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Original Article

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Endothelial cells modified by adenovirus vector containing nine copies hypoxia response elements and human vascular endothelial growth factor as the novel seed cells for bone tissue engineering

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Abstract

Vascularization is one of the hotspots during the development of new therapeutic strategies for bone tissue engineering, which can alleviate hypoxic circumstance and prevent transplant failure. Vascular endothelial growth factor (VEGF) gene transfection using recombinant adenovirus (Ad) vector can effectively promote angiogenesis, but uncontrolled long-term continuous expression of VEGF brings safety concern. Here we constructed a recombinant Ad vector containing nine copies of HRE promoter and the *hVEGF165* gene, which conserved the oxygen sensitivity of hypoxia-inducible factor-1/hypoxia response elements (HIF-1/HRE). After transfection into human umbilical vein endothelial cells (HUVEC), the hVEGF165 mRNA and protein levels were much higher in response to hypoxia, as revealed by RT-PCR and ELISA, respectively. Furthermore, Ad-9HRE-hVEGF165 vector effectively promoted proliferation, migration and tube formation of HUVEC under hypoxic conditions. Thus we believe that the Ad-9HRE-hVEGF165 vector can contribute to the regulation of vascularization, which may provide a new approach for a better control of the expression of hVEGF165 during bone tissue engineering.

Key words: vascular endothelial growth factor, hypoxic response element, bone tissue engineering, hypoxia

Introduction

It is fundamental for human body to maintain the oxygen homeostasis [1], as it influences the physiology of growth and development, energy metabolism, erythropoiesis, angiogenesis, and cell survival [2]. Fracture, acute injury and tumor sever local vasculature in bones, leading to regional hypoxia [3]. Meanwhile, these illnesses can cause bone defect as hypoxia zones. During the process of bone repair, hypoxia results in bone disunion, leading to transplant failure and limits the development of bone tissue engineering.

In order to cope with hypoxia, organisms undergo a variety of systemic and local changes to restore oxygen homeostasis and limit the effect of low O_2 [4]. Angiogenesis is a therapeutic approach for

maintaining oxygen homeostasis, providing oxygen to avoid cell death and relieve hypoxia. As a major promoter of vascular development and angiogenesis, vascular endothelial growth factor (VEGF) plays an important role in bone repair, since angiogenesis and osteogenesis are coupled processes. Neutralizing antibodies of VEGF receptor impair blood vessel formation and bone regeneration [5], whereas exogenous VEGF treatment enhances the bone formation within bone defects [6]. Thus, VEGF signaling is essential for the maintenance of bone remodeling.

Studies mentioned above demonstrated that the process of bone healing can be manipulated by control of VEGF levels. Thus, targeting VEGF in bone is of value in cases of defective bone repair and bone regeneration enhancement. A large body of studies showed that VEGF expression is increased under hypoxia, and hypoxia-inducible factor-1α (HIF-1α) is the key mammalian transcription factor controlling VEGF during low oxygen tension. HIF-1α binds to hypoxia response elements (HRE) in the enhancers of those genes to increase their transcription. Based on those pioneer studies, we have constructed HRE-containing vector to effectively control the expression of *VEGF* gene under hypoxia [7].

In this study, we constructed a promoter containing a concatemer of nine copies of the consensus sequence of HRE isolated from the erythropoietin enhancer and an SV40 minimal promoter. A recombinant Ad-9HRE-VEGF165 vector was then constructed with *hVEGF165* gene downstream driven by the new promoter. After transfection, we detected the expression of *VEGF165* in human umbilical vein endothelial cells (HUVEC) under hypoxia and normoxia conditions. Furthermore, we also explored whether Ad-9HRE-hVEGF165 affects *in vitro* angiogenic processes such as proliferation, migration and tube formation in human endothelial cells (ECs).

