Mol Cell Biochem (2018) 437:99–107
https://doi.org/10.1007/s11010-017-3098-6

HIF1A overexpression using cell-penetrating DNA-binding protein induces angiogenesis in vitro and in vivo

Mijeong Jeon[1] · Yooseok Shin[2] · Jaeeun Jung[1] · Ui-Won Jung[3] · Jae-Hoon Lee[4] · Jae-Seung Moon[5] · Ilkoo Kim[5] · Jin-Su Shin[5] · Sang-Kyou Lee[5,6] · Je Seon Song[1]

Received: 15 December 2016 / Accepted: 9 June 2017 / Published online: 28 June 2017

Abstract Hypoxia-inducible factor-1 alpha (*HIF1A*) is an important transcription factor for angiogenesis. Recent studies have used the protein transduction domain (PTD) to deliver genes, but the PTD has not been used to induce the expression of *HIF1A*. This study aimed at using a novel PTD (Hph-1-GAL4; ARVRRRGPRR) to overexpress the *HIF1A* and identify the effects on angiogenesis in vitro and in vivo. Overexpression of *HIF1A* was induced using Hph-1-GAL4 in human umbilical vein/vascular endothelium cells (HUVEC). The expression levels of genes were analyzed by the quantitative real-time polymerase chain reaction (qPCR) after 2 and 4 days, respectively. An in vitro tube formation was performed using Diff-Quik staining. *HIF1A* and Hph-1-GAL4 were injected subcutaneously into the ventral area of each 5-week-old mouse. All of the plugs were retrieved after 1 week, and the gene expression levels were evaluated by qPCR. Each Matrigel plug was evaluated using the hemoglobin assay and hematoxylin and eosin (HE) staining. The expression levels of *HIF1A* and *HIF1A* target genes were significantly higher in *HIF1A*-transfected HUVEC than in control HUVEC in vitro. In the in vivo Matrigel plug assay, the amount of hemoglobin was significantly higher in the *HIF1A*-treatment group than in the PBS-treatment group. Blood vessels were identified in the *HIF1A*-treatment group. The expression levels of *HIF1A*, vascular endothelial growth factor (*Vegf*), and *Cd31* were significantly higher in the *HIF1A*-treatment group than in the PBS-treatment group. These findings suggest that using Hph-1-G4D to overexpress *HIF1A* might be useful for transferring genes and regenerating tissues.

Keywords Hypoxia-inducible factor-1 alpha (*HIF1A*) · Protein transduction domain (PTD) · HUVEC · Angiogenesis · Tissue regeneration

Mijeong Jeon and Yooseok Shin have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11010-017-3098-6) contains supplementary material, which is available to authorized users.

✉ Sang-Kyou Lee
sjrlee@yonsei.ac.kr

✉ Je Seon Song
songjs@yuhs.ac

[1] Department of Pediatric Dentistry, Oral Science Research Center, College of Dentistry, Yonsei University, 250 Seongsanno, Seodaemun-gu, Seoul 03722, Korea

[2] Department of Conservative Dentistry, College of Dentistry, Yonsei University, Seoul, Korea

[3] Department of Periodontics, College of Dentistry, Yonsei University, Seoul, Korea

[4] Department of Prosthodontics, College of Dentistry, Yonsei University, Seoul, Korea

[5] Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Yonsei-ro 50, Seodaemun-gu, Seoul 03722, Korea

[6] Good T cells Inc., Seoul, Korea

Introduction

A viral vector such as a retrovirus or lentivirus or a non-viral vector such as a liposome is commonly used to control the expression of transcription factors. Each of these methods has both advantages and disadvantages. A non-viral vector is nonpathogenic, but low gene delivery efficiency is a problem compared to using a viral vector [1–8]. Lipofectamine is one of the most common reagents used

for transfection. However, the efficiency of transfection using lipofectamine varies with the experimental conditions and cell type, and increasing the lipofectamine treatment concentration increases the probability of cell death [9]. On the other hand, using a viral vector enhances the gene delivery efficiency relative to using a nonviral vector, but viral infection and an immunological response become potential problems. Also, the size of available DNA depends on the type of viral vector [10]. In addition, gene delivery has been found to be effective when using viral and nonviral vector systems in vitro, but the efficiency of gene transfer is low in vivo [6].

A method involving the use of a protein in gene delivery has recently been reported. The protein transduction domain (PTD) is composed of short amino acid sequences of less than 30 bp and can be obtained from various protein sources, such as the HIV TAT protein [11, 12], Oct4 protein [13], and heparin-binding protein [14]. The PTD has also developed as synthetic proteins [15–20]. A novel PTD (ARVRRRGPRR) was found from human transcription factor Hph-1, with its powerful protein transduction ability confirmed both in vitro and in vivo [21]. The advantage of the PTD is that it does not exhibit significant limitations in delivering genes, proteins, or other physiological factors.

