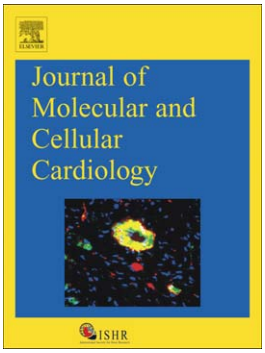

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Globular adiponectin improves high glucose-suppressed endothelial progenitor cell function through endothelial nitric oxide synthase dependent mechanisms

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Abstract

Aim: Plasma levels of adiponectin, an adipose-specific protein with putative anti-atherogenic properties, could be down-regulated in obese and diabetic subjects. Recent insights suggest that the injured endothelial monolayer is regenerated by circulating endothelial progenitor cells (EPCs), but high glucose reduces number and functions of EPCs. Here, we tested the hypothesis that globular adiponectin can improve high glucose-suppressed EPC functions by restoration of endothelial nitric oxide synthase (eNOS) activity.

Methods: Late EPCs isolated from healthy subjects appeared with cobblestone shape at 2-4 weeks. EPCs were incubated with high glucose (25 mM) and treatment with globular adiponectin for functional study. Migration and tube formation assays were used to evaluate the vasculogenetic capacity of EPCs. The activities of eNOS, Akt and concentrations of nitric oxide (NO) were also determined.

Results: Administration of globular adiponectin at physiological concentrations promoted EPC migration and tube formation, and dose-dependently upregulated phosphorylation of eNOS, Akt and augmented NO production. Chronic incubation of EPCs in high-glucose medium

significantly impaired EPC function and induced cellular senescence, but these suppression effects were reversed by treatment with globular adiponectin. Globular adiponectin reversed high glucose-impaired EPC functions through NO- and p38 MAPK-related mechanisms. In addition, nude mice received EPCs treated with adiponectin in high glucose medium showed a significant improvement in blood flow than those received normal saline and EPCs incubated in high glucose conditions.

Conclusion: The administration of globular adiponectin improved high glucose-impaired EPC functions in vasculogenesis by restoration of eNOS activity. These beneficial effects may provide some novel rational to the vascular protective properties of adiponectin.

Keywords: adiponectin; endothelial progenitor cell; nitric oxide

1. Introduction

Hyperglycemia is a key factor for the development of vascular complications in patients with type 2 diabetes [1]. However, the pathophysiology linking type 2 diabetes to atherosclerotic cardiovascular diseases is complex and still not fully clarified. Endothelial function has been shown to play an important role in the clinical manifestations of established atherosclerotic lesions [2]. Convincing evidence suggests that the injured endothelial monolayer is regenerated by circulating bone marrow derived-endothelial progenitor cells (EPCs), which accelerate reendothelialization and protect against the initiation and progression of atherosclerosis [3,4]. Levels of circulating EPCs reflect vascular repair capacity and may be directly related to endothelial function [5]. In diabetic subjects, the reduced number and function of EPCs have been suggested as the pathogenesis of vascular complication [6,7]. A progressive reduction in circulating EPCs under long-term exposure to high glucose may contribute to a vicious cycle resulting in endothelial dysfunction and the rapid progression of atherosclerosis [8,9]. Our recent work further indicated that long term exposure to high glucose may impair the proliferation and functions of both early and late (outgrowth) human EPCs through nitric oxide (NO)-related

mechanisms [10]. Thus, the novel therapeutic strategy to promote EPC function by enhancing its NO bioavailability could be potentially attractive for hyperglycemia-induced vascular complications in diabetic patients.

Adiponectin, an adipocyte-derived protein, is secreted from adipose tissue and was shown to have putative anti-atherogenic properties [11,12]. Clinically, plasma adiponectin may be decreased in obese and/or type 2 diabetic subjects. The clinical presence of hypoadiponectinemia is also associated with the increased risks of type 2 diabetes and related cardiovascular events [13,14]. Given the recent evidence that globular adiponectin may up-regulate NO production in vascular endothelial cells and suppress excess reactive oxygen species production under high glucose conditions, one may speculate the potential beneficial effects of adiponectin on EPCs for vascular repair as well as vasculogenesis [15-17]. However, it is not known whether globular adiponectin can improve high glucose-impaired EPC function and whether this improvement, if there were, is through up-regulation of endothelial NO synthase (eNOS) activity. This study was conducted to investigate whether administration of globular adiponectin can augment high glucose-suppressed EPC functions by eNOS activation.

2. Materials and methods

2.1. Human late EPC cultivation

Peripheral blood samples (20 ml) were obtained from healthy young volunteers, and total mononuclear cells (MNCs) were isolated by density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis, MO, USA). Briefly, MNCs (5×10^6) were plated in 2 ml endothelial growth medium (EGM-2 MV Cambrex, East Rutherford, NJ, USA) on fibronectin-coated 6-well plates. After 4 days of culturing, the medium was changed and nonadherent cells were removed; attached early EPCs appeared elongated with spindle shape. A certain number of cells were allowed to grow into colonies of late EPCs, which emerged 2-4 weeks after the start of the MNC culture. The late EPCs exhibited a “cobblestone” morphology and monolayer growth pattern typical of mature endothelial cells at confluence [10,18]. Late EPCs were collected and used for the functional assays in this study. The study was approved by the local research ethics committee, and the protocols of this study were consistent with ethical guidelines provided in the 1975 Helsinki Declaration.

2.2. EPC Characterization

The late EPC-derived outgrowth endothelial cell population was

characterized by immunofluorescence staining for the expression of VE-cadherin, platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD-31), and CD34 (Santa Cruz). The fluorescent images were recorded under a laser scanning confocal microscope [10,19].

2.3. EPC tube formation assay

An EPC tube formation assay was performed with an In Vitro Angiogenesis Assay Kit (Chemicon) [10]. ECMatrix gel solution was thawed at 4℃ overnight, mixed with ECMatrix diluent buffer, and placed in a 96-well plate at 37℃ for 1 hour to allow the matrix solution to sol idify. Late 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℃ for 16 hours. Tubule formation was inspected under an inverted light microscope (×100). Six representative fields were taken, and the average of the total area of complete tubes formed by cells was compared by using computer software, Image-Pro Plus.

2.4. EPC migration assay

The migratory function of EPCs was evaluated by a modified Boyden

chamber assay (Transwell, Coster) [10]. Briefly, isolated late EPCs were detached as described above with trypsin/EDTA, and then 4×10^4 late EPCs were placed in the upper chambers of 24-well transwell plates with polycarbonate membrane (8-μm pores) that contained serum-free endothelial growth medium; VEGF (50 ng/ml) was added to medium placed in the lower chambers. After incubation for 24 hours, the membrane was washed briefly with PBS and fixed with 4% paraformaldeyde. The upper side of the membrane was wiped gently with a cotton ball. The membrane was stained using hematoxylin solution and carefully removed. The magnitude of migration of late EPCs was evaluated by counting the migrated cells in six random high-power (×100) microscopic fields.

2.5. EPC senescence assay

Cellular aging was determined with a Senescence Cell Staining kit (Sigma) [10]. After washing with PBS, late EPCs were fixed for 6 minutes in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and then incubated for 12 hours at 37℃ without CO_2 with fresh X-gal staining solution. After staining, green-stained cells and total cells were counted and the percentage of β-galactosidase-positive cells was calculated.

