Angiogenic Factor AGGF1-Primed Endothelial Progenitor Cells Repair Vascular Defect in Diabetic Mice

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Hyperglycemia-triggered vascular abnormalities are the most serious complications of diabetes mellitus (DM). The major cause of vascular dysfunction in DM is endothelial injury and dysfunction associated with the reduced number and dysfunction of endothelial progenitor cells (EPCs). A major challenge is to identify key regulators of EPCs to restore DM-associated vascular dysfunction. We show that EPCs from heterozygous knockout *Aggf1*$^{+/-}$ mice presented with impairment of proliferation, migration, angiogenesis, and transendothelial migration as in hyperglycemic mice fed a high-fat diet (HFD) or *db/db* mice. The number of EPCs from *Aggf1*$^{+/-}$ mice was significantly reduced. Ex vivo, AGGF1 protein can fully reverse all damaging effects of hyperglycemia on EPCs. In vivo, transplantation of AGGF1-primed EPCs successfully restores blood flow and blocks tissue necrosis and ambulatory impairment in HFD-induced hyperglycemic mice or *db/db* mice with diabetic hindlimb ischemia. Mechanistically, AGGF1 activates AKT, reduces nuclear localization of Fyn, which increases the nuclear level of Nrf2 and expression of antioxidative genes, and inhibits reactive oxygen species generation. These results suggest that *Aggf1* is required for essential function of EPCs, AGGF1 fully reverses the damaging effects of hyperglycemia on EPCs, and AGGF1 priming of EPCs is a novel treatment modality for vascular complications in DM.

Diabetes mellitus (DM) is a chronic metabolic disorder; however, hyperglycemia-triggered vascular abnormalities, including cardiovascular disease and diabetic peripheral artery disease, are the most serious complications, contributing to numerous deaths, diabetic foot wounds, and lower-extremity amputation (1–3). The major causes of vascular dysfunction in DM are endothelial injury and dysfunctions associated with the reduced number and dysfunction of endothelial progenitor cells (EPCs) (4,5).

EPCs are progenitors of endothelial cells (ECs) and have the potential to proliferate, migrate, home into the disrupted endothelium, and differentiate into ECs to maintain endothelium integrity, restore endothelial dysfunction, promote neovascularization, and repair damaged vessels (5–8). Implantation of EPCs has emerged as a potential therapy for myocardial ischemia, brain ischemia, and pulmonary embolism (9,10). However, the efficacy of a therapy using EPC implantation for diabetic vascular complications is uncertain (11). In particular, autologous EPC transplantation may not work efficiently because of the impaired function of such EPCs in DM by hyperglycemia (4,5,11). Therefore, a major challenge for the next decades will be to identify key regulatory factors and mechanisms for EPC dysfunction in patients with DM and to develop strategies that can restore the angiogenic activity of EPCs in DM and increase the number (proliferation), migration, and homing of EPCs to injury sites for efficient vascular repair.

AGGF1 is a 714-amino acid angiogenic factor with an FHA domain and G-patch domain and plays a pivotal role in vasculogenesis, specification of venous ECs,

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angiogenesis, and vascular development (12–15). We have shown that *Aggf1* is the most upstream regulator for differentiation of hemangioblasts from the mesoderm in zebrafish (16). Hemangioblasts are multipotent progenitors for hematopoietic stem cells, EPCs, ECs, and blood cells (17). Therefore, it is possible that *Aggf1* plays an important role in the development and differentiation of EPCs in mice. In this study, we characterized the potential effects of *Aggf1* on function of EPCs and assessed whether AGGF1-primed EPCs have therapeutic potential for the treatment of vascular abnormalities associated with DM.

RESEARCH DESIGN AND METHODS

Animals

Wild-type (WT) C57BL/6J mice and *db/db* mice (The Jackson Laboratory) were used in the study. *Aggf1*$^{+/-}$ knockout (KO) mice with exons 2–11 deleted were described previously (13). Because homozygous *Aggf1*$^{-/-}$ KO mice die before embryonic day 8.5, heterozygous *Aggf1*$^{+/-}$ KO mice were studied. All animal procedures were approved by the ethics committee at Huazhong University of Science and Technology. A high-fat diet (HFD)–induced mouse model for type 2 DM (T2DM) was developed as previously described (18). Male C57BL/6J mice fed an HFD or *db/db* mice were used to create a hindlimb ischemia model associated with T2DM by ligation of the femoral artery with 6-0 Ethilon sutures as described previously by us and others (15).

Isolation and Culture of Bone Marrow–Derived EPCs

EPCs were isolated from the bone marrow of mice and cultured as previously described (5,19). Mice were anesthetized with chloral hydrate (3% mass/volume [m/v]) through intraperitoneal injection. Bone marrow mononuclear cells (MNCs) were washed out with PBS from the femurs and tibias of mice and then purified by density gradient centrifugation (2,000 rpm) with Histopaque-1083 (Sigma-Aldrich) for 30 min at room temperature with a horizontal rotor centrifuge (Anke LCJ-2B). The volume ratio of single-cell suspension and Histopaque-1083 separation liquid was 2:1. The cells were then washed twice using PBS, resuspended gently, plated in 0.1% m/v gelatin-coated cell culture dishes, and maintained in endothelial growth factor 2 (EGM-2)–supplemented media (EGM-2 BulletKit; Lonza) with 10% FBS. Cells were cultured at 37°C with 5% CO_2 in a humidified water jacket incubator (Thermo Fisher Scientific). The EGM-2 medium was replaced every 3 days after first plating, and cellular morphology was monitored every day.

Characterization of Bone Marrow–Derived MNCs

Characterization of MNCs was performed as previously described (20). Seven days after maintenance in endothelial-specific media and the removal of nonadherent MNCs, the remaining cells were subjected to immunostaining analysis. Cells were plated on gelatin-coated slides (ibidi) for 1 day and then incubated with 10 μg/mL of acetylated DiI lipoprotein from human plasma (Dil-Ac-LDL; Thermo Fisher Scientific) at 37°C for 4 h. The cells were washed three times with PBS and incubated with 10 μg/mL of FITC-labeled *Ulex europaeus* lectin 1 (Sigma-Aldrich) for 1 h at 37°C. The cells were rinsed three times with PBS again and visualized under a confocal microscope.

MNCs were also characterized by flow cytometry analysis. Cells were incubated with 5% BSA (Sigma-Aldrich) for 15 min to block nonspecific binding and then stained with anti-mouse CD34-phycoerythrin (PE), CD31-PE, CD45-PE, or CD144-PE (BD Biosciences) at room temperature for 1 h, respectively. The same fluorescein-labeled isotype IgG served as negative control. Cells were analyzed using a Beckman CytoFLEX, and data were analyzed using FlowJo version 10 software (Tree Star).

Cell Therapy With Implantation of EPCs Primed With and Without AGGF1

Recombinant AGGF1 protein was purified as described previously (14,21). EPCs isolated from mice were treated with purified AGGF1 protein (0.5 μg/mL) or negative control elution buffer for purification at 37°C for 12 h. Two days after the diabetic hindlimb ischemia surgery, AGGF1-primed EPCs ($\sim 1 \times 10^6$) were injected into mice through the tail vein. Blood flow in both legs was measured in mice anesthetized with chloral hydrate (3% m/v) using a Vevo 2100 high-resolution microultrasound system (VisualSonics) immediately before the ischemic surgery and at time points of 7, 14, and 28 days after ischemia. We measured the peak systolic velocity (Vs), the minimal end-diastolic flow velocity (Vd), and the temporal average velocity per cardiac cycle (Va) in the femoral artery. The blood flow ratio was computed with the equation (Vs − Vd) / Va using three to five continuous cardiac cycles (15,22).

Production of Lentiviruses and Infection

Human embryonic kidney 293 (HEK293) cells were transfected with a lentiviral vector with EGFP, the packaging plasmid psPAX2, and the envelope plasmid pMD2.G using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Viral supernatants were produced from the transfected HEK293 cells as previously described (5). HEK293 cells were maintained at 37°C in high-glucose (HG) DMEM supplemented with 10% v/v FBS (Gibco Life Technologies, New York, NY) in a humidified water jacket incubator with 5% CO_2. The supernatant was harvested 72 h after transfection, filtered through 0.45-μm polyvinylidene fluoride filters, and stored at −80°C until use.

