Deletion of FHL2 Gene Impaired Ischemia-Induced Blood Flow Recovery by Modulating Circulating Proangiogenic Cells

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Objective—The four and a half Lin11, Isl-1 and Mec-3 (LIM) domain protein 2 (FHL2) is a member of the four and a half LIM domain-only (FHL) gene family, and has been shown to play an important role in inhibiting inflammatory angiogenesis. Here, we tested the hypothesis that impaired ischemia-induced neovascularization in mice lacking FHL2 is related to a defect in proangiogenic cell mobilization and functions in vasculogenesis.

Approach and Results—Unilateral hindlimb ischemia surgery was conducted in FHL2−/− mice and wild-type (FHL2+/+) mice. After hindlimb ischemia surgery, expression of FHL2 protein was noted in ischemic tissues of wild-type mice. All FHL2-null mice (100%) suffered from spontaneous foot amputation, but only 20% of wild-type mice had ischemia-induced foot amputation after ischemic surgery. Blood flow recovery was significantly impaired in FHL2−/− mice when compared with that in wild-type mice as determined by laser Doppler imaging. Histological analysis revealed that the capillary density in the ischemic limb was increased in wild-type mice, whereas no such increase was noted in FHL2−/− mice. Flow cytometry demonstrated that the number of CD34+ or CD34+/Sca-1+/Flk-1+ in peripheral blood after ischemic surgery significantly decreased in FHL2-null mice than those in wild-type mice after hindlimb ischemia surgery. FHL2 deficiency impaired ex vivo angiogenesis in mouse aortic-ring culture assay, which revealed that the mean density of the microvessels was significantly higher in the wild-type aorta than in the FHL2−/− aorta. Western blot analysis showed that vascular endothelial growth factor (VEGF), interleukin-6, matrix metalloproteinase-2, matrix metalloproteinase-9, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 levels were significantly downregulated in ischemic muscles in FHL2-null mice compared with wild-type mice. Deletion of FHL2 protein by FHL2 small interfering RNA impaired VEGF production under hypoxia conditions, and also suppressed endothelial progenitor cell angiogenic functions, but these effects could be recovered by administration of VEGF.

Conclusions—Deficiency of FHL2 impairs ischemia-induced neovascularization, and these suppressive effects may occur through a reduction in proangiogenic cell mobilization, migration, and vasculogenesis functions.

Key Words: endothelial progenitor cell • four and a half LIM domain protein 2 • ischemia • proangiogenic cell
augments the neovascularization of ischemic tissue and may be clinically relevant in circumstances involving tissue ischemia. Many studies have indicated that inadequate angiogenic response to ischemia may result in severe organ damage attributable to poor collateral vessel formation and decreased new vessel formation.

The four and a half Lin11, Isl-1 and Mec-3 (LIM) domain (FHL) protein family is a newly identified group of proteins containing FHLs. This family consists of 6 members—FHL1, FHL2, FHL3, FHL4, FHL5, and activator of cAMP-responsive element modulator in testes. Recent studies have indicated that human four and a half LIM-only protein family members, including FHL2, are expressed in a cell- and tissue-specific manner and participate in various cellular processes, such as regulation of gene expression, cytoarchitecture, cell adhesion, cell survival, cell mobility, transcription, and signal transduction. Increasing evidence shows that FHL2 is abundantly expressed in the vascular system, including blood vessels, suggesting it might play a physiological or pathological role in the regulation of the circulatory system. In addition, our recent data have shown decreased chemically induced corneal angiogenesis in FHL2-deficient mice, implying that FHL2 protein plays an important role in inhibiting inflammatory angiogenesis. However, the relation between FHL2 and proangiogenic cell or EPC-related neovascularization in response to tissue ischemia remains unknown. Accordingly, in this study, we investigate the influence of targeted deletion of the FHL2 gene on ischemia-induced neovascularization and address the potential mechanisms.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