Materials and Methods

Recombinant Ad vector

Based on the published HRE sequence from the 3′ enhancer region of the *Epo* gene, we designed pairs of oligonucleotides that contain three tandem repeats of the HRE (G CCC TAC GTG CTG TCT CAC ACA GCG CCT GTC) [8]. And 9× HREs were generated by tandem ligation of the 3× HRE oligonucleotide pairs. We synthetized SV40 (5′-AAACGTTTTCGGATCCGGAGGTTTTTTCGGAGGAGTGATG AAGACCTTATCGAGTCTCCGGCTCCGCCGGAGCCGGAGAC GTATTTATTTTTTTTTAATCAGTCGCTACCCCGCCTCTTACCC GCCTTCACCCGCCTCAATCCCCGC-3′). These HRE elements were constructed upstream with SV40, and then HRE-SV40 sequences were cloned into pDC315 with replacing the original promoter by *Xba*I and *Eco*RI which situated upstream of the GFP. This construct was named as pDC315-9HRE-SV40-GFP (**Fig. 1**). To construct pDC315-9HRE-VEGF-GFP, the *VEGF165* gene was amplified using primer pair 5′-GTTCTGTCGTCTTTCAAGTATTAAG TTCGAAAAACGTTT-3′ and 5′-CTACACTGTTCGGCTCCG CCCTTAAGTACCACTCGTTCCC-3′ from template by PCR. The *VEGF165* cDNA fragments flanked by restriction enzyme sites *Eco*RI were inserted into pDC315-9HRE-GFP, followed by digesting with *Xba*I and *Kpn*I. Objective fragments were then connected with pShuttle vector. The shuttle vector pShuttle-HRE-SV40-VEGF-GFP was constructed and digested with *Pme*I. Linearized shuttle vector was transfected into BJ5183 cells with adenoviral genomic plasmid of pAdEasy (**Fig. 2**). The identified recombinant DNA was transfected into 293 cells to package adenovirus. Viral vectors were purified and concentrated by CsCl gradient centrifugation [7].

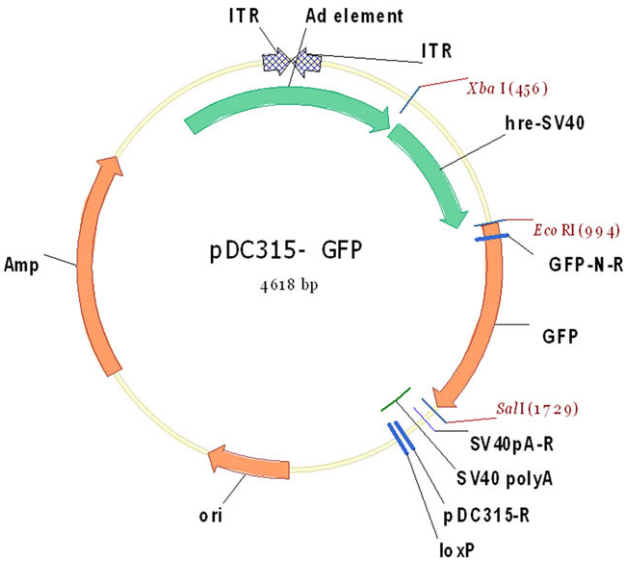

Figure 1. Structure of the pDC315-9HRE-SV40-GFP plasmid

Figure 2. Structural map of plasmid pDC315-9HRE-SV40-hVEGF165-GFP-Ad

Cell culture

HUVECs were maintained in M199 medium (Life Technologies, Grand Island, USA) supplemented with 10% FBS (Gibco, Grand Island, USA) and 100 ng/ml heparin (Life Technologies). Cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. For hypoxic condition, cells were cultured in an atmosphere containing 1% O_2 and 5% CO_2, achieved by substituting O_2 with N_2 in an O_2-controlled multi gas incubator.

Transient transfection

Cells were divided into four groups, transfection with Ad-9HRE-VEGF165-GFP group or control group, and culture in normoxia or hypoxia. Cells (2×10^5 cell/well) were seeded into six-well plates and grown for 16–18 h until cells reached log phase growth and the cell density was 50% confluent. Optimal conditions for adenovirus gene transfer concentration and virus incubation time were evaluated in preliminary experiments. The cells were transduced with 40 multiplicity of infection (MOI), then cultured in 1% O_2 or normoxic incubator for 48 h, respectively.

