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Locally Delivered Growth Factor Enhances the Angiogenic Efficacy of Adipose-Derived Stromal Cells Transplanted to Ischemic Limbs

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ABSTRACT

Ischemia is a potentially fatal medical event that is associated with as many as 30% of all deaths. Stem cell therapy offers significant therapeutic promise, but poor survival following transplantation to ischemic tissue limits its efficacy. Here we demonstrate that nanosphere-mediated growth factor delivery can enhance the survival of transplanted human adipose-derived stromal cells (hADSCs) and secretion of human angiogenic growth factors per cell, and substantially improve therapeutic efficacy of hADSCs. In vitro, in hypoxic (1% oxygen) and serum-deprived conditions that simulate in vivo ischemia, fibroblast growth factor-2 (FGF2) significantly reduced hADSC apoptosis and enhanced angiogenic growth factor secretion. In vivo, hADSCs delivered intramuscularly into ischemic hind limbs in combination with FGF2 resulted in significant improvements in limb survival and blood perfusion, as well as survival of the transplanted hADSCs and secretion of human angiogenic growth factors (i.e., vascular endothelial growth factor, hepatocyte growth factor, and FGF2). Interestingly, the majority of transplanted hADSCs were localized adjacent to the microvessels rather than being incorporated into them, suggesting that their major contribution to angiogenesis might be to increase paracrine secretion of angiogenic growth factors. This study demonstrates the potential of hADSCs in combination with growth factors for use in the treatment of ischemia. STEM CELLS *2009;27:1976–1986*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Adipose-derived stromal cells (ADSCs), multipotent stem cells residing in the stromal-vascular fraction of adipose tissues, are able to differentiate into multiple mesenchymal cell types [1–3]. Several studies have demonstrated that stem cells isolated from the stromal-vascular fraction of adipose tissue can differentiate into endothelial cells, incorporate into vessels, and promote postischemic neovascularization in vivo [4, 5]. Secretion of angiogenic and antiapoptotic factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) by ADSCs also enhances angiogenesis in animal models of hind limb or myocardial ischemia [6–10]. In addition, ADSCs are a favorable source of the large number of cells required for transplantation because they can be isolated from a small volume of adipose tissue or liposuction aspirates and expanded in vitro using standard cell culture technologies [11].

Despite the potential advantages of ADSCs, low therapeutic efficacy due to poor survival of cells transplanted into ischemic tissues remains the largest obstacle to overcome in stem cell therapy for angiogenesis. Cells transplanted into ischemic regions are exposed to hypoxia immediately after transplantation due to a lack of initial vasculature and are prone to undergo apoptosis [12, 13]. Indeed, a high level of cell death has been observed within a few days after transplantation into ischemic muscles [14, 15]. The inflammatory and proapoptotic environment in ischemic tissue also causes poor survival of transplanted cells [16]. Thus, administration of cells alone may not have a significant enough effect on angiogenesis induction.

To address these limitations, we investigated whether nanosphere-mediated, local delivery of fibroblast growth

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factor-2 (FGF2) could enhance the angiogenic efficacy of human ADSC (hADSC) therapy in treating mouse hind limb ischemia. A delivery system based on biodegradable polymer nanoparticles was used [17]. We previously demonstrated that heparin-conjugated poly(L-lactide-co-glycolide) nanospheres (HCPNs) suspended in fibrin gel can locally release FGF2 for up to 1 month [17]. One day after ischemia induction, athymic mice received HCPN injection alone, hADSC transplant, FGF2 delivery, or combined hADSC transplant and FGF2 delivery. The combination treatment of hADSC transplantation and FGF2 delivery led to a significant improvement in clinical markers including limb survival, angiogenesis, and blood perfusion.

Materials and Methods

An expanded Materials and Methods is provided in supporting information.

Isolation and Culture of hADSCs

hADSCs were isolated and cultured as described previously [18]. Informed consent was obtained from all patients included in this study according to approved procedures. Human lipoaspirates were collected from patients by elective liposuction. Liposuction tissue samples were washed extensively with phosphate-buffered saline (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and digested at 37°C for 30 minutes with 0.075% (wt/vol) collagenase type I (Sigma). After centrifugation at 1,200 rpm for 10 minutes, floating adipocytes were separated from the stromal-vascular fraction. The isolated stromal-vascular fraction was incubated overnight at 37°C with 5% CO_2 in an expansion medium of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco-BRL), 100 units/ml penicillin, and 100 μg/ml streptomycin. Following incubation, tissue culture flasks were washed to remove residual nonadherent cells and maintained at 37°C, 5% CO_2 in expansion medium. Cells were expanded for 7-10 days until they achieved confluence and were then subcultured. All experiments were performed using the third passage of hADSCs.

hADSC Culture Under Hypoxic and Serum-Deprived Conditions

When hADSC cultures reached 80% confluence, the culture medium (DMEM/F-12 medium with 10% FBS) was replaced with fresh DMEM/F-12 without FBS. hADSCs were placed in normoxic conditions (20% O_2), hypoxic (1% O_2) conditions, or hypoxic conditions with daily addition of 50 ng/ml FGF2 for 2 and 4 days. At each time point, the cells in each group were counted using a hemocytometer and conditioned medium was collected for enzyme-linked immunosorbent assay (ELISA).

Mouse Hind Limb Ischemia

Hind limb ischemia was induced in mice as previously described [19]. Four-week-old, female athymic mice (20 g body weight; Jungang Lab Animal, Seoul, Korea, http://www.labanimal.co.kr/) were anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg). The femoral artery and its branches were ligated through a skin incision using 5-0 silk suture (Ethicon, Somerville, NJ, http://www.ethicon.com). The external iliac artery and all of the above arteries were then ligated. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" [20].

Treatment of Limb Ischemia

HCPNs were prepared and loaded with FGF2 as described previously [17]. One day after arterial dissection, the mice were divided randomly into four experimental groups. The control group received an injection of fibrin gel (Greenplasts; Greencross PD Co., Yongin, Korea, http://www.greencross.com/) with HCPNs only (HCPN group, $n = 10$). Fibrin gel plus HCPNs loaded with 25 μg of FGF2 was injected intramuscularly into gracilis muscle in the medial thigh (FGF2 group, $n = 10$). hADSCs (5.0×10^6 cells per mouse) were suspended in fibrin gel with HCPNs and injected intramuscularly without (hADSC group, $n = 10$) or with (hADSC + FGF2 group, $n = 10$) 25 μg of FGF2. To identify the transplanted hADSCs, the cells were labeled with enhanced green fluorescence protein (eGFP) using a retroviral vector prior to transplantation as described previously [21]. Physiological status of ischemic limbs was followed up to 4 weeks after treatment.

Laser Doppler Imaging Analysis

Laser Doppler imaging analysis was performed as described previously [19]. A laser Doppler perfusion imager (Moor Instruments, Devon, U.K., http://www.moor.co.uk) was used for serial noninvasive physiological evaluation of neovascularization. Mice were monitored by serial scanning of surface blood flow in hind limbs on days 0, 3, 14, and 28 after treatment. Digital color-coded images were analyzed to quantify blood flow in the region from the knee joint to the toe and mean values of perfusion were subsequently calculated.

Statistical Analysis

Quantitative data are expressed as mean ± standard deviation. Statistical analysis was performed by analysis of variance using a Bonferroni test. A p value less than .05 was considered statistically significant.