### Deletion of FHL2 Gene Causes Auto-amputation and Impairs Blood Flow Recovery in Ischemic Limbs

To evaluate the role of FHL2 in ischemia-induced angiogenesis, we induced unilateral hindlimb ischemia by permanent right femoral artery ligation in FHL2−/− mice and wild-type mice (n=8 per group). As shown in Figure 1A, after hindlimb ischemia surgery, all FHL2−/− mice (100%) suffered from spontaneous foot amputation, but only 20% of wild-type mice had ischemia-induced foot amputation. We monitored the blood flow of the ischemic and nonischemic legs weekly by laser Doppler imaging (Figure 1B). In wild-type mice, the blood flow of the ischemic leg recovered gradually, reaching ≈50% of the flow of the untreated leg by 5 weeks, but blood flow recovery was significantly impaired in FHL2−/− mice (Figure 1B). Histological analysis revealed that the capillary density in the ischemic limb was significantly increased in FHL2−/− mice, whereas no such increase was noted in FHL2−/− mice (capillary/myofiber ratio: 0.93±0.13 versus 0.7±0.07 mm2; P<0.05; Figure 1C). Expression of FHL2 protein was demonstrated in ischemic tissues of wild-type mice but not in FHL2−/− mice (Figure 1D). These data indicate that new vessels formation and blood flow recovery were impaired in FHL2-deficient mice subjected to tissue ischemia.

### Deficiency of FHL2 Reduces Proangiogenic Cells Mobilization

To investigate proangiogenic cell mobilization in response to tissue ischemia in wild-type mice and FHL2−/− mice, we determined levels of CD34+ and CD34+/Sca-1+/Flk-1+ cells in peripheral blood by flow cytometry before and after the hindlimb ischemia surgery (24 hours). The basal number of CD34+ and CD34+/Sca-1+/Flk-1+ cells did not differ significantly between wild-type mice and FHL2−/− mice (n=6 for each group; Figure 2). As shown in previous studies, mobilization of CD34+ cells contributing to postnatal neovascularization was enhanced by tissue ischemia in wild-type mice but not in FHL2−/− mice. However, levels of CD34+ cells in peripheral blood were significantly decreased in FHL2−/− mice than those in wild-type mice (P<0.05).

Moreover, mobilization of CD34+/Sca-1+/Flk-1+ cells was enhanced after hindlimb ischemia in wild-type mice and in FHL2−/− mice. However, numbers of CD34+/Sca-1+/Flk-1+ cells in peripheral blood were significantly reduced in FHL2−/− mice than those in wild-type mice (P<0.05; n=6 for each group).

To further evaluate effects of FHL2 on bone marrow-derived proangiogenic cells homing and differentiation to endothelial cells, hindlimb ischemia surgery was conducted in wild-type and FHL2−/− mice that received enhanced green fluorescent protein mouse bone marrow cells. Immunofluorescence staining showed that wild-type mice had more green fluorescent protein+CD31+ double-positive cells (yellow color) in ischemic muscle than the diabetes mellitus control group (wild-type versus FHL2−/− mice: 8.2±1.1 versus 4.0±0.8/high power field; P<0.05; Figure 2C).

### FHL2 Deficiency Impairs Ex Vivo Angiogenesis in Mouse Aortic-Ring Culture Assay

The aortic rings isolated from wild-type and FHL2−/− mice were cultured in endothelial basal medium-2 with supplements at 37°C for 1 week. As shown in Figure 3, wild-type and FHL2−/− mouse aortic rings in endothelial basal medium (serum-free endothelial basal medium)-2 mounted a weak tubulogenic response. Administration of vascular endothelial growth factor (VEGF) (20 ng/mL) enhanced the tubulogenic response in aortic rings from both wild-type and FHL2−/− mice. However, quantitative analysis revealed that the mean density of the microvessels was significantly increased in the wild-type mouse aorta compared with the FHL2−/− mouse aorta (P<0.05; Figure 3). These data provided ex vivo evidence that deficiency of FHL2 protein impairs microvessel formation in mouse aortic-ring tissue.