After 48 h of transfection with Ad-9HRE-VEGF165-GFP, HUVECs were visualized and photographed by fluorescence microscopy. Transfected and control cells were then trypsinized, washed, and resuspended in phophate-buffered saline. Ten thousand cells were collected using a BD FACSCalibur Flow Cytometry System (BD Biosciences, Franklin Lakes, USA) for both transfected and control cells. The percentage of GFP-positive cells was calculated by defining a negative GFP fluorescence threshold on control cells and gating out debris. All experiments were performed in duplicate.

ELISA

The level of hVEGF165 secreted from the transfected HUVECs was tested using VEGF ELISA kit (R&D system, Minneapolis, USA). The supernatant of the transfected HUVECs was collected at 48 h. The supernatant of non-transfected HUVECs was used as the control. The assay was performed according to the manufacturer's instructions. The absorbance was measured by a multi-label counter at a wavelength of 450 nm.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted at 48 h from cells of four groups using the combination of Trizol Reagent (Invitrogen, Carlsbad, USA) and RNeasy Mini Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was generated from 1 μg of total RNA using M-MLV reverse transcriptase (Invitrogen). Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using SYBR Green Supermix (Bio-Rad, Hercules, USA), according to the supplier's recommendations. The reactions were run in triplicate in an iCycler iQ Real-Time PCR Detection System (BD Bioscience). PCR amplification was performed using the *hVEGF165* primer pair 5′-TGAACCGTACCACC TCCATC-3′ and 5′-TGAACCGTACCACCTCCATC-3′. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative expression level for each target gene.

MTT assay

MTT assay was used to measure the cell viability. Briefly, HUVECs (5000 cells/well) were seeded in 96-well plates in complete medium (M199 supplemented with 1% FBS and 100 ng/ml heparin) and cultured overnight. Then cells were exposed to hypoxia or normoxia for 48 h. MTT solution (10 μl) was added to a final concentration of 5 mg/ml, and the cells were incubated for another 4 h. The medium was then removed, and 150 μl of dimethyl sulfoxide (DMSO) was added and incubated for 30 min at room temperature. The absorbance of the samples was measured at 492 nm using a microplate reader.

Wound scratch assay

HUVECs were seeded into six-well plates, cultured to 90% confluence and scratched using a sterilized tip. After being washed with phosphate-buffered saline (PBS), cells were re-cultured with M199 medium supplemented with 1% FBS and 100 ng/ml heparin for 24 h in hypoxia or normoxia. Photographs were taken from three random fields. The wound areas were measured by Image J. The net wound closure under each condition was calculated by subtracting wound area at 24 h from wound area at time zero. The wound closure rate was expressed as percentage of net wound closure within wound area at time zero.

Tube formation assay

For *in vitro* tube formation assay, 96-well plates were coated with 60 μl/well Matrigel (BD Biosciences Pharmingen, San Diego, USA) and incubated at 37°C for 1 h. After solidification, HUVECs with different treatments were placed on the Matrigel and incubated in hypoxia or normoxia for 12 h to form the tubes. The average number of tubes was measured in five randomly selected fields per group under a microscope.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) unless otherwise indicated. The data were analyzed by analysis of variance (ANOVA) and '*T*' test using SPSS v11.0 (SPSS Inc. Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

Infection efficiency and validation of Ad-9HRE-VEGF165-GFP vectors

Cultured HUVECs were infected with 1×10^8 pfu/ml of Ad-9HRE-VEGF165-GFP and control vector under hypoxic conditions. To identify the infection efficiency, HUVECs were examined under a fluorescence microscope after 48 h of infection. As shown in **Fig. 3**, positive GFP signal was readily visible in up to 95% cells in both groups.