EPCs were infected with the EGFP lentiviruses overnight at a multiplicity of infection of 50 with 2.5 μg/mL polybrene supplemented, and the culture medium was replaced with the fresh growth medium 24 h after infection. After transfection for 72 h, the infection efficiency was determined by GFP expression under a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan).

Small Interfering RNAs and Inhibitors of PI3K

Small interfering RNAs (siRNAs) for NF-E2 p45-related factor 2 (*Nrf2*) and *AKT*, and the negative control siRNA were chemically synthesized by RiboBio. The control siRNA is

the silencer scrambled negative control siRNA (siNC) having no significant homology to any known gene sequences from the mouse genome. The sequences of siRNAs are as follows: Nrf2 sense, 5′-CGAGAAGUGUUUGACUUUATT-3′; Nrf2 antisense, 5′-UAAAGUCAAACACUUCUCGTT-3′; AKT sense, 5′-UGCCCUUCUACAACCAGGATT-3′; AKT antisense, 5′-UCCUGGUUGUAGAAGGGCATT-3′; siNC sense, 5′-UUCUCC GAACGUGUCACGUTT-3′; and siNC antisense, 5′-ACGUGA CACGUUCGGAGAATT-3′. EPCs were transfected with siRNA in Opti-MEM medium overnight using a transfection reagent (Santa Cruz Biotechnology, Dallas, TX) as previously described (23–25). The knockdown efficiency was determined by real-time RT-PCR analysis as described below. Wortmannin, an inhibitor of PI3K, was purchased from Selleck Chemicals.

Ex Vivo Studies

Assays for capillary tube formation (magnification: 40×) by EPCs were as previously described (26). Cell proliferation assays were carried out using a CCK-8 kit according to the manufacturer's instruction (Dojindo Laboratories, Kumamoto, Japan) (27,28). We evaluated transendothelial migration (TEM) (magnification: 200×) of EPCs with a transwell assay as described previously (5). EPC migration assays were carried out using scratch wound cell migration assays (magnification: 40×) as previously described (26).

In Vitro Studies

Immunostaining of paraffin-embedded sections of skeletal muscle for CD31 was performed using a rabbit polyclonal anti-CD31 antibody (1:200; Proteintech), and Western blot analysis was carried out as previously described (26). The primary antibodies against Nrf2 (1:1,000 dilution), heme oxygenase-1 (HO-1) (1:2,000), NAD(P)H dehydrogenase quinone 1 (NQO-1) (1:1,000), catalase (CAT) (1:1,000), and GAPDH (1:5,000) were from Proteintech. Antibodies for total protein kinase B or AKT and phosphorylated AKT (Ser473) (1:1,000) were purchased from Cell Signaling Technology. Quantitative real-time RT-PCR analysis was carried out using the FastStart Universal SYBR Green Master (Roche) as previously described (26). *GAPDH* served as an internal standard.

Statistical Analysis

Data are presented as mean ± SD. The comparison of means of two groups was made by a Student *t* test or a nonparametric Wilcoxon rank sum test when the sample size was small and/or the distribution was not normal. To compare the means of more than two groups, one-way ANOVA or the generalized linear regression approach was used for data with normal distribution, and the Kruskal-Wallis test was used for nonnormal distribution data or small samples. $P < 0.05$ was considered statistically significant.

RESULTS

Aggf1 Haploinsufficiency (Heterozygous *Aggf1*$^{+/-}$ KO) Significantly Reduces the Number of Bone Marrow–Derived EPCs

We isolated and characterized bone marrow–derived MNCs from WT and heterozygous *Aggf1*$^{+/-}$ KO mice. The isolated MNCs appeared to be centrally rounded EPCs with a peripheral spindle shape 4 days after culture (Supplementary Fig. 1*A*). The cells appeared to be spindle-shaped adherent EPCs, and some showed cobblestone-like morphology at day 7 (Supplementary Fig. 1*A*). The isolated EPCs were confirmed by a DiI-Ac-LDL endocytosis assay (red fluorescence) and a UEA-1 binding assay (green fluorescence). The isolated EPCs were able to endocytose Ac-LDL and bind UEA-1 (Supplementary Fig. 1*B*), suggesting that they are endothelial lineage cells with characteristics of EPCs as previously described (20,29,30). Previous studies indicated that early EPCs (<14 days) are positive for CD34, CD31, CD14, CD45, Scal-1, c-Kit, and vascular endothelial growth factor receptor 2 (VEGFR2) but are mostly negative for CD144 (5). Our flow cytometry analysis showed that the isolated EPCs were positive for CD31, CD34, and CD45 and negative for CD144 at day 10 of culture (Supplementary Fig. 1*C*). We compared the number of Dil-Ac-LDL/FITC-UEA-1–positive cells from heterozygous *Aggf1*$^{+/-}$ KO mice with the cells from WT mice. The number of Dil-Ac-LDL– and FITC-UEA-1–positive cells isolated from heterozygous *Aggf1*$^{+/-}$ KO mice was moderately but significantly reduced compared with WT mice (n = 6/group; $P < 0.05$) (Supplementary Fig. 2). These data suggest that *Aggf1* may be involved in differentiation of EPCs.

Aggf1 Is Required for Angiogenic and Other Functions of EPCs

Western blot analysis showed that AGGF1 expression was significantly increased in EPCs isolated from *db/db* mice and HFD-induced T2DM mice (Supplementary Fig. 3). Interestingly, we also found that *Aggf1* is required for angiogenic and other functions of EPCs. We studied *db/db* mice and a mouse model for T2DM by treating C57BL/6J mice with HFD. HFD treatment successfully induced a significant increase of blood glucose levels in mice (Supplementary Fig. 4). The EPCs isolated from *db/db* mice showed a significantly decreased angiogenic function in a matrigel-based capillary tube formation assay (Fig. 1*A* and *B*), decreased proliferation in a CCK8 assay (Fig. 1*C*), decreased TEM required for homing of EPCs to sites of endothelial disruption for repair and neovascularization in a transwell assay (Fig. 1*D* and *E*), and reduced migration in a scratch wound assay (Fig. 1*F* and *G*) compared with EPCs isolated form WT mice. Interestingly, *Aggf1*$^{+/-}$ showed similar effects as *db/db* mice (Fig. 1*A*–*G*). *Aggf1*$^{+/-}$ *db/db* double KO mice showed exacerbated effects compared with *Aggf1*$^{+/-}$ KO mice (Fig. 1*A*–*G*). We analyzed blood glucose levels between WT mice and *Aggf1*$^{+/-}$ KO mice at baseline, but no significant difference was detected (WT 6.54 ± 0.78 mmol/L, *Aggf1*$^{+/-}$ KO 7.14 ± 0.92 mmol/L). We analyzed the expression levels of *VEGF*, *VEGFR2*, and *CXCR4* mRNA in EPCs isolated from different groups of mice. Our real-time RT-PCR analysis showed that *Aggf1* haploinsufficiency did not affect the expression of *VEGF*, *VEGFR2*, and *CXCR4* in EPCs (Supplementary Fig. 5),