### Deletion of FHL2 Gene Downregulates VEGF, IL-6, IL-10, MMP-2, MMP-9, VCAM-1, and ICAM-1 Expression

We further evaluated the Western blots of ischemic-related proteins expression in ischemic muscle of wild-type and FHL2-null mice after hindlimb ischemia surgery. We assessed protein levels of VEGF, interleukin (IL)-6, IL-10, matrix metalloproteinase (MMP)-2, MMP-9, vascular cell adhesion...
molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), p-eNOS, eNOS, p-Akt, and Akt by Western blot analysis using protein extracts from different groups. As shown in Figure 4, VEGF, IL-6, IL-10, MMP-2, MMP-9, VCAM-1, and ICAM-1 expression levels were significantly downregulated in ischemic muscles in FHL2-null mice compared with those in wild-type mice. However, there were no significant differences in p-Akt, Akt, p-eNOS, eNOS, p-JNK, JNK, p-c-jun, and c-jun expression levels between the FHL2-null mice and the wild-type mice.

Figure 1. Deletion of FHL2 gene causes auto-amputation and impairs blood flow recovery in ischemic limbs. A, After hindlimb ischemia surgery, more FHL2−/− mice suffered from ischemia-induced foot amputation than wild-type mice. B, Hindlimb blood perfusion was measured with a laser Doppler perfusion imaging system before and after the surgery, and was then followed up weekly. Blood flow recovery was markedly impaired in FHL2−/− mice compared with that of wild-type mice. C, Mice were euthanized 5 weeks after surgery, and capillaries in the ischemic muscles were visualized by anti-CD31 immunostaining (*P<0.05 compared with wild-type mice; n=6–8 for each group). D, Expression of four and a half Lin11, Isl-1 and Mec-3 (LIM) domain protein 2 (FHL2) protein was demonstrated in ischemic muscles of wild-type mice by Western blot analysis (*P<0.05 compared with before operation and day 1; n=3 for each group). OP indicates hindlimb ischemia surgery.
Figure 2. Deficiency of four and a half Lin11, Isl-1 and Mec-3 (LIM) domain protein 2 (FHL2) reduces CD34+ and CD34+/Sca-1+/Flk-1+ cells mobilization. A, The number of CD34+ and CD34+/Sca-1+/Flk-1+ cells in peripheral blood mononuclear cells was examined by fluorescence-activated cell sorter. B, Circulating proangiogenic cells were quantified by enumerating CD34+ and CD34+/Sca-1+/Flk-1+ cells, and the number of proangiogenic cells was determined by flow cytometry before and after hind limb ischemia surgery (24 hours) in wild-type mice and FHL2−/− mice (n=6 for each group). C, Hindlimb ischemia surgery was conducted in wild-type and FHL2−/− mice that received enhanced green fluorescent protein (eGFP) mouse bone marrow cells. Immunofluorescence staining showed that wild-type mice had more green fluorescent protein+/CD31+ double-positive cells (yellow) in ischemic muscle than the FHL2−/− mice group. OP indicates hindlimb ischemia surgery; SSC, side scatter; FSC, forward scatter; and FITC, fluorescein isothiocyanate.
Characterization of Human EPCs

EPCs were isolated from peripheral blood mononuclear cells of healthy subjects as previously described. The peripheral blood mononuclear cells that initially seeded on fibronectin-coated wells were round in shape. After the medium was changed on day 4, attached early EPCs appeared to be elongated with a spindle shape. EPCs with a cobblestone-like morphology similar to mature endothelial cells were grown to confluence. We performed EPCs characterization by immunohistochemical staining, and most of the cells expressed mature endothelial markers, lectin, VE-cadherin, cluster of differentiation (CD)31, CD34, KDR, CD133, and eNOS (data not shown) which are considered critical markers of EPCs.