Next, we compared the percentages of GFP fluorescence-positive cells under hypoxia versus normoxia in Ad-9HRE-VEGF165-GFP-vector-infected group by flow cytometry assay. The percentage of GFP-positive cells in the hypoxia group was up to 99%, while only 30% cells expressed GFP in the normoxia group (**Fig. 4A,B**). These data indicated that SV40 minimal promoter and HRE increased the amount of gene expression tremendously under hypoxic condition.

Determination of hVEGF165 expression in HUVECs

After 48 h of exposure to hypoxia or normoxia, vector-infected cells were collected and *hVEGF165* mRNA expression was measured by RT-PCR. As shown in **Fig. 5A**, the expression of *hVEGF165* mRNA in the hypoxia group was obviously higher than that in the normoxia group ($P < 0.05$). hVEGF165 secretion from transfected HUVECs was determined by ELISA. Consistent with the mRNA result, hVEGF165 protein level in culture supernatant in the hypoxia group was significantly higher than that in the normoxia group ($P < 0.05$) (**Fig. 5B**). The mRNA and protein level of hVEGF165 expression were hardly detectable in the control group regardless of hypoxic or normoxic condition (**Fig. 5**). These data indicated that under hypoxic condition the expression of hVEGF165 mRNA and

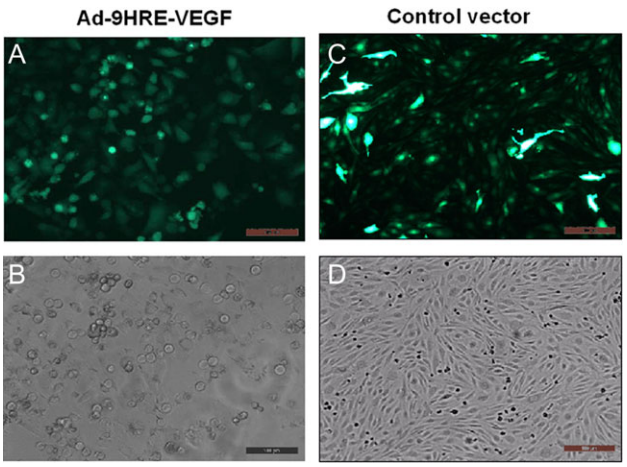

Figure 3. Transfection efficiency of cultured HUVECs under hypoxic condition Positive green fluorescence cells appeared in both groups observed under the fluorescent microscope. (A,B) Ad-9HRE-VEGF165-GFP vector. (C,D) Control vector. Scale bar, 100 μm.