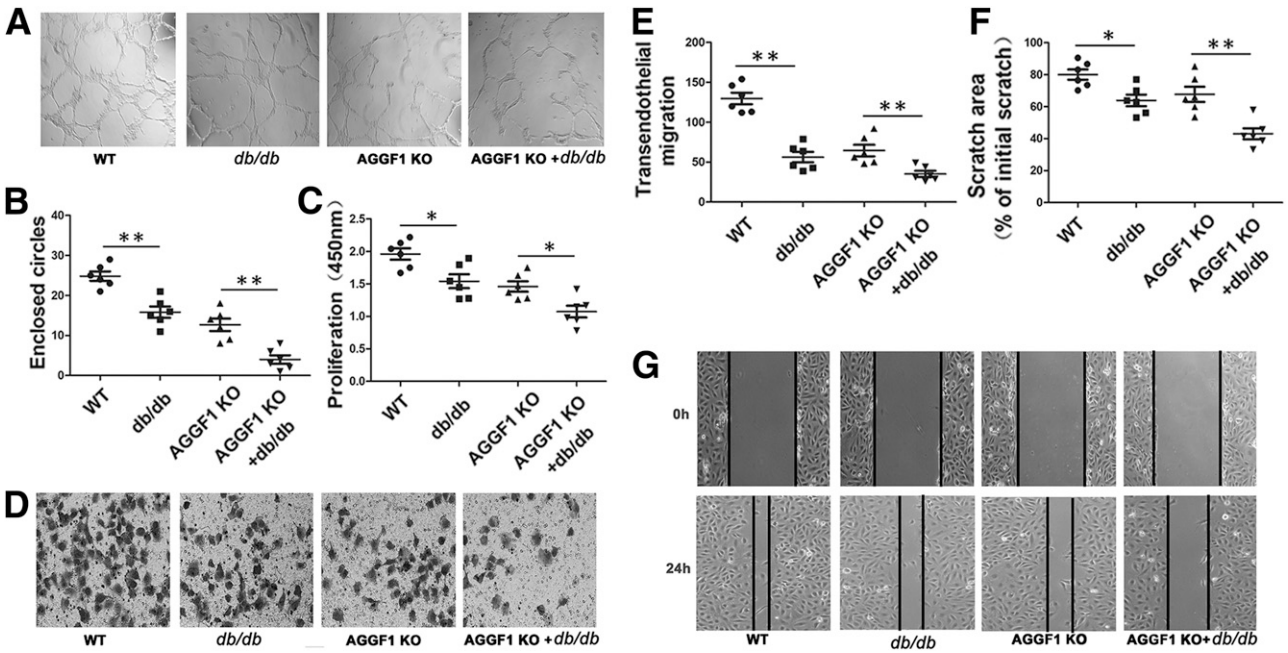


Figure 1—*Aggf1* is required for essential functions of EPCs in capillary tube formation, proliferation, TEM, and migration as in a diabetic mouse model (*db/db* mice). EPCs were isolated from bone marrow of WT, heterozygous *Aggf1*$^{+/-}$ KO mice, *db/db* mice, and *Aggf1*$^{+/-}$*db/db* mice and characterized. *A*: *Aggf1* haploinsufficiency inhibits angiogenesis mediated by EPCs. *B*: Images from panel *A* were analyzed, quantified, and plotted. *C*: *Aggf1* haploinsufficiency inhibits EPC proliferation. *D*: *Aggf1* haploinsufficiency inhibits TEM of EPCs in transwell assays. *E*: Images from panel *D* were quantified and plotted. *F*: *Aggf1* haploinsufficiency inhibits EPC migration in scratch wound migration assays. *G*: Representative images from EPC migration assays. Data are mean ± SD. *$P < 0.05$, **$P < 0.01$ (n = 6 mice/group).

suggesting that the effect of *Aggf1* haploinsufficiency is independent of *VEGF*, *VEGFR2*, and *CXCR4*. In addition, similar results were obtained in the HFD-induced mouse model for T2DM (Supplementary Fig. 6). These data suggest that *Aggf1* is required for essential functions of EPCs.

AGGF1 Protein Treatment Reverses Hyperglycemia-Impaired Angiogenic and Other Functions of EPCs

We found that the AGGF1 protein treatment reversed the impaired angiogenesis, proliferation, migration, and TEM of EPCs induced by HG. When cultured in HG media (30 mmol/L D-glucose mimicking the pathological environment of diabetic dyslipidemia or hyperglycemia, 30 mmol/L L-glucose as negative control), mouse EPCs showed significantly decreased angiogenic function (Fig. 2*B* and *C*), proliferation (Fig. 2*D*), TEM (Fig. 2*E* and *F*), and migration (Fig. 2*G* and *H*) compared with the EPCs cultured in control regular media. AGGF1 is an angiogenic factor that can be secreted outside of cells (14); therefore, we treated EPCs with the purified human AGGF1 protein in culture to determine the effects of AGGF1 on functions of EPCs (Fig. 2*A*). All the defects by HG on EPC functions were blocked by AGGF1 treatment (Fig. 2*B*–*H*). We analyzed the expression levels of *VEGF*, *VEGFR2*, and *CXCR4* mRNA in EPCs treated with or without AGGF1 in combination with or without HG. AGGF1 did not affect the expression levels of *VEGF*, *VEGFR2*, and *CXCR4* in EPCs (Supplementary Fig. 7). These data suggest that AGGF1 can counter the damaging effects of hyperglycemia on EPCs; however, its effects are independent of *CXCR4*, *VEGF*, or *VEGFR2*.

AGGF1 Boosts EPC-Mediated Angiogenesis and Blood Perfusion in T2DM

In a T2DM mouse model (*db/db* mice), AGGF1 was found to greatly boost the therapeutic effects of cell therapy with EPC implantation on vascular complications under ischemia in vivo. Because AGGF1 can reverse the damaging effects of T2DM on EPCs, we hypothesized that AGGF1-primed EPCs can serve as a successful treatment tool for vascular complications in T2D in vivo. We pretreated EPCs with recombinant AGGF1 and transplanted the AGGF1-primed EPCs into *db/db* mice after hindlimb ischemia surgery. Notably, AGGF1-primed EPCs were significantly more effective than elution buffer–pretreated EPCs in increasing blood flow in diabetic mice with ischemia (Fig. 3*A*). The rate of tissue necrosis was significantly decreased by AGGF1-primed EPCs compared with elution buffer–pretreated control EPCs (Fig. 3*B*). A similar significant inhibitory effect on ambulatory impairment was detected for AGGF1-primed EPCs (Fig. 3*C*). Immunostaining showed that the density of CD31$^+$ vessels was significantly higher for AGGF1-primed EPCs than for elution buffer–pretreated control EPCs at day 28 after transplantation (Fig. 3*D* and Supplementary Fig. 8*A*). Similar therapeutic efficacy for AGGF1-primed EPCs was obtained in HFD-induced hyperglycemic mice (Supplementary Fig. 9*A*–*E*). Together, these data demonstrate that AGGF1-primed EPCs improve blood perfusion and

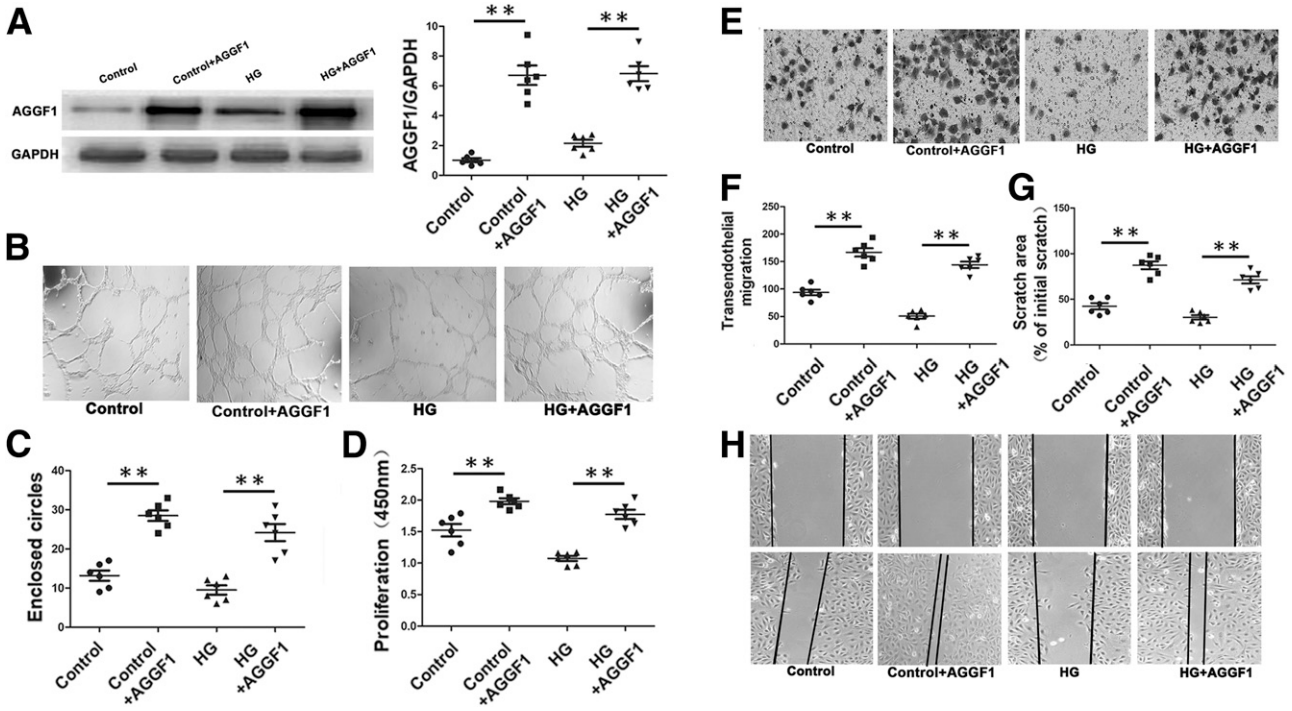


Figure 2—AGGF1 dramatically improves essential functions of EPCs impaired by HG. *A*: Western blot analysis showing increased AGGF1 expression in EPCs. *B*: AGGF1 treatment reversed the impairment of angiogenic function of EPCs by HG. *C*: Images from panel *B* were analyzed, quantified, and plotted. *D*: AGGF1 treatment reversed the reduced cell proliferation of EPCs by HG. *E*: AGGF1 treatment reversed the HG-impaired TEM of EPCs. *F*: Images from panel *E* were quantified and plotted. *G*: AGGF1 treatment reversed the HG-impaired migration of EPCs in scratch wound migration assays. *H*: Representative images from EPC migration assays. Data are mean ± SD. $^{**}P < 0.01$ (n = 6 mice/group).

angiogenesis in T2DM mice with hindlimb ischemia, suggesting that AGGF1 can boost the therapeutic effects of cell therapy with EPCs.

