteogenesis, neovascularization is considered as an important element in the repair of bone defects [26,27]. This study investigated the angiogenesis of HIF-1α-transduced BMSCs in tissue-engineered bone that was used to repair a CSD. Our results indicate that overexpression of HIF-1α may be a useful method to enhance angiogenesis and vascularization in tissue-engineered bone.

HIF-1 is a key transcription factor that mediates the adaptive responses to hypoxia and ischemia and regulates the expression of many angiogenic genes, including VEGF, PLGF, ANGPT1, and SDF-1 [8,28]. However, previous studies have found that HIF-1 is sensitive to oxygen levels and is easily degraded under normoxic conditions [20]. To maintain the stability and activity of HIF-1α in normoxic conditions, we constructed point mutations within HIF-1α (proline 564 to alanine, proline 402 to alanine and aminosuccinic acid 803 to alanine) and a mutant containing a truncation of HIF-1α (a deletion of amino acids 392–520 and 2 substitutions, Pro567Thr and Pro658Gln) [22,23]. Our previous studies showed that the truncated form of HIF-1α was more effective than the point mutant HIF-1α in terms of increasing the stability and activity of HIF-1α under non-hypoxic conditions. Therefore, we chose to use the truncated mutant allele of HIF-1α, CA5, as the target gene for increasing angiogenesis of BMSCs in tissue-engineered bone through gene therapy.

Angiogenic growth factors are implicated in neovascularization and in endochondral ossification. The angiogenesis is a co-

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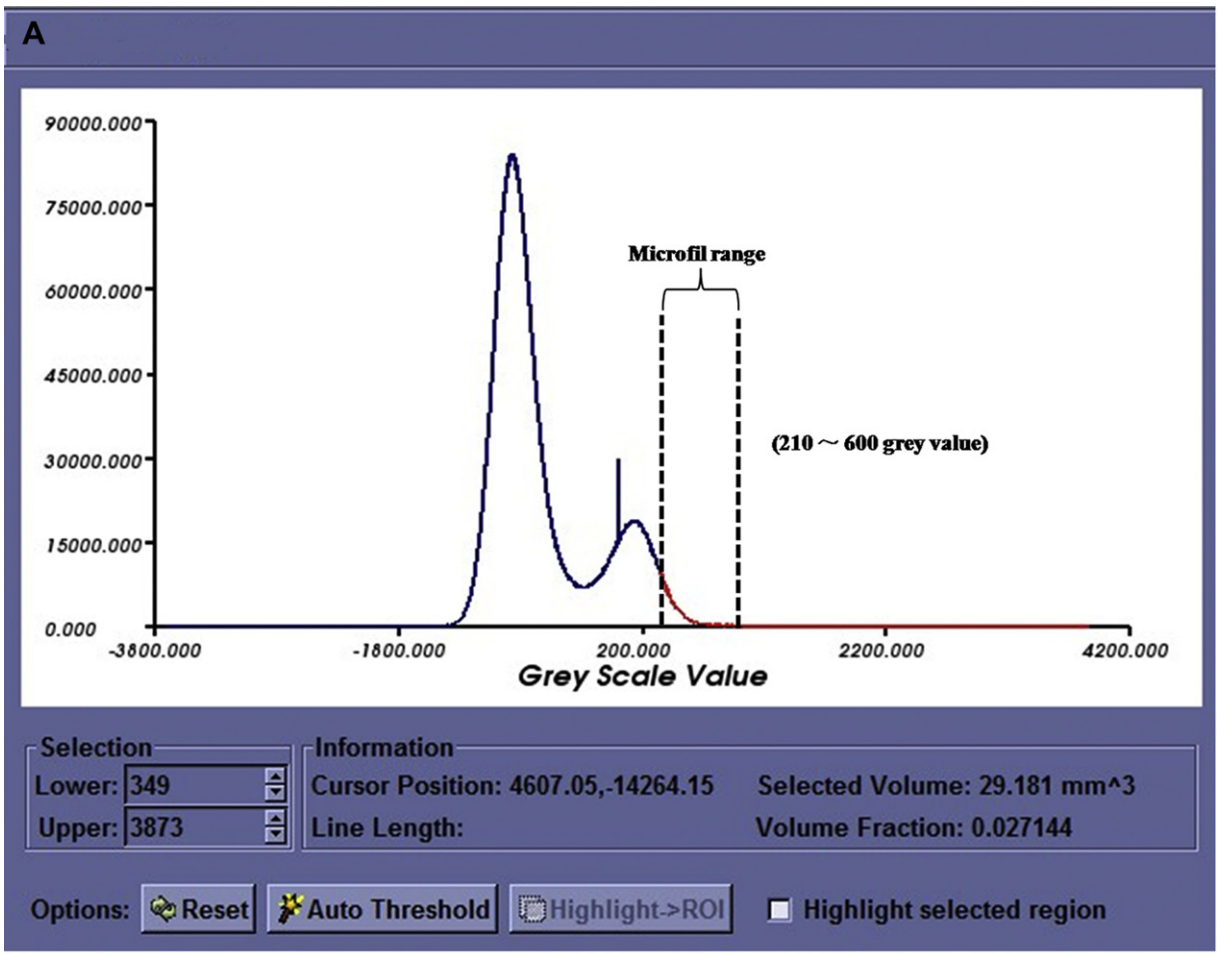

