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Enhancement of angiogenic and vasculogenic potential of endothelial progenitor cells by haptoglobin

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ABSTRACT

Endothelial progenitor cells (EPCs) were transfected with the haptoglobin (Hp) gene to investigate the effect of Hp on cell function. Hp potentiated the gene expression of various pro-angiogenic factors in the EPCs. The Hp-modified EPCs also increased in vitro tube formation on Matrigel compared with control cells. In hindlimb ischaemia models, Hp–EPCs showed a greater ability for improving blood perfusion and recovery from ischaemic injury. These results indicate that Hp improves EPC function in neovasculogenesis, which suggests that ex vivo modification of EPCs with the Hp gene can be applied to the treatment of vascular damage.

1. Introduction

Ischaemic tissue damage requires new blood vessel formation at damaged sites for tissue repair. Postnatal new vessels are formed by the processes of angiogenesis, arteriogenesis, and vasculogenesis. The angiogenic process involves proliferation and migration of the endothelial cells that sprout from pre-existing mature endothelial cells, and arteriogenesis is a process of remodelling pre-existing arteriolar connections into collateral vessels [1]. In contrast, vasculogenesis progresses via the differentiation of endothelial progenitor cells (EPCs) into mature endothelial cells [2]. The circulating EPCs, mobilized from bone marrow, home to the vascular injury sites and participate in neovascularization through direct incorporation into new-forming vessels, and/or secreting various angiogenic and trophic factors [3,4]. The EPCs, therefore, have been proposed as a potential target for therapeutic revascularization [3]. Recent studies have focused on ex vivo gene-modified EPCs to enhance cell functions, and vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α have been used for improving the pro-angiogenic capacity of EPCs [5,6].

Haptoglobin (Hp) is an acute-phase glycoprotein in the blood circulation, and haemoglobin capture is a well-known biological function of Hp. Hp prevents extravascular haemoglobin-stimulated oxidative tissue damage via the formation of a stable Hp–haemoglobin complex [7,8]. The Hp can be expressed in arteries [9], and acts as an angiogenic factor that induces proliferation and differentiation of endothelial cells, as well as a cell migration factor involved in arterial restructuring [10–12]. These findings suggest that Hp participates in the formation of new blood vessels and vascular remodelling. However, to date, the effect of Hp on EPC-promoted neovascularization has not been studied.

In the present study, EPCs were isolated from human umbilical cord blood and modified by the human Hp gene. Improved potential of Hp-modified EPCs for angiogenesis and recovery of blood perfusion in a mouse hindlimb ischaemia model were demonstrated.

Abbreviations: Hp, haptoglobin; EPCs, endothelial progenitor cells; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells; DiI-acLDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labelled acetylated low-density lipoprotein; UEA, *Ulex europaeus* agglutinin; GFP, green fluorescence protein; LDPI, laser Doppler blood perfusion imager

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2. Materials and methods

2.1. Cell culture

Cord blood and umbilical cords were obtained from donors who signed a written consent form at Kangnam St. Mary's Hospital in Seoul, Korea. Mononuclear cells were isolated from human cord blood by density gradient centrifugation over Histopaque-10771 (Sigma, St. Louis, MO), according to the manufacturer's protocol. The cells were plated into six-well plates coated with 0.1 mg/ml human fibronectin (Sigma) and incubated in EGM-2 BulletKit medium (Clonetics, San Diego, CA) supplemented with 5% foetal bovine serum (FBS; Gibco Life Technology, Gaithersburg, MD). After 3 days, non-adherent cells were removed and the medium was replaced. To obtain late EPCs, the adherent cells were cultured for 25–45 days by changing to fresh medium every 3 days.

Human umbilical vein endothelial cells (HUVECs) were isolated from cord vein and cultured as previously described [13].

2.2. Acetylated low-density lipoprotein uptake and Ulex europaeus lectin binding

After 14 days of culture, the cells were incubated with 2.5 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-perchlorate-labelled acetylated low-density lipoprotein (DiI-acLDL; Molecular Probes, Eugene, OR) for 1 h at 37 °C, and fixed with 1% paraformaldehyde for 10 min. The cells were incubated again with 10 μg/ml fluorescein-isothiocyanate-conjugated *Ulex europaeus* agglutinin lectin (UEA lectin; Sigma) for 1 h. Thereafter, the double-labelled cells were observed with a fluorescence microscope and photographed.

