

# Adiponectin Prevents Diabetic Premature Senescence of Endothelial Progenitor Cells and Promotes Endothelial Repair by Suppressing the p38 MAP Kinase/p16<sup>INK4A</sup> Signaling Pathway

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**OBJECTIVE**—A reduced number of circulating endothelial progenitor cells (EPCs) are casually associated with the cardiovascular complication of diabetes. Adiponectin exerts multiple protective effects against cardiovascular disease, independent of its insulin-sensitizing activity. The objective of this study was to investigate whether adiponectin plays a role in modulating the bioavailability of circulating EPCs and endothelial repair.

**RESEARCH DESIGN AND METHODS**—Adiponectin knockout mice were crossed with db<sup>+/-</sup> mice to produce db/db diabetic mice without adiponectin. Circulating number of EPCs were analyzed by flow cytometry. Reendothelialization was evaluated by staining with Evans blue after wire-induced carotid injury.

**RESULTS**—In adiponectin knockout mice, the number of circulating EPCs decreased in an age-dependent manner compared with the wild-type controls, and this difference was reversed by the chronic infusion of recombinant adiponectin. In db/db diabetic mice, the lack of adiponectin aggravated the hyperglycemia-induced decrease in circulating EPCs and also diminished the stimulatory effects of the PPAR $\gamma$  agonist rosiglitazone on EPC production and reendothelialization. In EPCs isolated from both human peripheral blood and mouse bone marrow, treatment with adiponectin prevented high glucose-induced premature senescence. At the molecular level, adiponectin decreased high glucose-induced accumulation of intracellular reactive oxygen species and consequently suppressed activation of p38 MAP kinase (MAPK) and expression of the senescence marker p16<sup>INK4A</sup>.

**CONCLUSIONS**—Adiponectin prevents EPC senescence by inhibiting the ROS/p38 MAPK/p16<sup>INK4A</sup> signaling cascade. The protective effects of adiponectin against diabetes vascular complications are attributed in part to its ability to counteract hyperglycemia-mediated decrease in the number of circulating EPCs. *Diabetes* 59:2949–2959, 2010

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Maintenance of an intact endothelial layer is essential for blood vessels to function properly and prevents the development of vascular disease such as atherosclerosis. Endothelial progenitor cells (EPCs), which were first discovered in 1997 as circulating immature cells in peripheral blood of humans (1), are now recognized as an important contributor to endothelial repair upon vascular damage (2). EPCs express the markers of both hematopoietic and endothelial lineages and reside mainly in the bone marrow. In response to stimuli such as tissue ischemia, EPCs can be mobilized into the bloodstream and then home or migrate toward the area of vascular damage, where they adhere, proliferate, and differentiate into mature endothelium, thereby leading to reendothelialization and neovascularization.

The number of circulating EPCs is considered to be a mirror of cardiovascular health. A reduced level of circulating EPCs is a cellular marker that independently predicts the outcome of vascular disease (3). In both type 1 and type 2 diabetic patients, the circulating number of EPCs is decreased compared with age- and sex-matched healthy subjects (4). In addition to diabetes, other major cardiovascular risk factors, including smoking, aging, hypertension, and dyslipidemia, have been associated with decreased number or dysfunction of circulating EPCs (5). On the other hand, therapeutic interventions capable of reducing cardiovascular risk factors, such as exercise, treatment with glucose- or lipid-lowering drugs, augment the number of EPCs and improve their functions in endothelial repair (6,7).

Adiponectin is an important adipocyte-secreted adipokine with insulin-sensitizing and antidiabetes properties (8). Unlike most proinflammatory adipokines/cytokines secreted by adipose tissue, the plasma concentrations of adiponectin are decreased in obese individuals and patients with type 2 diabetes, hypertension, and cardiovascular disease. Hypoadiponectinemia observed under these pathogenic conditions is attributed primarily to insulin resistance (9). On the other hand, the PPAR $\gamma$  agonists thiazolidinediones (TZDs) enhance adiponectin production in both animals and humans (10).

In addition to its metabolic functions, adiponectin exerts multiple protective effects against cardiovascular diseases, including alleviation of stroke (11), myocardial infarction (12), and diabetic cardiomyopathy (13). The endothelium is a major target of adiponectin, where the

adipokine promotes the production of nitric oxide (NO) by endothelial NO synthase (eNOS), depletes intracellular reactive oxygen species (ROS), and prevents inflammation and activation, consequently improving endothelial function and delaying atherosclerosis (14,15). Emerging evidence also demonstrates that adiponectin might be involved in regulating the functions of EPCs (16,17). A positive correlation between adiponectin and circulating EPCs has been observed in a cross-sectional study on Japanese (18). However, the precise roles of adiponectin in regulating EPCs under various pathophysiological conditions remain to be established.

The present study used adiponectin knockout (KO) mice to investigate the impact of adiponectin deficiency on the number of circulating EPCs during aging and under obese/diabetic conditions. The *in vivo* results demonstrate that lack of adiponectin aggravates the decrease in circulating EPCs under both circumstances. The *in vitro* study shows that adiponectin counteracts high glucose-induced senescence of EPCs isolated from both human peripheral blood and mouse bone marrow. Therefore, we further examined the signaling pathways that mediate the actions of adiponectin in both human and mouse EPCs.

## RESEARCH DESIGN AND METHODS

Male adiponectin KO mice (19) with a C57BL/6J background and their wild-type controls, leptin receptor<sup>-/-</sup> (*db/db*) mice and leptin receptor<sup>-/-</sup>/adiponectin<sup>-/-</sup> double knockout (DKO) mice (20) and their lean littermates were used for this study. The mice were housed in a room under controlled temperature (23 ± 1°C) and 12-h light-dark cycles, with free access to water and standard chow. All of the experiments were conducted under our institutional guidelines for the humane treatment of laboratory animals.