A PTD has the ability to deliver a drug that is difficult to deliver through absorption to the target cell [22]. Moreover, using a PTD has a higher gene transduction efficiency than using a viral vector [23], and large genes that are difficult to introduce using other methods can be efficiently delivered to cells using a PTD. Many studies have investigated using the GAL4 DNA-binding domain (DBD) to improve the efficiency of gene delivery [24–26]. The novel ARVRRRGPRR PTD combined with GAL4-DBD was used to efficiently deliver DNA both vitro and in vivo [27].

Hypoxia-inducible factor-1 alpha (HIF1A) is a transcription factor that acts as a major regulator to induce the expression of genes involved in the survival and adaptation of cells in hypoxia ($\sim$1% O_2) conditions [28, 29]. Angiogenesis plays an important role in the healing of injured tissue by supplying oxygen and nutrients [30, 31]. Increased HIF1A expression is known to play an important role in angiogenesis by stimulating the expression of related genes, such as VEGF [32].

Previous studies have demonstrated the role of HIF1A in tissue regeneration after inducing angiogenesis by using a viral vector to increase HIF1A expression [33, 34]. These studies have shown that HIF1A overexpression using a lentiviral vector increases tube formation and blood-vessel formation. Other previous studies found that HIF1A overexpression using an adenoviral vector increased the proliferation, migration, and viability of cell, and expression of proangiogenic genes, and in vitro capillary sprout formation [35, 36]. However, no previous study has used a PTD—which provides several advantages—to increase HIF1A expression. Therefore, in this study, HIF1A was overexpressed using a novel PTD, and in vitro tube formation was induced by HIF1A overexpression. We also confirmed the angiogenic effect of HIF1A using a novel PTD in vivo.

Materials and methods

Cell culture

Human umbilical vein/vascular endothelium cells (HUVEC; CRL-1730) were purchased from the American Type Culture Collection (Rockville, MD, USA). HUVEC were cultured in a cell culture medium comprising endothelial cell basal medium (EBM-2, Lonza, Walkersville, ML, USA) that included endothelial cell growth medium (EGM-2) containing fetal bovine serum, hydrocortisone, hFGF, VEGF, IGF-1, ascorbic acid, hEGF, gentamicin, amphotericin-B, and heparin (all from Lonza) at 37 °C in 5% CO_2. HUVEC at passages 12–15 were used in subsequent experiments.

Construction of pEGFPN1-*HIF1A* (*HIF1A*)

To generate the *HIF1A*, the human form of *HIF1A* (NM_001530.3) was amplified by the polymerase chain reaction (PCR). The conditions of amplification were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min and 30 s, and then 72 °C for 10 min. The PCR product was inserted into the pEGFPN1 plasmid vector (Invitrogen, Carlsbad, CA, USA) using restriction enzyme NheI (Takara Bio, Otsu, Japan) at 5′ termini and KpnI (Takara Bio) at 3′ termini of the PCR fragment. The fidelity of the reading frame was verified by DNA sequencing using the following 5′ and 3′ primers: 5′-CCTATGACCTGCTTGGTGCTG-3′, 5′-CCTTCCGATGGAAGCACTAGAC-3′, and 5′-CTACAGTTCCTGAGGAAGAAC-3′, respectively.

Expression and purification of Hph-1-G4D (GAL4-DBD) protein

The DNA of G4D combined with Hph-1 was transformed with *Escherichia coli* BL-21 Star (DE3) pLysS (Invitrogen). The protein expression was induced in 500 ml of Luria–Bertani media containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C for 4 h. Isopropyl-β-D-thiogalactopyranoside (1 mM; Duchefa Biochemie, Haarlem, Netherlands) was then added for protein induction, and after a further 4 h of incubation at 37 °C, cells were harvested and resuspended in native lysis buffer (50 mM

NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole; pH 8.0). The resuspended cells were sonicated at an amplitude of 30% for 10 min. Soluble fraction of lysates was obtained by centrifugation (10,000×*g* for 10 min at 4 °C) and then gently mixed with Ni–NTA resin (Qiagen, Valencia, CA, USA) for 1 h at 4 °C to bind the histidine tag of the protein. The proteins were loaded onto a chromatography column (Poly-Prep, Bio-Rad, Richmond, CA, USA). After nonspecifically bound proteins were washed away with native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 30 mM imidazole; pH 8.0), the recombinant proteins of interest were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole; pH 8.0) and then desalted by PD-10 Sephadox G-25 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with 10% glycerol phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA). The recombinant proteins were subsequently mixed with SP Sepharose Fast Flow (GE Healthcare, Milwaukee, WI, USA) for 1 h at 4 °C in SP binding buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 6.0). The proteins were loaded onto a Poly-Prep chromatography column and washed to remove endotoxins in *Escherichia coli* with SP wash buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 6.0), and eluted with the Hph-1-G4D protein by SP elution buffer (50 mM NaH_2PO_4 and 2 M NaCl, pH 6.0). The eluted proteins were desalted using PD-10 Sephadox G-25 with 10% glycerol PBS.

