

Ang-1 Gene Therapy Inhibits Hypoxia-Inducible Factor-1 α (HIF-1 α)-Prolyl-4-Hydroxylase-2, Stabilizes HIF-1 α Expression, and Normalizes Immature Vasculature in *db/db* Mice

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OBJECTIVE—Diabetic impaired angiogenesis is associated with impairment of hypoxia-inducible factor-1 α (HIF-1 α) as well as vasculature maturation. We investigated the potential roles and intracellular mechanisms of angiopoietin-1 (Ang-1) gene therapy on myocardial HIF-1 α stabilization and vascular maturation in *db/db* mice.

RESEARCH DESIGN AND METHODS—*db/db* mice were systemically administrated adenovirus Ang-1 (Ad-CMV-Ang-1). Myocardial HIF-1 α , vascular endothelial growth factor (VEGF), hemeoxygenase-1 (HO-1), endothelial nitric oxide synthase (eNOS), Akt, and HIF-1 α -prolyl-4-hydroxylase-2 (PHD)2 expression were measured. Vasculature maturation, capillary and arteriole densities, and cardiac interstitial fibrosis were analyzed in the border zone of infarcted myocardium.

RESULTS—Systemic administration of Ad-CMV-Ang-1 results in overexpression of Ang-1 in *db/db* mice hearts. Ang-1 gene therapy causes a significant increase in Akt and eNOS expression and HIF-1 α stabilization. This is accompanied by a significant upregulation of VEGF and HO-1 expression. Intriguingly, Ang-1 gene therapy also leads to a significant inhibition of PHD2 expression. Smooth muscle recruitment and smooth muscle coverage in the neovessels of the border zone of infarcted myocardium are severely impaired in *db/db* mice compared with wild-type mice. Ang-1 gene therapy rescues these abnormalities, which leads to a dramatic increase in capillary and arteriole densities and a significant reduction of cardiac hypertrophy and interstitial fibrosis at 14 days after ischemia. Taken together, our data show that Ang-1 increases myocardial vascular maturation and angiogenesis together with suppression of PHD2 and the upregulation of HIF-1 α signaling.

CONCLUSIONS—Normalization of immature vasculature by Ang-1 gene therapy may represent a novel therapeutic strategy for treatment of the diabetes-associated impairment of myocardial angiogenesis. *Diabetes* 57:3335–3343, 2008

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Received 15 April 2008 and accepted 17 September 2008.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 3 October 2008. DOI: 10.2337/db08-0503.

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Angiogenesis is mainly regulated by the interplay between two receptor tyrosine kinase families, specifically, the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) and angiopoietins/Tie-2 families (1,2). Similar to VEGF/VEGFR, Tie-2 is an endothelial-specific receptor tyrosine kinase predominantly expressed in the vascular endothelium. Ang-1 is an oligomeric-secreted glycoprotein that binds to the Tie-2 receptor and induces Tie-2 phosphorylation (3–5). Accumulating data demonstrate that dominant Ang-1/Tie-2 signaling is essential for the maintenance of endothelial integrity and vessel maturation (3,5–10). VEGF is required to initiate immature vascular formation, whereas Ang-1 is required for further remodeling and maturation of VEGF-initiated immature vessels during postischemic angiogenesis (1,2). Overexpression of Ang-1 in transgenic mice leads to larger and more mature neovessel formation (5).

Myocardial ischemia-induced angiogenesis is regulated by hypoxia-inducible factor-1 α (HIF-1 α) and VEGF. The expression of HIF-1 α and VEGF is significantly increased in the human heart during ischemia, which may contribute to the limitation of ischemic injury by promoting angiogenesis and collateral vessel formation (11). However, the expression of both HIF-1 α and VEGF is significantly decreased in diabetic patients (12). Similarly, in the streptozotocin-induced diabetic animal model, myocardial ischemia/reperfusion-induced HIF-1 α expression is impaired, indicating a critical role of hyperglycemia in HIF-1 α stabilization and the impairment of angiogenesis (13). So far, the intracellular molecular mechanisms by which hyperglycemia decreases HIF-1 α expression have not been identified. It is well known that HIF-1 α is regulated by HIF-1 α -prolyl-4-hydroxylases (PHDs), which target HIF-1 α for ubiquitination and proteasomal degradation (14–16). Most recent research demonstrates that nitric oxide (NO) inhibits PHD2 activity and increases HIF-1 α accumulation (17). In a previous study, we demonstrated that Ang-1 is an important component in regulating coronary artery endothelial NO production and that Ang-1 mediates myocardial angiogenesis in endothelial NO synthase (eNOS)/NO-dependent mechanisms (18). In the present study, we test whether Ang-1 gene therapy rescues defective HIF-1 α signaling and improves impaired myocardial angiogenesis by the inhibition of PHD2 in type 2 diabetic *db/db* mice.

RESEARCH DESIGN AND METHODS

Experimental diabetic mouse myocardial ischemia model. C57BLKS/J and *db/db* mice (12–14 weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME). Experimental mice were anesthetized with

ketamine (100–120 mg/kg) plus xylazine (15 mg/kg), intubated, and artificially ventilated with room air. A left thoracotomy was performed, and the left anterior descending coronary artery (LAD) was exposed. An 8-0 nylon suture was placed around the LAD. Myocardial ischemia was achieved by ligation of the LAD. The sham control underwent the surgery without the LAD ligation (19–21).

Systemic delivery of Ang-1 in experimental mice. After the surgery, *db/db* mice received an intravenous tail vein injection of Ad-Ang-1 (1×10^9 plaque-forming units [Pfu]) or Ad- β -gal (1×10^9 Pfu) (21).

Blood glucose and Ang-1 levels. Blood was obtained from *db/db* and Ad-Ang-1-treated *db/db* mice by tail snip, and blood glucose levels were measured with the use of One Touch SureStep test strips and a meter. Glucose levels are expressed as milligrams per deciliter. The serum Ang-1 level was measured with an Ang-1 immunoassay kit (R&D Systems, Minneapolis, MN).