Results

Inhibition of hADSC Apoptosis by FGF2 Under Hypoxic and Serum-Deprived Conditions In Vitro

FGF2 improved hADSC proliferation and inhibited hADSC apoptosis under hypoxic (1% oxygen) and serum-deprived conditions that simulate in vivo ischemia [13]. Proliferation of hADSCs cultured with daily addition of FGF2 to the culture medium for 2 and 4 days was significantly higher than that of hADSCs cultured without FGF2 ($p < .05$; Fig. 1A). Mitochondrial metabolic activity and cell viability were also significantly enhanced ($p < .05$; Fig. 1B, 1C). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining showed that hADSC apoptosis was significantly reduced at 2 and 4 days after culture under hypoxic and serum-deprived conditions by daily FGF2 addition ($p < .05$; Fig. 2A, 2B). Daily addition of FGF2 reduced p53 (proapoptotic factor) mRNA expression and enhanced Bcl-xL (antiapoptotic factor) mRNA expression in hypoxic hADSCs (Fig. 2C). When used in a 1-day hADSC culture together with apoptosis-inducing factors, conditioned medium from hypoxic hADSCs with FGF2 significantly reduced hADSC apoptosis compared with other conditioned media (from hypoxic hADSCs without FGF2 and normoxic hADSCs) and fresh medium ($p < .05$; Fig. 2D).

Enhancement of Angiogenic Factor Secretion from hADSCs by FGF2 Under Hypoxic and Serum-Deprived Conditions In Vitro

Addition of FGF2 to the culture medium promoted secretion of angiogenic growth factors from hypoxic hADSCs. Reverse-

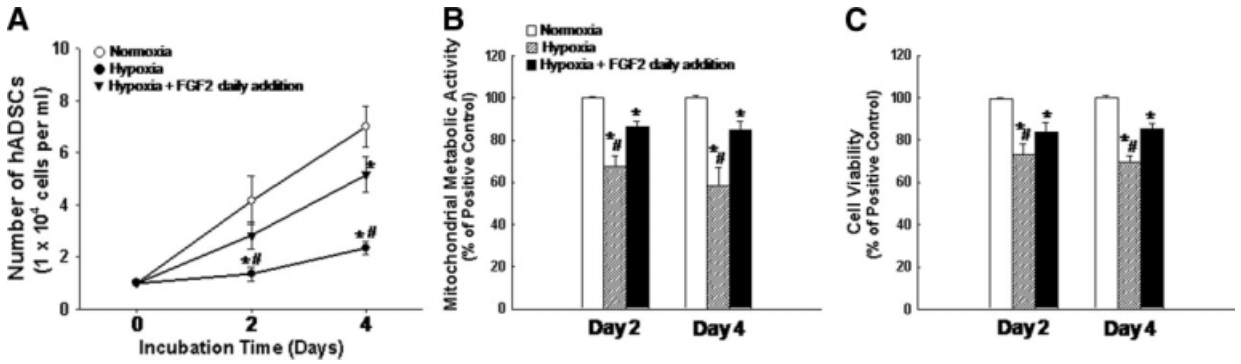


Figure 1. Enhancement of hADSC proliferation and viability by FGF2 addition under hypoxic and serum-deprived conditions in vitro. hADSCs were cultured for 2 and 4 days under normoxia (20% oxygen), hypoxia (1% oxygen), or hypoxia with daily FGF2 addition (50 ng/ml). **(A):** Number of hADSCs counted using a hemocytometer. **(B):** Mitochondrial metabolic activity of hADSCs determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **(C):** Viability of hADSCs determined by neutral red assay. (#, $p < .05$, compared with hypoxia + FGF2 daily addition group and *, $p < .05$, compared with normoxia group). Abbreviations: FGF2, fibroblast growth factor-2; hADSC, human adipose-derived stromal cell.