2.6. EPC fibronectin adhesion assay

EPC fibronectin adhesion test was assessed as previously described [20]. Briefly, after centrifugation and resuspension in basal medium with 5% fetal bovine serum, late EPCs (1×10^4 cell per well) were placed on fibronectin-coated 6-well plates and incubated for 30 minutes at 37 ºC. Gentle washing with PBS 3 times was performed after 30 minutes' adhesion, and adherent cells were counted by independent blinded investigators. Phenotyping of endothelial characteristics of adherent cells by indirect immunostaining was performed with Dil-acLDL and BS-1 lectin.

2.7. Western blotting analysis and measurement of nitrate levels

Protein extracts were prepared as previously described [21]. Briefly, EPCs were lysed in a 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 that directed to eNOS, phosphorylated eNOS (p-eNOS), p-Akt (Cell Signaling), and actin (Chemicon). Bands were visualized by chemiluminescence detection reagents. Densitometric analysis was conducted with ImageQuant

(Promega) software. After incubation of late EPCs with medications in high glucose medium for 4 days, the conditioned medium was measured for nitrate level by Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2% phosphoric acid].

2.8. Gene silencing of eNOS and adiponectin R1, R2 receptor using small inhibitory RNA

eNOS and adiponectin R1, R2 receptor small inhibitory RNA (siRNA) were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., CA, USA). Late EPCs were incubated with 25 or 50 nM siRNA, and scrambled siRNA (Dharmacon, Lafayette, CO) was used as a control. The silencing protocol used multiple transfection processing. Specific siRNAs were incubated with 8 μL Oligofectamine solution (Invitrogen, Carlsbad, CA, USA) and added to the antibiotic and serum-free medium for 6 hours followed by pulsing normal medium incubating for 2 days and repeated one time. The efficiency of specific eNOS siRNA inhibition was verified by Western blotting.

2.9. In vivo mouse ischemic hind limb model

Athymic nude mice at 6-8 weeks old were purchased from the National

Laboratory Animal Center, Taiwan. Briefly, the animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) [22]. The proximal and distal portions of the femoral artery were ligated. Nude mice were then randomly assigned to 3 treatment groups (n = 6 in each group) for intramuscular injection of normal saline, healthy EPC, EPC treated with high glucose for 4 days (EPC-HG), EPC treated with high glucose and globular adiponectin 5 μg/ml (24 hours following by treatment of EPCs in high-glucose conditions for 4 days) (EPC-HG+gAd). EPCs were labeled with fluorescent carbocyanine 1,1'-dioctadecyl-1 to 3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) dye (Molecular Probes) [23]. Intramuscular injection was performed 24 hours following arterial occlusion. A total volume of 200 μl normal saline or 5×10^6 EPC were injected at 6 sites into the ischemic hind limb distal to the arterial occlusion site. Three ventral injections were placed in the upper limb in proximity to the adductor and semimembranosus muscles. The remaining 3 injections were administered to the ventral lower limb involving the gastrocnemius and flexor digitorum muscles. In order to achieve maximal experimental uniformity, transplanted EPC treated with high glucose or adiponectin were derived from the same donors and used in parallel experiments. All experimental procedures and protocols involving animals

were approved by the institutional animal care committee of National Yang-Ming University (Taipei, Taiwan).

2.10. Laser Doppler blood perfusion imaging

Hind limb blood perfusion was measured with a Laser Doppler perfusion imager system (Moor Instruments Limited, Devon, UK) before and after the surgery and then followed weekly until 3 weeks after operation [19,22]. The animals were placed on a heating pad in order to maintain a constant body temperature during the entire measurement. To avoid the influence of ambient light and temperature, the results were expressed as the ratio of perfusion in the right (ischemic) versus left (non-ischemic) limb.

2.12. Statistical analysis

All data were expressed as mean ± SEM for continuous variables and as number (percent) for categorical variables. Intergroup comparisons were performed by Student's *t* test or one-way ANOVA. Probability values of $P < 0.05$ were considered statistically significant. The SPSS 9.0 (version 12, SPSS, Chicago, Illinois, USA) software package was used for all statistical analyses.

3. Results

3.1. Effects of globular adiponectin on EPC functions in vasculogenesis and migration

An in vitro angiogenesis assay was used to investigate the effects of adiponectin on EPC functions in vasculogenesis. EPCs were incubated with globular adiponectin at increased concentrations (2.5-40 μg/ml) for 24 hours. As shown in Figure 1A, adiponectin caused a significant increase in EPC tube formation on ECMatrix gel as compared to the control group (2.5-20 μg/ml, $p<0.05$; Figure 1A). A modified Boyden chamber assay with VEGF as a chemoattractic factor was used to evaluate the effects of adiponectin on EPC migration. After 24 hours incubation, administration of globular adiponectin with enhanced concentrations (2.5-40 μg/ml) significantly increased EPC migration (2.5-40 μg/ml, $p<0.01$; Figure 1B).

3.2. Effects of globular adiponectin on eNOS, Akt activation and nitrate production in EPCs

Recent studies indicated that adiponectin at physiological concentrations increases production of NO by eNOS activation from vascular endothelial cells [24], and the mobilization and function of EPCs are critically dependent

on eNOS activity [25]. Therefore, we evaluated the effects of globular adiponectin on eNOS and Akt activities in cultured EPCs. After incubation with globular adiponectin for 2 hours, eNOS phosphorylation (p-eNOS) at Ser^{1177} and Akt phosphorylation (p-Akt) at Ser^{473} were determined by Western blotting and revealed significantly up-regulation in EPCs in a dose-dependent manner (increased p-eNOS, 65%; p-Akt, 108% by adiponectin 5 μg/ml compared with control group; Figure 2A). Up-regulation of eNOS and Akt phosphorylation were also noted after administration of globular adiponectin for 24 hours (increased p-eNOS, 32%; p-Akt, 34 % by adiponectin 5 μg/ml compared with the control group; Figure 2B). As shown in Figure 2C, this activation of eNOS phosphorylation was associated with an increase in EPCs-derived nitrate production (652% enhancement of nitrate with 5 μg/ml of adiponectin compared with the control group, $p<0.001$).

3.3. Effects of globular adiponectin on high glucose-suppressed EPC functions in vasculogenesis and high glucose-induced senescence

We investigated whether treatment with globular adiponectin could improve high glucose-impaired EPC functions. As shown in Figure 3A, EPCs cultured in high-glucose medium showed significantly decreased tube

formation capacity (reduced 55%, $p<0.01$ compared with control group). Administration of globular adiponectin for 24 hours following treatment of EPC with high-glucose medium markedly augmented high glucose-reduced EPC tube formation capacity (all $p<0.01$ compared with the high-glucose group; Figure 3A).

Consistent with previous findings, incubation of EPCs in high-glucose medium (25 mM) for 4 days significantly suppressed EPC migration ($p<0.05$ compared with the control group; Figure 3B). However, administration of globular adiponectin for 24 hours following by treatment of EPCs in high-glucose conditions markedly improved high glucose-impaired EPC migration ($p<0.05$ compared with the high-glucose group; Figure 3B).