Analysis of Ang-1, PHD2, hemoxygenase-1, eNOS, Akt, HIF-1 α , and VEGF expression. At 24 h after myocardial ischemia, the hearts were harvested and homogenized in lysis buffer. Fifty micrograms of total protein were separated using SDS gel electrophoresis. The membranes were immunoblotted with HIF-1 α (1:1,000; GeneTex, San Antonio, TX), hemoxygenase (HO)-1 and eNOS (1:1,000; BD Transduction Laboratories, San Jose, CA), Akt (1:1,000; Cell Signaling Technology, Danvers, MA), PHD2, VEGF, and Ang-1 antibodies (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). For the liver Ang-1 expression, the membrane was immunoblotted with monoclonal anti-Ang-1 (1:1,000, Sigma, St. Louis, MO).

Analysis of myocardial capillary and arteriole densities. At 14 days after myocardial ischemia, the hearts were harvested and flash frozen immediately in SUPER FRIENDLY FREEZE-IT (Fisher, TX). Five-micrometer sections were cut and incubated with fluorescein-labeled Griffonia Bandeiraea Simplicifolia Isolectin B4 (1:200; LB4, Molecular Probe, Invitrogen, Eugene, OR) and Cy3-conjugated anti- α smooth muscle actin (SMA) (1:100; Sigma). The number of capillaries (LB4-positive EC) was counted and expressed as capillary density per square millimeter (mm^2). Myocardial arteriole (SMA-positive smooth muscle cells located in vascular walls) density was measured using image analysis software (Image J, National Institutes of Health, Bethesda, MD). To assess the acquisition of a muscular coat by infarct neovessels, the density of coated neovessels in the border zone of infarcted area was measured by using a section stained for SMA. Smooth muscle cell (SMC)/neovessel coverage in the border zone of infarcted area was measured using image analysis software (Image J, National Institutes of Health) (20,21).

Cardiac hypertrophy and interstitial fibrosis. Cardiac hypertrophy was assessed by measuring heart weight-to-body weight ratio. Each heart weight at 14 days postmyocardial ischemia was divided by the total body weight of the mouse, resulting in a ratio representative of cardiac hypertrophy. To determine cardiac fibrosis, sections were stained with Masson's trichrome (Sigma). Myocardial interstitial fibrosis was quantified by measuring the Masson's trichrome staining area in the remote zone of the infarcted area using National Institutes of Health image analysis software as previously described (22,23).

Mouse aortic ring sprouting and SMC recruitment assay in ex vivo model. Mouse aortas were isolated from C57BLKS/J and *db/db* mice under aseptic conditions and cut into rings ~1 mm in thickness. These rings were then placed in the middle of organ culture dishes, overlaid with 300 μl extracellular matrix (Sigma), and left to polymerize for 1–2 h at 37°C before the addition of 10% FBS endothelial growth medium. Vessel outgrowth at day 5 was examined using a Nikon TE-300 microscope. To characterize SMC recruitment, specific endothelial cell and SMC markers were directly applied to ex vivo aortic culture explants. Briefly, the cultured explants were fixed with 10% formalin for 20 min, washed with PBS, and incubated for 3 h with the following specific cell markers: fluorescein isothiocyanate-labeled mouse CD31 antibody (1:100, BD Biosciences, San Jose, CA) for EC and Cy3-conjugated anti- α SMA (1:100; Sigma) for SMCs. After incubation with these cell markers, the slides were washed three times with PBS (10 min) and mounted on an aqueous mounting medium. The immunostained explants of aortas were examined using confocal microscopy (19,20,24–26). SMC recruitment was quantified by measuring the relative area of SMC/endothelial cell coverage using image acquisition and analysis software (Image J, National Institutes of Health). All procedures were in conformance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Statistical analysis. The results are expressed as the mean \pm SD. Statistical analysis was performed using ANOVA followed by a *t* test corrected for multiple comparisons (Student-Newman-Keuls). Significance was set at $P < 0.05$.