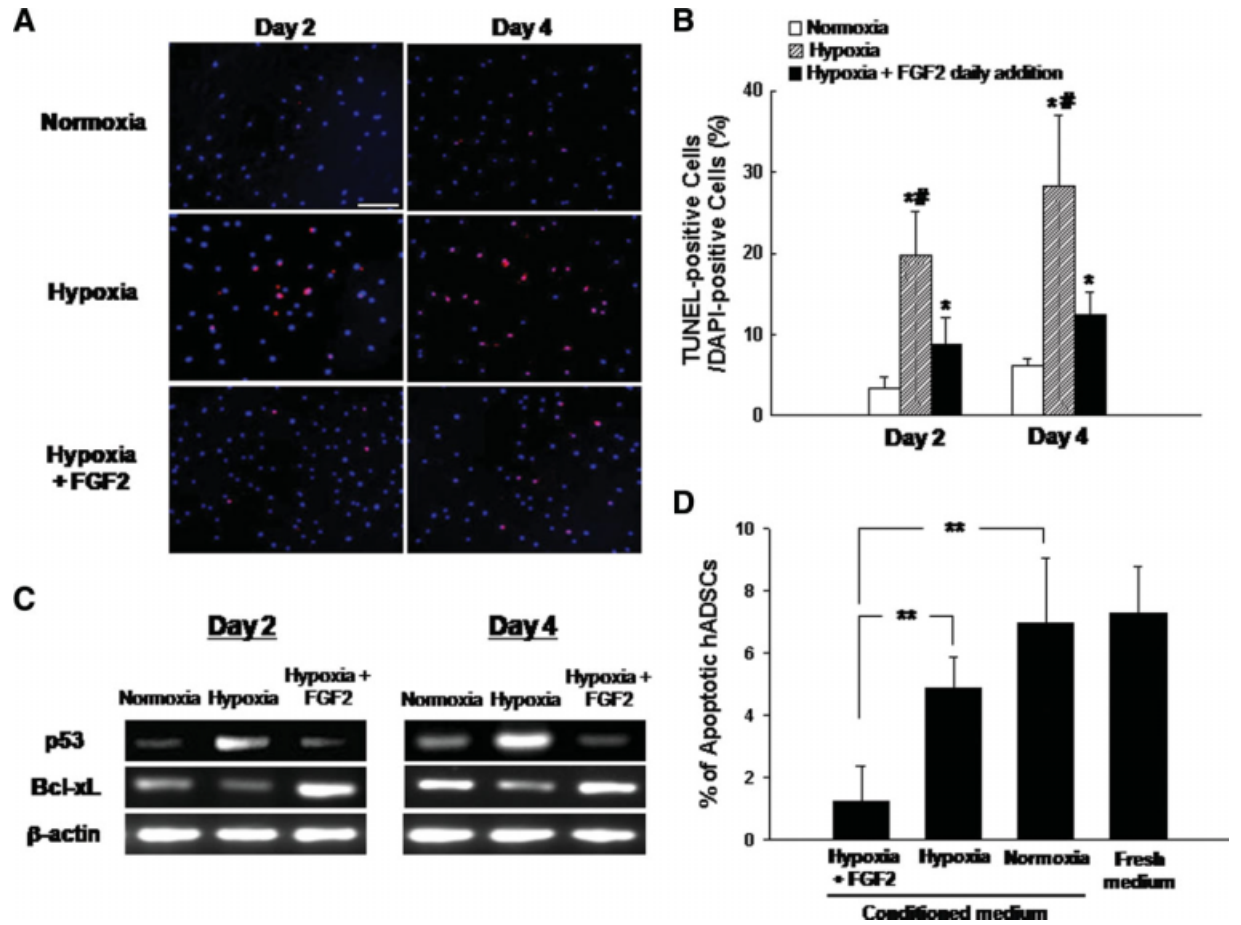


Figure 2. Inhibition of hADSC apoptosis by FGF2 addition under hypoxic and serum-deprived conditions in vitro. **(A):** TUNEL staining of hADSCs 2 and 4 days after culture under normoxia (20% oxygen), hypoxia (1% oxygen), or hypoxia with daily FGF2 addition (50 ng/ml). Scale bar indicates 100 μm. All photographs were taken at the same magnification. **(B):** Percentage ratio of TUNEL-positive cells (apoptotic cells) to DAPI-positive cells (total cells) (#, $p < .05$, compared with hypoxia + FGF2 daily addition group and *, $p < .05$, compared with normoxia group). **(C):** Reverse-transcription polymerase chain reaction of hADSCs for proapoptotic (p53) and antiapoptotic (Bcl-xL) factors. **(D):** Percentage ratio of TUNEL-positive cells in hADSCs cultured with either fresh medium or various conditioned media 1 day after apoptosis induction. Conditioned media were produced by culturing hADSCs under normoxia, hypoxia, or hypoxia with daily FGF2 addition (**, $p < .05$). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FGF2, fibroblast growth factor-2; hADSC, human adipose-derived stromal cell; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

transcription polymerase chain reaction (RT-PCR) showed that VEGF mRNA expression in hADSCs was enhanced by hypoxia (Fig. 3A). This result is consistent with a previous report that hypoxia increases VEGF expression in hADSCs [6]. Daily addition of FGF2 further increased VEGF mRNA expression in hypoxic hADSCs (Fig. 3A). Exogenous FGF2 increased

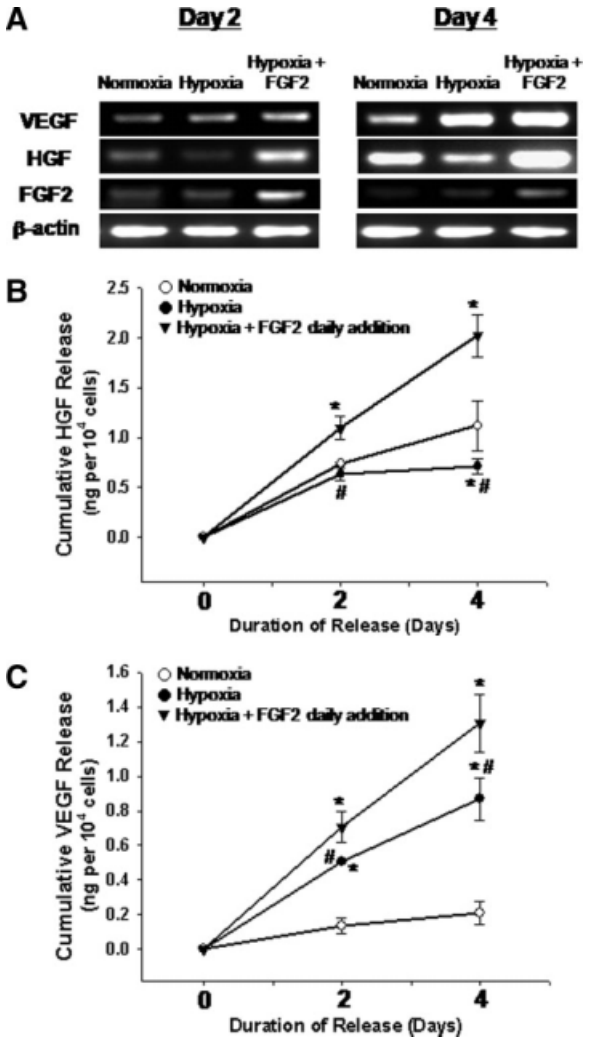


Figure 3. Enhancement of angiogenic factor secretion from human adipose-derived stromal cells (hADSCs) by FGF2 addition under hypoxic and serum-deprived conditions in vitro. **(A):** Reverse-transcription polymerase chain reaction to examine angiogenic factor expression in hADSCs 2 and 4 days after culture under normoxia (20% oxygen), hypoxia (1% oxygen), and hypoxia with daily FGF2 addition (50 ng/ml). Cumulative release profiles of **(B)** HGF and **(C)** VEGF from hADSCs at days 2 and 4, determined by enzyme-linked immunosorbent assay. Daily addition of FGF2 to the culture medium significantly enhanced HGF and VEGF secretion from hADSCs under hypoxia ($^{\#}$, $p < .05$, compared with hypoxia + FGF2 daily addition group and *, $p < .05$, compared with normoxia group). Abbreviations: FGF2, fibroblast growth factor-2; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor.

expression of endogenous FGF2 in hADSCs under hypoxic condition (Fig. 3A). HGF mRNA expression was lower in hypoxic hADSCs without FGF2 addition than in normoxic hADSCs, but was significantly enhanced in hypoxic hADSCs by the presence of FGF2 (Fig. 3A). Quantification of angiogenic proteins using ELISA confirmed that daily addition of FGF2 significantly increased secretion of angiogenic proteins (Fig. 3B, HGF; Fig. 3C, VEGF) by hADSCs cultured under hypoxic and serum-deprived conditions. Addition of FGF2 further enhanced hypoxia-inducible factor (HIF)-1α expression induced by hypoxia in hADSCs (supporting information Fig. 1).

Improvement of Transplanted hADSC Survival by Local Delivery of FGF2

Local delivery of FGF2 using HCPNs and fibrin gel promoted survival of hADSCs transplanted to ischemic regions of mouse hind limbs. TUNEL staining of ischemic muscle retrieved 3 days after hADSC transplantation showed that the proportion of TUNEL- and eGFP-positive hADSCs (apoptotic transplanted hADSCs, arrows) was significantly lower in the hADSC transplantation group with FGF2 delivery (hADSC + FGF2 group) than in the hADSC transplantation alone group (hADSC group) ($p < .05$; Fig. 4A, 4B). Human nuclear antigen (HNA)-positive cells (transplanted hADSCs) were more abundant in the hADSC + FGF2 group compared with the hADSC group ($p < .05$; Fig. 4C, 4D). RT-PCR using human-specific primers showed that local delivery of FGF2 enhanced mRNA expression of antiapoptotic factor (Bcl-xL) in hADSCs transplanted into ischemic regions while reducing proapoptotic factor (Bax) mRNA expression (Fig. 4E). Western blot for human HIF families (HIF-1α and HIF-3α) revealed that local delivery of FGF2 also increased the expression of HIF-1α and HIF-3α in hADSCs transplanted into mouse ischemic tissue (supporting information Fig. 2).

Enhancement of Angiogenic Factor Secretion from Transplanted hADSCs by Local Delivery of FGF2

Local delivery of FGF2 enhanced paracrine secretion of angiogenic factors by hADSCs transplanted to ischemic sites. Double immunofluorescent staining for HNA and angiogenic factors (VEGF, HGF, and FGF2) revealed that secretion of these factors was higher in the hADSC + FGF2 group than the hADSC group (Fig. 5A–5C). RT-PCR using human-specific primers confirmed that human angiogenic factor expression in ischemic muscles analyzed 3 days after hADSC transplantation was enhanced by local delivery of FGF2 (Fig. 5D). Enhanced expression of paracrine angiogenic factors was maintained for up to 28 days (Fig. 5E). This enhanced paracrine action of hADSCs activated host (mouse) cells to produce higher level of angiogenic growth factors (VEGF and FGF2) (supporting information Fig. 3). The expression of mouse adhesion molecules (intercellular adhesion molecule-1 [ICAM-1] and platelet-endothelial cell adhesion molecule-1 [PECAM-1]) was increased in mouse ischemic tissue treated with combined therapy of hADSC and FGF2, compared with either single therapy alone (supporting information Fig. 4).