**Antibodies and recombinant proteins.** Rabbit polyclonal antibody against human p16<sup>INK4A</sup> and goat anti-mouse p16<sup>INK4A</sup> polyclonal antibody were purchased from Santa Cruz Biotechnology. Rabbit anti-total p38 MAP kinase, anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-total extracellular signal-regulated kinases 1 and 2 (ERK1/2), anti-phospho ERK1/2 (Thr 202/Tyr 204, cat no. 4370) antibodies were from Cell Signaling Technology. Phycoerythrin (PE)-conjugated monoclonal antibody against CD31, von Willebrand factor (vWF), vascular endothelial growth factor (VEGF) receptor 2 (KDR), and vascular endothelial (VE)-cadherin (CD144) were obtained from Beckman Coulter. PE-conjugated anti-human CD14, fluorescein isothiocyanate (FITC)-labeled anti-mouse fetal liver kinase (Flk)-1, and PE-conjugated anti-mouse c-Kit were manufactured by BD Biosciences. Mouse full-length adiponectin was expressed in HEK293 cells and purified from a serum-free conditioned medium previously described (20). The endotoxin level was <0.1 EU/μg protein as determined by limulus amoebocyte lysate (LAL) chromogenic end point assay (Hycutt Biotech).

**Isolation and cultivation of human early EPCs from peripheral blood.** Human early EPCs were isolated from healthy volunteers aged from 21 to 35 years as previously described (21). In brief, mononuclear cells were isolated from peripheral blood by Ficoll density gradient centrifugation and cultured on fibronectin-coated dishes in Medium 199 supplemented with 20% FBS and VEGF. Four days after the growth, nonadherent cells were removed and were grown for another 3 days. The immunophenotypic characterization was conducted by fluorescence-activated cell sorter (FACS) analysis using the antibodies against a panel of endothelial and monocytic markers, including KDR, VE-cadherin, vWF, and CD14.

**Quantification of circulating EPC number by flow cytometry.** Total mononuclear cells were isolated from mouse peripheral blood as described above. After washing twice with Dulbecco's phosphate-buffered saline (DPBS) plus 2 mmol/l EDTA, cells were resuspended in DPBS/1% FBS/0.1% Na<sub>3</sub> and were then double stained with PE-conjugated anti-mouse c-Kit and FITC-conjugated anti-Flk-1 monoclonal antibodies or PE-conjugated rat anti-mouse CD31 and FITC-conjugated rat anti-mouse CXCR4 monoclonal antibodies at 4°C for 1 h in dark. Isotype-identical antibodies were used as negative controls. After staining, cells were washed twice with PBS and resuspended in DPBS/1% FBS/0.1% Na<sub>3</sub> for analysis on the flow cytometer (FC 500 with CXP software; Beckman Coulter). Cell viability was determined by staining with 7-AAD (Invitrogen). 50,000 viable cells were analyzed in each sample. Circulating EPCs and circulating angiogenic cells were defined as cells coexpressing c-Kit and Flk-1 or CD31 and CXCR4, respectively (22).

**Surgical procedures for wire-induced carotid denudation.** Mice were anesthetized, and the bifurcation of the right carotid artery was exposed via midline incision of the ventral side of the neck under a dissection microscope. Two ligatures were placed proximally and distally around the external carotid artery. After temporary occlusion of the internal, common, and external carotid artery, a transverse arteriotomy was performed on internal carotid artery and an angioplasty wire (0.39 mm) was introduced between the ligatures of the external carotid artery up to 4 mm from the bifurcation. The wire was passed along the common carotid artery four times under rotation. After removal of the wire, the proximal ligature was tied off proximal and distal to the incision hole of the external carotid artery. Normal blood flow was restored, and the skin was closed with single sutures using 5/0 silk. Mice were recovered on heated pads for the first 8 hours.

**Reendothelialization assay.** Four days after carotid injury, mice were infused with 60 μl of 5% Evans blue dissolved in saline via tail vein injection. Ten minutes after injection, mice were killed with an overdose of Hypnorm and Dormicum. The anesthetized mice were then perfused with saline to wash out whole blood and the excess dye and the stain was fixed via perfusion with 4% paraformaldehyde. Ten minutes after fixation, carotid arteries were dissected out and opened longitudinally for en face histological assessments. Regions of damaged endothelium incorporate the stain and appear blue, whereas areas with reendothelialization are resistant to the dye. Both stained and unstained areas were outlined and quantified with the NIH Image J software.