Effects of Deletion of FHL2 Gene on EPC Viability, Adhesion, and VEGF Production

To investigate the role of FHL2 in modulation EPC viability and functions, cultured EPCs were transfected with FHL2 small interfering RNA (siRNA) to inhibit FHL2 activity. As shown in Figure 5A, administration of FHL2 siRNA markedly inhibited FHL2 protein production. The effect of FHL2 siRNA on EPC viability and adhesive function were analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and fibronectin adhesion assay. As shown in Figure 5B and 5C, suppression of FHL2 activity by incubation of EPCs with FHL2 siRNA did not significantly affect EPC viability, adhesive function compared with scramble control. Moreover, suppression of FHL2 activity by incubation of EPCs with FHL2 siRNA did not significantly affect NO concentrations (Figure 5D).

VEGF was known to play a pivotal role in neovascularization after tissue ischemia. To further explore the effect of ischemia on EPCs, cultured EPCs were exposed to hypoxia (1% oxygen) conditions for 12 hours. As shown in Figure 5E, VEGF secretion was induced after hypoxia 12 hours in cultured medium, and incubation of EPCs with FHL2 siRNA decreased VEGF production.

Effects of FHL2 on EPC Migration, and Tube Formation

A modified Boyden chamber assay using VEGF as a chemotactic factor was performed to evaluate the effect of FHL2 siRNA on the migratory capacity of EPCs. As shown in Figure 6A, FHL2 siRNA suppressed the VEGF-induced migration of late EPCs (P<0.05), and administration of VEGF recovered the suppressed effect.

An in vitro angiogenesis assay was performed with late EPCs to investigate the effect of FHL2 inhibition on EPC neovascularization. After incubation of FHL2 siRNA for 24 hours, the functional capacity for tube formation of late EPCs on ECMMatrix gel was significantly attenuated in the FHL2 siRNA-treated group compared with the scramble.
control group ($P<0.05$), and these effects were improved by treatment with VEGF (Figure 6B). These results suggest FHL2 modulates VEGF-induced EPC functions.

**Discussion**

This study for the first time demonstrated that deletion of FHL2 gene impairs ischemia-induced neovascularization, which could be accounted for through a reduction in proangiogenic cells mobilization and functions in response to tissue ischemia. Western blot analysis showed that VEGF, IL-6, matrix metalloproteinase (MMP)-2, MMP-9, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) expression. Protein levels of ischemic-related protein expression were assessed by Western blot analysis using protein extracts of ischemic muscle from wild-type and FHL2−/− mice. VEGF, interleukin (IL)-6, MMP-2, MMP-9, VCAM-1, and ICAM-1 expression levels were significantly downregulated in ischemic muscles in FHL2-null mice compared with wild-type mice (n=3 for each group). eNOS indicates endothelial NOS; p-Akt, phosphorylated Akt; p-eNOS, phosphorylated eNOS; JNK, Jun N-terminal kinase; p-c-jun, phosphorylated c-Jun N-terminal kinase.

Increasing evidence indicates that human four and a half LIM-only protein family members, including FHL2, are expressed in a cell- and tissue-specific manner and participate in various cellular processes, such as regulation of gene expression, cytoarchitecture, cell adhesion, cell survival, cell mobility, transcription, and signal transduction. FHL2 protein has been shown to be abundantly expressed in the vascular system, including blood vessels, which suggests that it might play a pivotal physiological or pathological role in the regulation of the circulatory system. Aortic rings from FHL2-null mice display abnormalities in both endothelial-dependent and endothelial-independent relaxation, which suggests FHL2 is essential for the regulation of vasomotor tone. Our recent study also showed that decreased chemical-induced angiogenesis through modulation of proangiogenic cells and EPCs biological activities in response to tissue ischemia.

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corneal angiogenesis was observed in the FHL2-deficient mice, implying that FHL2 protein plays an important role in inhibiting inflammatory angiogenesis.15

In this study, we observed that more FHL2-null mice suffered from spontaneous foot amputation than wild-type mice, and blood flow recovery was markedly impaired in FHL2-null mice when compared with that in wild-type mice, suggesting ischemia-induced angiogenesis requires FHL2 protein. However, the biological mechanisms by which FHL2 contributes to ischemia-related neovascularization remain unclear.