2.3. Modification of EPCs with human Hp gene

To prepare a recombinant plasmid (MSCV-Hp), the cDNA of human *Hp*[2] gene [14] was subcloned into EcoRI and XhoI sites of MSCVneoEB retroviral vector that contained a green fluorescence protein (GFP) gene. 293T cells were transfected with the MSCV-Hp plasmid using FuGene 6 reagent (Roche Applied Science, Indianapolis, IN). At 48, 60 and 72 h after transfection, the virus-containing supernatants were collected and passed through a 0.45 μm syringe filter (Pall Corporation, East Hills, NY). The viral supernatants were added to the EPC culture in the presence of 5 μg/ml protamine sulphate (Sigma). Seventy-two hours after infection, GFP-positive cells were collected using a flow cytometric cell sorter (FACS Vantage SE; BD Biosciences, San Diego, CA).

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed using the following specific primers, as previously described [15]: Hp (F-ATGAGTGCCCTGGGAGCTGTCATT, R-GCATTAGTTCTCAGCTATGGTCTT), VEGF (F-AGGAGGGCAGAATCATCACG, R-CAAGGCCCACAGGGATTTTCT), KDR (F-CTGGCATGGTCTTCTGTG, R-AATGGGATTGGTAAGGATG), Flt-1 (F-AGCAAGTGGGAGTTTGC, R-AGGTCCCGATGAATGC), vWF (F-GAGGCTGAGTTTGAAGTGC, R-CTGCTCCAGCTCATCCAC), VE-cadherin (F-AAGACATCAATGACAACTTCC, R-CCTCCACAGTCAGGTTATACC), eNOS (F-AAGACATTTTCGGGCTCAC, R-GGCACTTTAGTAGTTCTCC), GAPDH (F-ACCACAGTCCATGCCATCAC, R-TCCACCACCCTGTTGCTGTA). The quantitative real-time RT-PCR was performed with FullVelocity SYBR Green QPCR master mix (Stratagene, La Jolla, CA) using a Real-Time PCR Machine (MX-3000P; Stratagene).

2.5. In vitro tube formation on Matrigel and EPC incorporation

Chilled Matrigel (BD Biosciences, San Jose, CA) was added to each well in 48-well plates and polymerized by incubating at 37 °C for 30 min. The GFP-tagged vector- or Hp-DNA-transduced EPCs (2×10^4 cells/well) were plated on the Matrigel and incubated for 20 h in EGM-2 medium that contained 3% FBS. To examine the incorporation of EPCs into the vascular structure, the EPCs (1×10^4 cells) and DiI-acLDL-labelled HUVECs (2×10^4 cells) were co-plated on Matrigel and co-cultured for 20 h in M199 medium that contained 3% FBS. The formation of a tubular network was observed with a fluorescence microscope and photographed.

2.6. Mouse hindlimb ischaemia

All animal procedures were approved by the Ethics Committee of the Catholic University of Korea. Male athymic nude mice of 18–22 g in weight (6 weeks old) were used. Under Zoletile 50® (10 mg/kg; Vibac, Carros, France) anaesthesia, the right proximal femoral artery and the distal saphenous artery were ligated with a 6.0 silk suture (Ethicon, Somerville, NJ), and the femoral artery and attached side branches were excised. The left hindlimb was kept intact and used as a non-ischaemic region. Twenty-four hours after induction of hindlimb ischaemia, the mice were divided randomly into three groups (1, 2, and 3), and 5×10^5 cells of vehicle vector–EPCs and Hp–EPCs per mouse were injected locally into the ischaemic thigh muscles at two different points in group 1 ($n = 8$) and 2 ($n = 8$) mice, respectively. Group 3 ($n = 4$) mice were injected with an equal volume of PBS. At 4 weeks after EPC transplantation, thigh blood flow was measured using a laser Doppler blood perfusion imager (LDPI; Perimed PeriScan PIM III, Järfälla, Sweden). After scanning three times, the LDPI index was determined as the ratio of ischaemic to non-ischaemic hindlimb blood perfusion values.