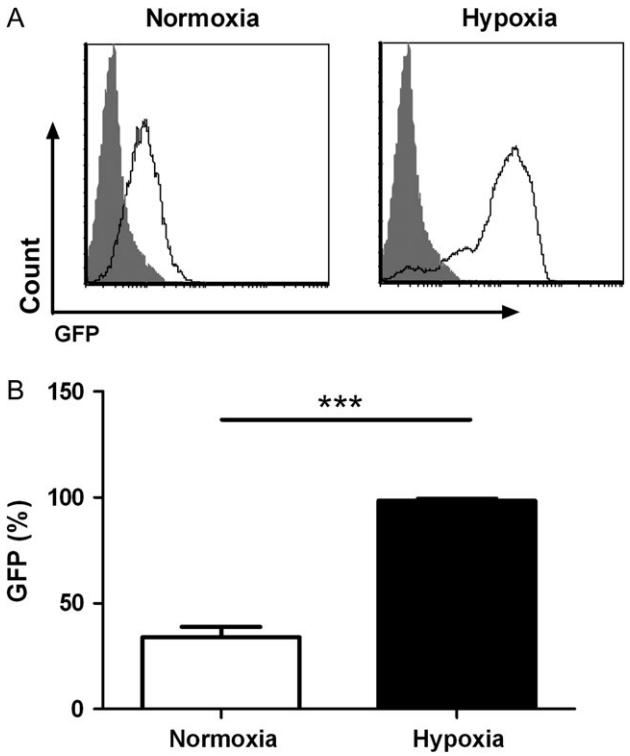

Figure 4. Transfection validation of Ad-9HRE-VEGF165-GFP vectors under hypoxic and normoxic condition After Ad-9HRE-VEGF165-GFP was transfected into cells, GFP-positive cells were detected by flow cytometry. (A) Representative histograms from at least four individual experiments are shown. non-GFP vector: gray filled histogram; Ad-9HRE-VEGF165-GFP vectors: open histogram. (B) The summarized results of GFP expression are shown as the percentage. Data are presented as the mean ± SEM from four independent experiments (***$P < 0.001$)

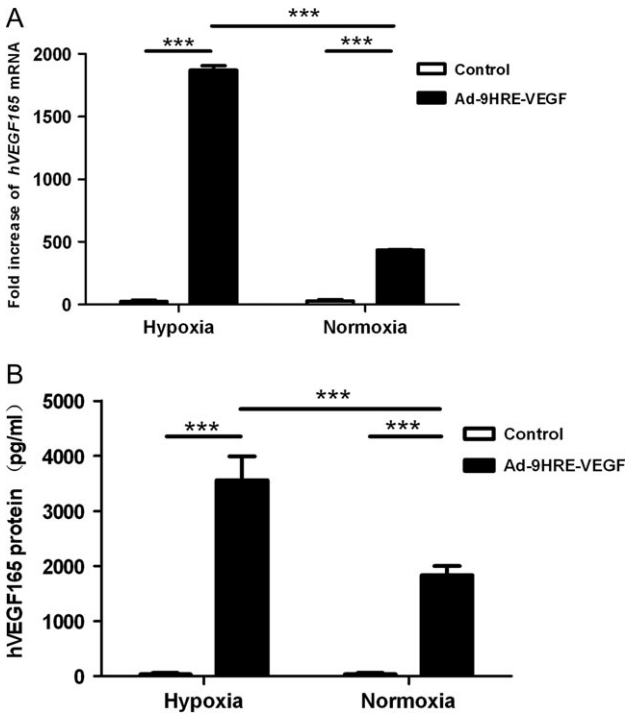

Figure 5. Determination of hVEGF165 mRNA and protein expression under hypoxic and normoxic condition (A) RT-PCR was used to detect the *hVEGF165* mRNA expression. Each value was expressed as the mean ± SD ($n = 3$). (B) Supernatants were collected from the indicated culture, and hVEGF165 protein level was evaluated by ELISA. Results are presented as the mean ± SEM from five experiments (***$P < 0.001$).

protein was up-regulated by HRE, whereas under normoxic condition the expression of hVEGF165 mRNA and protein was decreased.

Ad-9HRE-hVEGF165 promotes proliferation of HUVECs

To study the effect of Ad-9HRE-hVEGF165 transfection on proliferation of HUVECs *in vitro*, MTT assay was performed at 48 h after Ad-9HRE-hVEGF165 infection. Each condition was measured in triplicate. Our data showed that Ad-9HRE-hVEGF165 transfection significantly increased HUVEC proliferation (**Fig. 6**) under hypoxic condition compared with those under normoxic condition, thus indicates that Ad-9HRE-hVEGF165 transfection promotes HUVECs proliferation in hypoxia.

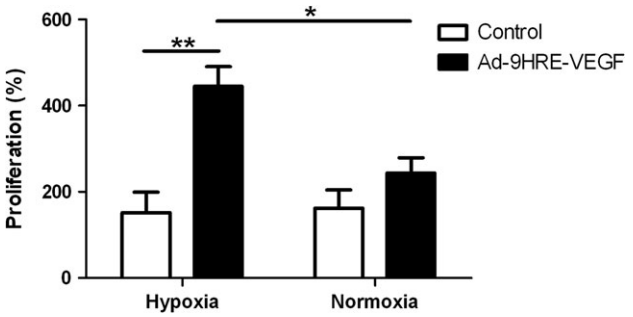

Figure 6. Ad-9HRE-VEGF165 transfection promotes cell proliferation in HUVECs After transfection, HUVECs were plated in 96-well plates and cultured for 48 h under hypoxic and normoxic condition. The effect of Ad-9HRE-VEGF165 transfection on HUVEC growth was analyzed by MTT assay. Data are presented as the mean ± SD ($n = 6$). *$P < 0.05$; **$P < 0.01$.