In vitro transfection

One microgram of *HIF1A* DNA was mixed with 5 μM Hph-1-GAL4 at room temperature for 15 min, after which HUVEC were incubated with this mixture under serum-free conditions for 6 h. The serum-free medium was replaced with complete medium after the incubation, and the expression levels of *HIF1A* and *HIF1A* target genes were analyzed by the quantitative real-time PCR (qPCR) after 2 and 4 days, respectively.

Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The integrity and concentration of the extracted RNA were evaluated using a spectrophotometer (NanoDrop ND-2000, Thermo Scientific, Waltham, MA, USA). One-microgram aliquots of RNA were reverse transcribed to synthesize cDNA using the Maxime RT Premix Kit [oligo $d(T)_{15}$ primer; Intron Biotechnology, Seongnam, Gyeonggi, Korea] according to the manufacturer's instructions. A qPCR assay was performed with SYBR Premix Ex Taq (Takara Bio) and a real-time PCR system (ABI 7300, Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The qPCR conditions were 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s, with a final 5-min extension at 72 °C. The application specificity was confirmed by visualizing PCR products on 1.5% agarose gels and using melting-curve analysis (from 60 to 95 °C). The specific primers for each gene are listed in Table 1. The values for each gene were normalized to the expression levels of the gene encoding beta actin (*ACTB*), and the relative expression levels of the studied genes were calculated using the $2^{-\Delta\Delta Ct}$ method [37].

In vitro tube formation assay

Matrigel (BD Biosciences, San Jose, CA, USA) was thawed at 4 °C and coated on 48-well culture plates (BD Falcon, Franklin Lakes, NJ, USA) at room temperature for at least 15 min to allow the Matrigel to gel. *HIF1A*-transfected HUVEC were seeded into Matrigel-coated wells at a density of 2.5×10^4 cells/well and then incubated at 37 °C. After 8 h, the medium was gently aspirated from each well and incubated with Diff-Quik fixative (Sysmex, Kobe, Japan) for 30 s. The fixative was then removed, and cells were stained for 2 min with Diff-Quik solution II (Sysmex). The tube structures were observed under a light microscope (Leica Microsystems, Wetzlar, Germany), and photographs were taken. The tube length was measured in

Table 1 qPCR forward (F) and reverse (R) primer sequences and sizes

Gene	Primer sequence (5′–3′)	Size (bp)
HIF1A	F: AGCTTGCTCATCAGTTGCCA	105
	R: CCAGAAGTTTCCTCACACGC	
VEGF	F: TCCTCACACCATTGAAACCA	131
	R: GATCCTGCCCTGTCTCTCTG	
IGF	F: GGACTTGAGTCCCTGAACCA	101
	R: TGAAAATTCCCGTGAGAAGG	
Vegf	F: TCCGAAACCATGAACTTTCTGC	157
	R: AGCTTCGCTGGTAGACATCC	
Cd31	F: TACGAGGTGAAGGTGCATGG	116
	R: ATCGGTGGCTTTTCTTCTTGC	
GAPDH	F: TCCTGCACCACCAACTGCTT	100
(Gapdh)	R: TGGCAGTGATGGCATGGAC	
ACTB	F: TCACCATGGATGATGATATCGC	161
(Actb)	R: GGAATCCTTCTGACCCATGC	

The annealing procedures were performed at 60°C for all primers. Abbreviations: *HIF1A*, hypoxia-inducible factor 1 alpha; *VEGF*, vascular endothelial growth factor; *IGF*, insulin-like growth factor; *Cd31*, cluster of differentiation 31; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, beta actin. Capital letters indicate human genes and small letters indicate mouse genes

the photographs using ImageJ software (version 1.45, NIH, Bethesda, MD, USA).