Enhancement of hADSC-Mediated Angiogenesis by Local Delivery of FGF2

Local delivery of FGF2 and HCPNs to ischemic limb tissues using fibrin gel enhanced angiogenesis mediated by hADSC transplantation. Immunofluorescent staining for mouse von Willebrand Factor (vWF) (Fig. 6A) and quantification of capillary density (Fig. 6C) revealed that the combined therapy of hADSC transplantation and FGF2 delivery significantly enhanced ($p < .01$) capillary formation ($184 \pm 25/mm^2$) compared with HCPN implantation ($5 \pm 5/mm^2$) or single therapy (FGF2 delivery, $38 \pm 15/mm^2$; hADSC transplantation, $69 \pm 13/mm^2$). Immunofluorescent staining for mouse smooth muscle (SM) α-actin (Fig. 6B) and quantification of arteriole density (Fig. 6D) revealed that arteriole formation was significantly enhanced ($p < .01$) by the combined therapy ($19 \pm 3/mm^2$) compared with HCPN implantation ($1 \pm 1/mm^2$) or single therapy (FGF2 delivery, $2 \pm 1/mm^2$; hADSC transplantation, $8 \pm 3/mm^2$). Many more HNA-positive hADSCs were detected in the vicinity of capillaries and arterioles in ischemic tissues from the hADSC + FGF2 group compared with the hADSC group (Fig. 6A, 6B). Most transplanted hADSCs

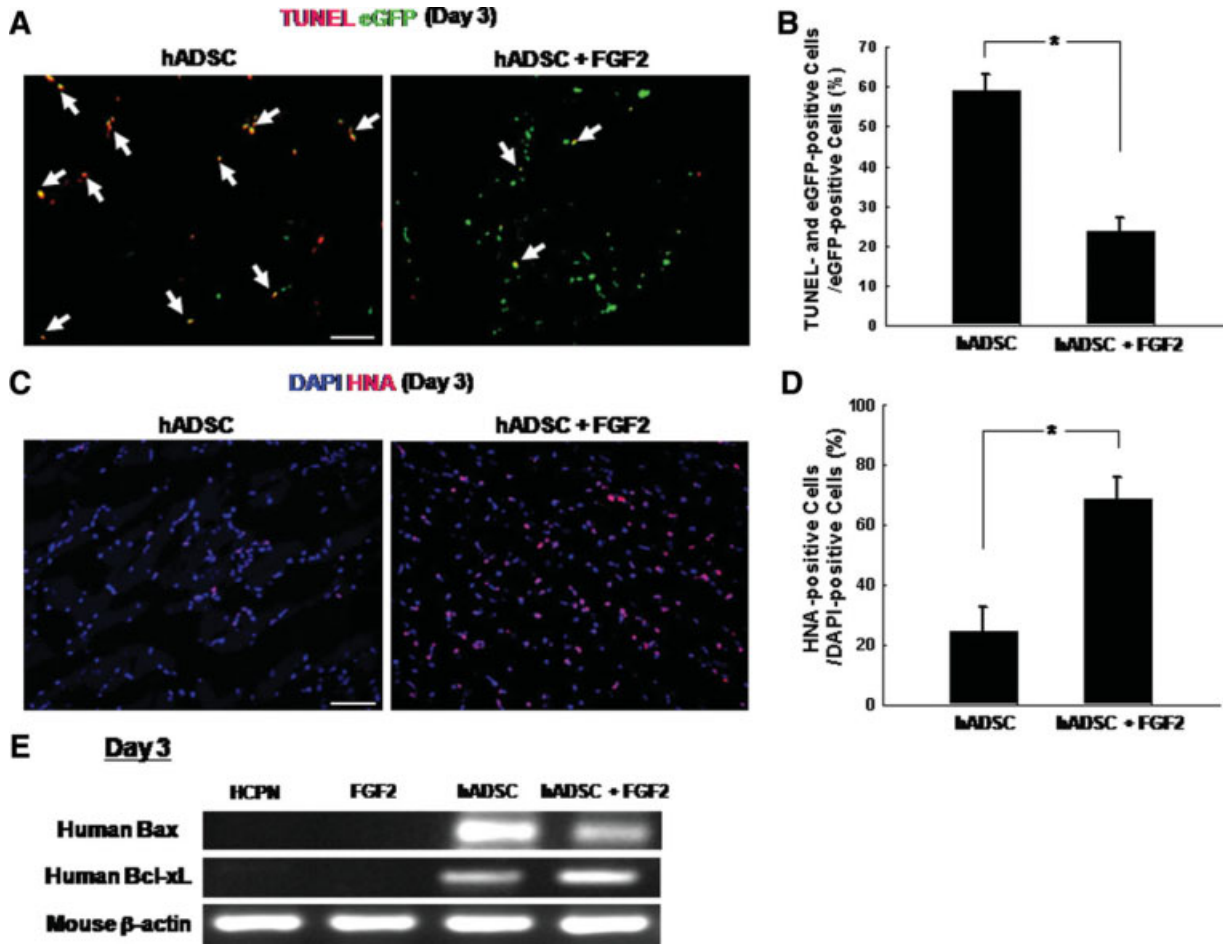


Figure 4. Enhancement of transplanted hADSC survival by locally delivered FGF2 3 days after transplantation into ischemic limb muscle. **(A):** TUNEL staining of hADSC and hADSC + FGF2 groups. Prior to transplantation, hADSCs were labeled with eGFP gene (green) using a retroviral vector. Apoptotic nuclei were stained with rhodamine (red) by the TUNEL method. Apoptosis in transplanted hADSCs (arrows) was reduced by FGF2 delivery. Scale bar = 100 μm. Both photographs were taken at the same magnification. **(B):** The ratio of TUNEL- and eGFP-positive cells (apoptotic transplanted hADSCs) to eGFP-positive cells (transplanted hADSCs) in ischemic regions (*, $p < .05$). **(C):** HNA staining of hADSC and hADSC + FGF2 groups. HNA-positive cells were more abundant in the hADSC + FGF2 group than in the hADSC group. Scale bar = 100 μm. Both photographs were taken at the same magnification. **(D):** The ratio of HNA-positive cells (transplanted hADSCs) to DAPI-positive cells (total cells) in ischemic regions (*, $p < .05$). **(E):** Reverse-transcription polymerase chain reaction for proapoptotic (Bax) and antiapoptotic (Bcl-xL) factors in ischemic limb muscles retrieved at 3 days. Human-specific primers were used. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FGF2, fibroblast growth factor-2; hADSC, human adipose-derived stromal cell; HCPN, heparin-conjugated poly(L-lactide-co-glycolide) nanosphere; HNA, human nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

were found adjacent to capillaries and arterioles in ischemic regions, although some hADSCs were incorporated into mouse microvessels (arrows, small figures in Fig. 6A, 6B). RT-PCR analysis using mouse-specific primers indicated that vWF and SM α-actin mRNA expression was higher in the hADSC + FGF2 group than in the other groups (Fig. 6E). RT-PCR analysis using human-specific primers showed that human vWF mRNA expression was also higher in the hADSC + FGF2 group than in the hADSC group (Fig. 6F), suggesting that local delivery of FGF2 promotes endothelial differentiation of hADSCs transplanted to ischemic limbs. The expression of human SM α-actin was detected in only combined therapy group (hADSC + FGF2) (supporting information Fig. 5).