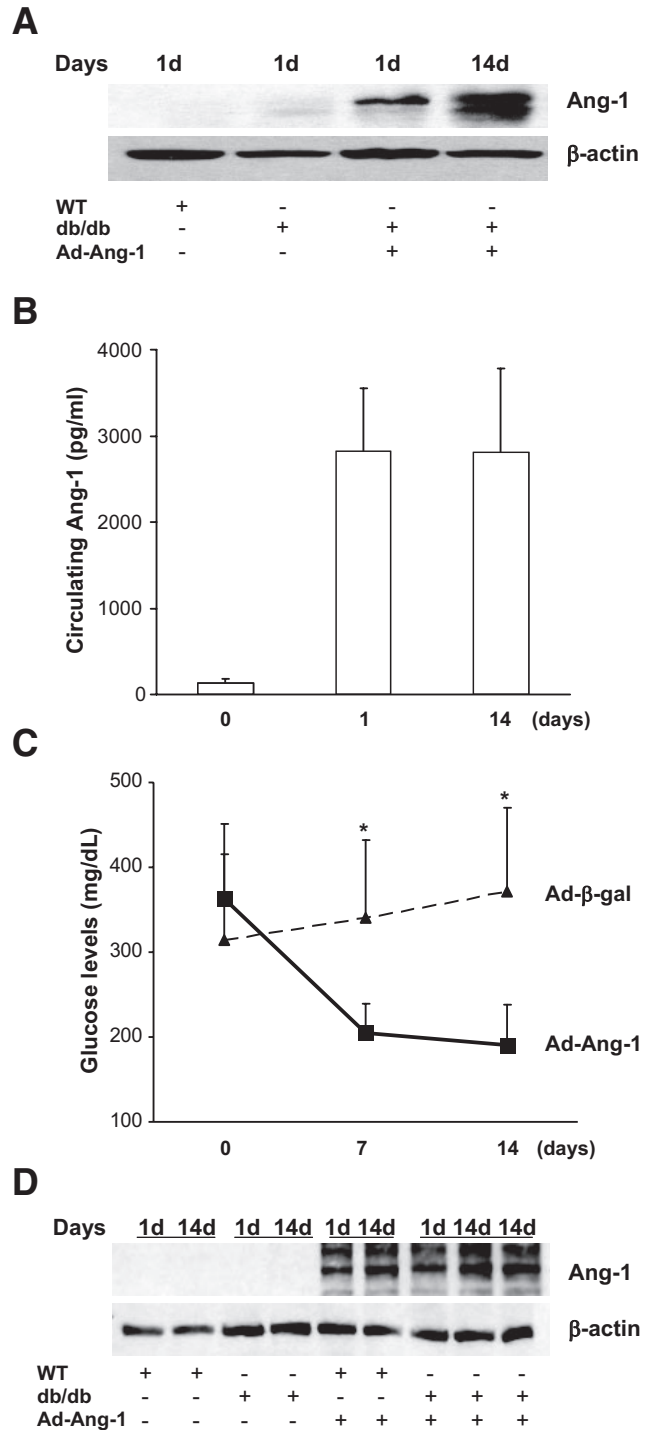


FIG. 1. A: Western blot analysis demonstrating that systemic administration of Ad-Ang-1 (1×10^9 Pfu Ad-Ang-1) resulted in overexpression of Ang-1 in the *db/db* mice liver at day 1 and day 14 ($n = 4-5$ mice). **B:** Serum level of Ang-1 after intravenous Ad-Ang-1 treatment in *db/db* mice. Values are expressed as mean \pm SD ($n = 6-7$ mice). **C:** Systemic delivery of Ad-Ang-1 in *db/db* mice led to a gradual decrease in glucose levels compared with Ad- β -gal-treated *db/db* mice during 14 days of the studies ($n = 6$) ($*P < 0.05$). **D:** Western blot analysis showing that myocardial Ang-1 protein expression was significantly increased at day 1 and remained elevated for 14 days in *db/db* mice after the systemic delivery of Ad-Ang-1. WT, wild type.

RESULTS

Systemic administration of Ad-CMV-Ang-1 by a single dose results in a sustained overexpression of Ang-1 in *db/db* mouse hearts. Intravenous administration of Ad-CMV-Ang-1 (1×10^9 Pfu) in *db/db* mice led to viral

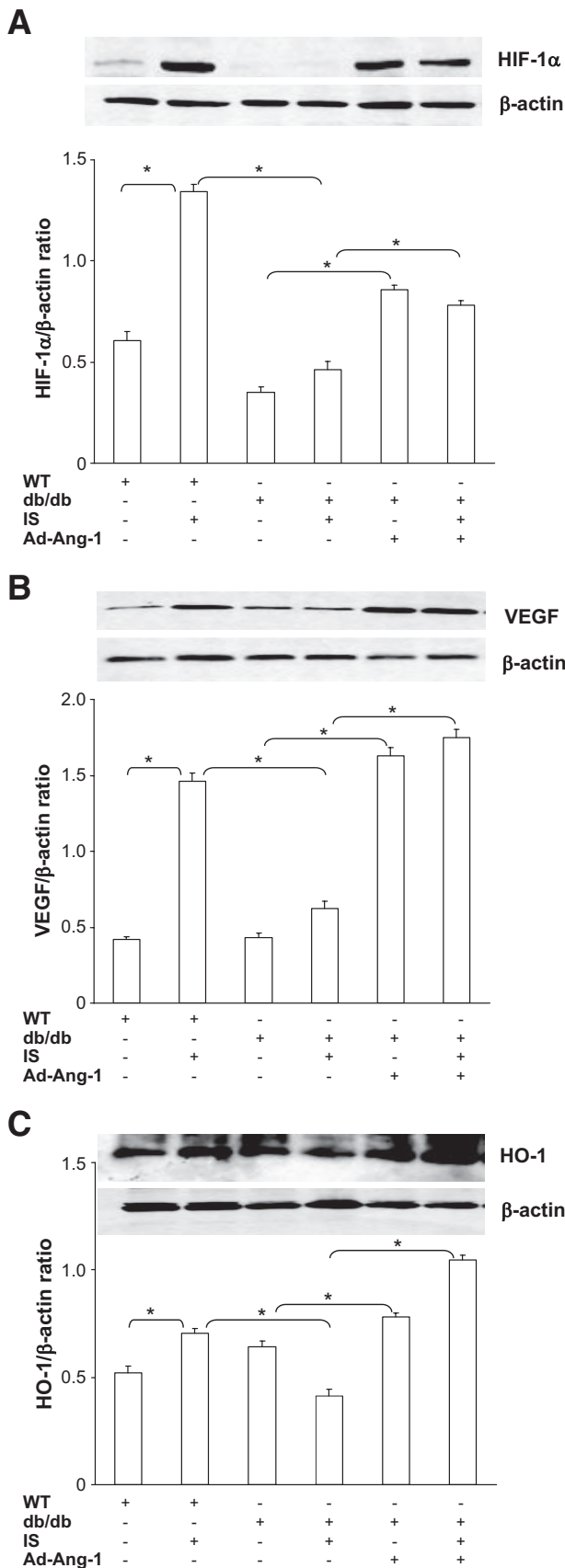


FIG. 2. *A*: Representative Western blot and densitometry analyses of myocardial HIF-1 α expression. Myocardial HIF-1 α expression was diminished in *db/db* mice compared with wild-type mice (WT). Overexpression of Ang-1 significantly enhanced HIF-1 α expression in *db/db* mice at the basal level and under ischemic conditions ($n = 6$ mice). *B*:

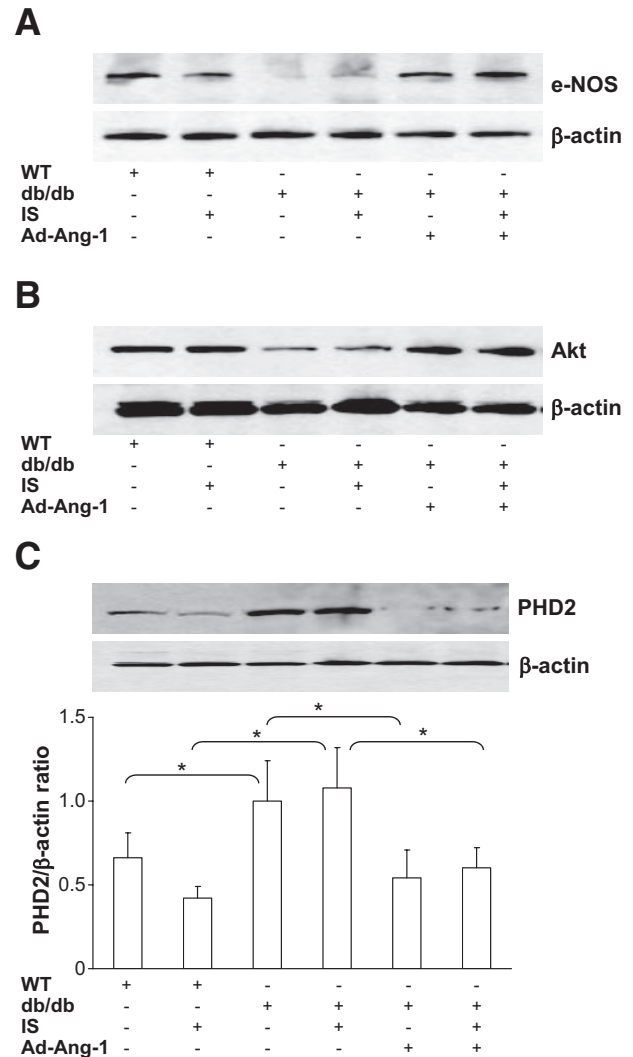
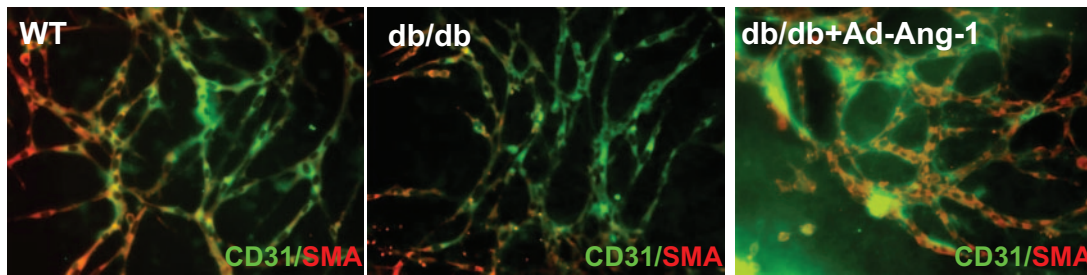


FIG. 3. Representative Western blot analysis of myocardial Akt and eNOS expression in wild-type, *db/db*, and Ad-Ang-1-treated *db/db* mice. *A*: eNOS protein expression was diminished in *db/db* mouse hearts, whereas overexpression of Ang-1 resulted in a significant increase in eNOS protein expression (Western blot data represented by four independent experiments). *B*: Total Akt protein expression was reduced in *db/db* mice compared with wild-type mice at the baseline level and at 24 h after myocardial ischemia. Systemic delivery of Ad-Ang-1 resulted in a significant increase in Akt expression in *db/db* mice. This increase was not observed in *db/db* mice treated with Ad-gal (Western blot data represented by six independent experiments). *C*: Representative Western blot and densitometry analyses of myocardial PHD2 protein expression at 24 h after myocardial ischemia. Western blot analysis showing decreased PHD2 expression in wild-type mice subjected to myocardial ischemia for 24 h. In *db/db* mice, basal PHD2 expression was increased without any further increase following myocardial ischemia. Systemic delivery of Ad-Ang-1 resulted in a significant decrease in PHD2 expression in *db/db* mice ($n = 6$). $*P < 0.05$. IS, ischemia; WT, wild type.

uptake in the liver; the level of Ang-1 expression in the liver started to increase at day 1, and the increase lasted for 2 weeks after intravenous administration of one bolus

Myocardial ischemia-induced VEGF expression was blunted in *db/db* mice compared with wild-type mice following myocardial ischemia. Overexpression of Ang-1 resulted in a significant increase in VEGF expression ($n = 6$). *C*: Western blot analysis showing that wild-type mice subjected to ischemia for 24 h demonstrated a significant increase in HO-1 expression; myocardial ischemia failed to induce HO-1 expression in diabetic *db/db* mice. Systemic delivery of Ad-Ang-1 led to a significant increase in HO-1 expression in *db/db* mouse hearts ($n = 6$). $*P < 0.05$. IS, ischemia; WT, wild type.

A



B

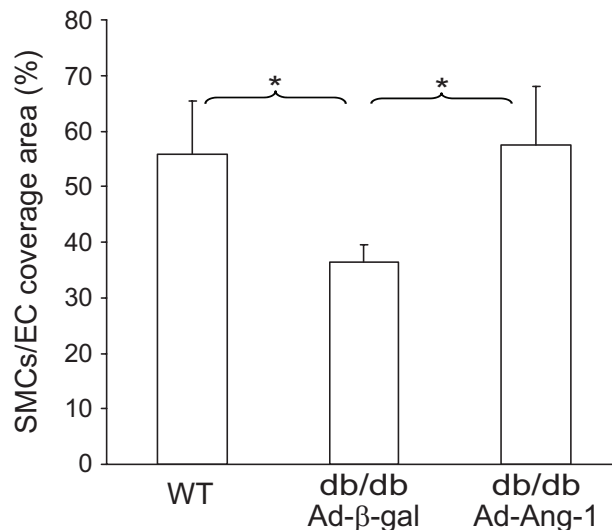


FIG. 4. A: Representative images of SMC recruitment in aortic ring explants from wild-type, *db/db*, or *db/db*⁺Ad-Ang-1 mice. ECs and SMCs in mouse aortic ring outgrowth explants were stained with CD31 (green, 40 \times) and SMA (red, 40 \times), respectively, at day 5. **B:** Quantitative analysis area of CD31/SMC coverage demonstrating that smooth muscle recruitment was significantly reduced in *db/db* mouse aortic ring explants. Systemic delivery of Ad-Ang-1 resulted in a significant increase in SMC recruitment in *db/db* mouse aortic ring explants ($n = 6$ mice). * $P < 0.05$. WT, wild type. (Please see <http://dx.doi.org/10.2337/db08-0503> for a high-quality digital representation of this figure.)