Compared with the control group, incubation of EPCs with high-glucose medium significantly increased the percentage of senescence-associated ß-galactosidase-positive EPCs ($p<0.01$; Figure 3C). Administration of globular adiponectin (5 μg/ml) significantly attenuated the percentage of senescence-associated ß-galactosidase-positive EPCs ($p<0.01$). Administration of L-NAME abolished the anti-senescence effect of adiponectin on EPC in high-glucose conditions.

As shown in Figure 3D, incubation of late EPCs with high glucose medium

markedly suppressed late EPC adhesion, and administration of globular adiponectin (5 μg/ml) significantly augmented adhesion in high glucose conditions ($p<0.01$). This effect was inhibited by treatment with L-NAME (100 μM) ($p<0.05$). Besides, treatment with NO donor sodium nitroprusside (SNP, 100 μM) exerted similar effects of adiponectin to improve high glucose-suppressed EPC adhesive function ($p<0.05$). These findings suggested supplementation with adiponectin may recover impaired EPCs functions in high-glucose medium, and this benefit may derive from the NO-related pathway.

3.4. Globular adiponectin recovered high glucose-attenuated eNOS activation and NO production

High glucose impairs eNOS activation and reduces NO bioavailability in cultured late EPCs [10]. We therefore tested the effects of globular adiponectin on high glucose-treated EPCs to determine whether adiponectin could recover impaired eNOS activation in EPCs. As shown in Figure 4A, EPCs incubated in high glucose medium (25 mM) for 4 days significantly down-regulated total form of eNOS and eNOS phosphorylation at Ser^{1177}, Akt phosphorylation at Ser^{473} by 68%, 71%, and 79%, respectively. The reduction of eNOS

phosphorylation was associated with decreased EPCs-derived nitrate production (39% reduction; Figure 4B). However, treatment with globular adiponectin in high-glucose conditions for 24 hours dose-dependently up-regulated eNOS, phosphorylation of eNOS and Akt, and increased NO production (Figure 4A).

To confirm the critical role of eNOS in promotion of high glucose-impaired EPC functions by adiponectin, EPCs were transfected with eNOS siRNA to inhibit eNOS activity. As shown in Figure 4C, administration of eNOS siRNA (25 and 50 nM) markedly inhibited eNOS activity by treatment with globular adiponectin in high-glucose conditions. Administration of eNOS siRNA with demonstrated inhibition of eNOS activity significantly suppressed benefit of adiponectin in enhancement of EPC tube formation capacity (Figure 4D).

3.5. Roles of Mitogen-activated protein kinase (MAPK) in adiponectin-modulated EPC function and eNOS activation

Previous study indicated that p38 MAPK have been implicated in the biological actions of adiponectin [26], leading to the hypothesis that adiponectin could act through the stimulation of these enzymes. After incubation of EPCs with indicated concentrations of adiponectin for 120

minutes, both p38 total protein and protein phosphorylation levels were analyzed. As presented in Figure 5A, EPCs cultured in globular adiponectin showed significantly increased phosphorylation of p38 MAPK, and administration of p38 MAPK inhibitor (SB203580, 10 μM) for 30 minutes inhibited globular adiponectin enhanced phosphorylation of eNOS and Akt (Figure 5B).

We further investigated the role of p38 MAPK in the process of adiponectin-mediated upregulation of eNOS and Akt activities in high glucose conditions. EPCs were incubated with p38 MAPK inhibitor (SB203580, 1 and 10 μM) before treatment with globular adiponectin (5 μM) for 24 hours in high glucose medium. As shown in Figure 5C and 5D, activation of phosphorylation of eNOS and Akt by treatment with adiponectin were significantly attenuated by SB203580 in high glucose conditions. In addition, the beneficial effect of adiponectin on high glucose-impaired EPC migration was significantly blocked by administration of p38 MAPK inhibitor (SB203580) (Figure 5E), suggesting adiponectin enhanced high glucose-suppressed EPC function thought p38 MAPK pathway.

3.6. Roles of adiponectin receptor 1 (adipoR1) and receptor 2 (adipoR2) in

adiponectin-mediated eNOS activation and EPC function

To test the role of adipoR1 and adipoR2 in adiponectin-stimulated eNOS activation and EPC function in high glucose conditions, EPCs were transfected with adipoR1 and adipoR2 siRNA to selectively inhibit adipoR1 and adipoR2 activity. Figure 6A shows that administration of adipoR1 siRNA (50 nM) markedly decreased activation of phosphorylation of eNOS, Akt and p38 MAPK, and attenuated EPC function by treatment with globular adiponectin in high-glucose conditions. These findings suggested that globular adiponectin upregulated high glucose-suppressed EPC function and eNOS activation through adipoR1.

3.7. EPC treated globular adiponectin transplantation improve hind limb perfusion

Nude mice were randomly assigned to 4 treatment groups for intramuscular injection of normal saline, healthy EPC, EPC treated with high glucose (EPC-HG), EPC treated with high glucose, and globular adiponectin (EPC-HG+gAd). In the animals receiving normal saline, blood flow remained constant throughout the study around 45% of that measured in the non-ischemic limb (46.0±5.5%, three weeks after operation). In contrast, the

mice treated with EPC-HG+gAd and healthy EPC alone but not EPC-HG showed a significant improvement in blood flow already by three weeks after injection (both $p<0.05$; Figure 7A,C). Besides, increased labeled EPCs were noted in ischemic tissues in mice transplanted with EPC and EPC-HG+gAd than those with EPC-HG (Figure 7B,D).

4. Discussion

The present study for the first time demonstrated that globular adiponectin could enhance NO bioavailability by up-regulation of eNOS activity, and promote the migration and the vasculogenesis of human late (outgrowth) EPCs. In addition, administration of globular adiponectin reversed high glucose-impaired EPC functions through NO- and p38 MAPK-related mechanisms, and increased high glucose-reduced eNOS activity by p38 MAPK-mediated pathways. Besides, globular adiponectin upregulated high glucose-suppressed EPC function and eNOS activation in high-glucose conditions was through adipoR1. Mice received EPC treated with adiponectin in high glucose conditions showed a significant improvement in blood flow recovery than those received normal saline and high glucose-treated EPC . These findings may explain some of the vasoprotective properties of adiponectin and provide a potential therapeutic rationale for vascular repair in type 2 diabetic subjects.

Type 2 diabetic patients frequently suffer from micro- and macrovascular abnormalities and accelerated atherosclerosis. The functional activity and integrity of the endothelial monolayer has been shown to play a pivotal role in

atherogenesis. In humans, extensive endothelial cell damage by cardiovascular risk factors can result in endothelial cell apoptosis with subsequent loss of integrity of the endothelium. The extent of endothelial injury may represent a balance between the magnitude of injury and the capacity for repair and predicts cardiovascular event rates. It has been suggested that bone marrow-derived circulating EPCs play an important role in endothelial cell regeneration [27]. Reduced levels of circulating EPCs independently predicts atherosclerotic disease progression and future cardiovascular events [9], which may support a crucial role for endogenous vascular repair by EPCs to modulate the clinical course of coronary artery disease. The increased prevalence of cardiovascular complications in type 2 diabetes may be also attributed to the disturbed balance between increased endothelial injury and hampered endothelial repair processes. Reduced numbers and functions of EPCs are associated with the pathogenesis of diabetic vascular complications in either Type 1 or Type 2 diabetes [28,29]. A progressive reduction in circulating EPCs when long-term exposure to high glucose and/or other risk factors may contribute to a vicious cycle resulting in endothelial dysfunction and the rapid progression of atherosclerosis [8-10]. Accordingly, EPCs could be the potential therapeutic target for vascular

protection in patients with Type 2 diabetes.