Accumulating evidence suggests that angiogenesis may start with degradation of nonfibrillar collagens in basement membrane, followed by migration and proliferation of pre-existing, fully differentiated vascular endothelial cells, and more importantly, incorporation and differentiation of circulating EPCs into endothelial cells in situ.2–5 These circulating EPCs or proangiogenic cells, originally derived from bone marrow, could be mobilized endogenously as triggered by tissue ischemia or exogenously by cytokine stimulation.4,5 Enhanced mobilization of EPCs augments the neovascularization of ischemic tissue and may be clinically relevant in such situations.5–8

Furthermore, it has become clear that the role of MMPs in angiogenesis is more complex than simply degrading the extracellular matrix to facilitate invasion of endothelial cells.17 Recent reports have suggested that MMP-2 and MMP-9 play a critical role in initiating angiogenesis,18 and is upregulated after tissue ischemia,19 which could promote the release of extracellular matrix-bound cytokines, such as VEGF, and can regulate angiogenesis. Mice lacking MMP-9 have impaired releasing of the soluble form of Kit-ligand in bone marrow after hindlimb ischemia surgery, and attenuated mobilization and homing process of EPCs contributed to postnatal neovascularization in ischemic tissues.19 In this study, we used a gene-targeting strategy to demonstrate that FHL2 plays an important role in ischemia-induced neovascularization, and deletion of FHL2 markedly reduced MMP-2 and MMP-9 expression in ischemic tissues. These findings are in line with previous reports, showing that MMP-2 and MMP-9 expression in ischemic tissues required FHL2 protein, and down-regulation of MMP-2 and MMP-9 after hindlimb ischemia in FHL2-deficient mice may further impair EPC mobilization and subsequent neovascularization.

VEGF, a potent angiogenic stimulator, has been reported to promote proliferation, migration, proteolytic activity, and
capillary tube formation in endothelial cells. In this study, we observed the protein level of VEGF was significantly lower in ischemic muscle of FHL2-deficient mice than that in the wild-type mice. Based on our data, it can be inferred that FHL2 protein has an antiangiogenic effect by preventing ischemia-induced angiogenesis. VEGF secretion was induced after hypoxia in cultured medium, and decreased VEGF production was noted by incubation of EPCs with FHL2 siRNA. The migratory function and tube formation capacity of EPCs in response to VEGF play a critical role during neovascularization. It is also known that the members of the FHL subclass of LIM-only proteins function as transcriptional cofactors via protein–protein interaction. Therefore, the phenotype of FHL2-null mice in ischemia-induced angiogenesis can be explained by transcriptional regulation via protein–protein interaction.

Importantly, our data showed downregulated adhesion molecules, ICAM-1 and VCAM-1, in ischemic hindlimbs of FHL2−/− mice. These findings are consistent with previous report showing that inactivation of FHL2 leads to impaired assembly of extracellular matrix proteins on the surface and to impaired bundling of focal adhesions. Furthermore, previous report showed an essential role of CD18 and its ligand ICAM-1 in mediating EPC recruitment and the subsequent functional effects on the infarcted tissues. Using in vitro assays, we have for the first time demonstrated the detrimental effect of FHL2 siRNA on the migration and vasculogenesis activity of EPCs, which implies a multifunctional role for FHL2 in ischemia-induced neovascularization. However, multiple functions, mainly based on observations of cell cultures, have been ascribed to FHL2, but the molecular mechanisms by which this protein exerts such effects are incompletely understood. Further studies are needed to make clear the underlying mechanisms of action of FHL2 protein in ischemia-related neovascularization and how it modulates biological activity of proangiogenic cells and EPCs.
Conclusions
These findings suggest that deficiency of FHL2 impairs ischemia-induced neovascularization, and these effects may occur through a reduction of proangiogenic cells mobilization and EPC functions. These biological effects of FHL2 on proangiogenic cells and EPCs provide a novel mechanism, in addition to the current understanding about FHL2 in ischemia-induced neovasculogenesis, and may be exploited as a potential therapeutic target of neovascularization in clinical tissue ischemia.