In vivo matrigel plug assay

In vivo Matrigel plug assay was performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Yonsei University (Protocol No. #2014-0223). Two micrograms of *HIF1A* DNA was mixed with 100 μg of Hph-1-GAL4 at room temperature for 15 min, after which Matrigel was incubated with the mixture on ice. A negative control was produced using PBS at the same volume as Matrigel in the experimental group under the same conditions. Aliquots (0.5 ml) of the solutions were loaded into a syringe and injected subcutaneously into the ventral area of 5-week-old female mice ($n = 60$; C57BL/6N, SLC, Shizuoka, Japan). All of the plugs were retrieved after 1 week, with 36 of them were used for qPCR and the other 24 divided into two fragments for the hemoglobin assay and histological analysis.

qPCR in the Matrigel plugs

The relative expression levels of the genes encoding *Hif1a*, *Vegf*, *Cd31*, and *Gapdh* were evaluated using qPCR. Immediately after retrieval, the plug fragments were homogenized using 0.5-mm-diameter stainless steel beads (Next Advance, Averill Park, NY, USA) and a bullet blender (Next Advance) in RLT buffer, which is a component of the RNeasy Mini Kit (Qiagen). Total RNA was isolated using the kit according to the manufacturer's instructions. The integrity and concentration of extracted RNA were evaluated using a spectrophotometer (NanoDrop ND-2000, Thermo Scientific). RNA (500 ng) was reverse transcribed to synthesize cDNA using the Maxime RT Premix Kit [oligo $d(T)_{15}$ primer, Intron Biotechnology] according to the manufacturer's instructions. A qPCR assay was performed using the aforementioned procedure. The sequences and sizes of the primers are given in Table 1. The values for each gene were normalized to the expression levels of the gene encoding beta actin (*Actb*), and the relative expression levels of the studied genes were calculated using the $2^{-\Delta\Delta Ct}$ method [37]. The expression level of each gene was calculated relative to that in the negative-control plugs.

Hemoglobin assay of the Matrigel plugs

The concentration of hemoglobin in each Matrigel plug was measured using a hemoglobin assay kit (Sigma-Aldrich) according to the manufacturer's instructions. In brief, the retrieved Matrigel plugs were rinsed and soaked in PBS (pH 7.4; Invitrogen) containing 0.16 mg/ml heparin. They were then homogenized and centrifuged at $10{,}000 \times g$ for 15 min at 4 °C. Reagent from the kit was added to the collected supernatant of the lysates, and the colorimetric change was measured at 400 nm. The concentration of hemoglobin was calculated according to the manufacturer's instructions, and normalized to the weight of each Matrigel plug.

Histological analysis of Matrigel plugs

The Matrigel plugs were fixed with 10% buffered formalin (Sigma-Aldrich) for 1 day. The fixed Matrigel plugs were embedded in paraffin, sectioned at a thickness of 3 μm, and stained with hematoxylin and eosin (HE). The newly formed vessels were identified using a light microscope (BS40, Olympus, Tokyo, Japan). Sections were examined by light microscopy, and the total number of microvessels containing red blood cells in 10 high-power fields (×400 magnification) was counted in a blinded fashion. Only microvessels that contained red blood cells were counted. Results shown represent the average of counts from twelve Matrigel plugs per group.

Statistical analysis

All experiments were performed at least in triplicate. The normality of the data was evaluated using the Shapiro–Wilk test ($p < 0.05$). The Mann–Whitney U test ($p < 0.05$) was performed for all experiments using SPSS software (version 20.0, SPSS, Chicago, IL, USA).

Results

Identification of *VEGF*, *IGF1*, and *GAPDH* expression levels in *HIF1A*-transfected HUVEC

HIF1A was transfected into HUVEC using the PTD, and HIF1A gene expression was measured 2 days later using qPCR. *HIF1A* expression was significantly higher in *HIF1A*-transfected HUVEC than in the *UAS*-transfected control group of HUVEC (Fig. 1). *VEGF*, *IGF1*, and *GAPDH*, which are known as *HIF1A* target genes, were identified at 4 days after transfection. Their expression levels were significantly higher in *HIF1A*-transfected HUVEC than in *UAS*-transfected control HUVEC (Fig. 1).

Tube formation in *HIF1A*-transfected HUVEC

Tube formation was identified in *HIF1A*-transfected HUVEC seeded on Matrigel. *HIF1A* transfection was performed using the PTD. The total tube length did not