In the present study, the function of human late EPCs is impaired in high glucose status and globular adiponectin could directly improve the dysfunction of EPCs with or without the presence of high glucose, supporting the universal role of adiponectin in vascular protection. Adiponectin is the most abundant adipokine secreted by adipose cells with putative anti-atherosclerotic properties [30-32]. Adiponectin exists in the circulation as a full-length protein and a putative proteolytic cleavage fragment consisting of the globular C-terminal domain. This globular adiponectin is pharmacologically active and can regulate body weight and fatty acid oxidation in mice [33]. At physiological levels (2 to 20 μg/ml), adiponectin exhibits specific and saturable binding to aortic endothelial cells and readily binds to the walls of catheter-injured vessels more than to intact vascular walls [34]. Adiponectin could also reduce the expression of adhesion molecules in endothelial cells and decrease cytokine production from macrophages by inhibiting NF-κB signaling through cAMP-dependent pathway [35]. Clinically, although secreted from adipose tissues, plasma levels of adiponectin are paradoxically decreased in obesity and diabetes subjects [10]. Besides, plasma adiponectin levels were also significantly

reduced in patients with coronary artery disease and immediately after acute coronary syndrome [36,37]. It is suggested that hypoadiponectinemia may contribute to endothelial injury and coronary plaque vulnerability [38]. Finally and most interestingly, though the detailed mechanisms were not known, adiponectin knockout mice portray negligible EPC mobilization, which can be restored by systemic administration of adiponectin [39]. Given the evidence mentioned above, our current findings further indicated the direct beneficial effects of globular adiponectin on human EPCs, particularly in high glucose conditions, which may provide some novel rationales to its potential clinical impact to vascular protection.

It is evident that decreased bioavailability of NO produced from eNOS plays a crucial role in the development and progression of atherosclerosis. Under various pathological conditions such as type 2 diabetes eNOS may become dysfunctional or its expression may be decreased. Recent evidence showed that adiponectin could directly stimulate NO production in endothelial cells through phosphatidylinositol (PI) 3-kinase-dependent pathways involving phosphorylation of eNOS by AMPK [22], suggesting that enhancement of vascular NO bioavailability by adiponectin may convey vasoprotective effects on endothelium. Of note, expression and

phosphorylation of eNOS are known to be essential for the survival, migration, and angiogenesis of either EPCs or endothelial cells [24]. Our recent work further indicated that the administration of NO donor could reverse the impairments in proliferation and functions of both early and late EPCs induced by long-term exposure to high glucose, suggesting the critical role of NO to reverse hyperglycemia-induced EPC down-regulation [10]. Therefore, enhancement of EPCs and their functional capacity by upregulation of NO bioavailability through some novel pharmacological strategies could be of potential clinical benefits especially in diabetic subjects. In the present study, we demonstrated that administration of globular adiponectin could activate eNOS and Akt, and upregulate the migration and tube formation capacities of late EPCs. This is in line with previous report showing that adiponectin directly stimulate phosphorylation of Akt in endothelial cells [24]. Akt is downstream from PI 3-kinase and is capable of directly phosphorylating eNOS at Ser^{1179}, resulting in its activation [40]. Recent evidence indicated that p38 MAPK plays a key role in downregulating EPCs by hyperglycemia in diabetic patients [41]. In our study, it is interesting to find that EPCs cultured in globular adiponectin showed significantly increased phosphorylation of p38 MAPK, and administration of p38 MAPK inhibitor inhibited globular

adiponectin enhanced phosphorylation of eNOS and Akt. Besides, the beneficial effect of adiponectin on high glucose-impaired EPC migration was significantly blocked by administration of p38 MAPK inhibitor (SB203580), and adiponectin stimulated-phosphorylation of eNOS was significantly attenuated by p38 MAPK inhibitor. These findings suggest that adiponectin could increase eNOS activation through PI 3-kinase-dependent pathways involving the phosphorylation of eNOS by p38-MAPK [24]. Adiponectin acts through its two receptors, designated AdipoR1 and AdipoR2, which are primarily expressed in skeletal muscle and liver, respectively [42]. Subsequent studies have shown that AdipoR1 is also present in endothelial cells, cardiomyocytes, and pancreatic-β cells, whereas AdipoR2 is also expressed in endothelial cells [43,44]. In this study, we showed that administration of adipoR1 siRNA decreased activation of eNOS and attenuated EPC function by treatment with globular adiponectin in high-glucose conditions, suggesting, globular adiponectin augment high glucose-suppressed EPC function through adipoR1. Thus, our findings provided novel mechanism of adiponectin in vascular protection by enhancement of late EPC function. Given the similarity between human late

EPCs and matured arterial endothelial cells, it is then interesting to confirm our *in vitro* findings in an *in vivo* model in the future.

5. Conclusions

Administration of globular adiponectin may enhance high glucose-impaired EPC functions by restoration of eNOS activity in vitro, and these novel findings may explain some of vasoprotective properties of adiponectin and provide the therapeutic rationales for clinical vascular protection in patients with diabetes.

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Competing interest statement

None

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FIGURE LEGENDS

Figure 1. Effects of globular adiponectin on EPC tube formation and migration. (A) Cultured EPCs were incubated with globular adiponectin (gAd) and an in vitro angiogenesis assay was used to investigate the effect of adiponectin on EPC neovascularization. Representative photomicrographs showed the number of branch points in adiponectin-treated EPCs. (B) A Boyden chamber assay was performed to evaluate the effect of gAd on EPC migration. The migrated cells were stained with hematoxylin and counted under a microscope. (n=4, *p<0.05, compared with control group)

Figure 2. Effects of globular adiponectin on eNOS, Akt phosphorylation, and NO production in EPCs. (A,B) Effects of globular adiponectin (gAd) on eNOS, Akt activities and nitrite levels were determined in cultured late EPCs. Administration of gAd (2 hours and 24 hours) dose-dependently upregulated phosphorylation of eNOS (p-eNOS) and Akt (p-Akt). (C) Nitrate production (as NO content) in culture medium was measured by Griess reagent. (n=4, *p<0.05, compared with control group).

Figure 3. Adiponectin recovers high glucose-impaired EPC functions and attenuated cellular senescence. After incubation of EPCs with indicated concentrations of adiponectin for 24 hours, cultured EPC were assessed functional assays after treatment with high glucose medium for 4 days. (A) An in vitro angiogenesis assay was performed with EPCs to investigate the effect of adiponectin (gAd) on EPC neovascularization in high-glucose medium. (B) A modified Boyden chamber assay was used to assess the effect of adiponectin on EPC migration in high-glucose conditions.