Ad-9HRE-hVEGF165 promotes migration of HUVECs

The effect of Ad-9HRE-hVEGF165 on HUVEC migration was determined by generating a 'scratch' in a confluent monolayer of HUVECs and then measuring the degree of 'wound closure' during 24 h. As shown in **Fig. 7A,B**, HUVECs transfected with Ad-9HRE-hVEGF165 in hypoxia showed more rapid wound closure than those under normoxic condition. Meanwhile, Ad-9HRE-hVEGF165 transfection effectively enhanced the degree of wound closure compared with the control vector under hypoxic condition. Data from wound healing assay showed that the migration of HUVECs was significantly promoted after Ad-9HRE-hVEGF165 transfection in hypoxia without loss of cell viability.

Ad-9HRE-hVEGF165 enhances tube formation of HUVECs

The effect of Ad-9HRE-hVEGF165 on the HUVEC angiogenesis was determined by 12 h tube formation assay. **Figure 8A** showed the tubular structures formed by HUVECs in each group. The level of tube formation was quantified by counting branch points

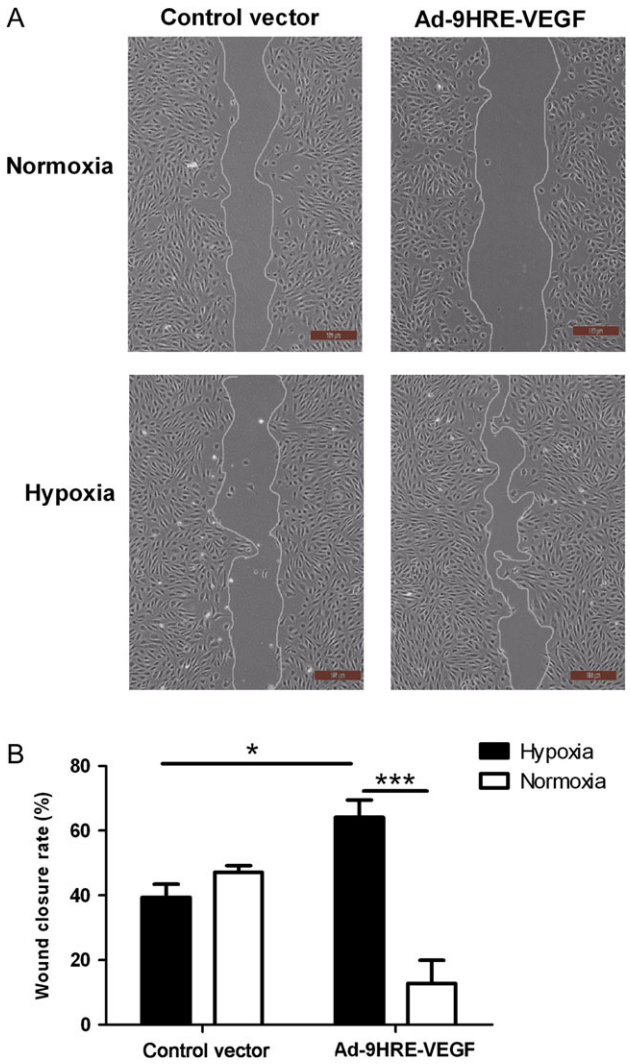

Figure 7. Ad-9HRE-hVEGF165 transfection enhances the rate of wound closure in HUVECs (A) Representative images of HUVEC scratch assays at 24 h were shown. Scale bar: 100 μm. (B) Quantification of wound closure was calculated. Results are expressed as the mean ± SD. Data are representative of three independent experiments. $*P < 0.05$; $***P < 0.001$.