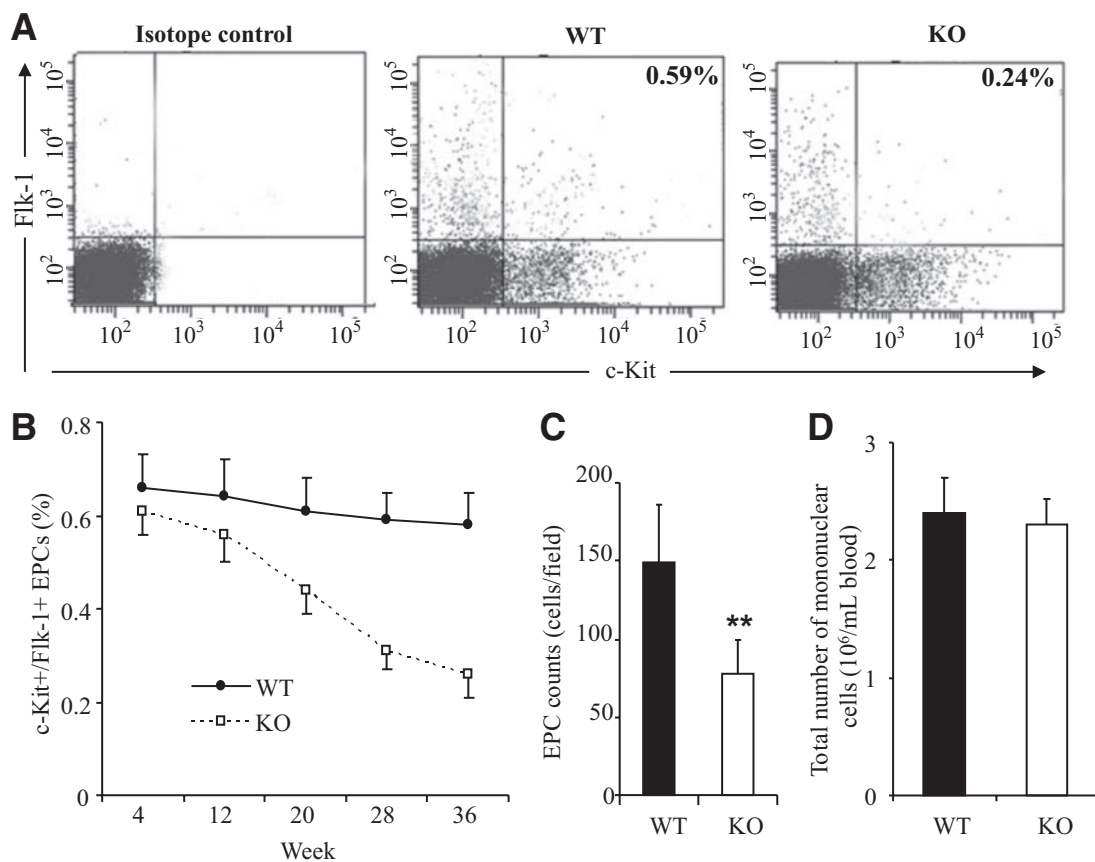
**Isolation and cultivation of bone marrow-derived EPCs from mice.** Lin<sup>-</sup>c-Kit<sup>+</sup>Flk-1<sup>+</sup> bone marrow cells were isolated using the magnetic sorting method as described in the supplemental data, available in an online appendix (<http://diabetes.diabetesjournals.org/cgi/content/full/db10-0582/DC1>). The isolated cells were seeded (2 × 10<sup>5</sup> cells/well) on 8-well glass chamber slides precoated with 20 μg/ml fibronectin (Sigma-Aldrich) in M199 medium supplemented with 20% FBS. Over 90% of the Lin<sup>-</sup>c-Kit<sup>+</sup>Flk-1<sup>+</sup> cells expressed CD31, c-kit and Flk-1 and were positively stained for Dil-ac-LDL (molecular probes) and FITC-labeled lectin (sigma). These bone marrow-derived EPCs were used for further functional characterization.

**Isolation of bone marrow-derived EPCs from mice using a magnetic cell sorting method.** Total mononuclear cells were isolated from mouse bone marrow using Histopaque-1083 (Sigma) density gradient centrifugation, and mature hepatopoietic cells were depleted using Mouse Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's instruction. The lineage negative cells were subjected to separation using CD117 (c-Kit) microbeads. The resulting Lin<sup>-</sup>c-Kit<sup>+</sup> cells were stained with FITC-conjugated primary antibody against Flk-1, followed by magnetic labeling with an anti-FITC MultiSort kit and separation using a mini-MACS column.

**Quantification of the EPCs number in peripheral blood by culture assay.** Total mononuclear cells were isolated from 800 μl peripheral blood of mice using Histopaque-1083 (Sigma) density gradient centrifugation and cultured in endothelial growth medium-2 (Clonetics) medium including antibiotics, growth factors, and 10% FBS. To determine the number of EPCs, cells were grown on 4-well glass chamber slides coated with rat plasma vitronectin (Sigma) in 0.5% gelatin (Sigma). After 4 days of culture, nonadherent cells were removed by washing with DPBS (Sigma) thoroughly and attached EPCs were characterized by Dil-acLDL (Invitrogen) and FITC-Bandeiraea simplicifolia-1 lectin (Sigma) double staining. Double-positive cells were counted in ten randomly selected high-power fields of each cultured slide under a fluorescent microscope (×100).

**Quantification of p16<sup>INK4A</sup> mRNA expression by quantitative PCR analysis.** Total RNA was isolated from human early EPCs using TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min to remove genomic DNA. For reverse transcription, 1 μg total RNA was converted into first-strand complementary DNA in 20-μl reactions using the ImProm-II Reverse Transcriptase kit (Promega). The mRNA abundance of p16<sup>INK4A</sup> was determined by quantitative real-time PCR in duplicate in a total reaction volume of 20 μl with SYBR Green PCR Master Mix on an ABI Prism 7000 instrument (Applied Biosystems), using the following primers: 5'-GAC CTG GCT GAG GAG CTG-3' (forward) and 5'-TTC AAT CGG GGA TGT CTG A-3' (reverse). The PCR conditions were set as follows: 50°C for 2 min and 95°C for 10 min, followed by 35 two-step cycles at 95°C for 15 s and 60°C for 1 min. β-actin (forward primer, 5' TGA CCC AGA TCA TGT TTG AGA 3', and reverse primer, 5' AGT CCA TCA CGA TGC CAG T 3') was amplified at the same plate for each sample for normalization purpose. Analysis was performed with ABI Prism 7000 SDS software.

**Measurement of intracellular cAMP levels.** The effects of adiponectin on intracellular cAMP levels in human early EPCs were measured with the cAMP Biotrak Enzymeimmunoassay system using the protocol recommended by the manufacturer (GE Healthcare). In brief, cells grown in 24-well dishes for 48 h were dissolved with the lysis reagents provided in the kit to release intracellular cAMP. The cell lysates were transferred immediately into a 96-well



**FIG. 1. Circulating EPCs are progressively decreased in adiponectin KO mice.** *A*: Representative FACS analysis of circulating EPCs from 25-week-old adiponectin KO and wild-type (WT) controls double stained for c-Kit (*x*-axis) and Flk-1 (*y*-axis). *B*: Comparison of the percentage of c-Kit/Flk-1 double-positive EPCs in peripheral blood between adiponectin KO mice and wild-type controls at different ages (in weeks). Data are expressed as % of total mononuclear cells in blood. *C*: Quantification of circulating EPCs by culture assay. *D*: Quantification of total mononuclear cells in adiponectin KO mice and wild-type controls via a hemocytometer. \*\**P* < 0.01 vs. wild-type controls (*n* = 5–7).

microplate precoated with rabbit anti cAMP for the subsequent assay. The protein concentration was determined by a bicinchoninic acid assay (Pierce). The intracellular cAMP concentration was expressed as picomoles per microgram of cellular protein.