Improvement of Ischemic Limb Salvage by hADSC Transplantation with FGF2 Local Delivery. We investigated whether the therapeutic potential of hADSC transplantation in an athymic mouse model of hind limb ischemia could be enhanced by local delivery of FGF2. After treatment, the control group (HCPN implantation) showed rapid and extensive necrosis of the ischemic hind limbs, resulting in complete limb loss via autoamputation by 28 days (Fig. 7A). All mice receiving HCPN injection underwent limb loss (90%, 9 of 10) or severe limb necrosis (10%, 1 of 10) without limb salvage (0%, 0 of 10) (Fig. 7B). Single therapies (FGF2 delivery or hADSC transplantation) slowed limb necrosis after treatment (Fig. 7A), but more than 60% of the mice ultimately underwent limb loss by 28 days (Fig. 7B). Intramuscular injection of hADSCs with FGF2 significantly reduced the rate of limb loss compared with the other groups (Fig. 7A, 7B). Most mice receiving the combined therapy exhibited limb salvage (40%, 4 of 10) or mild limb necrosis (30%, 3 of 10), although some lost limbs (30%, 3 of 10) (Fig. 7B).

Inhibition of Muscle Degeneration and Fibrosis in Ischemic Limbs by Angiogenic Therapies. Histological examination of ischemic limbs retrieved 28 days after treatment showed

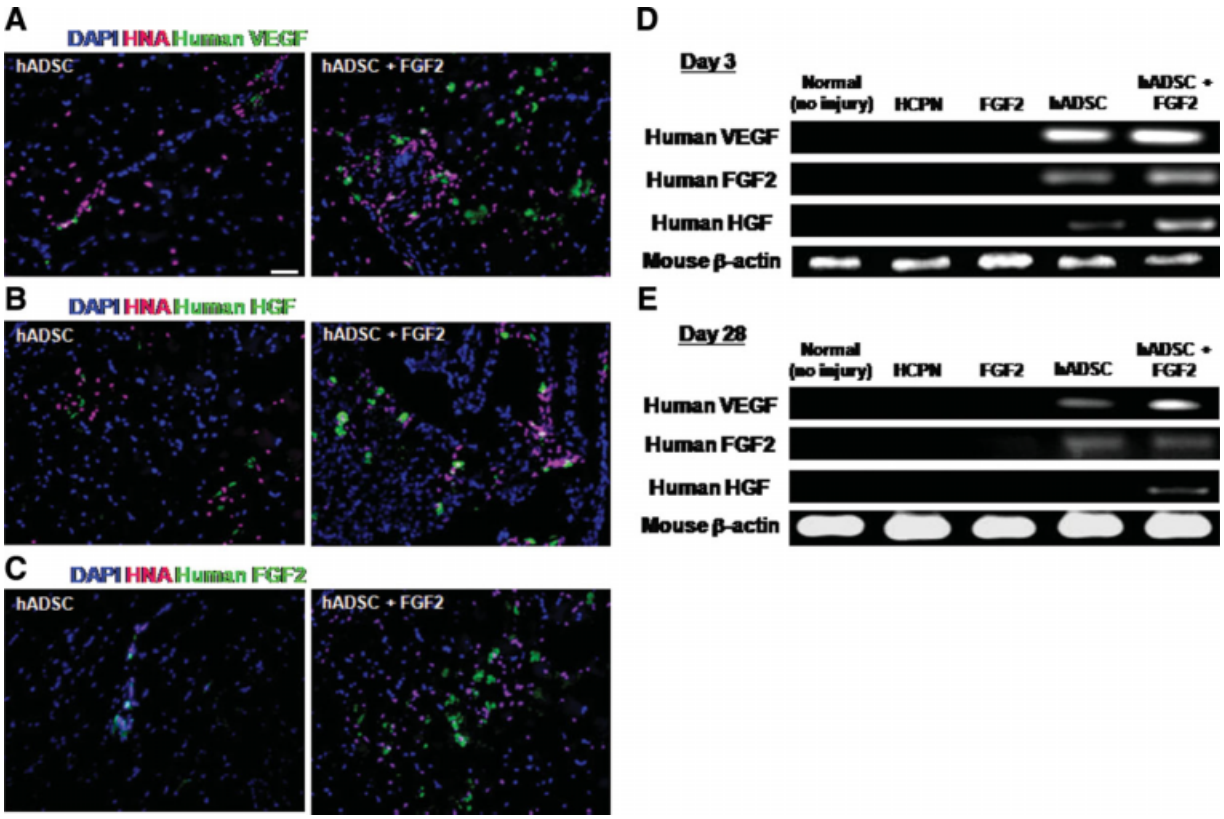


Figure 5. Enhancement of angiogenic growth factor secretion from transplanted hADSCs by locally delivered FGF2. Double immunofluorescent staining for HNA and human-specific **(A)** VEGF, **(B)** HGF, and **(C)** FGF2 of ischemic hind limb muscle at 28 days. Significant expression of human angiogenic factors (green) was detected in the vicinity of hADSCs (pink) transplanted with FGF2 delivery compared with hADSCs transplanted without FGF2 delivery. Scale bar = 100 μm. All photographs were taken at the same magnification. Reverse-transcription polymerase chain reaction analysis of angiogenic growth factors using human-specific primers. Ischemic limbs were retrieved at **(D)** 3 days and **(E)** 28 days. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FGF2, fibroblast growth factor-2; hADSC, human adipose-derived stromal cell; HCPN, heparin-conjugated poly(L-lactide-co-glycolide) nanosphere; HGF, hepatocyte growth factor; HNA, human nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; VEGF, vascular endothelial growth factor.

that angiogenic therapies (hADSC transplantation, FGF2 delivery, or combined therapy) protected limb muscles from necrotic damage by ischemia. Hematoxylin and eosin staining of the control group (HCPN injection) showed massive muscle degeneration in the ischemic regions and infiltration of numerous granulocytes and neutrophils, indicative of tissue inflammation after ischemia (Fig. 7C). In contrast, muscles in the ischemic limbs of groups receiving angiogenic therapies were protected from degeneration after treatment (Fig. 7C), and Masson's trichrome staining showed that fibrosis was markedly attenuated (Fig. 7D).

Improvement of Blood Perfusion in Ischemic Limbs by hADSC Transplantation with Local FGF2 Delivery

Laser Doppler perfusion imaging analysis (Fig. 7E) revealed that blood perfusion in ischemic limbs was significantly improved in the hADSC + FGF2 group compared with the other groups. Fourteen days after treatment, the relative ratios of blood flow (ischemic to normal limb) were 46.9% ± 7.5% in the hADSC + FGF2 group and 11.5% ± 2.9% in the HCPN group ($p < .05$; Fig. 7F). Perfusion in the FGF2 group (19.5% ± 8.2%) and hADSC group (28.9% ± 8.3%) was significantly lower than in the hADSC + FGF2 group ($p < .05$; Fig. 7F). hADSC transplantation with FGF2 delivery significantly improved the relative ratio of blood perfusion (56.1% ± 5.7%) compared with the other groups (HCPN injection, 14.8% ± 4.1%; FGF2 delivery, 26.3% ± 7.4%; and hADSC transplantation, 36.3% ± 6.2%) 28 days after treatment ($p < .05$; Fig. 7F).