The therapeutic effect of AGGF1-primed EPCs may be related to the possibility that AGGF1 increases the homing of transplanted EPCs to the ischemic areas. To test this hypothesis, we infected EPCs with EGFP lentivirus (Supplementary Fig. 10) and transplanted EPCs into *db/db* mice. Four weeks after transplantation, the gastrocnemius muscle of T2DM mice with hindlimb ischemia was characterized by immunostaining with an anti-GFP antibody. As shown in Supplementary Fig. 8*B*, EPCs labeled by overexpression of EGFP and primed by AGGF1 induced significantly more homing or mobilization of GFP-labeled EPCs in the vascular injury site in the muscle than elution buffer–pretreated control EPCs. Similar results were obtained in HFD-induced hyperglycemic mice (Supplementary Fig. 9*F*).

AGGF1 Attenuates Hyperglycemia-Induced Oxidative Stress in EPCs

To identify the molecular mechanism by which AGGF1 boosts the therapeutic effects of EPCs, we analyzed the effect of AGGF1 on oxidative stress induced by hyperglycemia in EPCs. Oxidative stress is a key factor accounting for endothelial dysfunction in diabetic EPCs (31–34). Therefore, we analyzed the effect of AGGF1 on HG-induced oxidative stress in EPCs. The level of reactive oxygen species (ROS) in EPCs after treatment with an HG solution was significantly increased, but the effect was attenuated by treatment with AGGF1 (Supplementary Fig. 11). However, AGGF1 treatment did not fully reverse the HG-induced ROS increase ($P < 0.05$ between the control group and HG + AGGF1 group) (Supplementary Fig. 11). These data suggest that AGGF1 can attenuate the production of ROS in EPCs, which may be a factor for its effects on boosting the therapeutic effects of EPCs on endothelial dysfunction in DM.

AGGF1 Activates Nuclear Localization of Nrf2 Through the AKT-Fyn-Nrf2 Signaling Pathway in EPCs

To identify the molecular mechanism by which AGGF1 inhibits ROS generation, we characterized the effect of AGGF1 on antioxidative transcription factor Nrf2 (35). We found that AGGF1 promoted nuclear accumulation of Nrf2, thereby activating the Nrf2 pathway in EPCs. When cultured in HG media, the nuclear accumulation of Nrf2 was significantly reduced, but the effect was abolished by treatment with AGGF1 (Fig. 4). Nrf2 activates the transcription from many downstream genes, including *HO1*, *NQO-1*, and *CAT* (5,36). Western blot analysis showed that AGGF1 increased the expression levels of HO1, NQO-1, and CAT (Fig. 4*A*). Real-time RT-PCR analysis showed that AGGF1 increased the expression levels of *HO1*,

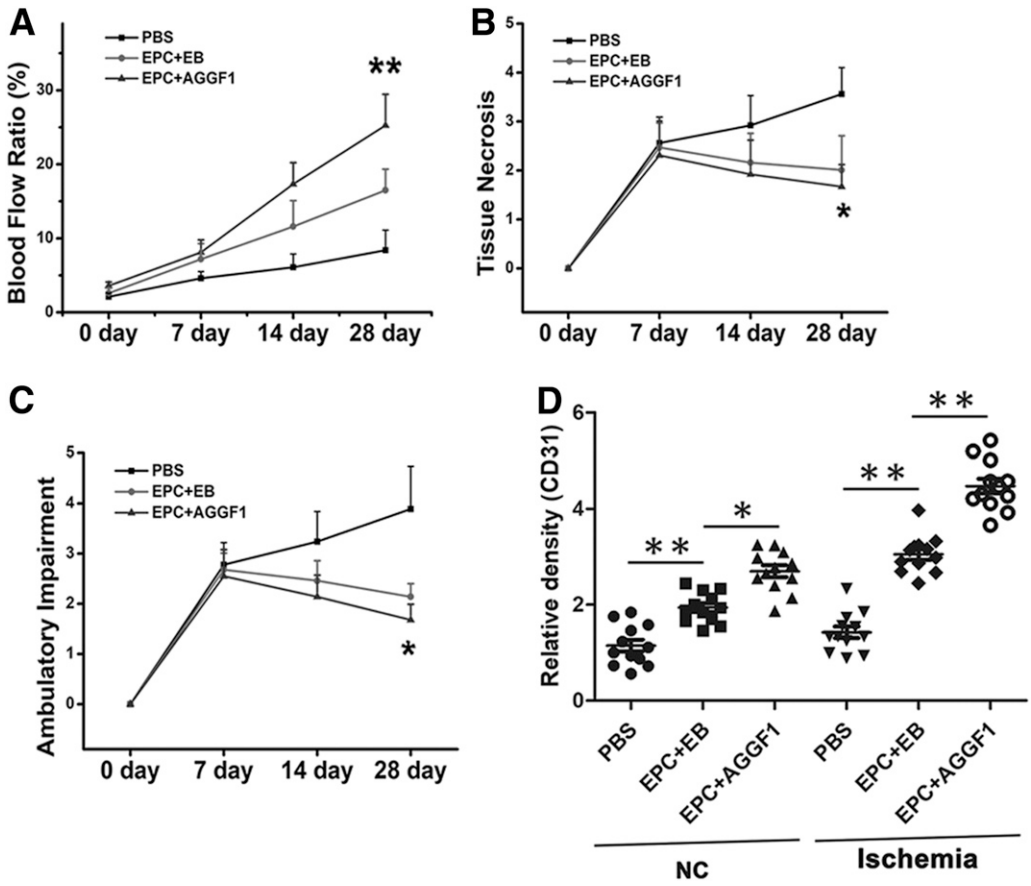


Figure 3—AGGF1 protein treatment robustly potentiates the therapeutic effects of EPCs on peripheral vascular complications in a hindlimb ischemia model in *db/db* mice. A hindlimb ischemia model was created in *db/db* mice. *A*: Transplantation of AGGF1-pretreated EPCs dramatically improved blood perfusion compared with EPCs without AGGF1 pretreatment in *db/db* mice. *B*: Therapeutic effects of AGGF1-pretreated EPCs on necrosis compared with EPCs without AGGF1 pretreatment. *C*: Therapeutic effects of AGGF1-pretreated EPCs on ambulatory impairment compared with EPCs without AGGF1 pretreatment. *D*: Effects of AGGF1-pretreated EPCs on the density of $CD31^+$ capillary vessels compared with EPCs without AGGF1 pretreatment. $^*P < 0.05$, $^{**}P < 0.01$. EB, elution buffer; NC, negative control.

NQO-1, and *CAT* mRNA (Fig. 4*B*). Furthermore, immunostaining with an anti-Nrf2 antibody confirmed the finding of increased nuclear immunofluorescence/localization for Nrf2 by AGGF1 (Supplementary Fig. 12). Interestingly, real-time RT-PCR analysis showed that AGGF1 did not affect the expression level of *Nrf2* mRNA (Supplementary Fig. 13*A*). Western blot analysis also showed that AGGF1 did not affect the expression level of Nrf2 and KEAP-1 (binding to Nrf2 to facilitate its ubiquitination) (37) (Supplementary Fig. 13*B* and *C*). These data demonstrate that AGGF1 increases the nuclear accumulation of Nrf2, but not the expression levels of Nrf2 and KEAP-1, and results in transcriptional activation of Nrf2 downstream target genes *NQO-1* and *CAT*.