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Disclosures
None.

References

Significance
The FHL2 protein is necessary for vasculogenesis through proangiogenic cells mechanisms, and FHL2 is related to ischemia-induced neovascularization.
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Materials and methods

Animals

FHL2-null mice were generated as previously described.\(^1,2\) Briefly, the endogenous ATG start codon of FHL2 was replaced by a cDNA encoding LacZ and a pGKneo cassette. In this manner, the LacZ cDNA was brought under the control of the endogenous FHL2 promoter, while it ablated the endogenous FHL2 gene. All animals used in this study (FHL2-null and wild-type littermates) were of a C57BL/6 genetic background and were genotyped by PCR. All mice were kept in microisolator cages on a 12-h day/night cycle. All experimental procedures and protocols involving animals were in accordance with the local institutional guidelines for animal care of National Yang-Ming University (Taipei, Taiwan), and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

Mouse hindlimb ischemia model

In this study, we used eight-week-old male FHL2\(^{-/-}\) mice and wild-type mice (FHL2\(^{+/+}\) mice). After a two-week stabilization period, we induced unilateral hindlimb ischemia by excising the right femoral artery, as previously described.\(^3\) Briefly, the animals were anesthetized by intraperitoneal injection...
of pentobarbital (50 mg/kg). The proximal and distal portions of the right femoral artery and the distal portion of the right saphenous artery were ligated. After that, the arteries and all side branches were dissected free and excised. Hindlimb blood perfusion was measured with a laser Doppler perfusion imager (LDPI) system (Moor Instruments Limited, Devon, UK) before and after the surgery, and was then measured weekly. To avoid the confounding influence of ambient light and temperature, the results were expressed as the ratio of perfusion in the right (ischemic) versus left (nonischemic) limb.

Measurement of capillary density in the ischemic leg

Five weeks after surgery, the mice were sacrificed by intraperitoneal injection of an overdose of pentobarbital. We fixed the whole limbs in methanol overnight. The femora were carefully removed, and the ischemic thigh muscles were embedded in paraffin. We de-paraffinized sections (5 μm) and incubated them with a rat-monoclonal antibody against murine CD31 (clone MEC13.1, BD PharMingen, San Diego, CA, USA). Antibody distribution was visualized with the use of the avidin-biotin-complex technique and Vector Red chromogenic substrate (Vector Laboratories, Burlingame, CA, USA), followed by counterstaining with hematoxylin. Capillaries were identified by positive
staining for CD31 and morphology. We analyzed three cross-sections for each animal and randomly selected ten different fields from each tissue preparation, counting visible capillaries. Capillary density was expressed as the number of capillaries per myofiber.\(^4\)

**Mobilization of pro-angiogenic cells in wild-type mice and FHL2 knockout mice**

To examine the role of FHL2 in pro-angiogenic cells mobilization in response to tissue ischemia (24 hours after surgery), we used Alexa 647 anti-mouse CD34 (BD Biosciences, California, USA), fluorescein isothiocyanate (FITC) anti-mouse Sca-1 (eBioscience, San Diego, CA, USA) and phycoerythrin (PE) anti-mouse Flk-1 (VEGFR-2, eBioscience) antibodies. The numbers of CD34\(^+\) and CD34\(^+\)/Sca-1\(^+\)/Flk-1\(^+\) cells in peripheral blood mononuclear cells (MNCs) were examined by a fluorescence-activated cell sorter (FACS calibur; Becton Dickinson, San Jose, CA, USA). We quantified the circulating pro-angiogenic cells by enumerating CD34\(^+\) and CD34\(^+\)/Sca-1\(^+\)/Flk-1\(^+\) cells (n=6 for each group).\(^3\)
Mouse aortic ring assay for angiogenesis

Briefly, the thoracic aorta was isolated and cleaned of perivascular adipose tissue, and 1-mm-long aortic rings were embedded in Matrigel supplemented with 20 U/ml heparin. The aortic rings from wild-type and FHL2 mice were then cultured in EBM-2 (Cambrex Bioscience) with supplements and supplemented with VEGF at 37°C. The sprouting microvessels from aortic rings were identified by incubation with 10 μg/ml BS-1 lectin-FITC (Sigma). The number of sprouting microvessels from aortic rings was determined under the microscope once per day for one week.