Discussion

Transplantation of ADSCs could be an alternative therapeutic approach for treatment of ischemic heart and peripheral tissue diseases. Although endothelial progenitor cells from peripheral blood [22, 23] or bone marrow [24, 25] have shown angiogenic potential, these cell sources have limitations for therapeutic angiogenesis. A large amount of autologous bone marrow or peripheral blood is required to obtain a sufficient number of endothelial progenitor cells to induce satisfactory reperfusion of ischemic tissues [26, 27]. This could cause practical complications in some patients with severe cardiovascular disease such as myocardial infarction or atherosclerosis. In contrast, ADSCs can be easily isolated from a small volume of adipose tissue harvested by a simple, minimally invasive method and expanded in vitro to the numbers required for transplantation [8, 11]. ADSCs can proliferate rapidly and retain their mesenchymal pluripotency even after long-term culture over several passages [8]. ADSC transplantation can enhance angiogenesis in animal models of severe hind limb or myocardial ischemia [8–10]. However, whether

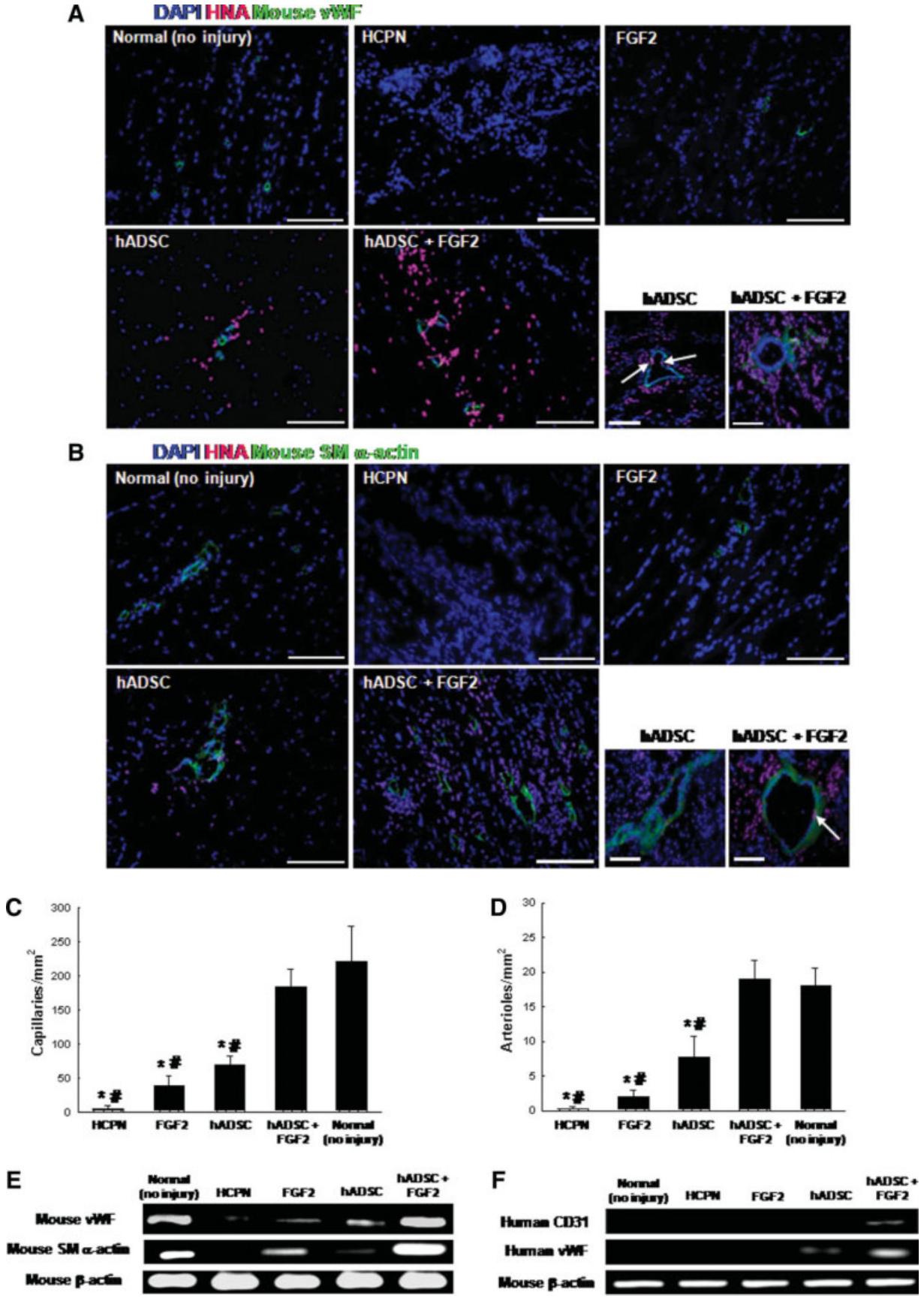
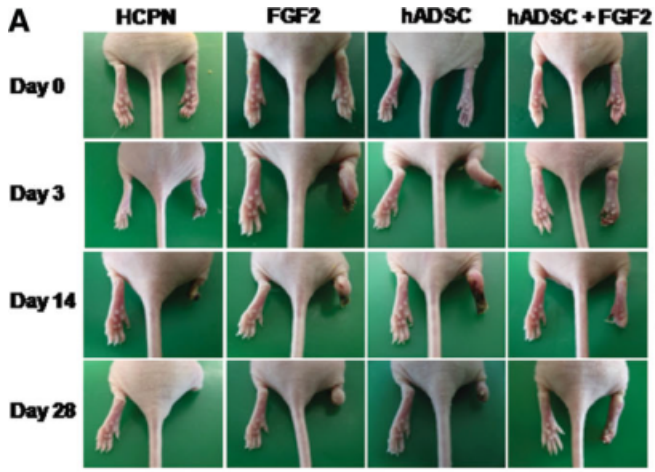
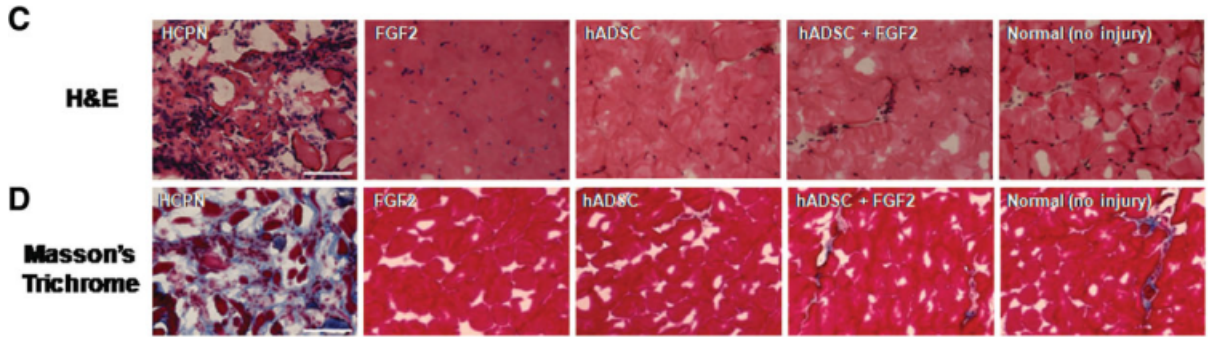