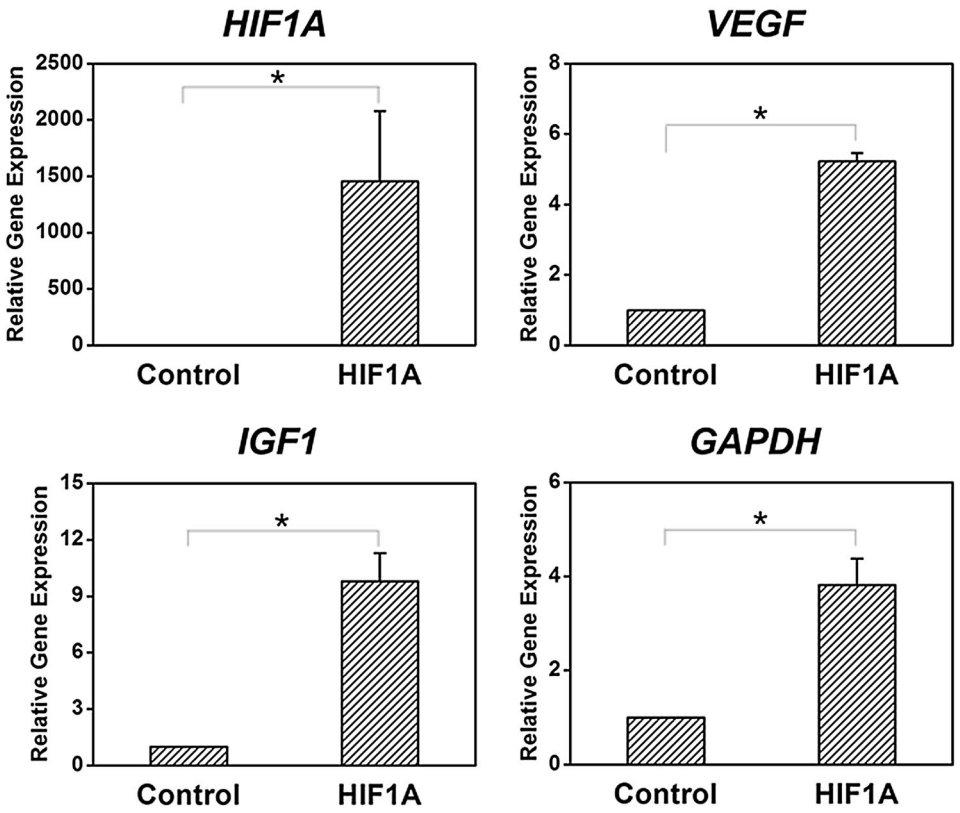

Fig. 1 Relative gene expression levels for *HIF1A* transfected into HUVEC using the novel PTD Hph-1-G4D and qPCR. *HIF1A* expression was measured at 2 days after transfection, while *VEGF*, *IGF1*, and *GAPDH* expression levels were measured at 4 days after transfection. Control indicates *UAS*-transfected HUVEC and *HIF1A* indicates *HIF1A*-transfected HUVEC. Data were obtained from three separate experiments, with all samples run in duplicate. The data are mean and standard deviation values. The relative expression levels of all of the genes differed significantly between control and *HIF1A*-transfected HUVEC (Mann–Whitney *U* test, $p < 0.05$)

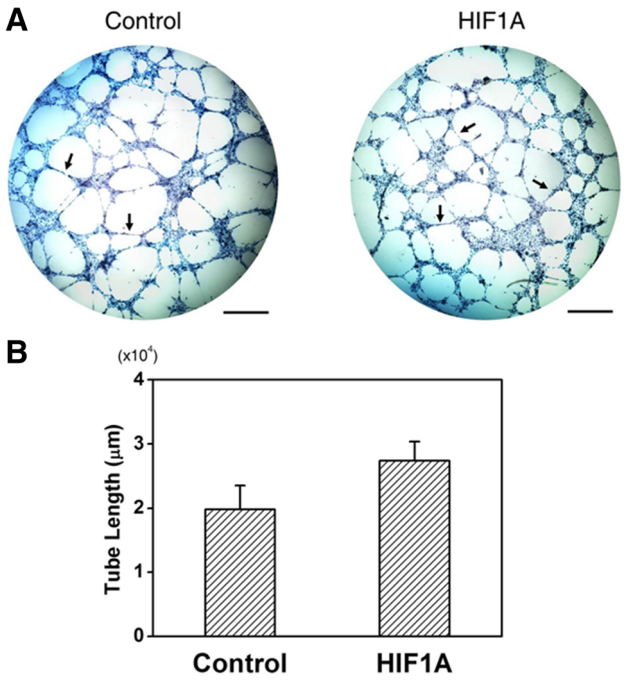

Fig. 2 Tube formation assay in HUVEC. **a** HUVEC stained by Diff-Quik to identify tube formation. *Black arrows* indicate the tubes. **b** Total tube length as calculated using ImageJ software. Data were obtained from three separate experiments, and are indicated as mean and standard deviation values. The tube length did not differ significantly between the two groups (Mann–Whitney *U* test, $p > 0.05$). *Scale bars* 0.2 cm in A

differ significantly between *HIF1A*-transfected HUVEC and the control group (Fig. 2).