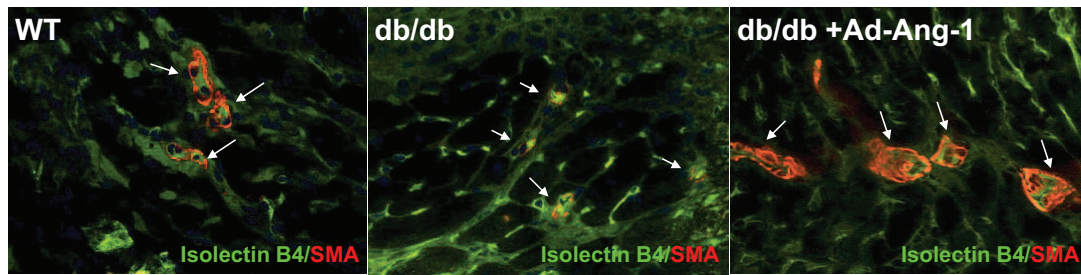
of Ad-CMV-Ang-1 (Fig. 1A). Similarly, the serum level of Ang-1 was increased 1 day after the Ad-CMV-Ang-1 injection and remained at the elevated level for 2 weeks in diabetic *db/db* mice (Fig. 1B). Intriguingly, glucose levels were significantly decreased in Ad-Ang-1-treated *db/db* mice during 14 days of the study (Fig. 1C). To further examine whether systemic administration of Ad-CMV-Ang-1 results in overexpression of Ang-1 in the heart, we examined myocardial Ang-1 levels in *db/db* mice. Our Western blot analysis data showed that myocardial Ang-1 protein expression was significantly increased at day 1 and remained elevated for 14 days in *db/db* mice after the systemic delivery of Ad-Ang-1 (Fig. 1D). Additionally, we conducted fluorescent immunohistochemistry and confocal imaging to discern the location of Ang-1 expression in Ad-Ang-1-treated *db/db* mouse hearts. Our merged images revealed that Ang-1 localized with Isolectin B4 (LB4) on ECs and SMA on vessel SMCs (supplemental Fig. 1 in the online appendix available at <http://dx.doi.org/10.2337/db08-0503>).

Ang-1 gene therapy stabilizes HIF-1 α expression and rescues impaired HIF-1 α signaling in response to ischemia in *db/db* mice. Our Western blot analysis revealed that wild-type mouse hearts exposed to ischemia for 24 h resulted in a significant increase in HIF-1 α expression, whereas myocardial ischemia-induced HIF-1 α expression was significantly blunted in *db/db* mouse hearts

(Fig. 2A). Diabetic *db/db* mice treated with Ad-Ang-1 showed a significant increase in HIF-1 α expression after myocardial ischemia compared with *db/db* mice that received the control vector (Fig. 2A). Next, we examined the expression of the HIF-1 α downstream angiogenic signaling molecules VEGF and HO-1 in response to myocardial ischemia in *db/db* mice. Wild-type mouse hearts subjected to ischemia for 24 h experienced a significant increase in both VEGF and HO-1 expression. Myocardial ischemia-induced VEGF and HO-1 expression was diminished in *db/db* mouse hearts subjected to ischemia for 24 h (Fig. 2B and C). Systemic administration of Ad-Ang-1 resulted in a significant increase in VEGF and HO-1 expression in *db/db* mice (Fig. 2B and C). Our colocalization immunohistochemical studies revealed that HIF-1 expression, but not VEGF, was colocalized with Tie-2 (supplemental Fig. 2). Furthermore, Ang-1 was colocalized to only a small extent with VEGF and HO-1 in Ad-Ang-1-treated *db/db* mouse hearts (supplemental Fig. 3). These data suggested that other mechanisms may be involved in regulation of VEGF and HO-1 expression in Ad-Ang-1-treated *db/db* mice.

Ang-1 upregulates eNOS and Akt and downregulates PHD2 expression. To explore the potential intracellular molecular mechanism by which overexpression of Ang-1 rescues the impaired HIF-1 signaling in diabetes, myocardial eNOS, Akt, and PHD2 expression in *db/db* mice was examined. Myocardial eNOS and Akt expression was signif-

A



B

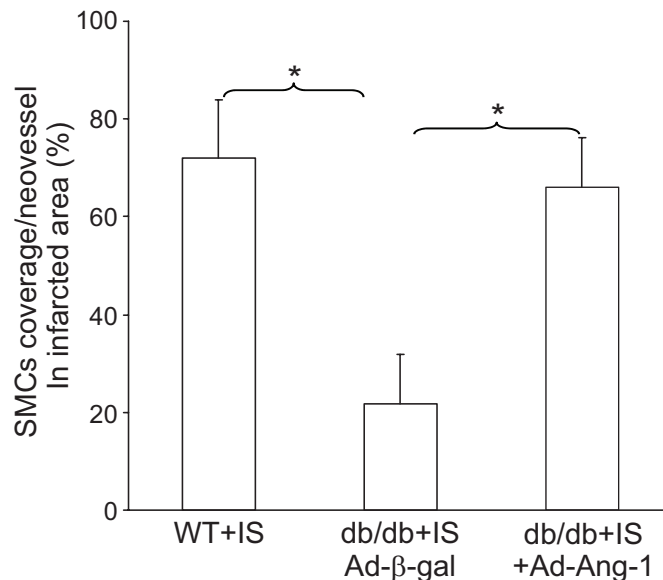


FIG. 5. A: Representative images of SMC coverage of neovessels in the border zone of infarcted myocardial of wild-type, *db/db*, and *db/db*+ Ad-Ang-1 mice at 14 days after myocardial ischemia. ECs and SMCs were stained with LB4 (green, 40 \times) and SMA (red, 40 \times) in the border zone of the infarction myocardial area. Overexpression of Ang-1 led to a significant number of coated, mature SMC neovessel formations. **B:** Quantitative analysis area of SMC coverage/neovessels showing that myocardial ischemia-induced smooth muscle recruitment was significantly reduced in *db/db* mouse hearts. Systemic delivery of Ad-Ang-1 resulted in a significant increase in the relative ratio of SMC coverage/neovessel in *db/db* mice ($n = 6$ mice). * $P < 0.05$. IS, ischemia; WT, wild type. (Please see <http://dx.doi.org/10.2337/db08-0503> for a high-quality digital representation of this figure.)

icantly decreased in *db/db* mouse hearts subjected to ischemia compared with hearts from wild-type mice. Systemic delivery of Ad-Ang-1 strikingly increased eNOS and Akt expression (Fig. 3A and B). This was accompanied by a significant suppression of PHD2 protein expression in *db/db* mice under both basal and ischemic conditions (Fig. 3C).

Overexpression of Ang-1 increases smooth muscle recruitment and improves myocardial ischemia-induced vasculature maturation. Using the ex vivo aortic ring sprouting explants model, we first examined whether overexpression of Ang-1 rescues impaired SMC recruitment in *db/db* mice. As shown in Fig. 4A and B, SMC coverage was significantly less in aortic ring explants isolated from *db/db* mice than in those from wild-type mice. Overexpression of Ang-1 significantly increased SMC recruitment in *db/db* mouse aortic ring explants (Fig. 4A and B).