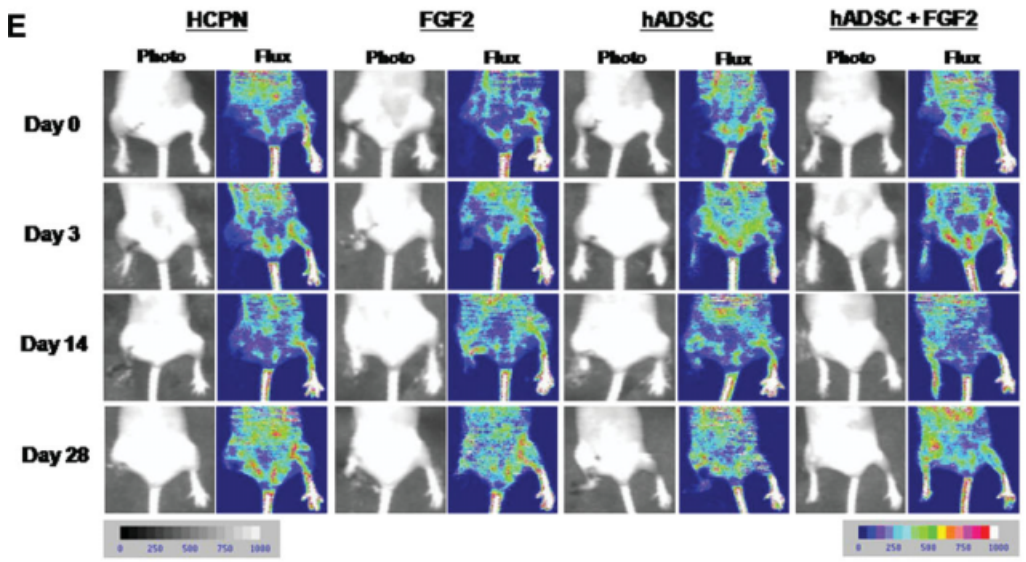
Figure 6. Enhancement of hADSC-mediated angiogenesis by local FGF2 delivery in ischemic limbs 28 days after treatment. Double immunofluorescent staining for HNA and mouse-specific **(A)** vWF and **(B)** SM α-actin. Scale bars = 100 μm. Quantification of **(C)** capillary density and **(D)** arteriole density in ischemic regions (*, $p < .01$, compared with hADSC + FGF2 group and #, $p < .05$, compared with normal group). **(E)** Reverse-transcription polymerase chain reaction (RT-PCR) analysis of vWF and SM α-actin using mouse-specific primers. **(F)** RT-PCR analysis of CD31 and vWF using human-specific primers. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FGF2, fibroblast growth factor-2; hADSC, human adipose-derived stromal cell; HCPN, heparin-conjugated poly(L-lactide-co-glycolide) nanosphere; HNA, human nuclear antigen; SM, smooth muscle; vWF, von Willebrand factor.



B

Day 28 (n=10)	HCPN	FGF2	hADSC	hADSC + FGF2
Limb salvage	0/10	1/10	2/10	4/10
Foot necrosis	1/10	2/10	2/10	3/10
Limb loss	9/10	7/10	6/10	3/10





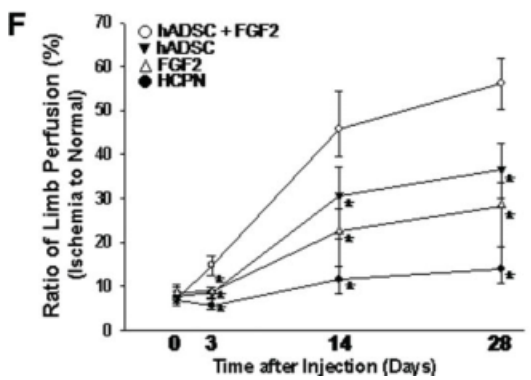


Fig. 7

the major benefits of ADSC therapy are due to direct vascular differentiation of the ADSCs themselves or result from indirect mechanisms such as paracrine support of ischemic tissues remains controversial [4–10].

Local delivery of FGF2 enhanced the angiogenic efficacy of transplanted hADSCs, likely by enhancing their survival. Our results showed that compared with either hADSC transplantation or FGF2 delivery alone, hADSC transplantation with local FGF2 delivery significantly enhanced microvessel formation and improved ischemic limb salvage and blood perfusion in ischemic limbs (Figs. 6, 7). It is well known that poor viability of stem cells transplanted to ischemic sites limits their therapeutic potential [14]. Importantly, our results showed that FGF2 significantly enhanced viability and proliferation and reduced apoptosis of hADSCs cultured in vitro under hypoxic conditions (Figs. 1A, 1C, 2). Similarly, local delivery of FGF2 significantly enhanced viability (Fig. 4C, 4D) and reduced apoptosis (Fig. 4A, 4B) of hADSCs transplanted to ischemic limbs. These results are consistent with those of previous reports in which overexpression of FGF2 enhanced ADSC proliferation and improved the viability of bone marrow-derived mesenchymal stem cells under hypoxic conditions [28, 29]. FGF2 seems to improve cell survival by activating several signaling components related to cell survival, including mitogen-activated protein kinase, Src, and protein kinase C [30, 31], and by stimulating secretion of antiapoptotic VEGF and HGF (Fig. 5E) [6].

Local delivery of FGF2 also enhanced the angiogenic efficacy of transplanted hADSCs, likely by enhancing paracrine factor secretion. FGF2 significantly enhanced the secretion of HGF and VEGF by hADSCs cultured in vitro under hypoxic conditions (Fig. 3). Following hADSC transplantation to ischemic limbs, local delivery of FGF2 also significantly enhanced mRNA expression of human HGF, FGF2, and VEGF (Fig. 5D, 5E). Local delivery of FGF2 to ischemic regions resulted in better hADSC survival and paracrine factor secretion, probably through secretion of angiogenic growth factors (Fig. 5A--5C), thus enhancing the therapeutic efficacy of hADSC transplantation. The enhanced paracrine action of hADSCs activated host cells to produce higher level of angiogenic growth factors (VEGF and FGF2) (supporting information Fig. 3).

FGF2 could contribute to improvement of hADSC therapy via the following potential signaling pathways. First, FGF2 might enhance the angiogenic efficacy of hADSCs via activation of HIF families. Addition of FGF2 further enhanced HIF-1α and HIF-3α expression induced by hypoxia in hADSCs (supporting information Figs. 1, 2). This result is consistent with the finding from a previous study showing that FGF2 enhances hypoxia-induced HIF-1α expression in many types of cells [32]. Several studies have shown that HIF-1α activity is essential for hypoxic induction of paracrine factor (VEGF, FGF2, and HGF) expression and angiogenic signaling pathway [33, 34]. Thus, exogenously added FGF2 might facilitate secretion of paracrine factors from hADSCs by enhancing the activity of HIF-1α. HIF-3α plays a role as a complement to HIF-1α in protecting cells or tissues against hypoxic damage [35]. Together these results (supporting information Figs. 1, 2) elucidate the relevance of HIF families to the action of FGF2 on hADSC survival and paracrine secretion. Exogenous FGF2 might enhance survival and paracrine action of hADSCs by triggering intrinsic signaling pathway via HIF families as well as directly affecting extrinsic pathway relating to cell proliferation. Second, adhesion molecules might be involved in enhanced angiogenesis by hADSCs treated with FGF2. The expression of adhesion molecules (ICAM-1 and PECAM-1) was increased in mouse ischemic tissue receiving combined therapy of hADSC and FGF2, compared with either single therapy (supporting information Fig. 4). Enhanced paracrine actions of hADSCs stimulated by FGF2 could activate host cells to express adhesion molecules [36]. The increase in ICAM-1 and PECAM-1 expression on activated host cells might promote the mobilization of endothelial progenitor cells to ischemic sites and contribute to neovascularization [36]. This may explain another mode of enhanced paracrine action of hADSCs by FGF2 delivery.