Effect of *HIF1A* in the in vivo Matrigel plug assay

More blood vessels were detected in the *HIF1A*-treatment group than in the PBS-treatment group (Fig. 3a). The HE staining revealed blood vessels in the *HIF1A*-treatment group (as indicated by red arrows in Fig. 3c), whereas they were barely detected in the PBS-treatment group. In the hemoglobin analysis, the amount of hemoglobin normalized to the weight of Matrigel was significantly greater in the *HIF1A*-treatment group than in the PBS-treatment group (Fig. 3b). The number of microvessels was also higher in HIF1A-treatment group than PBS-treatment group (Fig. 3d).

Gene expression analysis in the in vivo Matrigel plug assay

HIF1A expression was higher in the *HIF1A*-treatment group than in the PBS-treatment group. The expression levels of angiogenesis and vessel-related markers, such as *Vegf* and *Cd31*, were higher in the *HIF1A*-treatment group than in the PBS-treatment group. The expression levels of three genes (*HIF1A*, *Vegf*, and *Cd31*) were significantly higher in the *HIF1A*-treatment group than in the PBS-

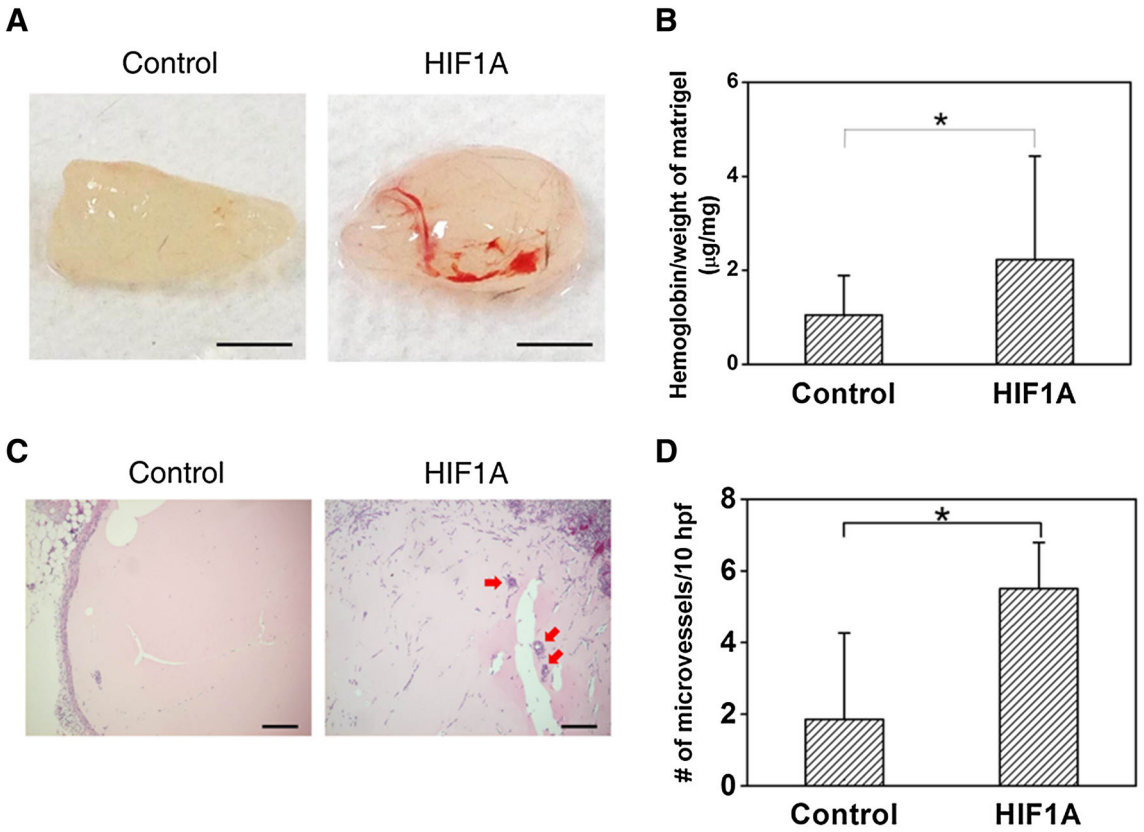

Fig. 3 In vivo Matrigel plug assay using *HIF1A*. **a** Identification of newly formed blood vessels in Matrigel plugs. **b** The amount of hemoglobin normalized to the weight of the Matrigel plug was analyzed in the two groups. Data were obtained from 24 plugs, with all samples run in duplicate. The data are mean and standard deviation values. The amount of hemoglobin differed significantly between the two groups (Mann–Whitney *U* test, $p < 0.05$). **c** Histological analysis of Matrigel plugs. HE staining was performed to identify the blood vessels (indicated by *red arrows*). **d** Quantitation of in vivo neovascularization assessed with the Matrigel plug assay. Twelve plugs per group (one plug per mouse) were examined. The total number of microvessels containing red blood cells from 10 high-power fields was counted and averaged. The data are mean and standard deviation values. The number of microvessels/10 hpf was significantly differences between two groups (Mann–Whitney *U* test, $p < 0.05$). *Scale bars* 0.5 cm in (**a**) and 200 μm in (**c**). (Color figure online)

treatment group, while there was no significant intergroup difference in the expression of one of the *HIF1A* target genes, *Gapdh* (Fig. 4).