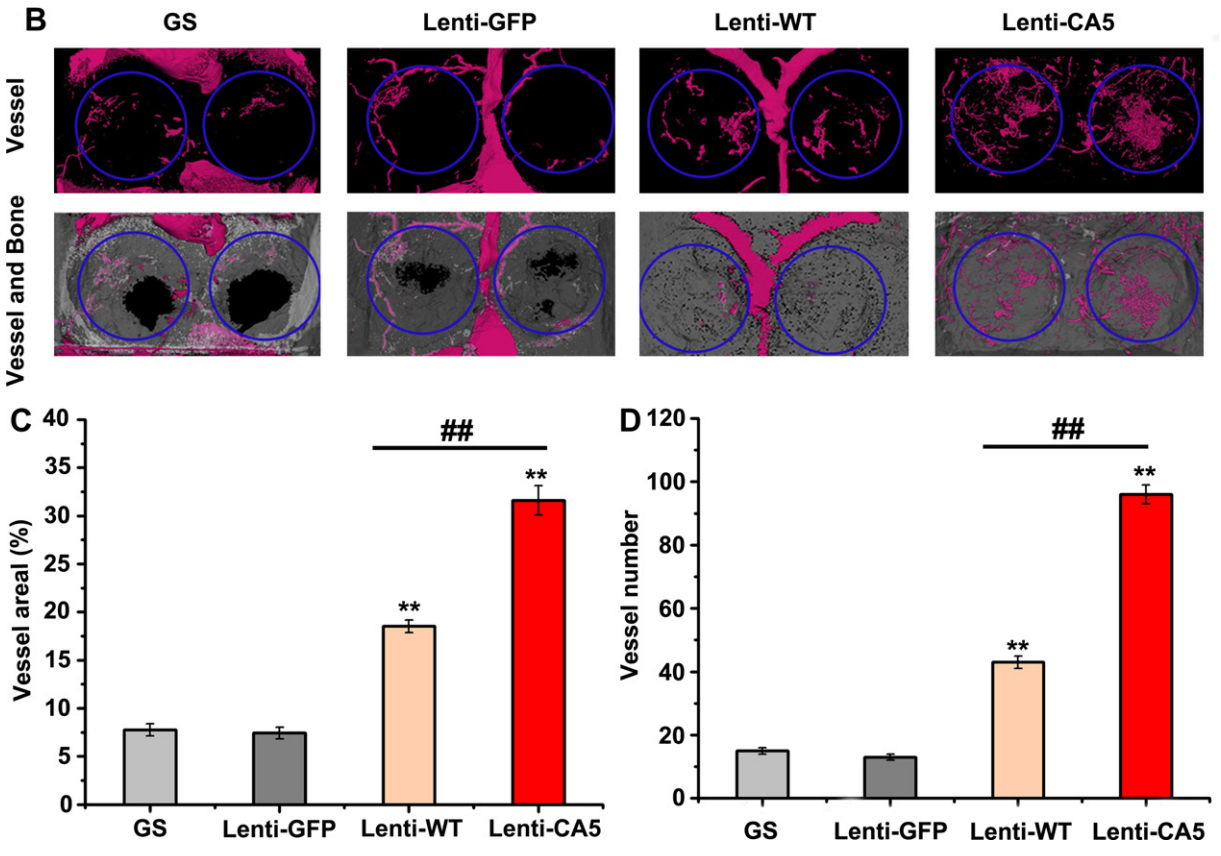

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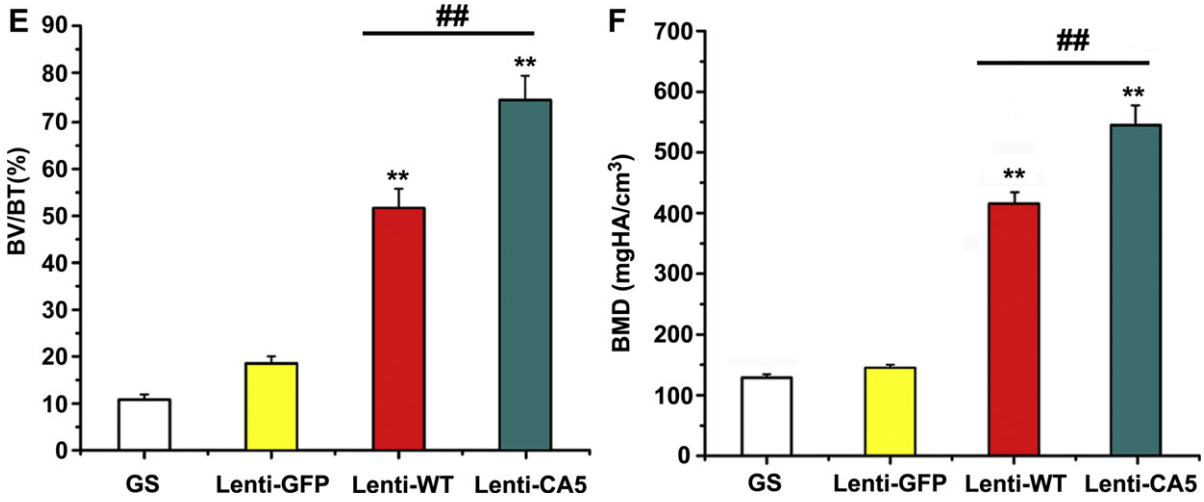

Fig. 4. (*continued*).

ordinated process that requires the participation of multiple angiogenic factors. It is important to detect whether HIF-1α is in fact inducing BMSCs overexpression of a series of angiogenic factors *in vitro*. The results from analyzing mRNA and protein levels have confirmed that CA5 can enhance BMSCs angiogenesis *in vitro*. VEGF is regarded as a key angiogenic factor that has the strongest and most significant biological activity in enhancing blood formation [29]. The VEGF mRNA level was increased two- to seven-fold in the target gene-treated BMSCs group from day 4 to day 21. SDF-1, which induces neovascularization and angiogenesis by recruiting endothelial progenitor cells (EPCs) to the site of injury and ischemia [30], showed the greatest response with a ten- to twenty- fold increase on day 21. Additionally, bFGF, an important factor in angiogenesis, has been reported to have an even more profound effect on vessel growth than VEGF at this early time point [31]. From days seven to twenty-one, bFGF levels increased three- to four- fold in the HIF-1α-transduced groups compared to the GFP group. Moreover, other important factors were also significantly up-regulated in the target gene-transduced group. These factors included ANGPT1, which plays an important role in regulating endothelial cell survival and also reduces VEGF-mediated vascular permeability [32]; PLGF, which is a close homolog of VEGF and shares VEGF's function in stimulating angiogenesis [33]; and SCF, which has an important role in hematopoiesis [34]. The protein levels of these factors had a nearly identical pattern to the mRNA levels. These data show that HIF-1α can induce significant angiogenesis of BMSCs *in vitro*. Furthermore, CA5 demonstrated higher HIF-1α stability under normoxic conditions as measured by both mRNA and protein levels. The expression of the angiogenic markers was stronger in the CA5 group than in the WT group. This result suggests that the CA5-modified BMSCs achieved better angiogenic function than the WT group *in vitro*. It will be interesting to further explore the effects of angiogenesis in tissue-engineered bone.