The present study shows that the paracrine effect of factors secreted by transplanted hADSCs, rather than differentiation of hADSCs into vascular endothelial cells and direct participation in vessel formation, may be its major contribution to the repair of ischemic tissues. Some transplanted hADSCs expressed endothelial or smooth muscle marker (Fig. 6F and supporting information Fig. 5) and were incorporated into vascular networks in ischemic sites, but the frequency was quite low (small figures in Fig. 6A, 6B). Most hADSCs were found in the vicinity of microvessels (Fig. 6A, 6B). Those hADSCs might be present as pericyte-like cells in perivascular regions to support formation and stabilization of blood vessels [37, 38]. Previous studies have also reported that vascular differentiation of ADSCs transplanted into ischemic tissues was rarely observed [8, 9]. Human angiogenic growth factors (VEGF, HGF, and FGF2) were expressed in ischemic limbs receiving hADSC transplant (Fig. 5). These results suggest that transplanted hADSCs enhance angiogenesis through secretion of human angiogenic growth factors. Interestingly, treatment with small interfering RNA for HGF significantly inhibited ADSC-mediated angiogenesis [7], and treatment with VEGF antibodies completely abolished mesenchymal stem cell-mediated angiogenesis in ischemic tissues [39]. These findings suggest that ADSC transplantation could be used in the context of cell-based cytokine therapy for therapeutic angiogenesis.

Physical incorporation and persistence of transplanted cells are critical for neovascularization and functional tissue recovery. A previous study has shown that deletion of transplanted human cells via cell death induction at 1 or 2 weeks after administration leads to a failure in improvement of neovascularization and cardiac function in treating myocardial

Figure 7. Improvement of ischemic limb salvage by hADSC transplantation with local FGF2 delivery. (A) Representative photographs of HCPN-, FGF2-, hADSC-, and hADSC + FGF2-treated ischemic hind limbs on days 0, 3, 14, and 28 after treatment. (B) Physiological status of ischemic limbs 28 days after treatment. hADSC transplantation with local FGF2 delivery significantly improved the salvage of ischemic limbs compared with the other treatments. (C) H&E staining (×400) showed massive muscle degeneration in the ischemic regions of control limbs (HCPN injection group). (D) Masson's trichrome staining (×400) showed that fibrosis in the ischemic regions was markedly attenuated by hADSC transplantation, local delivery of FGF2, and hADSC transplantation with FGF2 delivery. Scale bars = 100 μm. All photographs were taken at the same magnification. (E) Serial analysis of laser Doppler perfusion imaging performed 0, 3, 14, and 28 days after treatment. A greater increase in blood perfusion was observed in ischemic limbs of mice receiving combined therapy compared with the other treatments ($n = 10$ in all groups). (F) Blood perfusion ratio of ischemic limbs measured by laser Doppler imaging 0, 3, 14, and 28 days after treatment. The ratio of ischemic to normal limb blood perfusion was significantly improved by combined therapy at all time points (*, $p < .05$, compared with hADSC + FGF2 group). Abbreviations: FGF2, fibroblast growth factor-2; hADSC, human adipose-derived stromal cell; HCPN, heparin-conjugated poly(L-lactide-co-glycolide) nanosphere.

infarction [40]. Our staining data (Figs. 5A–5C, 6A, 6B) revealed that a number of HNA-positive cells (transplanted hADSCs) were present in ischemic regions 4 weeks after transplantation. Although incorporation of hADSCs into vascular networks is low, the persistence of hADSCs in perivascular sites not only contributes to formation of blood vessels but also stabilizes generated vascular networks in ischemic tissue through extended paracrine secretion over 4 weeks. Our FGF2 delivery system may increase the persistence of transplanted hADSCs for long-term paracrine activities, resulting in improved angiogenesis and functional recovery of ischemic limb.

The local delivery system composed of HCPN and fibrin hydrogel was designed to enhance the angiogenic efficacy of hADSC transplantation by controlling the delivery of growth factor and cells. FGF2 can be released from HCPNs suspended in fibrin hydrogel at a controllable rate over 1 month [17]. Long-term, local release of growth factor at a constant rate can allow transplanted cells to be exposed to a steady concentration over a long period [17]. Considering that most angiogenic growth factors have a short half-life in vivo, controlled and localized exposure to FGF2 could prolong survival of transplanted cells and, in turn, improve the angiogenic efficacy of stem cell therapy for the treatment of ischemia. Delivery of cells to ischemic tissue using fibrin hydrogel could localize the transplanted cells in hydrogel matrix and maintain their viability by separating them from the inflammatory and proapoptotic environment [16]. In addition, cells in fibrin matrix may avoid anoikis or apoptosis following transplantation through cell-matrix interactions, because fibrin can provide substrates that bind to cell adhesion receptors [41].

Our delivery system could be used for stem cell therapy with other growth factors that have binding affinity to heparin. For example, VEGF-A, an important signaling protein enhancing cell proliferation and angiogenesis, has heparin binding domains as FGF2 does [42], and thus could be loaded onto HCPNs. VEGF-A delivery using HCPNs could improve the therapeutic potential of hADSCs for ischemia treatment. Other growth factors with binding affinity to heparin (e.g., bone morphogenetic protein [BMP]-2) can be also applied to promote tissue regeneration of transplanted stem cells. It was reported that ectopic bone formation of transplanted mesenchymal stem cells is enhanced using HCPN-mediated BMP-2 delivery [43].

Several studies have demonstrated the benefits of combining stem cell therapy and protein or gene therapy on the angiogenic efficacy of stem cell therapy. Local delivery of an appropriate growth factor to the transplantation site could allow transplanted cells to adapt to in vivo hypoxic and inflammatory microenvironments by stimulating signal pathways required for cell survival, thus promoting their therapeutic efficacy. The present study shows that protein delivery can enhance the angiogenic efficacy of ADSC transplantation. Genetically modified hADSCs may exhibit an enhanced therapeutic angiogenesis via both autocrine and paracrine action. Previous studies have shown that genetic modification of bone marrow mesenchymal stem cells with Akt or Bcl-2 genes improved their therapeutic efficacy in treating myocardial infarction through enhanced paracrine effects (Akt [44, 45]) or reduced cell apoptosis (Bcl-2 [46]). Combining bone marrow mononuclear cell transplantation and in vivo angiopoietin-1 gene transfer promoted functional neovascularization in hind limb ischemic tissues [47]. However, most of studies have largely relied on viral vectors to deliver those genes to stem cells, which are associated with significant safety risks including immunogenicity, mutagenesis, and possible toxicity [48]. Nonviral vectors that can offer a safer delivery method still provide significantly lower delivery efficiency than viral vectors [49]. Therefore, novel delivery systems ensuring both safety and efficiency should be developed for genetic modification to be another strategy of enhancing the therapeutic efficacy of stem cells.

Summary

In summary, our study suggests that local delivery of FGF2 enhances the therapeutic angiogenic efficacy of hADSC transplantation through improvement of transplanted cell survival and paracrine angiogenic factor secretion. The delivery system used in this study could be applied to deliver dual or multiple angiogenic factors that have affinities for heparin, such as VEGF and HGF [50], which could synergistically enhance therapeutic angiogenesis using various types of stem cells [51]. The local delivery system could also used be to engineer large-volume tissues (e.g., bone, adipose, or skeletal muscle) by facilitating angiogenesis.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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