**Quantification of angiogenic growth factors secreted from human early EPCs.** Four days after isolation of early EPCs, cells were grown in a growth factor-free basal M199 medium supplemented with 5% FCS for 48 h. Conditioned media were analyzed to determine the concentrations of VEGF, granulocyte-macrophage colony stimulating factor, and hepatocyte growth factor using the immunoassay kits from R&D systems.

**Measurement of intracellular ROS.** The fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA) was used to determine the intracellular generation of ROS in human EPCs. EPCs in 24-well plates were grown in 5 or 25 mmol/l glucose for 72 h under various treatments. Thereafter, the cells were incubated with 5 μmol/l CM-H<sub>2</sub>DCFDA for 30 min at 37°C. After removal of the media and washing of the cells, the fluorescence intensity (relative fluorescence units) was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively, using a spectrofluorometer (Hitachi).

**Senescence-associated β-galactosidase activity assay.** Human early EPCs or mouse bone marrow-derived EPCs were isolated and cultured as described above. The cells were incubated with recombinant adiponectin or 25 mmol/l glucose for 72 h. The cellular senescence was detected using a Senescent Cells Staining Kit (Sigma). Blue-stained cells and total cells were counted respectively and the percentage of β-galactosidase-positive cells was calculated.

**Western blot analysis.** Cellular proteins from EPCs under various treatments were resolved by SDS-PAGE and probed with different primary and secondary antibodies as specified in the figure legends. The proteins were visualized by the chemiluminescence detection. The relative band densities were quantified using the MultiAnalyst software package (Bio-Rad).

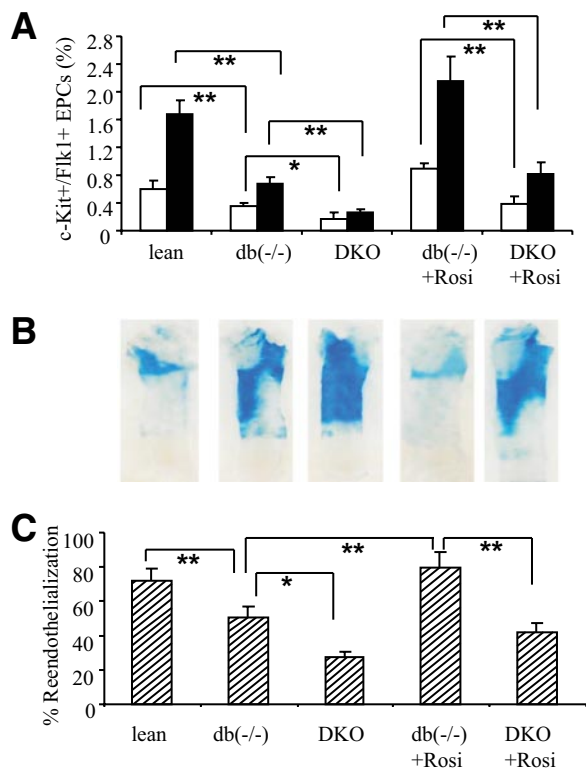
**Statistical analysis.** Data are expressed as means ± SD. Statistical significance was determined by one-way ANOVA or Student's *t* test. *P* values < 0.05 were accepted as indicative of statistically significant differences.

## RESULTS

**Circulating numbers of EPCs are modulated by adiponectin in mice.** Flow cytometry analysis showed that the number of circulating c-Kit<sup>+</sup>Flk-1<sup>+</sup> EPCs in adiponectin KO mice progressively decreased in an age-dependent manner (Fig. 1*A* and *B*). At 28 weeks old, adiponectin KO mice had a circulating number of EPCs ~50% lower than that in wild-type controls. A similar result was also observed when the EPC number was determined by the culture assay (Fig. 1*C*). On the other hand, there was no obvious difference in the number of total circulating mononuclear cells between the two groups (Fig. 1*D*).

We next investigated the effects of adiponectin supplementation on the number of circulating c-Kit<sup>+</sup>Flk-1<sup>+</sup> EPCs in mice. To this end, recombinant adiponectin produced from mammalian cells was delivered into both adiponectin KO mice and wild-type littermates using Alzet osmotic pumps as previously described (23). Serum levels of adiponectin started to increase at the second day of the treatment in both types of mice (supplemental Fig. 1*A* in the online appendix). Noticeably, the increase in serum adiponectin was accompanied by significant elevation of the number of circulating EPCs in both wild-type and adiponectin KO mice (supplemental Fig. 1*B*).

**Rosiglitazone-mediated increase in circulating EPCs and improvement in reendothelialization are dependent on adiponectin.** To further explore the role of adiponectin in modulating the circulating number of EPCs



**FIG. 2.** An indispensable role of adiponectin in rosiglitazone (Rosi)-induced increase in circulating EPCs and reendothelialization in  $db^{-/-}$  mice. Eight-week-old male  $db^{-/-}$  mice or DKO mice were treated with rosiglitazone or vehicle control for 4 weeks, followed by wire-induced carotid denudation. **A:** The circulating number of EPCs in lean mice,  $db^{-/-}$  mice, and DKO mice treated with rosiglitazone or saline were measured at days 0 (□) and 4 (■) after carotid injury, respectively. **B:** Representative *in situ* Evans blue staining of carotid arteries 4 days after denudation. Note that reendothelialization regions are resistant to the dye staining and appear white. **C:** Quantification of reendothelialization in Evans blue-stained carotid arteries 4 days after surgical operation. \* $P < 0.05$ ; \*\* $P < 0.01$  ( $n = 5-6$  in each group). (A high-quality digital representation of this figure is available in the online issue.)

under the pathological conditions, adiponectin KO mice were crossed with  $db^{-/-}$  mice (a genetically inherited obese/diabetic mouse model due to the lack of functional leptin receptors) to generate  $db^{-/-}$ /adiponectin $^{-/-}$  DKO mice (20). As expected,  $db^{-/-}$  mice displayed typical diabetic phenotypes, including hyperglycemia, hyperinsulinemia, and dyslipidemia (supplemental table 1). Compared with the lean controls, the number of circulating EPCs in  $db^{-/-}$  mice was significantly reduced at both baseline and 4 days after carotid injury (Fig. 2A). Noticeably, the number of circulating EPCs in DKO mice were further decreased by 50.2% at baseline and 61.4% at 4 days after carotid injury, respectively, compared with  $db^{-/-}$  obese/diabetic mice, despite the fact that DKO mice and  $db^{-/-}$  mice had comparable blood levels of glucose, insulin, and body weight gains.