To test the effectiveness of HIF-1α modified BMSCs in inducing angiogenesis in tissue-engineered bone, an *in vivo* study on angiogenesis in a CSD was performed in a rat model. GS is an organic material; specifically, it is a gelatin-based sponge that is prepared from purified, type A pork skin gelatin [35]. GS has been widely used in clinical applications, such as to control bleeding and dress wounds. Furthermore, GS has been also widely used as a scaffold material because of its flexibility, biocompatibility, biodegradability, low residue once osteofied, and lack of a foreign-body reaction [36,37]. In this study, GS was used as a scaffold to detect angiogenesis induced by HIF-1α-modified BMSCs in a CSD. The specimens, which were treated with alcohol-methyl salicylate, indicated that the CA5 group had the greatest angiogenic effect compared to the other groups (WT, GFP, and GS) (**, $P < 0.01$; #, $P < 0.05$). The percentage of new vessel area after 8 weeks was 20.11 ± 2.16% in the CA5 group, 14.18 ± 3.47% in the WT group, 3.63 ± 1.15% in the GFP group, and 2.81 ± 0.54% in the GS-alone group. Consistent with observations of the specimens, a quantitative analysis by micro-CT of both the vessel area and the vessel number revealed that there was more vascularization in the CA5 group than in the WT, GFP, and GS groups (##, $P < 0.01$; **, $P < 0.01$). The number of blood vessels was 96 ± 6 in the Lenti-CA5 group, 42 ± 5 in the Lenti-WT group, 12 ± 2 in the Lenti-GFP group, and 13 ± 3 in the GS group. The percentage of new vessel area was 7.68% ± 2.21% in the GS group, 7.46% ± 2.35% in the GFP group, 17.82% ± 4.33% in the WT group, and 31.23% ± 5.27% in the CA5 group. A histological examination also confirmed that CA5 effectively enhanced the vascular response in tissue-engineered bone and bone regeneration in the CSD. BMSCs have been widely used as seed cells in tissue engineering studies because they are multipotent stem cells. In this study, the presence of the implanted BMSCs and the target gene was detected using immunohistochemistry. The results were consistent with our hypothesis that new blood vessels increased following overexpression of the target gene in BMSCs implanted in a CSD. The *in vivo* results systematically validated our hypothesis that the overexpression of CA5 in BMSCs can enhance angiogenesis in tissue-engineered bone.

Angiogenesis is regulated by activating a variety of signaling pathways including prostaglandins E1 and E2, TGF-β, bone morphogenetic proteins (BMPs), bFGF, IGF-1, and endothelin-1

Fig. 4. Micro-CT evaluation of the blood vessels 8 weeks after implantation. The binarization threshold was set to a grey value of 2000. (A) The GS constructs, Lenti-GFP-transduced BMSCs/GS constructs, Lenti-WT-transduced BMSCs/GS constructs, and Lenti-CA5-transduced BMSCs/GS constructs are shown from left to right. (B) Micro-CT images of the calvarial defects were taken 8 weeks after implantation. Morphometric analysis was completed for the local vessel area (C), the vessel number (D) in the bone defect area, new bone formation (E) and local bone mineral density (F). There were significant differences between the target gene groups and the GS or GFP groups (*, $P < 0.05$, the target gene groups compared with the GFP group or the GS group).