(C) Acidic ß-galactosidase was detected as a biochemical marker for acidification typical of EPC senescence. Administration of eNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) abolished the effects of gAd on EPC adhesion and senescence. (D) Fibronectin adhesion assay was used to evaluate the effect of gAd on EPC adhesive function. (E) (n=4, *p<0.05 vs control, $^{\#}$p<0.05 vs high-glucose group, §p<0.05 vs high-glucose+adiponectin)

Figure 4. Adiponectin recovered high glucose-impaired EPC eNOS activation and NO production. (A) Effects of globular adiponectin (gAd) on total form of eNOS, Akt and eNOS, Akt phosphorylation were determined in EPCs. (B) Nitrate production (as NO content) in culture medium was measured by Griess reagent. (*p<0.05 compared with control group). (C) Administration of eNOS siRNA (25 and 50 nM) markedly inhibited accumulation of eNOS by treatment with gAd in high-glucose medium. (D) An in vitro angiogenesis assay was performed with EPCs to investigate the effect of adiponectin on EPC neovascularization in high-glucose conditions after ablation of eNOS activity. (n=4, *p<0.05 vs control, $^{\#}$p<0.05 vs high-glucose group, §p<0.05 vs high-glucose+adiponectin group)

Figure 5. Roles of Mitogen-activated protein kinases (MAPK) in adiponectin-modulated EPC function and eNOS activation. (A) After incubation of EPCs with indicated concentrations of adiponectin for 120 minutes, p38 total protein and protein phosphorylation levels were analyzed in EPCs. (B) EPCs incubation with the p38 MAPK inhibitor (SB203580, 10 μM) for 30 minutes followed by incubation with globular adiponectin (gAd, 5

μg/ml) for 1 day. Effects of adiponectin on total form of eNOS, Akt and eNOS, Akt phosphorylation were determined in EPCs after pretreatment with SB203580. (C,D) Effects of adiponectin on total form of eNOS, Akt and eNOS, Akt phosphorylation were measured in EPCs after pretreatment with SB203580 in high glucose conditions. (E) Scratch test was performed with EPCs to investigate the effect of adiponectin on EPC migration. (n=4, *p<0.05 vs control, $^{\#}$p<0.05 vs high-glucose group, §p<0.05 vs high-glucose+adiponectin group)

Figure 6. Roles of adiponectin receptor 1 (adipoR1) and receptor 2 (adipoR2) in adiponectin-upregulated eNOS activation and EPC function. Cultured EPCs were transfected with adipoR1 and adipoR2 siRNA to selectively inhibit adipoR1 and adipoR2 activity. (A) Administration of adipoR1 siRNA (50 nM) markedly decreased activation of eNOS by treatment with globular adiponectin (gAd) in high-glucose conditions. (B) Incubation with adipoR1 siRNA abolished adiponectin enhanced EPC migration in high-glucose conditions. (n=4, *p<0.05 vs control, $^{\#}$p<0.05 vs high-glucose group, §p<0.05 vs high-glucose+adiponectin group)

Figure 7. Adiponectin treated-EPC transplantation improved blood perfusion in the ischemic hind limb. (A,C) Representative images of hind limb blood flow measured by laser Doppler and quantitative analysis of blood flow expressed as perfusion ratio of the ischemic to the contralateral (non-operated) hind limb immediately after hind limb ischemia surgery and 3 weeks after intramuscular injection of normal saline, EPC-treated with high glucose (EPC-HG), or EPC-treated with high glucose and globular

adiponectin (EPC-HG+gAd). (B,D) Labeled EPCs were determined in ischemic tissues in mice received normal saline or transplanted EPCs. (n=6 in each group, *p<0.05 vs control & EPC-HG)