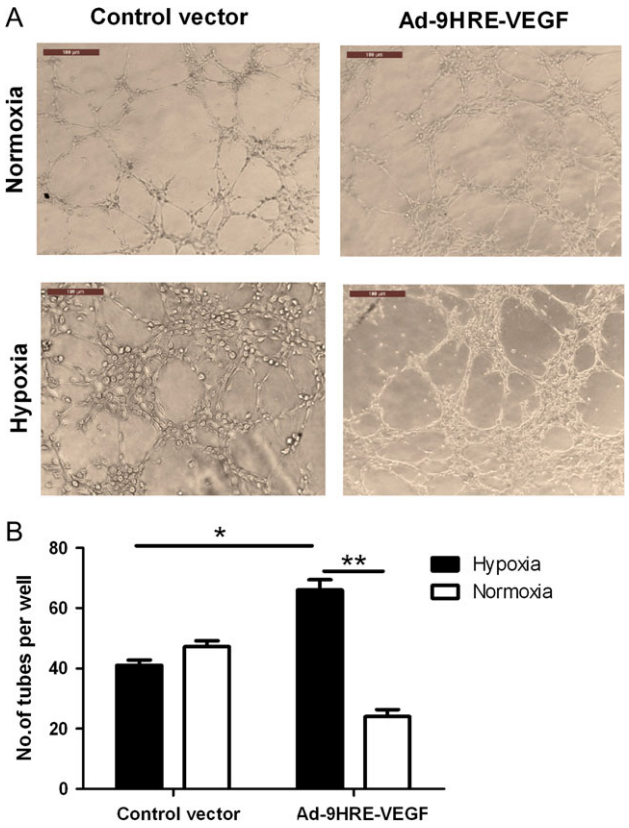

Figure 8. Ad-9HRE-hVEGF165 transfection results in increased tube formation of HUVECs (A) Tube formation of HUVECs with Ad-9HRE-hVEGF165 or control vector in hypoxia and normoxia. Images are representative of five different experiments. Scale bar: 100 μm. (B) Quantification of tube formation. Cells from five random fields per group were counted under a microscope.$*P < 0.05$; $**P < 0.01$.

between discrete ECs. Compared with the cells under normoxic condition, HUVECs with Ad-9HRE-hVEGF165 transfection induced tube formation by 67% ± 5%, while control vector transfection had no effect on tube formation regardless oxygen circumstance (**Fig. 8A,B**). These data showed that tube formation of HUVECs transfected with Ad-9HRE-hVEGF165 was significantly promoted in hypoxia.

Discussion

Angiogenesis represents a crucial step in skeletal development, bone fracture repair, as well as in bone tissue engineering [9]. Pioneering studies demonstrated that sufficient nutrition and oxygen supply is confined to 150 μm for the capillary [10], in the center of the large bone defect there is almost no blood supply. The heterogeneity in vascularity after bone damage could be responsible for the local differences in bone formation in normal, delayed, and mal-unions [11]. Thus, vascularity is the most obvious barrier that needs to be crossed to improve the outcome of bone healing. The strategy to provide sufficient blood supply is pivotal for the success of bone tissue engineering. A great number of studies have indicated that VEGF expression is increased in ischemic and hypoxia regions. Endogenous VEGF secretion, however, is inadequate to fully maintain tissue regeneration. Effective control of exogenous VEGF expression plays a pivotal role in promoting angiogenesis and accelerating the healing of defects.