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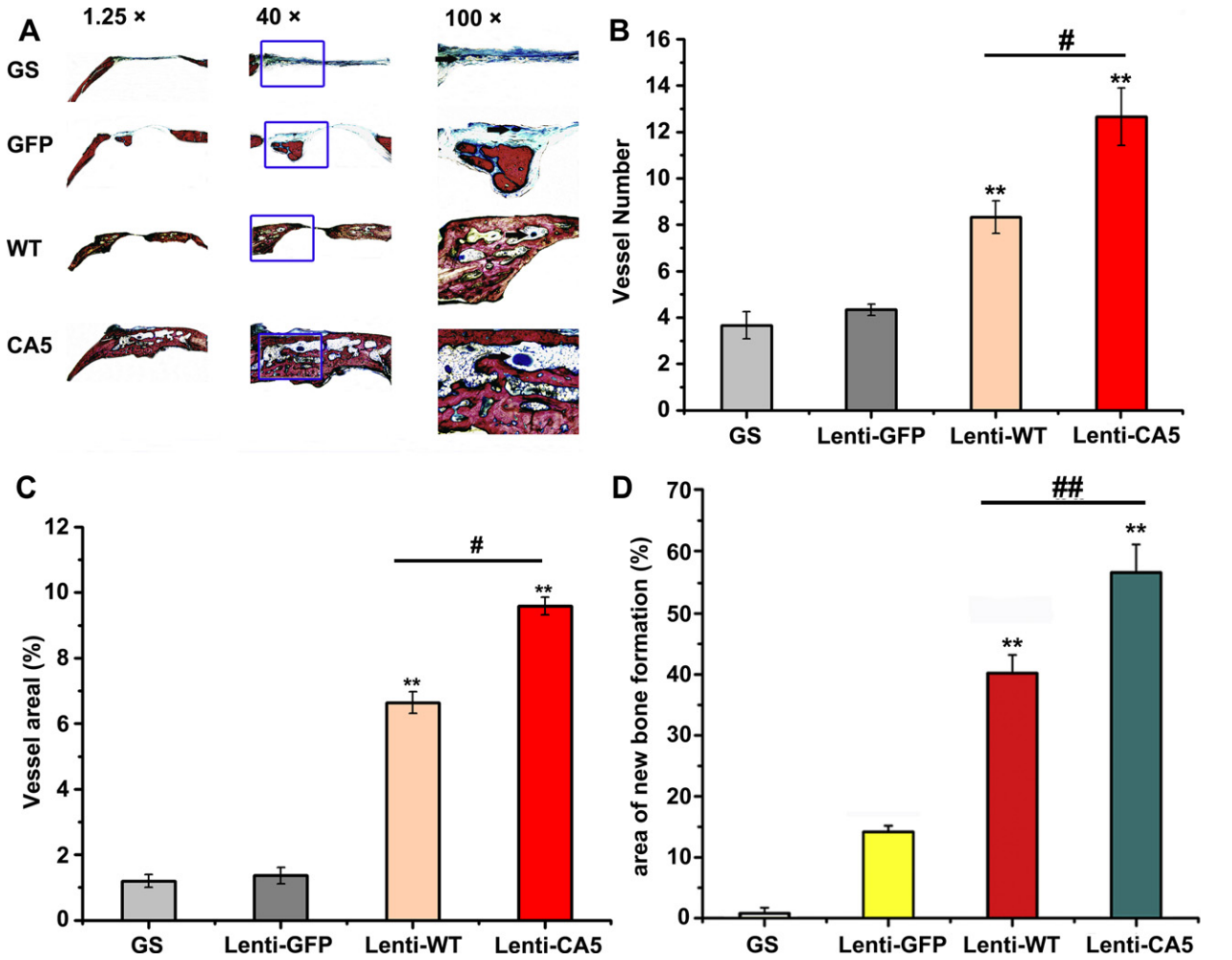

Fig. 5. Histological analysis of new blood vessel formation 8 weeks after implantation. (A) The undecalcified specimens were sectioned, and the sections were stained with van Gieson's picrofuchsin. From top to bottom: GS constructs, Lenti-GFP-transduced BMSCs/GS constructs, Lenti-WT-transduced BMSCs/GS constructs, and Lenti-CA5-transduced BMSCs/GS constructs (1.25× 40× and 100×). The number of new blood vessels (B), the total blood vessel area formed (C) and new bone formation area (D) per 100× field in the sections. There were significant differences between the WT or CA5 groups and the GS or GFP groups (**, $P < 0.01$, the target gene groups compared to the GFP or GS groups). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