We previously showed that AGGF1 can activate PI3K and AKT signaling in ECs and in zebrafish (12,13). PI3K and AKT were shown to regulate phosphorylation and nuclear localization of Fyn (38,39). Fyn was shown to phosphorylate Nrf2 at Y568, resulting in nuclear export and degradation of Nrf2 (38). Therefore, we hypothesized that AGGF1 regulates ROS generation and EPC functions through an AGGF1-AKT-Fyn-Nrf2 signaling pathway. When cultured in HG media, EPCs showed a significantly reduced level of phosphorylation of AKT; however, the effect was reversed by AGGF1 treatment (Fig. 5*A*). On the other hand, the effect of AGGF1 was blocked by siRNA for *AKT* or wortmannin, a specific inhibitor of PI3K upstream of AKT (Fig. 5). EPCs treated with HG showed a significantly increased level of nuclear Fyn (n-Fyn), but the effect was reversed by AGGF1 treatment (Fig. 5*A* and *B*). The effect of AGGF1 was blocked by siRNA for *AKT* or wortmannin (Fig. 5*A* and *B*). Similarly, siRNA for *AKT* or wortmannin or siRNA for *Nrf2* blocked the rescue effects of AGGF1 on the HG-induced decrease of Nrf2 nuclear accumulation and downregulation of Nrf2 downstream genes *NQO-1* and *CAT* (Fig. 5*C*–*E*). Furthermore, the nuclear accumulation of nuclear Nrf2 (n-Nrf2) was decreased by siRNA for *AGGF1* under either a normal or an HG condition; however, Fyn knockdown with siRNA blocked the effect of AGGF1 (Supplementary Fig. 14). These data suggest that AGGF1 promotes Nrf2 nuclear localization and activates Nrf2 downstream target genes by regulating the AKT-Fyn signaling pathway.

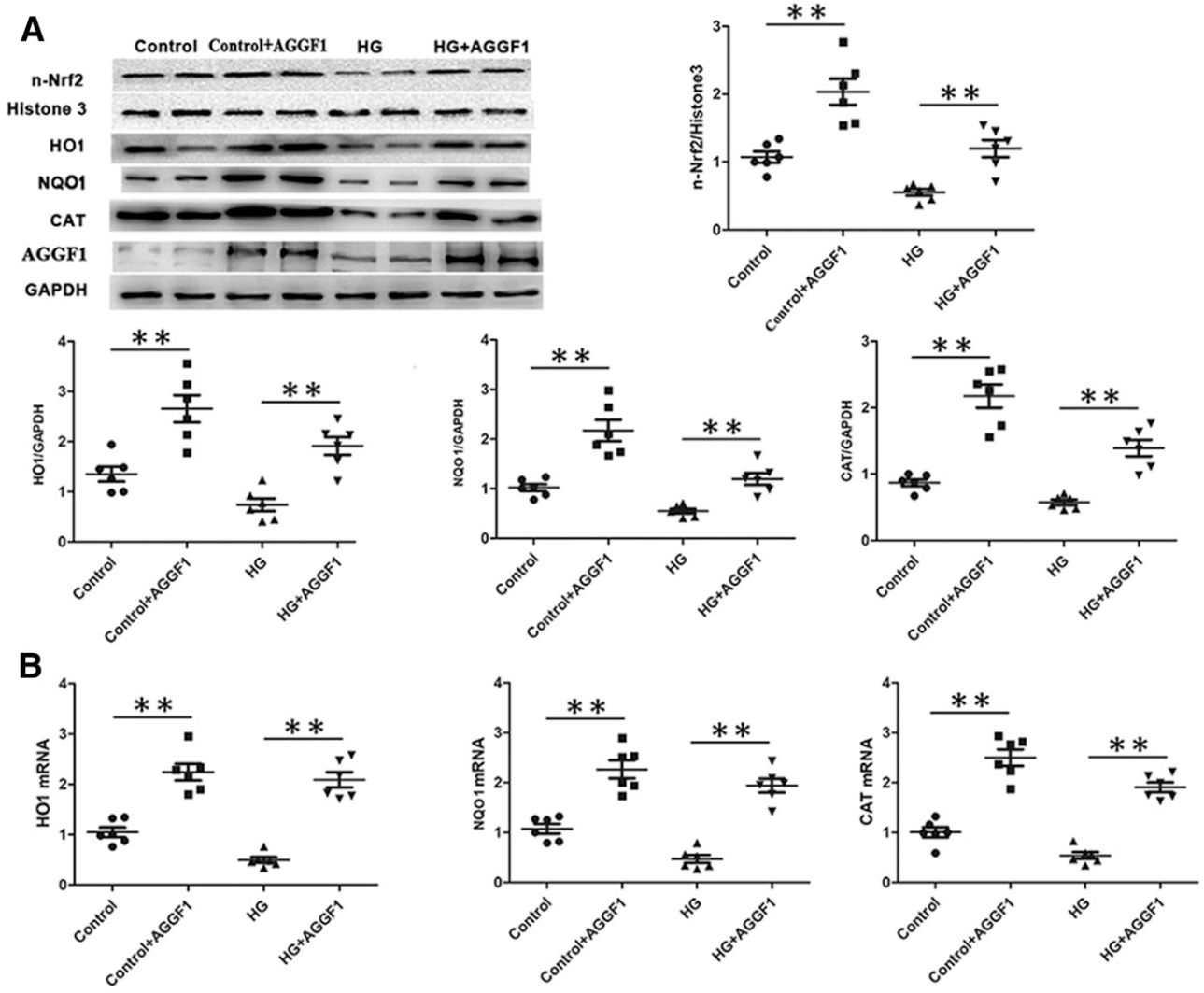


Figure 4—AGGF1 regulates the nuclear accumulation of Nrf2. *A*: Western blot analysis for the effect of AGGF1, HG, and HG + AGGF1 on the expression levels of n-Nrf2 and its downstream signaling molecules, including HO-1, NQO-1, and CAT. GAPDH was used as loading control. *B*: AGGF1 protein treatment induces expression of *Nrf2* downstream antioxidative genes in EPCs. Data are mean ± SD. $^{**}P < 0.01$ ($n = 3$/group).

To further demonstrate that AGGF1 regulates the function of EPCs by Nrf2, we studied the effects of wortmannin and siRNAs for *AKT* and *Nrf2* on EPCs treated with AGGF1 (Fig. 6*A*, *F*, and *K*). Treatment of EPCs with AGGF1 reversed the impaired angiogenesis (Fig. 6*C*, *H*, and *M*), proliferation (Fig. 6*B*, *G*, and *L*), TEM (Fig. 6*D*, *I*, and *N*), and migration (Fig. 6*E*, *J*, and *O*) by hyperglycemia. However, these effects of AGGF1 were attenuated by wortmannin, knockdown of *AKT*, and knockdown of *Nrf2* (Fig. 6). These data further indicate that AGGF1 regulates the functions of EPCs by affecting Nrf2.

DISCUSSION

Our study identifies a novel molecular mechanism underlying the regulation of EPC functions. We show that AGGF1 activates the AKT-Fyn-Nrf2 signaling pathway in EPCs (Supplementary Fig. 15). Nrf2 is a transcription factor important to cellular defense against oxidative stress. Nrf2 needs to be translocated into the nucleus to execute its function, and its nuclear localization is regulated by AKT-Fyn signaling. AGGF1 activates AKT, which leads to dephosphorylation of Fyn, resulting in reduced translocation of Fyn into the nucleus and decreased n-Fyn. Decreased n-Fyn leads to decreased phosphorylation of Nrf2 and inhibits the nuclear export, ubiquitination, and degradation of Nrf2, increasing the expression levels of n-Nrf2 and downstream cytoprotective genes, such as *NQO-1* and *CAT* (Fig. 4). This blocks hyperglycemia-induced ROS generation in EPCs and potentiates the function of EPCs. Oxidative stress was considered to be a critical factor accounting for EPC dysfunction in T2DM (31,32). Our results suggest that AGGF1 protects EPCs