Western blotting analysis

Briefly, homogenates muscle tissues were lysed in buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.5 mM PMSF, 2 μg/ml aprotinin, pepstatin and leupeptin), and the protein lysates were subjected to SDS-PAGE, followed by electroblotting onto a PVDF membrane. Membranes were probed with monoclonal antibodies against matrix metalloproteinase-2 (MMP-2, Calbiochem, Merck, Darmstadt, Germany), MMP-9 (Calbiochem), vascular cell adhesion protein-1 (VCAM-1, R&D Systems, Minneapolis, MN USA), intercellular adhesion molecule-1 (ICAM-1, R&D Systems), vascular
endothelial growth factor (VEGF, R&D Systems), Interleukin-6 (IL-6, Serotec, Oxford, UK), Interleukin-10 (IL-10, Serotec), phosphorylated endothelial NO synthase (p-eNOS), eNOS (Cell Signaling), p-Akt, Akt (Cell Signaling), p-JNK, JNK (Santa Cruz Biotechnology, California, USA), p-c-jun, c-jun (Millipore, Billerica, MA, USA), and α-tubulin (Sigma). Bands were visualized by chemiluminescence detection reagents. Densitometric analysis used ImageJ (NIH Image software).3,4

**Bone Marrow Transplantation Model**

Briefly, recipient FHL2−/− mice and wild-type mice at 8 weeks of age were lethally irradiated with a total dose of 9.0 Gy. eGFP transgenic mice (C57BL/6 background) that ubiquitously expressed enhanced GFP (Level Biotechnology Inc., Taipei, Taiwan) were used as the donors.3 After being irradiated, the recipient mice received unfractionated bone marrow cells (5×10^6) from eGFP mice and FHL2−/− mice by a tail vein injection. Eight weeks after the bone marrow transplantation, the chimeric mice were rendered a hindlimb ischemic injury (n=8 per group). Repopulation by eGFP-positive bone marrow cells was measured by flow cytometry to be 95%. Two weeks after the induction of
hindlimb ischemic surgery in the bone marrow-reconstituted mice, tissues were harvested for confocal immunofluorescent and histological analysis. Tissue neovascularization was assessed in frozen sections (5 μm) of the gastrocnemius muscle from the ischemic limbs. Bone marrow-derived pro-angiogenic cells were stained with antibodies directed against eGFP (Chemicon) and CD31 (BD PharMingen). Bone marrow-derived pro-angiogenic cell density was estimated by counting eGFP⁺CD31⁺ double-positive cells (yellow color) under HPF (×100) in at least six different cross-sections from different animals.

**Human EPC isolation and cultivation**

Total MNCs were isolated from 20 ml peripheral blood collected from healthy young human volunteers by density gradient centrifugation with Histopaque-1077 (1.077 g/ml; Sigma, St. Louis, MO, USA) as previously described. Briefly, MNCs (5 × 10⁶) were plated in 2 ml endothelial growth medium (EGM-2 MV; Cambrex, East Rutherford, NJ, USA), with supplements on fibronectin-coated six-well plates. Under daily observation, after four days of culturing, medium was changed and nonadherent cells were removed; attached early EPCs appeared to be elongated with a spindle shape. Culture
medium was replaced every four days, and each colony/cluster was followed up. A certain number of cells could continue to grow into colonies of EPCs, which emerged two to four weeks after the start of MNC culture. The EPCs exhibited a cobblestone morphology and monolayer growth pattern typical of mature endothelial cells at confluence. EPCs were collected and used for all assays in this study.