Our morphological analysis further showed that the border zone of infarcted myocardial area contained a significant number of mature, coated neovessels in wild-type mice subjected to myocardial ischemia, whereas few coated neovessels were found in *db/db* mouse hearts after 14 days of ischemia (Fig. 5A). Overexpression of Ang-1 significantly increased the number of mature, coated

neovessels in *db/db* mice (Fig. 5A). To further identify whether overexpression of Ang-1 improves myocardial smooth muscle recruitment, the relative ratio of SMA to neovessel coverage in the border zone of infarcted myocardial area was investigated in *db/db* mouse hearts after 14 days of ischemia. In the border zone of infarcted myocardial area of wild-type mice, 71.9% of the neovessels were stained positive for SMA after 14 days of myocardial ischemia. The relative ratio of SMC to neovessel coverage was considerably reduced, and only 21.8% of neovessels were covered with SMA in the *db/db* mouse hearts. Overexpression of Ang-1 resulted in a significant increase in the ratio of SMC to neovessel coverage in the *db/db* mouse hearts (Fig. 5B).

Overexpression of Ang-1 increases myocardial ischemia-induced capillary and arteriole densities. To investigate whether overexpression of Ang-1 in *db/db* diabetic mouse hearts improves myocardial angiogenesis in vivo, myocardial capillary and arteriole densities in the border zone of infarcted myocardial area were examined at 14 days after myocardial ischemia. Our immunohistochemical studies revealed that overexpression of Ang-1 in *db/db* mouse hearts resulted in a significant increase in myocardial capillary density in the border zone of in-

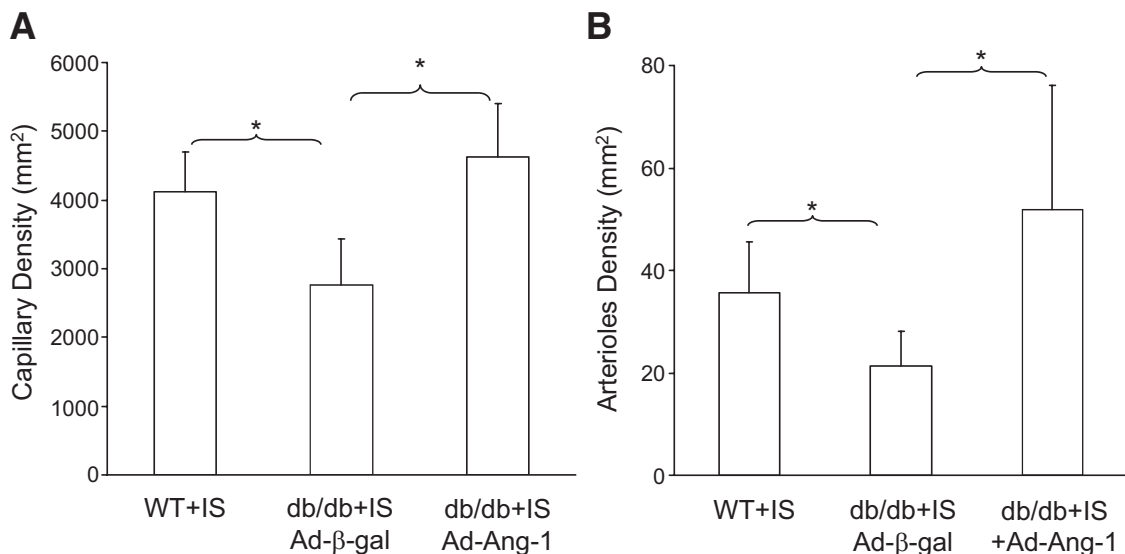


FIG. 6. A: Representative quantitative analysis by LB4 staining showing that, in Ad-β-gal-treated *db/db* mice, myocardial ischemia-induced myocardial capillary density was significantly decreased compared with that of wild-type mice. Treatment with Ad-Ang-1 significantly increased capillary formation in *db/db* mice subjected to ischemia ($n = 6$). **B:** Representative quantitative analysis by SMC staining showing that myocardial ischemia-induced arteriole density was reduced in Ad-β-gal-treated *db/db* mice compared with wild-type mice subjected to myocardial ischemia for 14 days. Treatment with Ad-Ang-1 caused a greater increase in arteriole formation in *db/db* mice subjected to ischemia than treatment with Ad-β-gal ($n = 6$). * $P < 0.05$. IS, ischemia; WT, wild type.

farcted myocardium in response to ischemia (Fig. 6A). Furthermore, the number of arterioles in the healing myocardium was also significantly increased compared with the Ad-β-gal-treated *db/db* mice (Fig. 6B).

Overexpression of Ang-1 attenuates cardiac hypertrophy and interstitial fibrosis. The heart weight-to-body weight ratio and myocardial interstitial fibrosis were evaluated to further investigate the consequence of Ad-Ang-1-induced vasculature maturation and angiogenesis on cardiac remodeling. The heart weight-to-body weight ratio was measured at 14 days after myocardial ischemia. As shown in Fig. 7A, myocardial ischemia resulted in a significant increase in the heart weight-to-body weight ratio in *db/db* mice compared with wild-type mice. Treatment with Ad-Ang-1 led to a 27% decrease in the heart weight-to-body weight ratio in *db/db* mice subjected to myocardial ischemia (Fig. 7A). Myocardial fibrosis was significantly increased in *db/db* mice compared with wild-type mice after myocardial ischemia (Fig. 7B and C). Myocardial interstitial fibrosis in the remote zone was significantly reduced in Ad-Ang-1-treated *db/db* mice compared with Ad-β-gal-treated *db/db* mice at 14 days after myocardial ischemia (Fig. 7B and C).