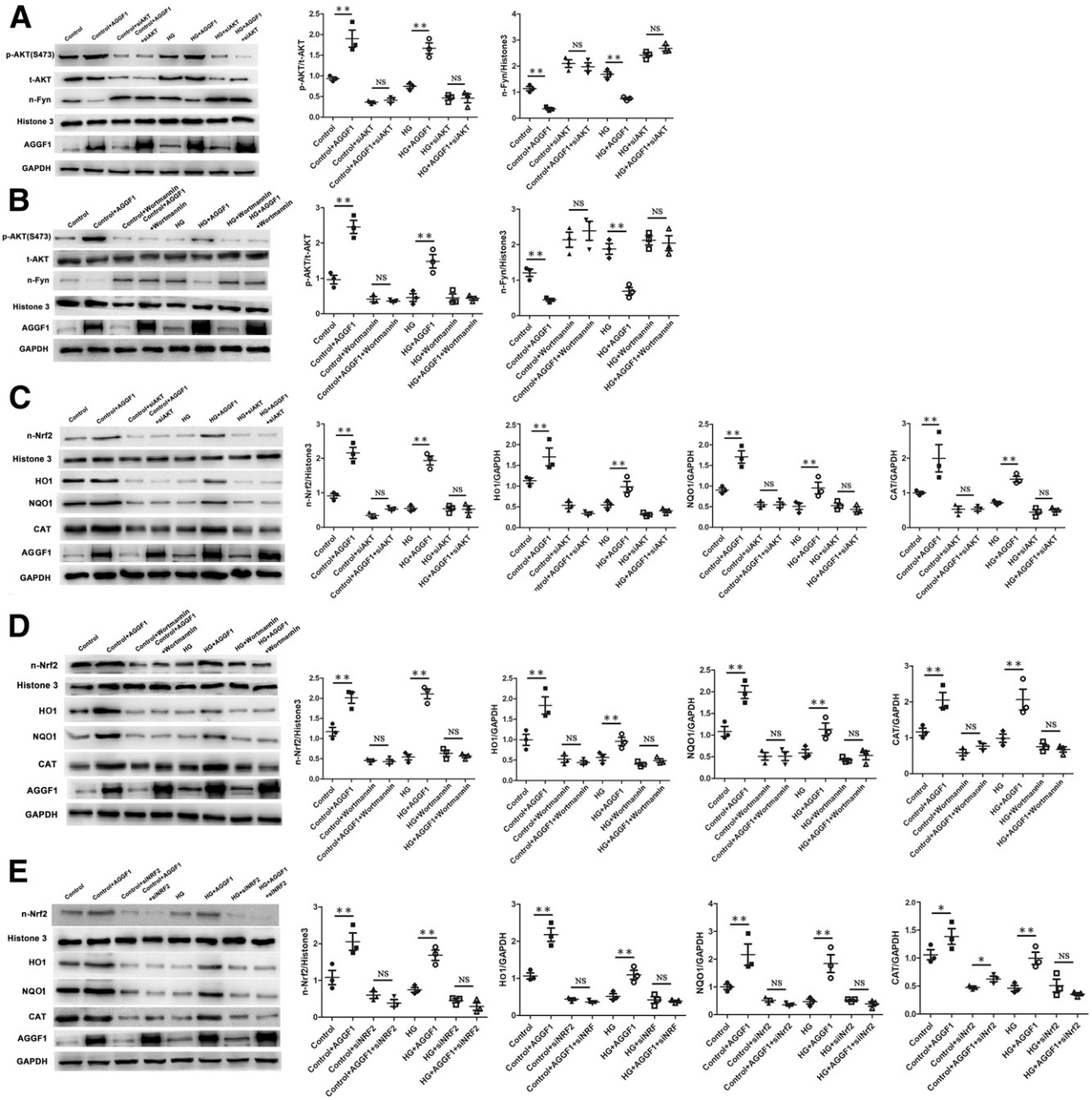


Figure 5—AGGF1 activates AKT-Fyn-Nrf2 signaling in EPCs. *A* and *B*: Western blot analysis showing the effect of siRNA for *AKT* (siAKT) and wortmannin on AGGF1-activated phosphorylation of AKT (p-AKT) and n-Fyn. *C–E*: Western blot analysis showing the effect of siAKT, wortmannin, and siRNA for *Nrf2* (siNrf2) on AGGF1-induced increases of n-Nrf2 and its downstream signaling molecules. Data are mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$ (n = 3/group). t-AKT, total AKT.

partly by inhibiting hyperglycemia-induced ROS generation through a novel antioxidative stress signaling pathway (Supplementary Fig. 15).

Endothelial dysfunction is a major problem in T2DM, and lack of endothelial regeneration and impaired angiogenesis by EPCs are responsible for the vascular abnormalities in DM (5,40). Hence, there is an unmet need for therapeutic interventions to accelerate the repair of dysfunctional endothelium and restore blood flow by EPCs in the treatment of patients with DM. Robust therapeutic effects were observed for AGGF1-primed EPCs in boosting angiogenesis, restoring blood flow, and reducing tissue necrosis and ambulatory impairment in vivo in a diabetic hindlimb ischemia model in *db/db* mice and HFD-induced hyperglycemic mice (Fig. 3 and Supplementary Figs. 8 and 9). Although some hypoglycemic agents (e.g., metformin), lipid-lowering drugs (e.g., statins), and renin-angiotensin system inhibitors were shown to increase circulating EPC levels, the increases were moderate compared with the levels in healthy study