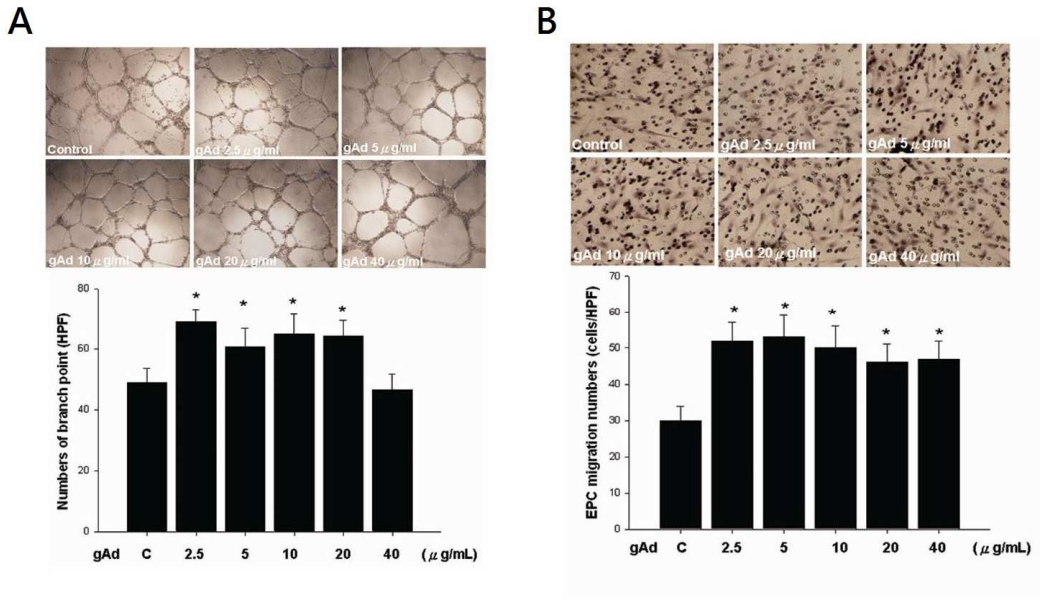


Fig. 1

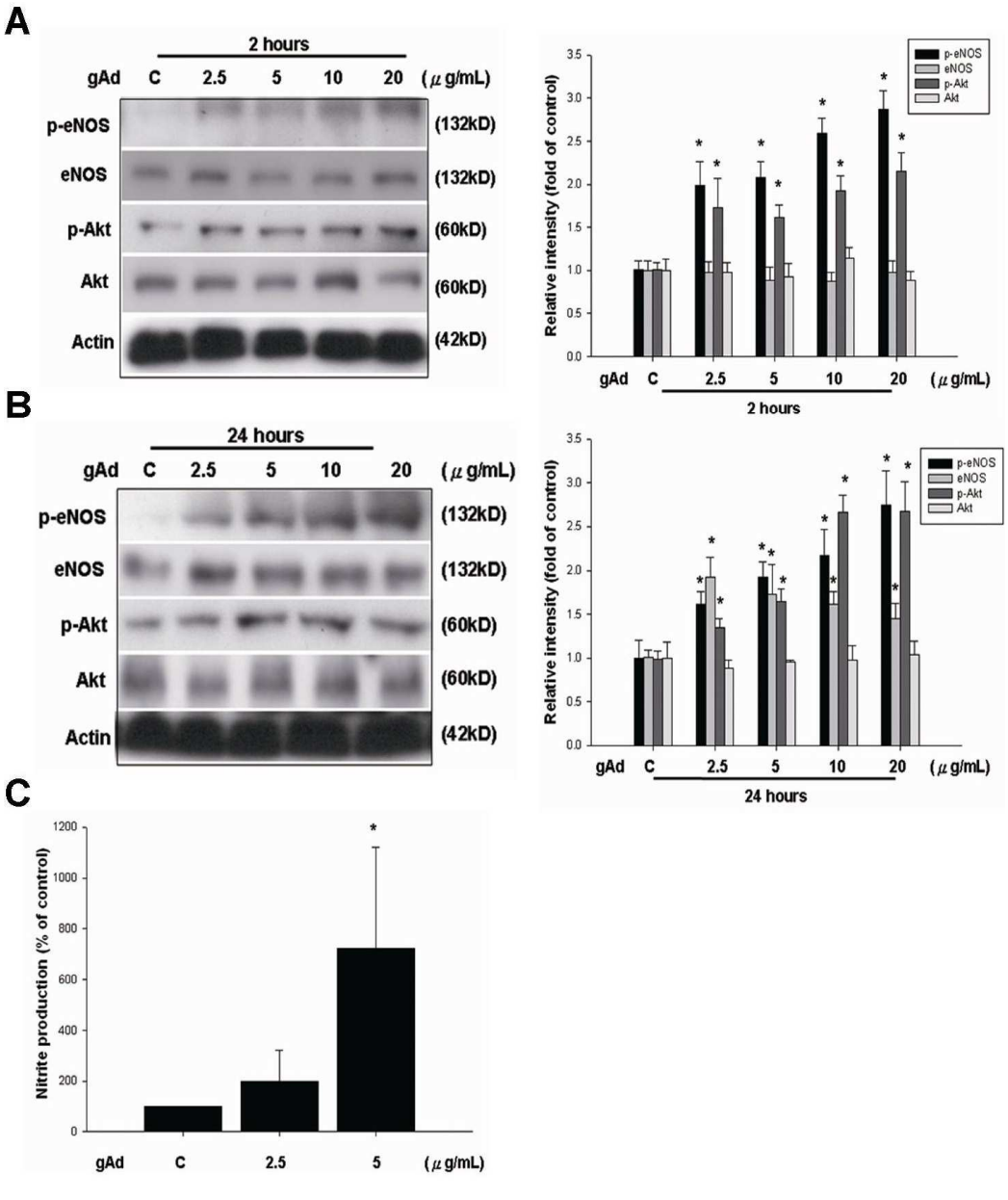


Fig. 2

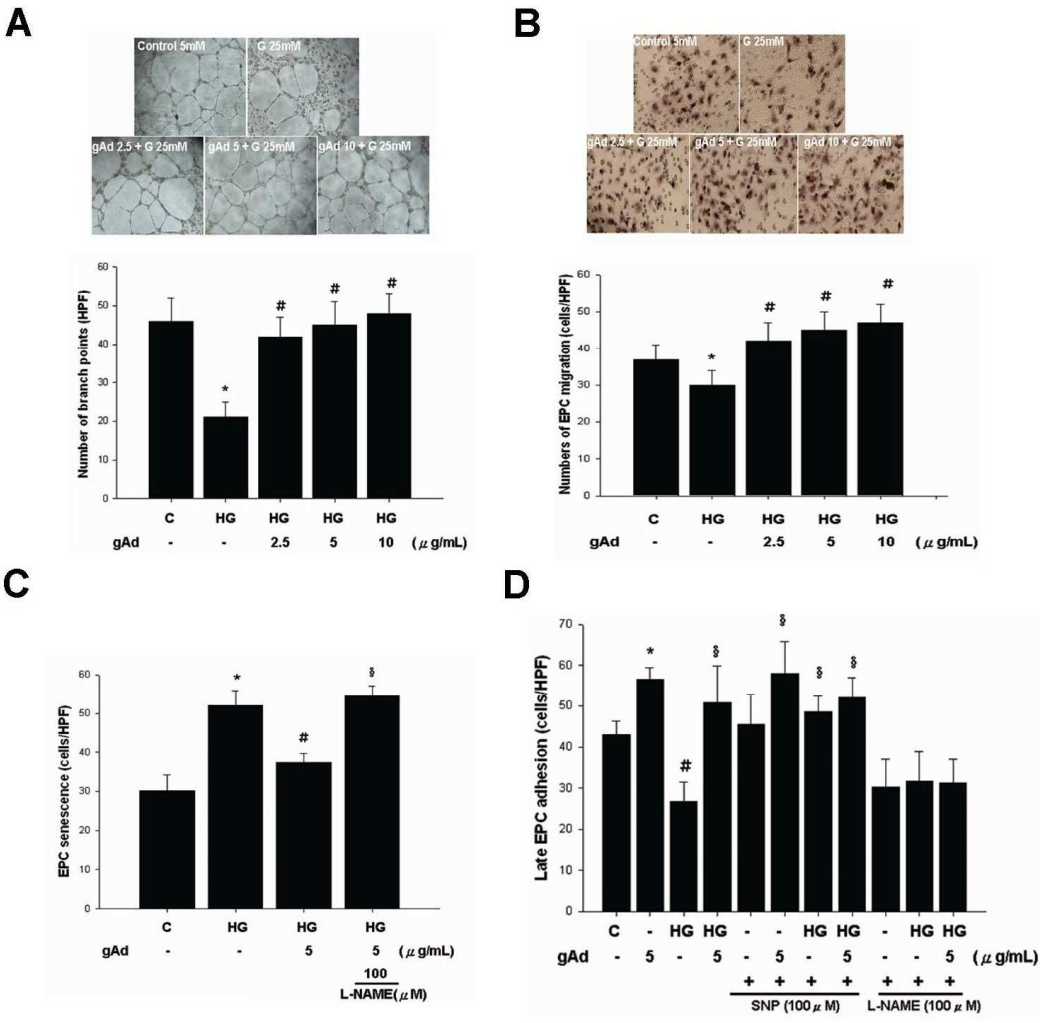
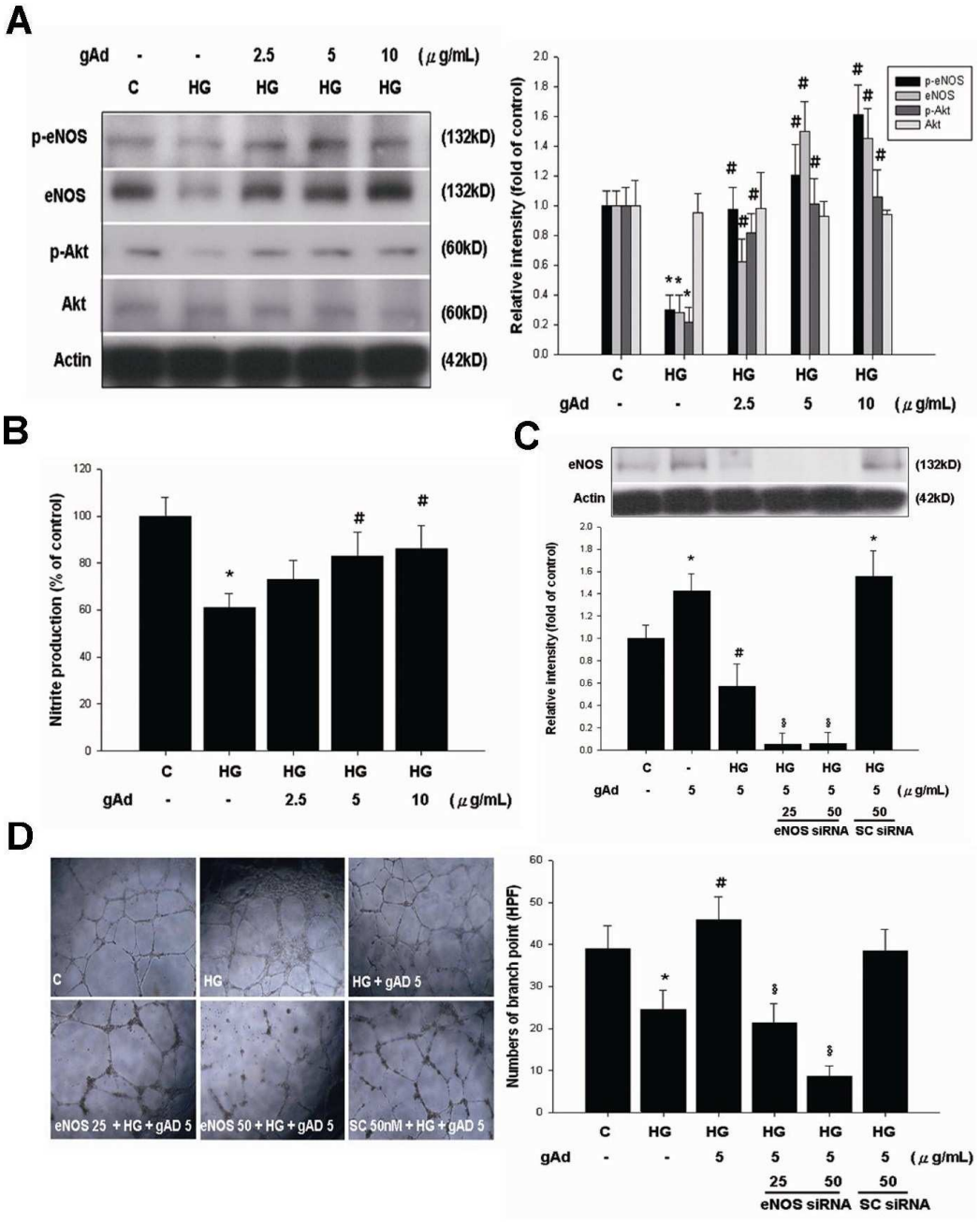