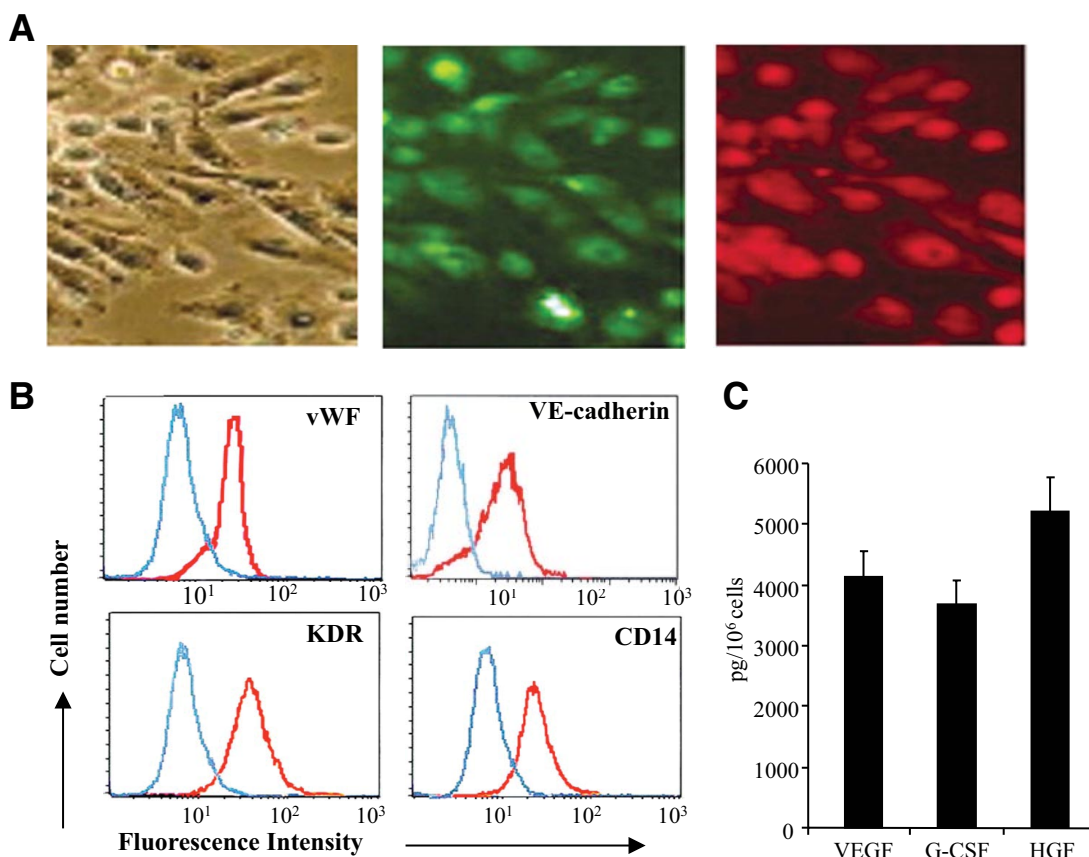
Administration of the PPAR $\gamma$  agonist rosiglitazone (20 mg  $\cdot$  kg $^{-1}$  body wt  $\cdot$  day $^{-1}$ ) to  $db^{-/-}$  obese/diabetic mice for a period of 4 weeks increased serum levels of adiponectin by approximately twofold. This change in serum adiponectin was accompanied by a significant elevation in the number of circulating EPCs at both baseline and 4 days after carotid injury (Fig. 2A). On the other hand, the magnitude of rosiglitazone-mediated increase in circulating EPCs in DKO mice was significantly smaller than that

observed in  $db^{-/-}$  mice ( $1.64 \pm 0.22$  vs.  $2.53 \pm 0.31$  fold;  $P < 0.01$ ;  $n = 6$ ), although the effects of this drug on decreasing hyperglycemia and hyperinsulinemia were comparable between  $db^{-/-}$  mice and DKO mice (supplemental table 1).

In line with the changes in the number of circulating EPCs,  $db^{-/-}$  mice exhibited an impaired reendothelialization compared with their lean littermates, and this impairment was further aggravated in DKO mice (Fig. 2B and C). Treatment with rosiglitazone completely reversed the impairment in reendothelialization in  $db^{-/-}$  mice, whereas this effect of rosiglitazone was largely abolished in DKO mice. **Adiponectin counteracts hyperglycemia-induced induction of cellular senescence.** To further study the underlying mechanism whereby adiponectin increases circulating EPCs, we evaluated the direct effect of recombinant adiponectin on human EPCs isolated from peripheral blood of healthy subjects. The cells exhibited a typical spindle-shaped, endothelial cell-like morphology (Fig. 3A). Over 90% of adherent cells were double positive for uptake of acetylated LDL and binding to lectin Ulex europaeus agglutinin-1 (UEA-1), a characteristic feature of EPCs. Further FACS analysis showed that >75% of adherent cells expressed endothelial cell-specific markers, including KDR ( $81.3 \pm 5.6\%$ ), vWF ( $84.1 \pm 6.7\%$ ), and VE-cadherin ( $76.2 \pm 7.9\%$ ) (Fig. 3B). In addition, positive immunostaining for the monocytic marker CD14 was identified in  $89.6 \pm 3.2\%$  of the cells. However, these cells did not proliferate (as determined by both BrdU incorporation assay and direct cell counting) and did not have the capacity for tube formation (data not shown). Consistent with previous findings (24,25), the cells secreted high levels of several angiogenic growth factors, including VEGF, granulocyte-macrophage colony stimulating factor, and hepatocyte growth factor (Fig. 3C). Taken together, these cells showed the characteristic features of early EPCs (24), which are also defined as circulating monocyte-related angiogenic cells (25), or endothelial cell colony-forming units (26).