DISCUSSION

The novel findings of our present studies reveal that: 1) overexpression of Ang-1 stabilizes HIF-1α protein expression and rescues impaired endogenous angiogenic growth factors in *db/db* mouse hearts subjected to myocardial ischemia; 2) overexpression of Ang-1 leads to a significant increase in eNOS and Akt expression and the suppression of PHD2 expression; 3) overexpression of Ang-1 promotes SMC recruitment, normalizes immature vasculature, and increases mature neovessel formation, which is accompanied by a significant improvement in ischemia-induced capillary and arteriole densities in the border zone of the infarcted area of *db/db* mouse hearts; and 4) overexpression of Ang-1 reduces myocardial ischemia-induced cardiac hypertrophy and interstitial fibrosis. Our data strongly

suggest that Ang-1 gene therapy promotes vascular maturation and stabilization and rescues impaired myocardial angiogenesis and myocardial remodeling via a mechanism involving the suppression of PHD2 and the upregulation of HIF-1α signaling.

HIF-1α is a transcriptional activator that is expressed in response to hypoxia and ischemia (27,28). HIF-1α has been shown to bind to a hypoxia-response element and to regulate VEGF expression (28,29). Recent studies demonstrate that hyperglycemia impairs HIF-1α and VEGF expression in human microvascular ECs and rat proximal tubule cells (30,31). Furthermore, defective hypoxic signaling and the loss of HIF expression have been shown to contribute to the dysfunction of pancreatic β-cells in diabetic patients (32). Recent research shows that the inhibition of PHD2 with small interfering RNA (siRNA) protects against ischemia/reperfusion-induced myocardial injury (33). Most recent research also demonstrates that the deficiency of PHD2 promotes the formation of mature new blood vessels in mouse hearts (34). These data raise the possibility that PHD2 may be a novel therapeutic target for the treatment of diabetic impaired HIF-1α signaling and the impairment of angiogenesis. Our present studies demonstrate for the first time that overexpression of Ang-1 suppresses PHD2 expression and attenuates myocardial ischemia-induced impairment of HIF-1α signaling and its downstream VEGF and HO-1 expression in *db/db* mice. Further studies are needed to test whether the inhibition of PHD2 activity by using PHD2 siRNA could rescue HIF-1α signaling and improve impaired angiogenesis in diabetes.

Our present studies clearly demonstrate that Ang-1 stabilizes HIF-1α protein expression and suppresses PHD2 expression; however, the intracellular mechanism by which Ang-1 mediates this effect in diabetic hearts still remains unknown. NO has been shown to regulate HIF-1α expression and activity under normoxic conditions. Recent studies also reveal that NO donors or the increase of NO formation promotes HIF-1α stabilization and increases VEGF gene expression. Furthermore, both NO-induced

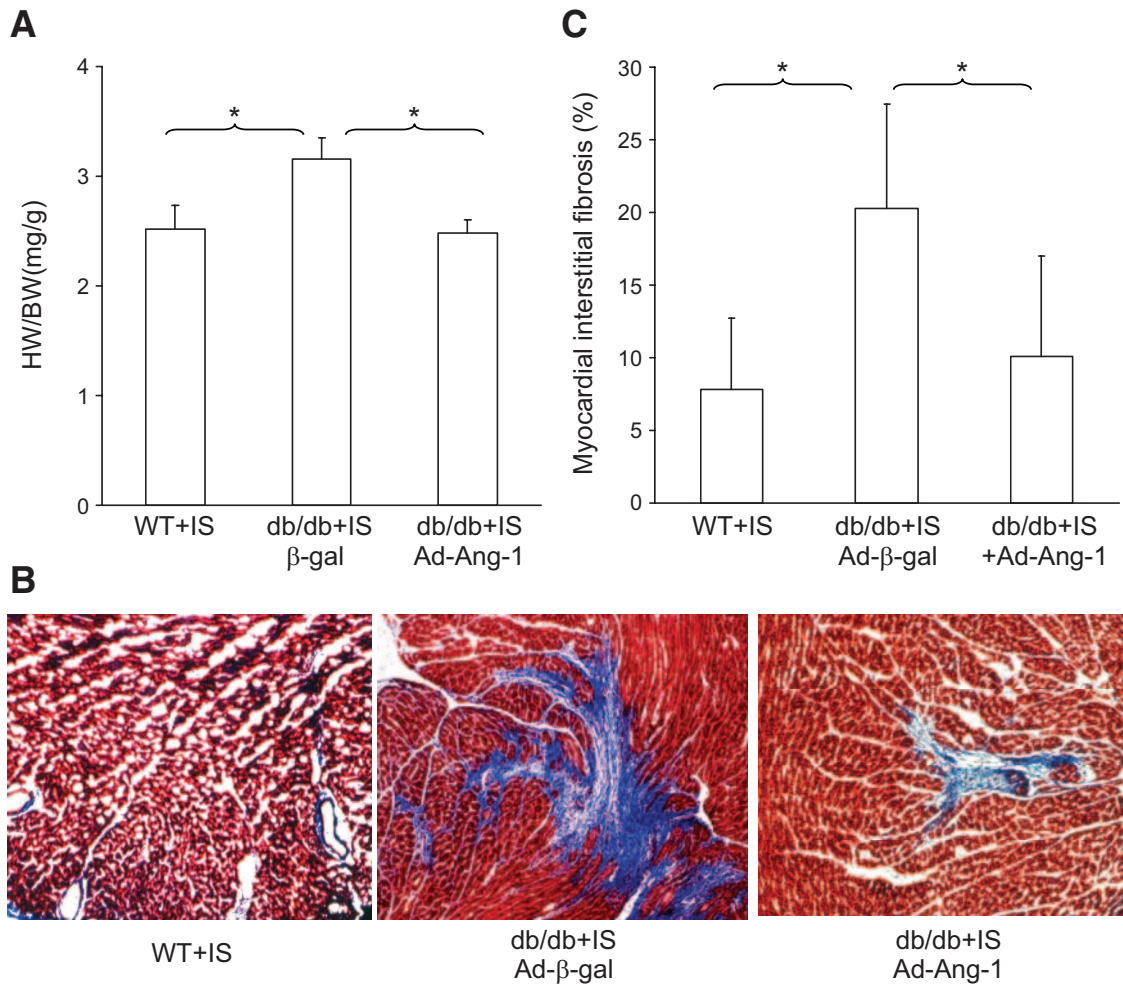


FIG. 7. A: Heart weight-to-body weight (HW/BW) ratio in wild-type, *db/db*, and Ad-Ang-1-treated *db/db* mouse hearts at 14 days after myocardial ischemia. Cardiac hypertrophy was determined by measuring the heart weight-to-body weight ratio. Significant increases in cardiac hypertrophy were observed in *db/db* diabetic mice after 14 days of ischemia. Treatment with Ad-Ang-1 significantly attenuated myocardial ischemia-induced cardiac hypertrophy in *db/db* mouse hearts ($n = 6$). **B and C:** Representative images of Masson's trichrome staining and quantitative analysis of myocardial interstitial fibrosis of heart sections. Myocardial interstitial fibrosis in the remote zone was significantly increased in *db/db* mice compared with wild-type mice at 14 days after myocardial ischemia. Myocardial interstitial fibrosis was significantly reduced in Ad-Ang-1-treated mice compared with Ad- β -gal-treated *db/db* mice ($n = 6$). IS, ischemia; WT, wild type. (Please see <http://dx.doi.org/10.2337/db08-0503> for a high-quality digital representation of this figure.)