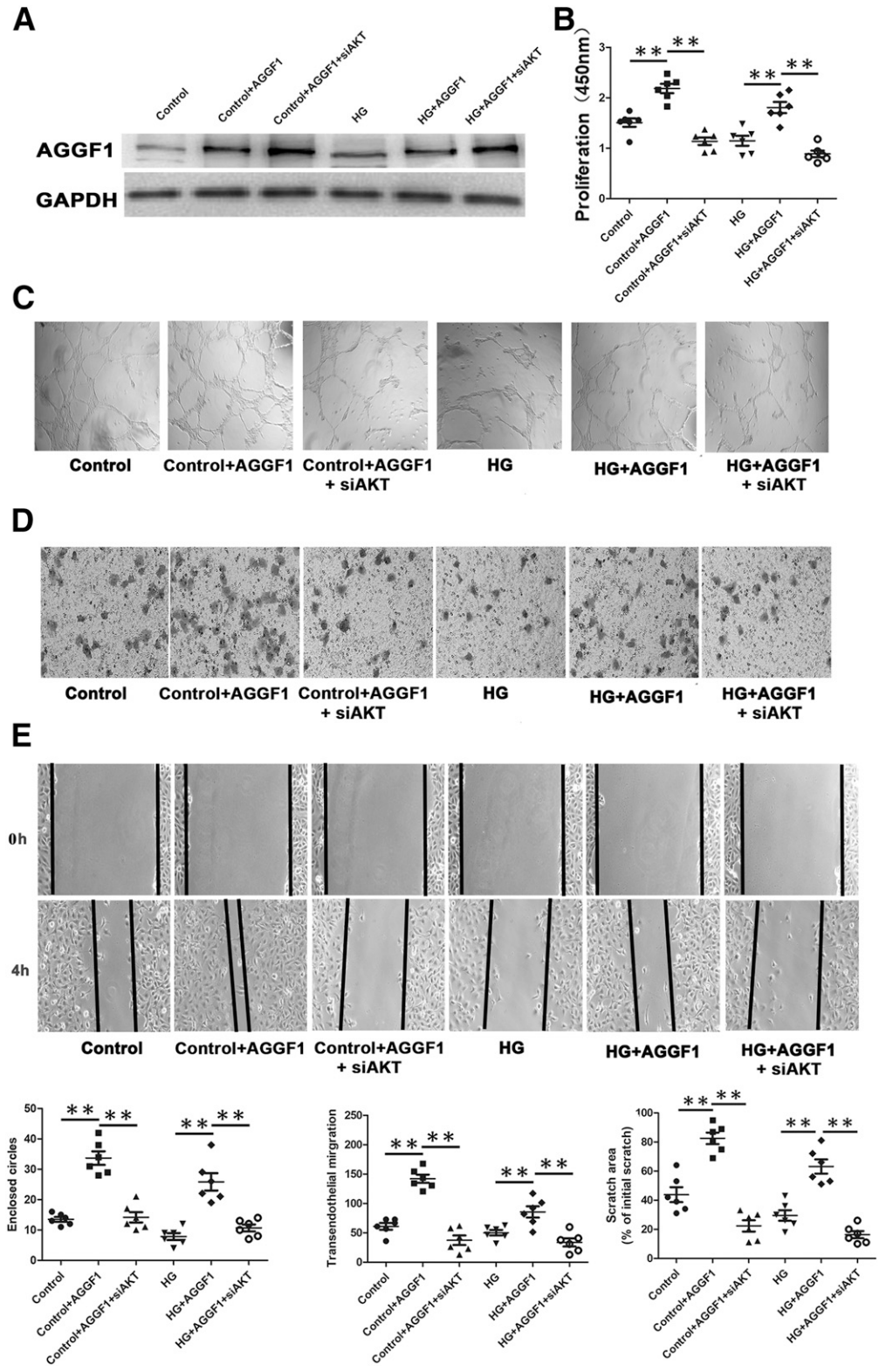
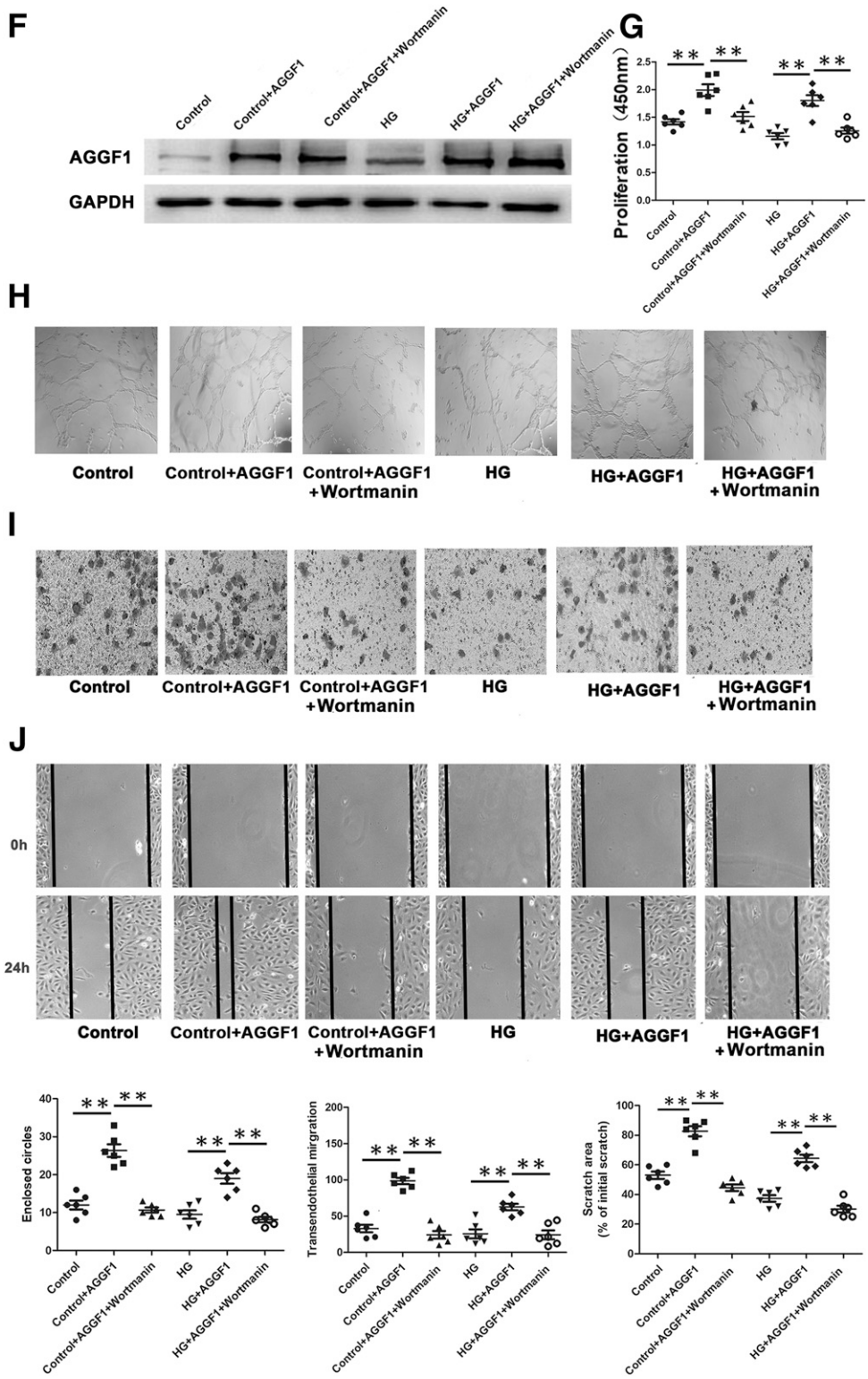


Figure 6—Knockdown of *AKT* expression, wortmannin treatment, and knockdown of *Nrf2* expression attenuate the protective effects of AGGF1 on EPCs. *A*: Western blot analysis for AGGF1 in EPCs. *B–E*: Effects of *AKT* siRNA on AGGF1-mediated rescue of HG-impaired cell proliferation (*B*), tube formation (*C*), TEM (*D*), and cell migration (*E*) by EPCs. *F*: Western blot analysis for AGGF1 in EPCs. *G–J*: Effects of wortmannin on AGGF1-mediated rescue of HG-impaired cell proliferation (*G*), tube formation (*H*), TEM (*I*), and cell migration (*J*) by EPCs. *K*: Western blot analysis for AGGF1 in EPCs. *L–O*: Effects of *Nrf2* siRNA on AGGF1-mediated rescue of HG-impaired cell proliferation (*L*), tube formation (*M*), TEM (*N*), and cell migration (*O*) by EPCs. Data are mean ± SD. $^{**}P < 0.01$ ($n = 6$ mice/group).

subjects (41,42). Moreover, EPC functions in patients with DM are severely impaired; therefore, the therapeutic effects may be limited even with the increased level of autologous EPCs by these drugs. We showed that AGGF1 fully reversed hyperglycemia-impaired functions of EPCs (Fig. 2); therefore, one potential advantage of the AGGF1-based EPC therapy is that dysfunctional EPCs from patients with DM may be repaired and/or their functions



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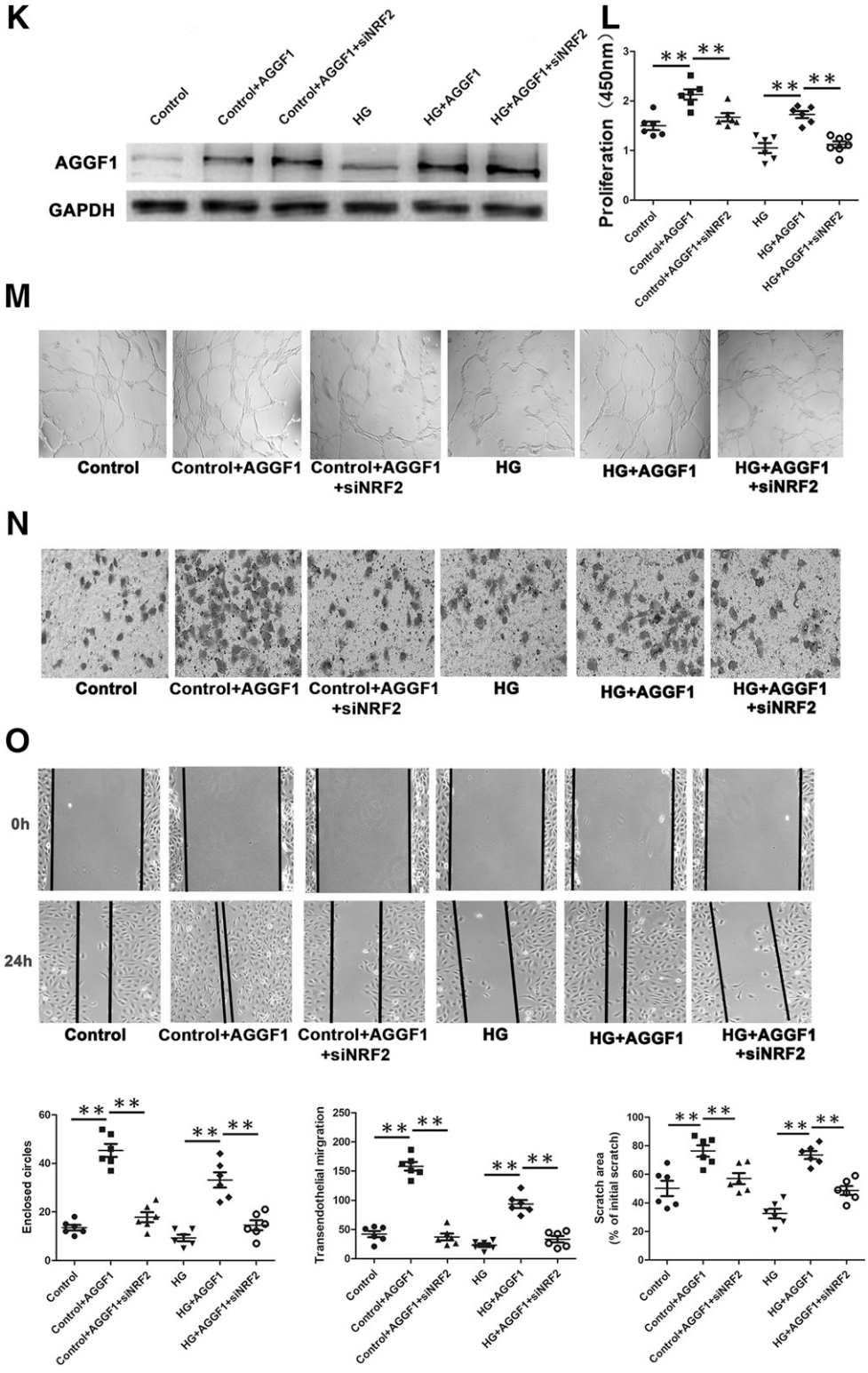
enhanced, which may achieve a maximum efficacy for the treatment for DM-associated vascular abnormalities. Moreover, we recently showed that AGGF1 protein therapy can successfully treat coronary artery disease and myocardial infarction (26) as well as cardiac hypertrophy and heart failure (21) and blocks neointima formation after vascular injury (43). In addition, AGGF1 was found to inhibit ischemia-reperfusion–induced cardiac apoptosis