2.7. Capillary density determination

After 4 weeks of ischaemia, the ischaemic and non-ischaemic leg muscles were removed from the mice, embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands). Frozen 5 μm-thick tissue sections were prepared by cutting with a cryostat (Leica CM1800; Wetzlar, Germany), and then stained for alkaline phosphatase using the fast BCIP/NBT solution (Sigma). The tissue sections were also incubated with a monoclonal rat anti-mouse CD31 antibody (Abcam, Cambridge, UK) overnight at 4 °C. After washing with PBS, the samples were incubated with Alexa-Fluor-555-labelled secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature.

2.8. Statistical analysis

Student's *t*-test and one-way analysis of variance were used to analyse the differences between values obtained in the various experimental and control conditions. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Enhancing effect of Hp on EPC differentiation

To identify the isolated cells with typical characteristics of EPCs, at day 14 of culture, DiI-acLDL uptake and UEA-lectin binding were detected. As shown in Fig. 1A, the cells were positive for DiI-acLDL uptake and UEA-lectin binding, which suggested that the isolated cells had characteristics of EPCs. At 25–40 days after cell plating, the EPCs had grown to confluence and showed a cobblestone-like shape (Fig. 1B). EPCs are a heterogeneous cell population. Hur et al. have classified EPCs into two types according to their time-dependent appearance: spindle-shaped early EPCs with a low proliferative capacity, and cobblestone-shaped late EPCs with a high

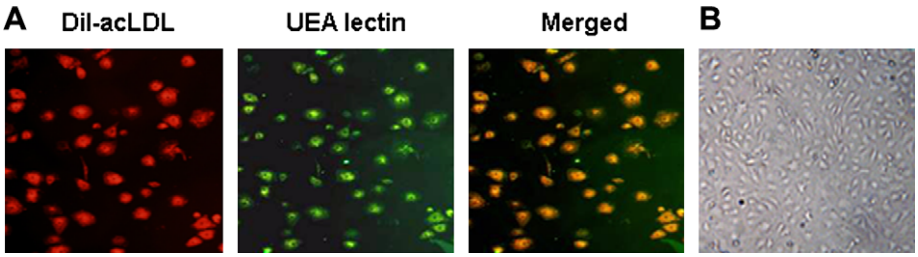

Fig. 1. Characterization of isolated EPCs. (A) At 14 days of culture, the adherent cells were double-labelled with DiI-acLDL and FITC-UEA lectin, observed by fluorescent microscopy, and photographed (×100). The merged images demonstrate that the isolated cells were dual positive for uptake of DiI-acLDL and binding to FITC-lectin. (B) Representative photomicrograph of cobblestone-like EPCs grown to confluence.

expansive capacity [16]. Here, we used the outgrowing cobblestone-like late EPCs, which have the potential to differentiate into mature endothelial cells.

When the late EPCs were transfected with human Hp cDNA, Hp protein was expressed in the cells and secreted into culture medium (Fig. 2A). The Hp-expressing EPCs showed greater increases in mRNA levels of various endothelial markers, VEGF, KDR, Flt-1, vWF, VE-cadherin, and eNOS, compared with the vehicle vector-transfected EPCs (Fig. 2B and C). Hp overexpression, however, did not significantly affect EPC growth (Fig. 2D). These results indicate that Hp promotes differentiation of EPCs but not their proliferation.

3.2. Improvement in the angiogenic property of EPCs by Hp

When EPCs were cultured on growth-factor-reduced Matrigel, the Hp-expressing EPCs showed a higher capability of capillary-like tube formation than did vector-transfected control EPCs (Fig. 3A). To assess the incorporation of EPCs into the vascular structure of endothelial cells, GFP-containing EPCs were co-cultured with DiI-AcLDL-labelled HUVECs on Matrigel. Incorporation of EPCs (green) into the network structure of HUVECs (red) was enhanced in the Hp–EPCs (Fig. 3B).