Exposure of human early EPCs to high glucose (25 mmol/l) for 72 h had no obvious effect on apoptosis (data not shown) but led to a marked induction of cellular senescence, as determined by staining for senescence-associated  $\beta$ -galactosidase (Fig. 4A and B). Treatment of cells with recombinant adiponectin reversed high glucose-induced cellular senescence in a dose-dependent manner. However, adiponectin had no obvious effect on senescence when human early EPCs were cultured in the presence of a normal glucose concentration.

**Adiponectin suppresses hyperglycemia-induced activation of p38 MAP kinase and expression of p16<sup>INK4A</sup> in human early EPCs.** p38 MAP kinase (MAPK) plays a key role in downregulating EPCs by hyperglycemia in diabetic patients (27). This kinase has been shown to inhibit EPCs proliferation and to promote EPCs senescence through inducing the expression of the senescence-associated cyclin-dependent kinase inhibitor p16<sup>INK4A</sup> (28,29). We next tested the effect of adiponectin on the p38 MAPK/p16<sup>INK4A</sup> pathway in human EPCs. Incubation of EPCs with high glucose significantly increased the phosphorylation of both p38 MAPK and ERK1/2 and also caused a significant elevation of p16<sup>INK4A</sup> mRNA expression compared with control cells (Fig. 5A-C). The expression of p16<sup>INK4A</sup> protein was barely detectable when cultured under normal glucose conditions but was mark-



**FIG. 3.** Characterization of EPCs isolated from human peripheral blood. **A:** Mononuclear cells were grown on a fibronectin-coated culture dish for 7 days and were then visualized under a light microscope (*left panel*) or stained for uptake of acetylated LDL (*middle panel*) or for fluorescein isothiocyanate UEA-1 (lectin) binding (*right panel*). **B:** Representative FACS analysis for the endothelial markers KDR, VE-cadherin, and vWF and the monocytic lineage marker CD14 (shown in red). Isotope controls are shown in blue. **C:** Secretion of angiogenic growth factors by EPCs in growth factor-free medium over 48 h. (A high-quality digital representation of this figure is available in the online issue.)

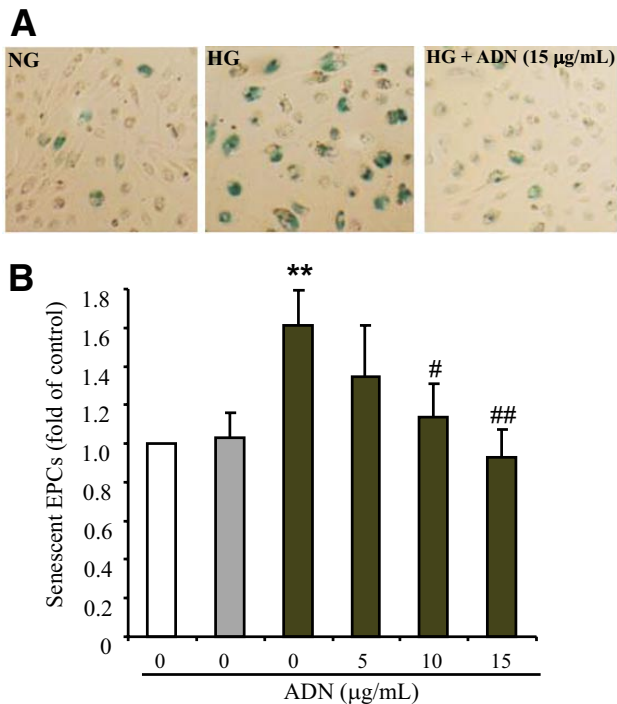
edly induced following prolonged exposure to high glucose (Fig. 5D). Treatment of human early EPCs with adiponectin significantly reduced high glucose-induced increase in phosphorylation of both p38 MAPK and ERK1/2 and prevented the high glucose-induced mRNA and protein expression of p16<sup>INK4A</sup> but did not affect those parameters significantly under normal glucose conditions.

Incubation of cells with the p38 MAPK selective inhibitor SB203508, but not the ERK1/2 MAPK selective inhibitor PD98059, abrogated high glucose-induced expression of p16<sup>INK4A</sup> as well as cellular senescence (supplemental Fig. 2). The combined treatment of cells with SB203508 and adiponectin did not result in additive effects on the inhibition of high glucose-induced p16<sup>INK4A</sup> expression and cellular senescence. Adiponectin treatment also suppressed high glucose-induced phosphorylation of c-Jun NH<sub>2</sub>-terminal kinases (JNKs). However, incubation of cells with the synthetic inhibitor of JNK (SP600125) had no effect on high glucose-induced p16<sup>INK4A</sup> expression or senescence of EPCs (data not shown).

**Adiponectin decreases high glucose-induced ROS accumulation in EPCs through a mechanism dependent on both AMP-activated protein kinase and cAMP.** Intracellular accumulation of ROS is a major risk factor for inducing premature senescence of hematopoietic stem cells through activation of p38 MAPK pathway (30). Hyperglycemia can increase ROS production in many cell lines, including EPCs (21). We next investigated whether adiponectin counteracts high glucose-induced cellular se-

nescence through suppressing ROS production. Chronic exposure of EPCs to high glucose significantly increased intracellular dichlorofluorescein (DCF)-sensitive ROS levels compared with cells grown in normal glucose or in osmotic control medium (Fig. 6). Treatment of the cells with recombinant adiponectin reversed the effects of hyperglycemia on intracellular ROS accumulation in a dose-dependent manner. The suppressive effect of adiponectin on high glucose-induced ROS accumulation was partially attenuated by treatment with compound C (a selective inhibitor of AMP-activated protein kinase) or by H-89 (a cell-permeable inhibitor of protein kinase A) and was abolished by combination of the two pharmacological inhibitors. Noticeably, compound C exerted a much greater inhibitory effect than H-89 on adiponectin-induced decrease in intracellular ROS accumulation.