**EPC characterization**

As previously described, the EPC was characterized by immunofluorescence staining for the expression of lectin, VE-cadherin, platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD-31), CD34, Kinase insert domain receptor (KDR), CD133, and eNOS (Santa Cruz Biotechnology, Inc., California, USA).\(^3\) The fluorescent images were recorded under a laser scanning confocal microscope.

**EPC proliferation assay**

The proliferation of EPCs were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.\(^6\) After incubation with FHL2 siRNA and scrambled siRNA (Santa Cruz), EPCs
were supplemented with MTT (0.5 mg/ml, Sigma) and incubated for four hours for the proliferation assay. Blue formazen was dissolved with dimethyl sulfoxide and measured at 550/650 nm.

**EPC fibronectin adhesion assay**

EPCs were washed with PBS and gently detached with 0.5 mM EDTA in PBS. After centrifugation and resuspension in basal medium with 5% fetal bovine serum, identical cells (1×10⁴ cells per well) were placed on fibronectin-coated 6-well plates and incubated for 30 min at 37ºC. We performed gentle washing with PBS three times after 30 minutes' adhesion, and independent blinded investigators counted adherent cells. Phenotyping of endothelial characteristics of adherent cells was done by indirect immunostaining using Dil-acLDL and BS-1 lectin.

**EPC migration test**

The migratory function of EPCs was evaluated by a modified Boyden chamber assay (Transwell, Coster, San Diego, CA, USA). Briefly, isolated EPCs were detached as described above with trypsin/EDTA and then 4×10⁴ EPCs were placed in the upper chambers of 24-well Transwell plates with
polycarbonate membrane (8-µm pores) with serum-free endothelial growth medium; VEGF (50 ng/ml) in medium was placed in the lower chamber. After incubation for 24 hours, the membrane was washed briefly with PBS and fixed with 4% paraformaldehyde. We then stained the membrane using hematoxylin solution and carefully removed it. The magnitude of migration of the EPCs was evaluated by counting the migrated cells in six random high-power (×100) microscopic fields.

**EPC tube formation assay**

In vitro tube formation assay was performed with an In Vitro Angiogenesis Assay Kit (Chemicon). Briefly, we thawed ECMatrix gel solution at 4°C overnight, mixed it with ECMatrix diluent buffer, and placed it in a 96-well plate at 37°C for one hour to allow the matrix solution to solidify. EPCs were harvested as described above with trypsin/EDTA, and then 1×10^4 EPCs were placed on a matrix solution with EGM-2 MV medium and incubated at 37°C for 16 hours. Tubule formation was inspected under an inverted light microscope (×100). We took four representative fields, and the average of the total area of complete tubes formed by cells was compared using Image-Pro Plus computer software.
VEGF ELISA and measurement of nitrate levels

Human VEGF production in culture supernatants was determined by sandwich ELISA. Antibody pairs were obtained from R&D system (Minneapolis, USA). Briefly, antibody coated plates were incubated with the supernatants and recombinant protein standards were added for 2 hours, followed by detection antibody for 2 hours at room temperature. Following incubation, wells were washed 4 times, and stop solution was added to stop the reaction. The optical density of each well was read at 450 nm with reference absorbance at 540 nm. Concentration of VEGF in supernatants was calculated according to an appropriate standard curve.

The conditioned medium was also measured for nitrate level by Griess reagent <1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2% phosphoric acid>. The fluorescence intensity (relative fluorescence units) was assessed at 485-nm excitation and 530-nm emission using a fluorescence microplate reader.

Statistical analysis

Quantitative data are expressed as means ± standard error of the mean
(SEM). Statistical analysis was adequately performed by the unpaired Student’s \( t \) test or analysis of variance followed by Scheffe’s multiple-comparison post hoc test. Data were analyzed using SPSS software (version 14; SPSS, Chicago, IL, USA). A \( p \) value of < 0.05 was considered to indicate statistical significance.
References