As shown in Fig. 3B (middle images), the tubular network structure of HUVECs was better formed in the presence of Hp–EPCs than

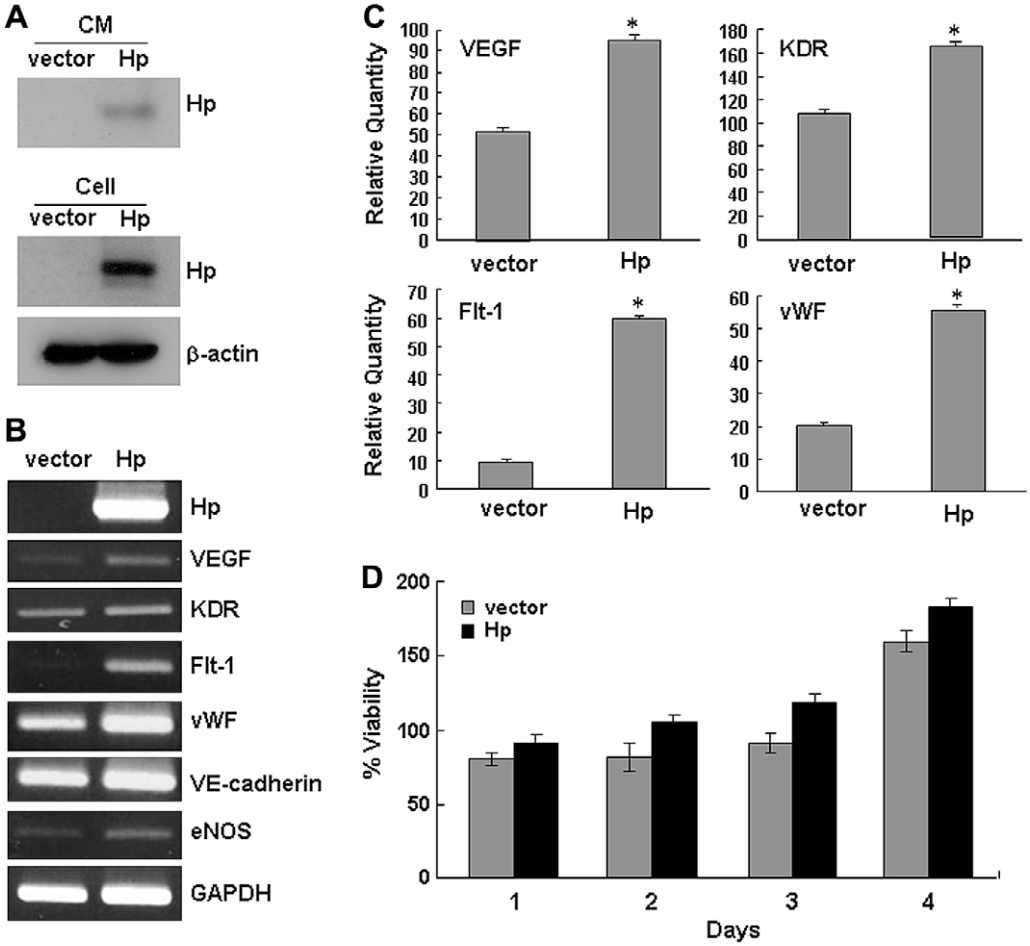

Fig. 2. Effect of Hp on EPC differentiation and proliferation. (A) Hp expression in the Hp gene-transduced EPCs and secretion into culture medium (CM) were analyzed by Western blotting. Expression of endothelial-cell-specific markers was analyzed by RT-PCR (B) and quantitative real-time RT-PCR (C) in vector– and Hp–EPCs. (D) Growth of modified EPCs was determined by MTT assay. $^{*}P < 0.05$ compared with vector-transfected EPCs. The results represent means ± S.D. of the data from triplicate experiments. The experiments were done three times and the results from three experiments were similar.