In line with the changes in intracellular ROS accumulation, the inhibitory effects of adiponectin on high glucose-induced phosphorylation of p38 MAPK was reduced by either compound C or H-89 and was abrogated by combined treatment with the two inhibitors (Fig. 7A). When the cells were treated with compound C and H-89 alone, the inhibitory effect of adiponectin on high glucose-induced phosphorylation of p38 MAPK was attenuated by ~57 and 26%, respectively. Incubation of human early EPCs with compound C, but not with H-89, significantly attenuated the suppressive effects of adiponectin on high glucose-induced p16<sup>INK4A</sup> expression and cellular senescence (Fig. 7B and C). Furthermore, combined treatment



**FIG. 4.** Adiponectin prevents high glucose-induced cellular senescence of human EPCs. Cells isolated as in Fig. 3 were grown in normal glucose (NG) (open bar), normal glucose plus 20 mmol/l mannitol as an osmotic control (gray bar), or high glucose (HG) (black bar) in the absence or presence of different concentrations of recombinant adiponectin (ADN) for 72 h. EPC senescence was evaluated by senescence-associated  $\beta$ -galactosidase staining. **A:** Representative photographs of senescence-associated  $\beta$ -galactosidase-stained blue EPCs under normal glucose, high glucose, and high glucose plus 15  $\mu$ g/ml adiponectin. **B:** The degree of cell senescence was quantified as the percentage of senescence-associated  $\beta$ -galactosidase-positive cells and expressed as fold of normal glucose control. \*\* $P < 0.01$  vs. normal-glucose group. # $P < 0.05$ , ## $P < 0.01$  vs. the high-glucose group without adiponectin treatment ( $n = 5-6$ ). (A high-quality digital representation of this figure is available in the online issue.)

with compound C plus H-89 did not cause further attenuation in adiponectin-mediated suppression of p16<sup>INK4A</sup> expression or cellular senescence when compared with that treated with compound C alone.

Real-time PCR analysis showed that amp-activated protein kinase (AMPK)  $\alpha 1$ , but not AMPK  $\alpha 2$ , was the predominant isoform expressed in human early EPCs (data not shown). The inhibitory effect of adiponectin on high glucose-induced ROS production was reduced by ~51 and 24% by knocking down of AMPK  $\alpha 1$  and the catalytic subunit of protein kinase A (PKA)- $\alpha$  respectively, and by ~68% by simultaneous knockdown of these two kinases (supplemental Fig. 3). Knockdown of AMPK  $\alpha 1$  significantly attenuated adiponectin-mediated suppression of high glucose-induced cellular senescence of EPCs, whereas knockdown of PKA alone had little effect.

Treatment of cells with the antioxidant N-acetylcysteine (a thiol-containing radical scavenger) prevented high glucose-induced phosphorylation of p38 MAPK, p16<sup>INK4A</sup> expression, and cellular senescence (Fig. 7A-C).

**Adiponectin suppresses hyperglycemia-induced p16<sup>INK4A</sup> expression and senescence of EPCs by decreasing ROS accumulation in diabetic mice.** Consistent with the findings in human early EPCs, mouse EPCs isolated from bone marrow of *db*<sup>-/-</sup> diabetic mice exhibited a significant elevation in intracellular ROS accumulation, p38 phosphorylation, and p16<sup>INK4A</sup> ex-

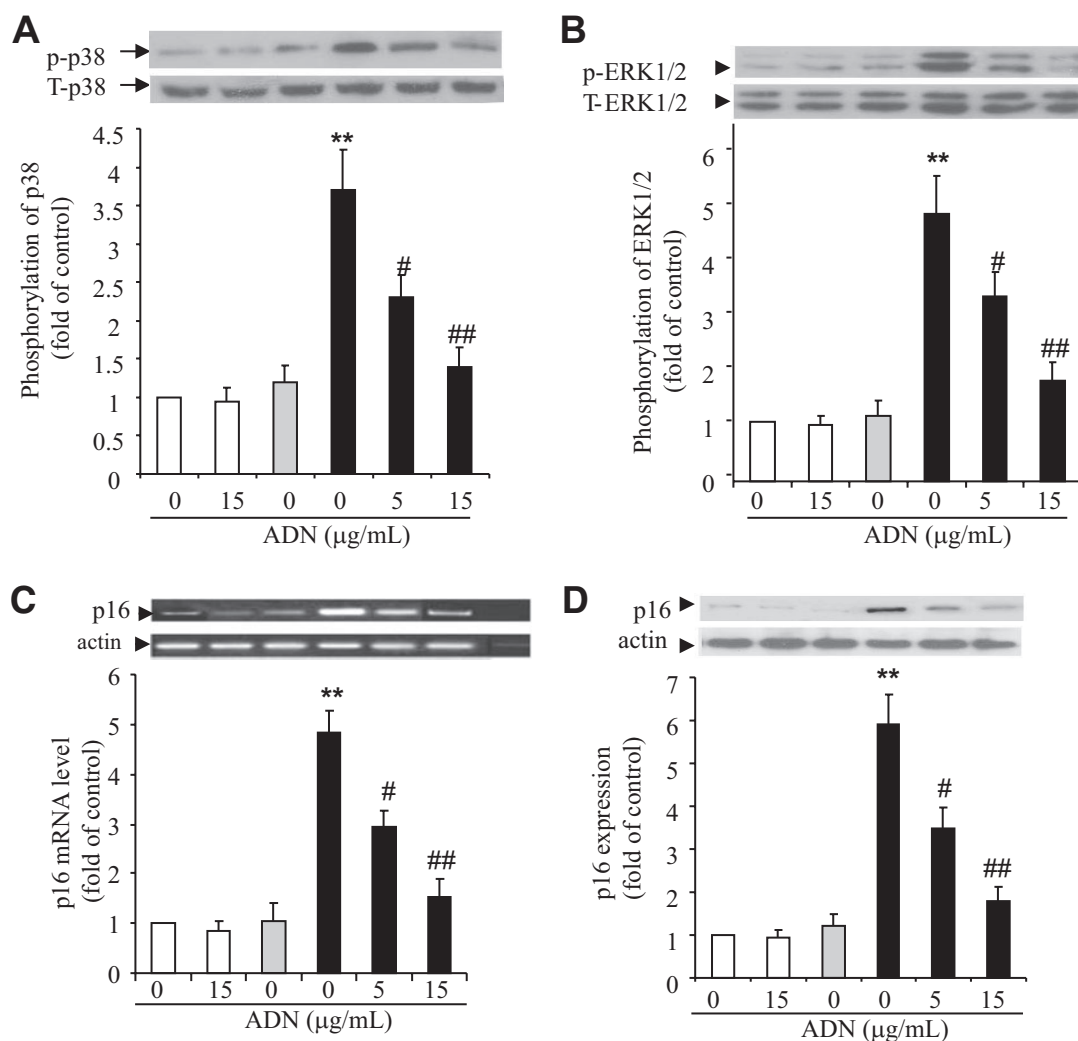
pression compared with those from the lean littermates (Fig. 8A-C), and these changes were completely reversed by treatment of *db*<sup>-/-</sup> diabetic mice with rosiglitazone for a period of 4 weeks. The intracellular ROS accumulation, p38 phosphorylation, and p16<sup>INK4A</sup> expression in bone marrow-derived EPCs from DKO mice were significantly higher than those from *db*<sup>-/-</sup> mice. Furthermore, the ability of rosiglitazone to reverse these diabetes-induced changes were significantly compromised, suggesting that rosiglitazone-mediated inhibition of ROS production, p38 phosphorylation, and p16<sup>INK4A</sup> expression is mediated at least in part by adiponectin. Bone marrow EPCs isolated from DKO mice were more susceptible to high glucose-induced senescence compared with those from *db*<sup>-/-</sup> mice (Fig. 8D and E). On the other hand, recombinant adiponectin was able to inhibit high glucose-induced senescence of EPCs isolated from DKO mice to a level comparable with that observed in *db*<sup>-/-</sup> mice, indicating that EPCs originated from DKO mice are hypersensitive to adiponectin.