Fig. 3


A
gAd - - 2.5 5 10 (μg/mL)
C HG HG HG HG
p-eNOS (132kD)
eNOS (132kD)
p-Akt (60kD)
Akt (60kD)
Actin (42kD)
Relative intensity (fold of control)
p-eNOS
eNOS
p-Akt
Akt
B
Nitrite production (% of control)
C HG HG HG HG
gAd - - 2.5 5 10 (μg/mL)
C
eNOS (132kD)
Actin (42kD)
Relative intensity (fold of control)
C - HG HG HG HG
gAd - 5 5 5 5 5 (μg/mL)
25 50 50
eNOS siRNA SC siRNA
D
C
HG
HG + gAD 5
eNOS 25 + HG + gAD 5
eNOS 50 + HG + gAD 5
SC 50nM + HG + gAD 5
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25 50 50
eNOS siRNA SC siRNA

Fig. 4

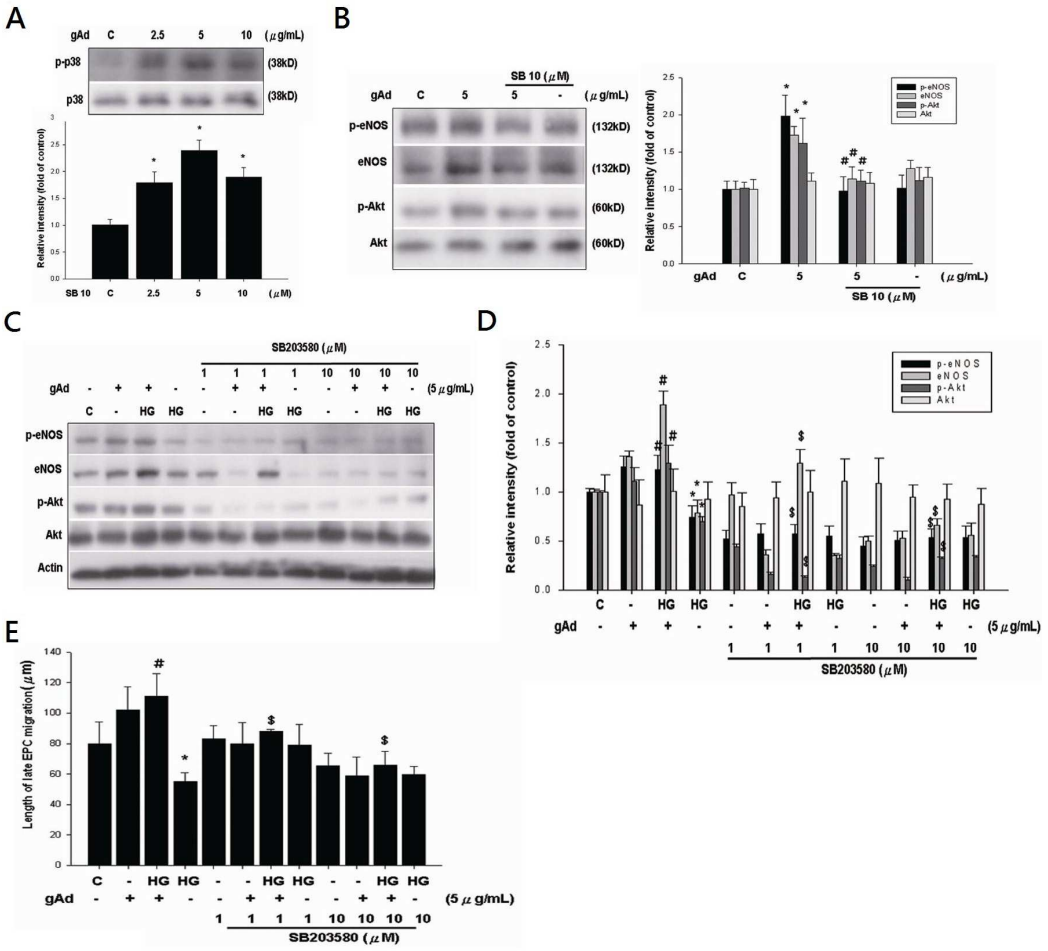
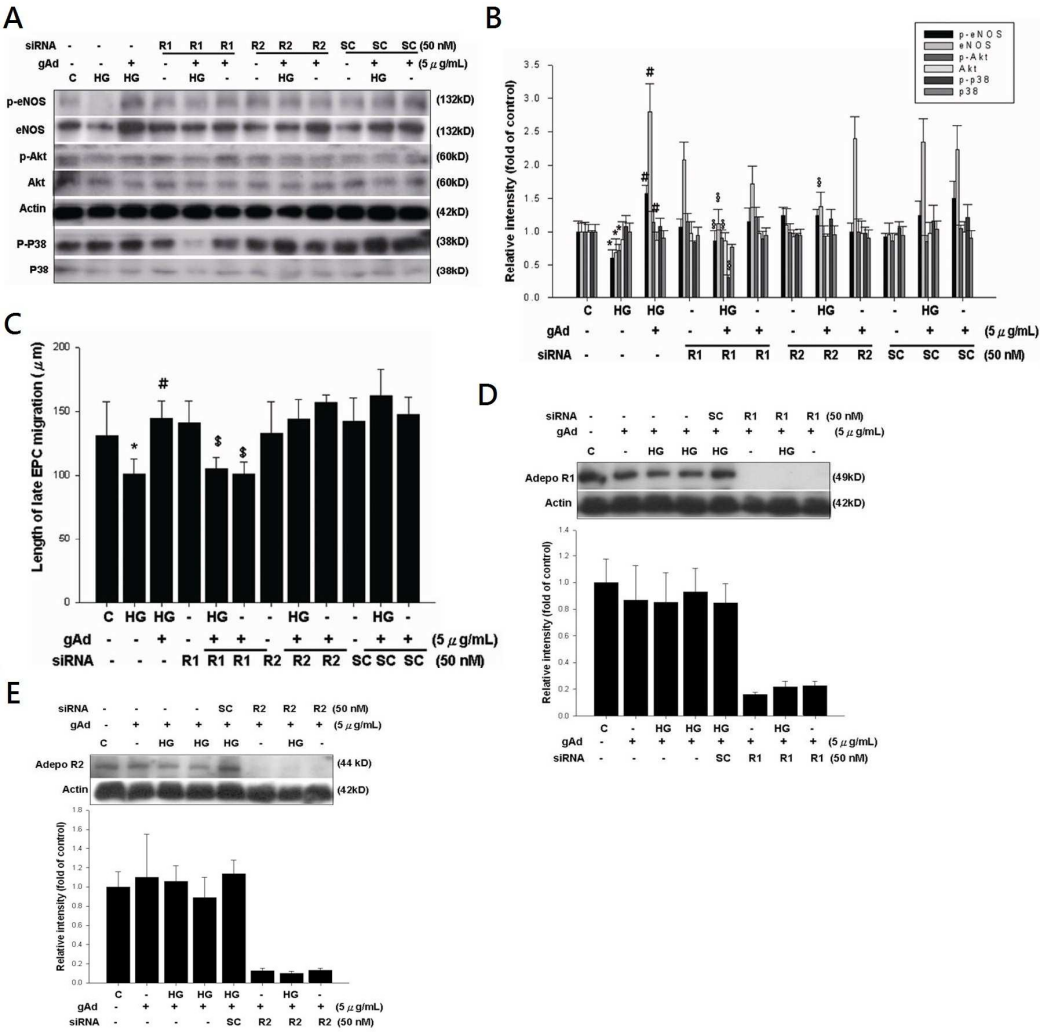