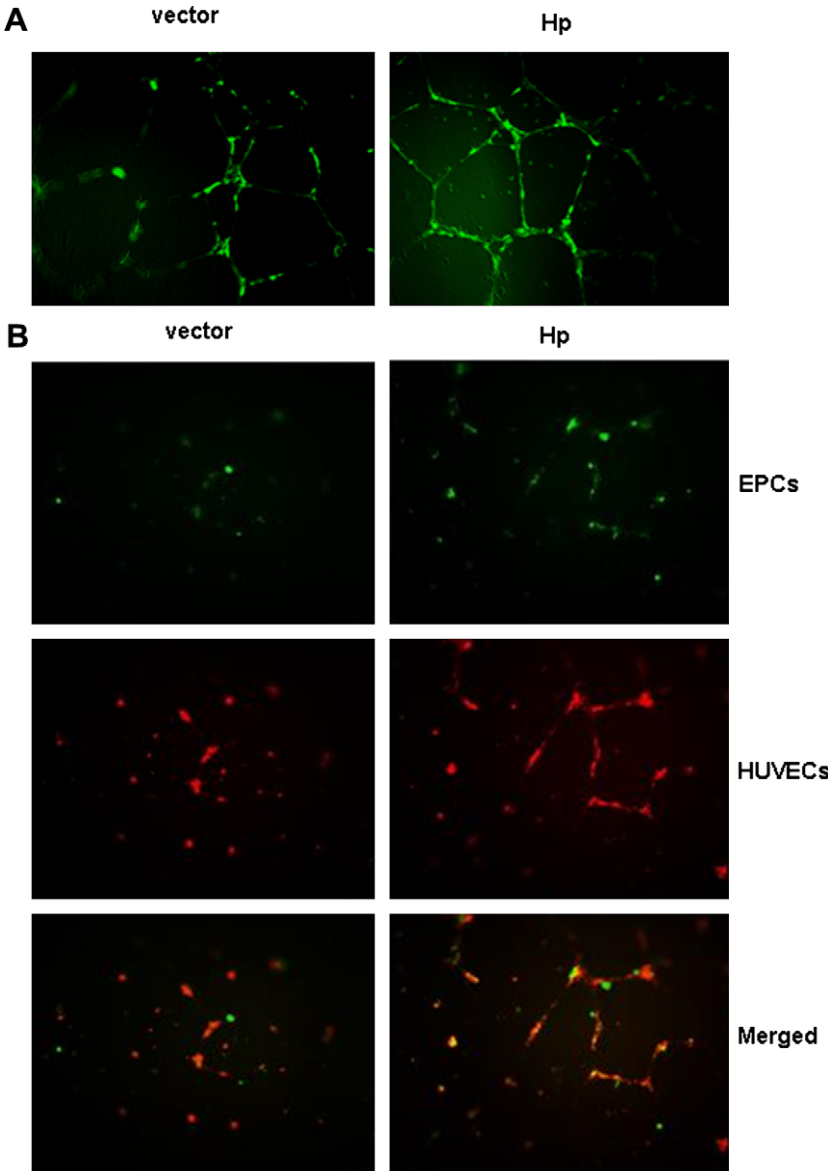

Fig. 3. Improvement of the angiogenic property of EPCs by Hp. Capillary-like tube formation on Matrigel (A) and the incorporation of EPCs into the tubular structure (B) were examined. The cells were observed under a fluorescent microscope and photographed (×100). The red and green colours correspond to HUVECs and EPCs, respectively. The merged images show that Hp–EPCs were more integrated into the vascular structure than control vector–EPCs were.

that in vector–EPCs. This seems to have resulted from the angiogenic activity of Hp, which was expressed in Hp–EPCs and secreted into the Matrigel. The result was consistent with the previous study of Cid et al. [10], in which Hp stimulated in vitro tube formation by HUVECs. However, the action mechanism of Hp on HUVEC stimulation remains to be established.

3.3. Improving activity of Hp on EPC-induced neovascularization in the mouse hindlimb ischaemia model

To investigate the effect of Hp on in vivo neovascularization, Hp-modified EPCs were transplanted locally into the hindlimb after induction of ischaemia in athymic nude mice. According to their outcomes, we classified the experimental mice into three groups: limb salvage, mild loss of limb, and severe loss of limb (Fig. 4A). The rate of limb salvage was elevated in Hp–EPCs transplantation (4/8 mice) compared with the control EPC mice (1/8) and the PBS group (0/4). Mild loss of limb was also high in the Hp–EPC group (3/8 mice with Hp–EPCs, 1/8 mice with control EPCs, and 1/4 mice with PBS). Consistent with this, LDPI index was higher in the Hp–EPC group (0.68 ± 0.22) than in the control EPC group (0.44 ± 0.19) or PBS group (0.35 ± 0.08) (Fig. 4B and C). To assess the neovascularization in the ischaemic hindlimb, capillary density was measured by staining against alkaline phosphatase and CD31 in tissue sections. Histochemical staining showed that the blood vessel density was significantly increased in tissue sections obtained from limbs treated with Hp–EPCs (Fig. 5). These findings suggest that Hp improves the potential of EPCs for repairing ischaemic injury by neovascularization.