Discussion

Overexpression of *HIF1A* induced using Hph-1-G4D increased angiogenesis both in vitro and in vivo. Application of the qPCR also confirmed that the levels of angiogenesis-related genes such as *VEGF* and *EGF* were increased.

Hph-1-G4D as used in this present study is the novel PTD containing GAL4-DBD, and its effectiveness and safety have been demonstrated previously. It was found that cell viability was maintained at above 90%, and that the gene transfer efficiency was similar to or a little higher than that when using Lipofectamine, although this varies with the cell type [27]. The present study also found that the gene transfer efficiency for HUVEC was higher when using Hph-1-G4D than when using Lipofectamine (Supplementary Fig. 1). Many previous studies have used a viral vector system rather than a nonviral vector system such as Lipofectamine to increase the transfer efficiency when transferring DNA into cells. Although the present study did not directly compare between a viral vector system and Hph-1-G4D, it was considered that using Hph-1-G4D might be better than a viral vector system for delivering genes to HUVEC. Using Hph-1-G4D might be very useful in terms of both safety and efficiency in cases where Lipofectamine cannot be applied because of its lower efficiency or when a viral vector is used.

Hph-1-G4D has many advantages as a novel PTD containing GAL4-DBD. Various studies have investigated gene delivery proteins obtained from diverse sources, such as HIV TAT, Oct4, heparin-binding, and synthetic proteins [11–13, 38, 39]. Hph-1-G4D as used in the present study showed a better gene delivery ability than the TAT-GAL4

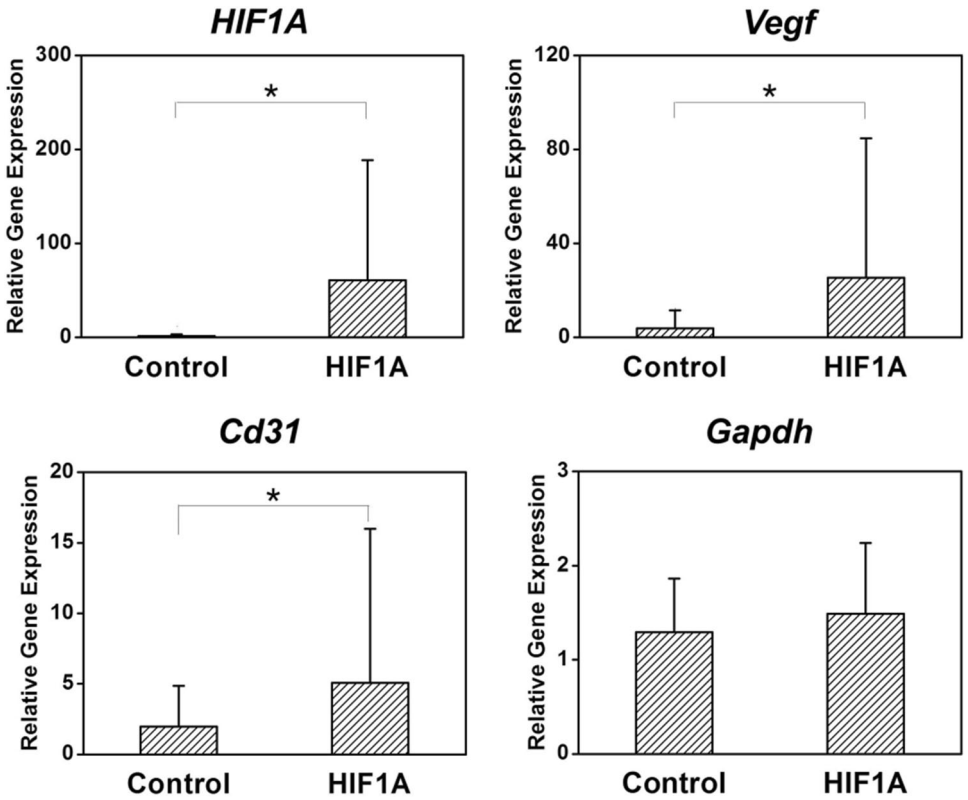

Fig. 4 qPCR analysis of Matrigel plugs. The expression levels of angiogenesis and blood-vessel-related markers, such as *VEGF* and *CD31*, were identified in Matrigel plugs. The *HIF1A* target gene *GAPDH* was also identified in these plugs. Data were obtained from 36 plugs, with all samples run in duplicate. The relative expression levels of *HIF1A*, *VEGF*, and *CD31* genes differed significantly between the two groups (Mann–Whitney *U* test, $p < 0.05$)