Using adenovirus vectors to deliver VEGF gene have been tested in some ischemic diseases, showing potential therapeutic value. The encouraging results of angiogenesis gene therapy, along with the side effects associated with long-term expression of the VEGF gene have led to clinical attention. For example, a continuous systematic elevation of VEGF protein level often results in hemangioma formation, which has been found in the heart and limbs. In addition, VEGF gene applications are also associated with arthritis, retinopathy, and occult tumor occurrences [12]. Effective control of exogenous VEGF expression has become a research hotspot. Hypoxia could activate hypoxia-inducible genes, such as *endothelin 1*, *adrenomedullin*, and *NO synthase* for vasomotor control, *transferrin receptor* for iron metabolism, growth factors like *PDGF* and *TGF-β* and angiogenic factors like *VEGF*. Among these factors, HIF-1 is a heterodimeric protein composed of two subunits, HIF-1α which is accumulated under hypoxic conditions, and HIF-1β which is a constitutive subunit. The level of HIF-1β is stable regardless of

tissue oxygen concentration. However, the expression of HIF-1α changes rapidly in response to oxygen concentration alteration. The regulation of HIF-1α level is an important regulatory step for specific gene expression. HIF-1α is specially stabilized under hypoxic conditions and degraded rapidly under normoxia. In hypoxia, the accumulated HIF-1α binds to specific *cis*-regulatory elements HRE in order to switch on the expression of hypoxia-inducible genes. HRE has been proved to be a piece of DNA fragment located at the 5′ or 3′ flanking regions of genes including *VEGF* and *Epo*. Analysis of the *VEGF* promoter reveals that a single HRE is located at nucleotide positions −947 to −39 (5′-TACGTG-3′) relative to the common transcription start site [13]. Experimentally, the binding of HIF-1α to HRE in the *VEGF* promoter is a predominant enhancer of VEGF production. Based on the previous research, HRE/HIF system may be a switch to regulate gene expression for the maintenance of oxygen homeostasis.

Ruan *et al.* [8] compared the induction of gene expression from constructs containing an SV40 minimal promoter and 3×, 6×, or 9× HRE, and demonstrated that 9× HRE increased the expression of genes markedly. Their results indicate that multi-copy HRE can amplify the signal, and the number of HRE gene copies subsequently determines the efficiency of its target gene control [14].

In our experiment, we reconstructed the promoter of *hVEGF165* which consisted of SV40 minimal promoter and 9× HRE. After 48 h of infection, the results determined by fluorescence microscopy and flow cytometry indicated that the efficiency of infection was up to 99% under hypoxic condition, but 30% under normal oxygen. We also found that both hVEGF165 mRNA and protein level were obvious up-regulated in HUVECs transfected with Ad-9HRE-hVEGF165 cultured in hypoxia, and low VEGF165 expression was observed under normoxic condition. These data suggested that the recombined promoter up-regulated hVEGF165 expression effectively in hypoxia, but was almost ineffective when cultured in normal oxygen tension.

In the present study, we chose the ECs as the target cells because they have many advantages. First of all, the circulatory system is initially formed only by EC [15]. During the process of exploring vascularised tissue engineering, ECs serve as the seed cells for preexisted blood vessels *in vitro* to ensure sufficient vascularization of engineered tissues [16]. Second, ECs co-cultured with osteoblastic lineages such as osteoprogenitor and osteoblast, which determines osteogenic differentiation, enhances the production of alkaline phosphatase (ALP) and accelerates osteogenesis by producing specific growth factors such as VEGF [17,18]. When the Ad-9HRE-hVEGF165 modified ECs are implanted to bone defect region, more ECs will be recruited to this region by over-expression of hVEGF165.

In summary, the introduction of 9× HRE promoter induced hVEGF165 expression under hypoxic condition and regulated the sensitivity of this gene in response to oxygen environment, keeping low expression of hVEGF165 under normoxic condition. Notably, Ad-9HRE-hVEGF165 transfection under hypoxia significantly enhanced HUVEC angiogenesis activity, as evidenced by increased proliferation, migration and tube formation compared with that under normal circumstance as well as with the control group. No significant difference in tube formation of HUVECs was observed at the presence of control Ad infection either in normoxia or in hypoxia. Our results further confirmed that HIF-1/HRE is a switch to control the expression of a delivered gene. Hence, improved understanding of the relationship between HIF-1/HRE and oxygen tension will help to develop novel gene therapies which are more effective for bone tissue engineering.

Funding

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