In the in vivo study, the control EPCs without Hp (5×10^5cells per mouse) showed a low activity for recovery of ischaemic tissue (Figs. 4 and 5), despite 5×10^5–10^6 EPCs per mouse commonly being used for repair of hindlimb ischaemia. It is thought that EPC activity is reduced slightly during the processes of ex vivo modification and cell sorting by FACS. However, the stimulating effect of Hp on EPC function, which was the focus of the present study, was obvious.

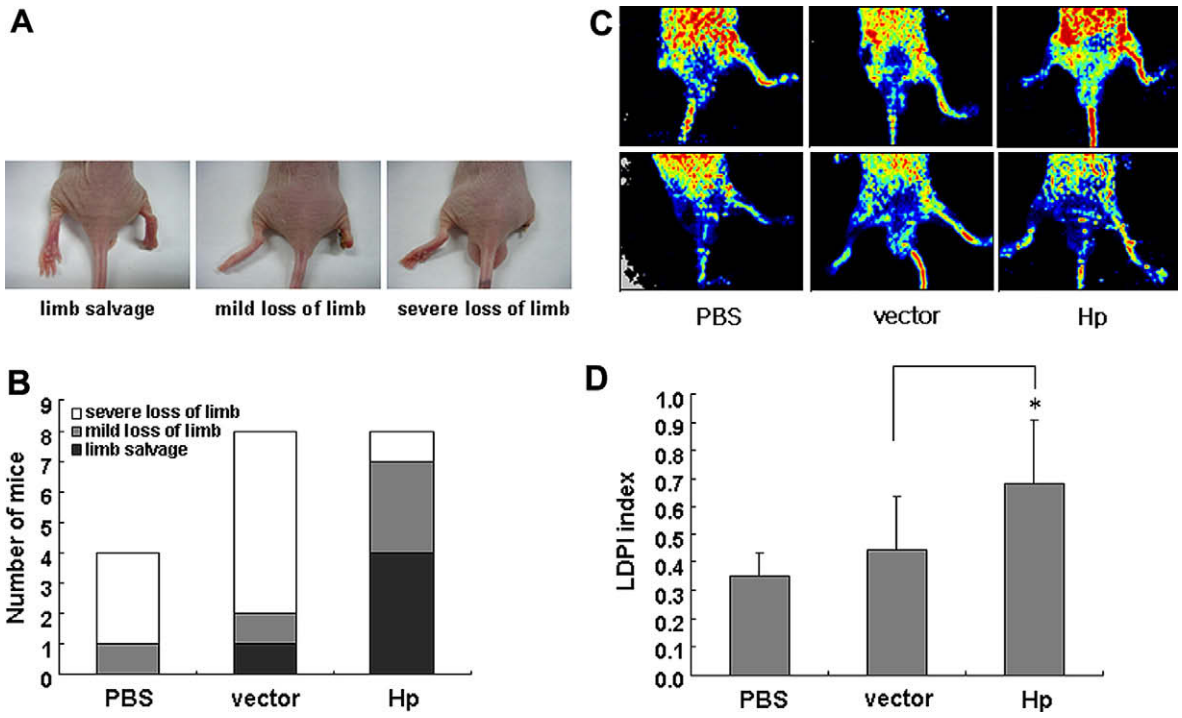

Fig. 4. Improvement of blood flow recovery by Hp-modified EPCs in the mouse hindlimb ischaemia model. (A) and (B) Representative three different outcomes (limb salvage, mild loss of limb, and severe loss of limb) of mice at day 28, and rate of the three outcomes in each group of mice. (C) Representative LDPI images of the ischaemic mice. (D) LDPI index was expressed by the blood perfusion ratio of ischaemic/non-ischaemic limbs in each group. $^{*}P < 0.05$ compared with vehicle vector-transfected EPCs.