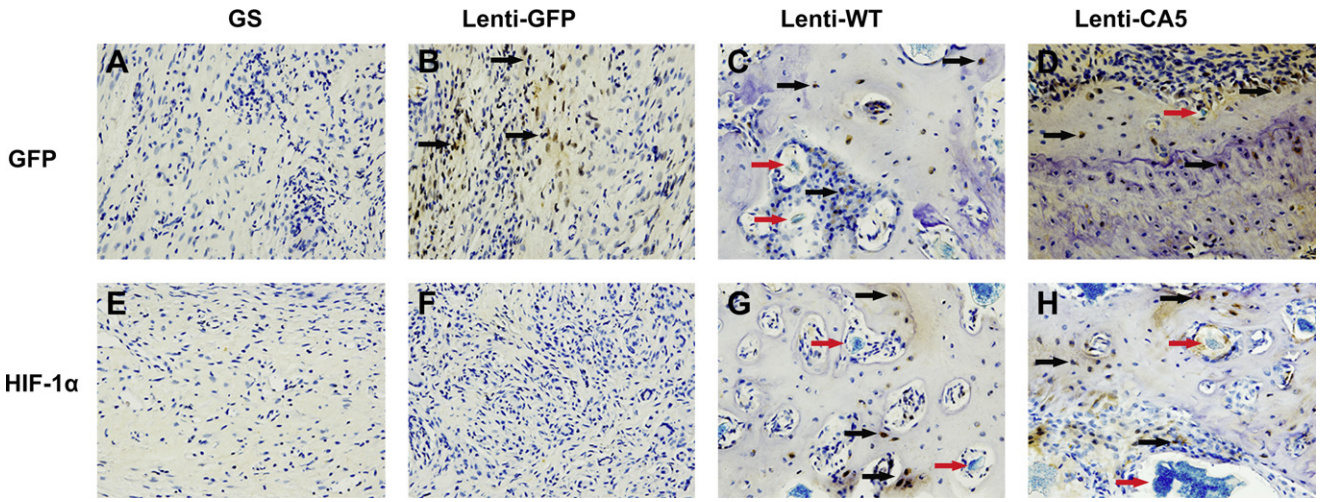

Fig. 6. Immunohistochemical analysis of GFP and HIF-1α expression in each group 8 weeks post-operation. Immunostaining for GFP of (A) the scaffold alone group, (B) the Lenti-GFP group, (C) the Lenti-WT group, and (D) the Lenti-CA5 group. The GFP, WT, and CA5 groups showed positive, brown staining in the fibroblast-like tissue and the bone matrix (black arrow). HIF-1α staining demonstrates high levels of HIF-1α in both the bone and the surrounding fibroblast-like tissue (black arrows) in (G) the Lenti-WT group and (H) the Lenti-CA5 group. There was no obvious positive staining in (E) the GS group or (F) the GFP group. The red arrows indicate the blood vessels in the sections (a–h, 400×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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[38]. And all of these pathways involve the HIF-1α pathway [39]. Due to the close association between angiogenesis and osteogenesis, angiogenesis is an indispensable element of bone regeneration [40,41]. In this study, the results of newly formed bone in a CSD were coupled with blood vessels in tissue-engineered bone. Previous studies have confirmed that HIF-1α can markedly enhance both the vessel number and the luminal area in ischemic tissue following a local injection of HIF-1α vectors [42–44]. However, to our knowledge, there lacks reports about angiogenesis in tissue-engineered bone using HIF-1α over-expressed in BMSCs. This study shows that the HIF-1α gene is able to stimulate angiogenesis in tissue-engineered bone, thus enhance osteogenesis in a CSD. Angiogenesis of HIF-1α gene-transduced BMSCs mainly exerts its function via direct effects on BMSCs. After the downstream target genes of HIF-1α are activated, under the participation of multiple angiogenic factors, new blood vessels formation are promoted in tissue-engineered bone which in turn led to enhance bone regeneration by providing nutrients, osteogenic cues, and numerous types of cells. It would be a challenge, however, for future work to identify the clear signals emanating from BMSCs that promote angiogenesis.

5. Conclusions

In summary, this study demonstrates that HIF-1α overexpression in BMSCs by gene therapy can increase angiogenesis in tissue-engineered bone, thereby promoting bone regeneration in a CSD *in vivo*. At the molecular level, the mRNA and protein expression of angiogenic factors in BMSCs were significantly up-regulated by CA5 *in vitro*. These results provide preclinical data for the potential applications of BMSCs engineered with CA5 to promote vascularization, vascular remodeling, and bone defect repair.

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Appendix. Supplementary information

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.11.053.

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