phosphatidylinositol 3-kinase signaling (35,36) and the NO-dependent inhibition of PHD activity (17,37) have been shown to contribute to HIF-1 α stabilization. Additionally, Ang-1 gene transfer has been shown to increase eNOS expression and reverse pulmonary hypertension (38). Ang-1 gene transfer has also been reported to prevent diabetic retinal vascular changes and to attenuate the increased retinal permeability by the upregulation of eNOS expression in the streptozotocin-induced diabetic rat model (39). Consistent with these findings, our Western blot analysis also shows that eNOS and Akt protein expression was attenuated in *db/db* mice hearts. Ang-1 gene therapy results in an approximately twofold increase in eNOS and Akt protein expression in the hearts of *db/db* mice, indicating that Ang-1 might inhibit PHD2 and stabilize HIF-1 α protein by a mechanism involving the upregulation of Akt and eNOS expression.

Ang-1 is an important vascular stabilizing factor that controls SMC recruitment and neovessel maturation. Although VEGF initiates neovessel formation during tissue ischemia, Ang-1 promotes subsequent remodeling, maturation, and stabilization (4,5). During the progression of myocardial ischemia, the neovessels of the ischemic area

acquire a muscular coat to form a mature vasculature and stabilize the myocardium, whereas uncoated neovessels undergo regression (40–42). Therefore, the recruitment of SMC is crucial for the growth of mature neovessels and the prevention of neovessel regression (43). Abnormal diabetic angiogenesis is characterized by both structural and functional derangements, and diabetic neovessels are leaky, tortuous, and immature. These structural abnormalities may cause or contribute to many of the clinical manifestations of diabetes. Our previous studies show that disruption of Ang-1 and Tie-2 signaling has been attributed to the impairment of myocardial angiogenesis in diabetes (20,21). Our data demonstrated that SMC recruitment and myocardial ischemia-induced neovessel maturation were severely impaired in *db/db* mice, implicating that the formation of immature neovessels might be novel mechanisms responsible for reduced myocardial angiogenesis in diabetes (20). This notion was further validated by our present data that overexpression of Ang-1 significantly increases SMC recruitment, normalizes diabetic immature vasculature, and improves ischemia-induced capillary and arteriole formation in diabetic *db/db* mouse hearts.

Progressive cardiac hypertrophy and interstitial fibrosis that occur in response to myocardial ischemia are known to increase the risk of heart failure in diabetes (22,23,44). Agents that promote angiogenesis have been shown to be beneficial to cardiac remodeling after chronic myocardial ischemia (22,23,44). Most recent studies have demonstrated that the systemic administration of Ang-1 significantly reduced cardiac hypertrophy and myocardial fibrosis in phenylephrine-induced cardiac hypertrophy (45). Furthermore, overexpression of Ang-1 decreased transforming growth factor-1 β expression and attenuated interstitial fibrosis progression in the kidneys of *db/db* mice (46). Consistent with these findings, our present study showed that Ang-1 gene therapy resulted in significant decreases in both myocardial fibrosis and in the heart weight-to-body weight ratio (hypertrophy) following myocardial ischemia in *db/db* mice, implicating the favorable effects of Ang-1 gene therapy on myocardial ischemia-induced adverse remodeling. So far, the intracellular molecular mechanisms responsible for these alterations and whether such alterations are associated with reduction of myocardial transforming growth factor-1 β expression in diabetes remain unknown. Additionally, our data demonstrated that the systemic administration of Ad-Ang-1 led to a dramatic increase in the serum level of Ang-1; this was accompanied by a significantly lower fasting blood glucose level in *db/db* mice, suggesting the metabolic effect of Ad-Ang-1 in diabetes. Although our present studies have focused on the intracellular mechanisms and therapeutic potential of Ad-Ang-1 on diabetic myocardial angiogenesis and remodeling, the metabolic effects of Ang-1 may also play a critical role in its cardioprotective effects. The metabolic effects resulting from the systemic administration of Ad-Ang-1 in diabetic mice and the exact mechanisms responsible for these effects warrant further investigation. Previous studies have demonstrated that the intravenous administration of Ad-Ang-1 in normal mice led to specific viral gene uptake and expression in the liver, resulting in very high circulating levels of Ang-1 for several weeks (8). Our finding that the systemic administration of Ad-Ang-1 in diabetic *db/db* mice started to increase Ang-1 production at day 1 but caused no change at day 14 in the mouse liver, serum, and myocardium was somewhat surprising, suggesting that pathological conditions such as diabetes may influence viral gene uptake and Ang-1 expression.

In summary, our present data demonstrate that myocardial ischemia-induced HIF-1 α expression is significantly decreased in *db/db* mouse hearts; this is accompanied by a significant impairment of VEGF and HO-1 expression and vascular maturation. Overexpression of Ang-1 increases Akt and eNOS expression and inhibits PHD2 expression, thus leading to HIF-1 α stabilization and the improvement of immature vasculature, and rescues the impairment of angiogenesis in diabetes. Based upon these findings, we propose the novel concept that diabetic impaired angiogenesis is not only caused by impaired neovessel growth but is involved in the formation of immature vasculature, which may result in neovessel regression. Therefore, we propose to treat diabetic abnormal angiogenesis by normalizing and stabilizing immature vasculature, thereby preventing neovessel destabilization and regression.

ACKNOWLEDGMENTS

This work was supported by American Heart Association Grant 0565196B and National Institutes of Health Grant DK074995 to J.-X.C.

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