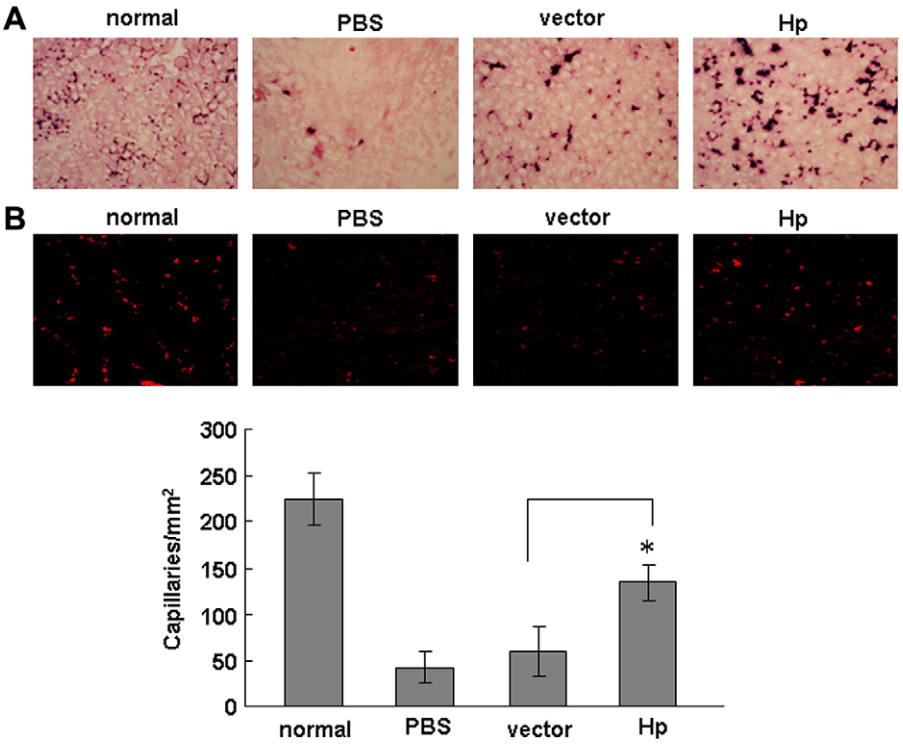

Fig. 5. Capillary density determination in the ischaemic and non-ischaemic tissues. After 4 weeks of ischaemic operation, tissue sections of the muscles obtained from ischaemic and healthy hindlimbs were stained for alkaline phosphatase (A) and CD31 (B). Four fields from each CD31 staining were selected randomly and counted. $^{*}P < 0.05$ compared with vector-transfected EPCs. Normal means the results from non-ischaemic healthy hindlimbs.

To identify the integration of transplanted GFP-containing EPCs into the repaired vessels, we examined green fluorescence in the tissue sections obtained from recovered muscles after ischaemia. However, we could not find the fluorescent cells, despite confirming their integration in the in vitro model (Fig. 3B). Hp perhaps participates in neovascular formation in vivo by enhancing pro-angiogenic factors rather than EPC integration. The underlying mechanism of Hp action on transcriptional activation of these factors is unknown. We are continuing to study the mechanism.

Humans show an allelic polymorphism for Hp. According to two major alleles, Hp^1 and Hp^2, Hp is expressed as three major phenotypes: Hp 1-1 (Hp^1/Hp^1), Hp 2-1 (Hp^1/Hp^2), and Hp 2-2 (Hp^2/Hp^2) [17]. Hp 2-2 has more potent angiogenic activity compared with that of Hp 1-1 [10], therefore, in our study, human Hp^2 gene was transduced and the effects of expressed Hp 2-2 on EPC function were investigated. More recently, Rouhl et al. have reported that Hp 1-1 inhibits EPC cluster formation and decreases the endothelial repair potential in cerebral small vessel disease with silent ischaemic lesions [18]. Further studies to confirm phenotype-dependent Hp functions on the angiogenic and neovasculogenic activities of EPCs are required.

In summary, we demonstrated that Hp improves the pro-angiogenic property of EPCs and their ability to promote recovery of blood perfusion after ischaemic injury. These results indicate that Hp can participate in neovascularization by accelerating the function of EPCs. It suggests that ex vivo modification of EPCs by Hp gene transfection is a potential strategy for improving the capability of EPCs for therapeutic revascularization.

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