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through hypoxia-inducible factor-1α/VEGF and attenuate neuroinflammation and blood-brain barrier dysfunction through the PI3K/AKT/nuclear factor-κB pathway (44,45). Therefore, an AGGF1-based EPC therapy for diabetic ischemia may have additional benefits for patients with DM who often are affected by coronary artery disease/myocardial infarction, heart failure, peripheral vascular disease, and other conditions. Furthermore, DM can cause many other diseases, including diabetic cardiomyopathy, end-stage renal disease, and vascular aging, which are all associated with abnormal angiogenesis and vascular dysfunction. On the basis of our data, it is likely that AGGF1-primed EPC therapy may serve as a new strategy not only for treating DM-associated vascular abnormalities but also for many other DM complications such as diabetic cardiomyopathy, end-stage renal disease, and vascular aging. Our study showed that in *db/db* mice or HFD-induced diabetic mice with dysfunctional EPCs, AGGF1-primed EPCs attenuated vascular complications by increasing blood flow and $CD31^+$ capillary density. Because AGGF1-primed EPCs showed increased capillary formation capability, they may be able to increase blood flow and capillary density, even in lean and nondiabetic mice with functional EPCs. It may be interesting to test this hypothesis in the future.

Similar to AGGF1, other growth factors were also reported to reverse the impairment of the proangiogenic potential of EPCs caused by T2DM, including the platelet-derived growth factor (PDGF), VEGFA, fibroblast growth factor 2 (FGF2), granulocyte colony-stimulating factor (G-CSF), and stromal cell–derived factor 1 (SDF-1) (46–48). However, PDGF and FGF2 were shown to increase the proliferation and dedifferentiation of vascular smooth muscle cells, and SDF-1 was shown to enhance recruitment of smooth muscle progenitor cells, all of which are known to cause restenosis after vascular injury. In a clinical trial with 27 patients undergoing coronary stenting, patients treated with G-CSF followed by EPC infusion showed improved exercise capacity, myocardial perfusion, and systolic function; however, G-CSF treatment was found to be associated with an unexpectedly elevated rate of in-stent restenosis (48). On the other hand, we have shown that AGGF1 blocks vascular smooth muscle cell proliferation and migration and restenosis after vascular injury (43). Moreover, the therapeutic effect of AGGF1-primed EPCs would be better than EPCs primed with a combination of VEGFA, FGF2, and PDGF or EPCs primed with SDF-1 (46,47). VEGFA treatment was associated with problems of increased vascular permeability, which causes no-reflow associated with microvascular obstruction and increased risk of vascular dysfunction and death. We have reported that AGGF1 blocks vascular permeability through inhibition of vascular endothelial cadherin phosphorylation (13). Together, these studies suggest that AGGF1 is a better therapeutic target for treating vascular dysfunction associated with T2DM.

In the past several decades, extensive studies were carried out to identify the molecular mechanism underlying the reduced number and impaired functionality of EPCs in DM. Although the underlying mechanisms are complicated and remain poorly understood, the findings of the SDF-1/CXCR-4/CXCR-7/NO pathway and the p53/SIRT1/p66Shc pathway have provided important insights (5,40). We found that *Aggf1* haploinsufficiency impaired the essential functions of EPCs to similar levels as in *db/db* mice or HFD-induced diabetic mice (Fig. 1 and Supplementary Fig. 6). The data suggest that *Aggf1* is required for angiogenic and other functions of EPCs. However, the expression level of AGGF1 was significantly increased in EPCs isolated from *db/db* mice and HFD-induced T2DM mice (Supplementary Fig. 3). The data suggest that *Aggf1* is not directly involved in hyperglycemia-induced EPC dysfunction in diabetic mice. On the other hand, because of the important regulatory role of *Aggf1* in EPC functions, diabetic mice may have developed a remodeling system to upregulate *Aggf1* expression to attenuate the effect of hyperglycemia on EPCs. It should be interesting to investigate how hyperglycemia induces *Aggf1* expression in the future.

The AGGF1 concentration we used in this study (0.5 μg/mL) was empirically determined using in vitro studies with ECs. Using an ELISA kit, we detected AGGF1 in human plasma samples at a concentration of 64.9 ± 29.1 ng/mL. The AGGF1 concentration we used in this study was higher than what was found in a physiological setting. There are several factors that may explain why a much higher concentration than is found in a physiological setting was warranted. First, EPCs may have a low response to AGGF1 in culture. Another cause may be the reduced half-life of AGGF1 during the long period of the in vitro experiments. It is also possible that EPCs may have evolved to require a greater concentration of AGGF1 than would other cell types. It would be important in the future to identify an optimal EPC treatment regimen by analyzing various doses of the AGGF1 protein or by identifying mutant AGGF1 proteins (e.g., deletions, point mutations, posttranslational modifications) with more potent effects. Also equally interesting would be to study the therapeutic effects of AGGF1 on human EPCs in immunosuppressed mice to increase their translational relevance. Other areas of important studies may include the identification of the other molecular mechanisms by which AGGF1 promotes EPC differentiation, proliferation, migration and homing, secretion of EPC-derived factors, incorporation of EPCs in or around capillaries, and other aspects of EPC functions in vivo.

In summary, we show that haploinsufficiency of *Aggf1* in mice damages the functions of EPCs, such as differentiation, proliferation, migration, angiogenesis, and TEM (homing), to a similar level as that in HFD-induced hyperglycemic mice and *db/db* mice, suggesting that *Aggf1* is required for essential functions of EPCs. We further show that the AGGF1 protein can reverse all damaging effects of hyperglycemia on EPCs. Most importantly, in an in vivo model for diabetic hindlimb ischemia in HFD-induced hyperglycemic mice and *db/db* mice, transplantation of

AGGF1-primed EPCs successfully boosts angiogenesis, restores blood flow, and blocks tissue necrosis and ambulatory impairment. In addition, the studies reported here identify a novel signaling pathway (i.e., AGGF1-AKT-Fyn-Nrf2-antioxidative genes) involved in the function of EPCs. These studies highlight the identification of AGGF1 as a new therapeutic target for treating cardiovascular complications associated with DM.

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Author Contributions. Y.Y., Y.L., Q.S., and W.X. performed experiments. Y.Y., Y.L., Q.C., and Q.K.W. critically revised the manuscript. Y.Y., Y.L., and Q.K.W. conceived and designed the study. Y.L., C.H., C.X., Q.C., and Q.K.W. analyzed data. Y.L. and Q.K.W. drafted the manuscript. All authors reviewed the manuscript. Q.K.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data and Resource Availability. The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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