PTD that has mostly been used in previous studies [27]. The Hph-1-G4D PTD also has an advantage of delivering drugs effectively, since it can be used to improve the efficiency of delivering drugs that are normally administered intravenously due to them being difficult to absorb through the skin [22]. The advantage of using a known PTD is that the PTD system can transfer not only DNA but also RNA, protein, drugs, and various biologically active substances into a cell. The use of a viral vector enhances the gene delivery efficiency relative to using a nonviral vector, but viral infection and an immunological response become potential problems. Moreover, the size of the available DNA depends on the type of viral vector [10]. The delivery potential of the PTD system has already been demonstrated, where TAT-linked magnetic nanobeads with a diameter of 45 nm were delivered into cells and these beads did not affect cell proliferation and differentiation [40]. It might be possible to apply the PTD system clinically for transportation through the skin barrier. Cyclosporine A is a systemically effective drug that is ineffective *when applied topically* due to its poor penetration into the skin. However, conjugating the PTD system with cyclosporine A could facilitate the topical delivery into the skin in vivo [22]. In other words, using the PTD provides the advantage that absorption through the skin is effective while being easier than an intravenous injection or oral administration. Choi et al. applied Hph-1 as an ointment to the skin of hairless mice, and found that it was absorbed through the skin [21]. Therefore, using the PTD system might allow large biologically active substances including DNA, RNA, proteins, and drugs to be delivered effectively and thereby facilitate clinical applications.

Angiogenesis involves the formation of new blood vessels from pre-existing blood vessels [41, 42]. In wound healing, angiogenesis is the normal procedure for the formation of granulation tissue containing new blood vessels and connective tissues. Moreover, angiogenesis is also a normal process for promoting tissue regeneration including wound healing. Angiogenesis is promoted using various angiogenic proteins containing certain growth factors. FGF, VEGF, angiopoietin, and MMP are well known to promote angiogenesis [43–49]. VEGF is a potent mitogen of endothelial cells and is known to help the migration of endothelial cells and the proliferation and migration of smooth-muscle cells [50]. FGF-2 is known to up-regulate VEGF [51], while MMP is involved in extracellular matrix remodeling. Angiopoietin plays an important role in angiogenesis, but it does not affect the migration of endothelial cells [48]. In particular, angiopoietin-2 is known to be involved in angiogenesis in the presence of VEGF [48]. In summary, VEGF can be considered to be the key factor in angiogenesis.

Hypoxia induces the expression of VEGF, and overexpression of VEGF promotes angiogenesis [32]. A hypoxic

environment induces the creation of blood vessels to receive the oxygen supply, and therefore, the expression of VEGF is increased [52]. HIF1A is easily degraded in the normoxia condition, but it is activated in the hypoxia condition, in which it is known to increase the expression of VEGF [52]. It is found that VEGF expression was increased in HIF1A transgenic mice and that skin regeneration was induced through angiogenesis in vivo [53]. In the present study, overexpression of HIF1A increased VEGF expression both in vitro and in vivo, and it was confirmed that new blood vessels were generated.

Angiogenesis plays a key role in tissue regeneration, which can be promoted by supplying oxygen and nutrients to injured tissues. Angiogenesis is an essential process in the re-establishment of a microvascular network for supplying nutrients and oxygen [30, 31]. Many studies have investigated the effect of HIF1A on bone regeneration. HIF1A overexpressed by bone-marrow-derived mesenchymal stromal cells promoted osteogenesis and angiogenesis so as to repair a critically sized rat calvarial defect [54].

Hph-1-G4D as a novel PTD and HIF1A might be useful in tissue regeneration. Viral vectors or nonviral vectors have had limitations in clinical use due to safety problems and low efficiencies in vivo. But this study has shown that HIF1A can be delivered effectively into cells using Hph-1-G4D and promote angiogenesis. And we consider that Hph-1-G4D might be much more useful for tissue regeneration, because it exhibits good cell permeability. In particular, the promotion of angiogenesis by HIF1A could also play a role in facilitating the supply of various factors and nutrients needed in tissue regeneration such as osteogenesis. Future studies should therefore investigate the effects of HIF1A in both angiogenesis and osteogenesis.

Acknowledgements This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science, ICT& Future Planning) (NRF-2014R1A2A1A10052466, NRF-2015037075 and NRF-2017R1A2B2002537)

Funding All authors have no financial and personal relationships that could influence their work.

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