Fig. 5



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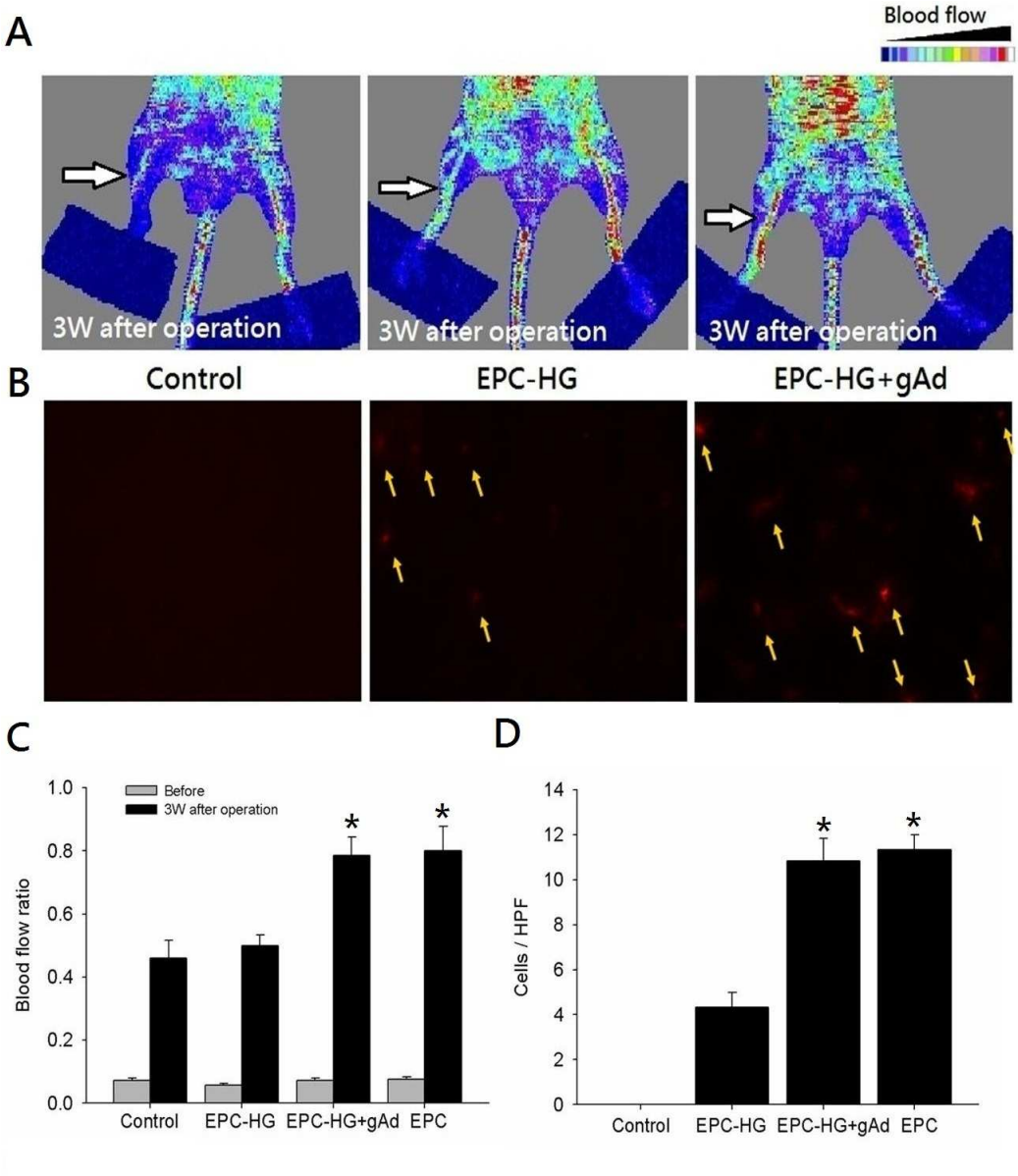

Fig. 7