In DKO mice, the expression of p16<sup>INK4A</sup> in bone marrow-derived EPCs was elevated by 2.6 folds from the age of 8-12 weeks, and this change was accompanied by ~52% decline in circulating EPCs (supplemental Fig. 4). Treatment of DKO mice with either the antioxidant NAC or recombinant adiponectin for 4 weeks prevented the aging-associated elevation of p16<sup>INK4A</sup> expression and stopped the decline in circulating EPCs in DKO mice.

## DISCUSSION

The present study demonstrates that adiponectin protects against both aging- and diabetes-induced decrease in circulating EPCs in mice. This finding corroborates a recent clinical observation showing a positive association between the plasma levels of adiponectin and the number of EPCs in patients with coronary heart disease (18), suggesting that the vasculoprotective effects of adiponectin are attributed in part to its ability to increase the availability of circulating EPCs.

Another novel observation of this study is that the beneficial effect of the PPAR $\gamma$  agonist rosiglitazone (a thiazolidinedione class of antidiabetic drug) in elevating the number of circulating EPCs is diminished in *db/db* diabetic mice without adiponectin. In addition to their well-established efficacy in improving insulin sensitivity and glycemic control, thiazolidinediones have potent protective effects against the vascular complications of diabetes by reducing both classical and nonclassical risk factors for cardiovascular disease (31). A growing body of evidence shows that treatment with rosiglitazone or pioglitazone increases the circulating number of EPCs and normalizes their impaired endothelial repair capacity in patients with type 2 diabetes or coronary heart disease (6,7,21). Noticeably, the elevation of circulating EPCs by the PPAR $\gamma$  agonists is accompanied by an increased plasma level of adiponectin in these patients. The PPAR $\gamma$  agonists have been shown to induce adiponectin production in adipocytes through transcriptional activation as well as by enhancing its secretion (32). Taken in conjunction, these findings suggest that the beneficial effects of PPAR $\gamma$  agonists in increasing the number and improving the functions of circulating EPCs are mediated in part by their ability to induce adiponectin production from adipocytes.

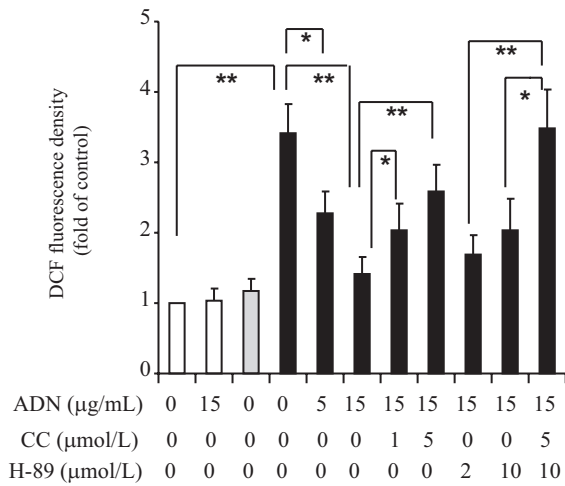


**FIG. 5. Adiponectin suppresses high glucose–induced phosphorylation of p38 MAPK (A) and ERK1/2 (B), and mRNA and protein expression of p16<sup>INK4A</sup> (C and D).** Human early EPCs isolated from peripheral blood were grown in normal glucose (5 mmol/l) (open bar), normal glucose plus 20 mmol/l mannitol (gray bar), or high glucose (25 mmol/l) (black bar) in the absence or presence of different concentrations of recombinant adiponectin (ADN) for 72 h. A and B: Equal amounts of cellular proteins were separated by SDS-PAGE and probed with anti-phospho- (p-p38) or anti-total (T-p38) MAPK or anti-phospho or anti-total ERK1/2 as specified. The bar charts (lower panel) are the quantitative analysis of the corresponding immunoblots, and the data are expressed as fold over normal glucose control. C: The mRNA levels of p16<sup>INK4A</sup> were quantified by real-time PCR and normalized against  $\beta$ -actin. The representative gel is shown on the top of the bar chart. D: The protein level of p16<sup>INK4A</sup> was determined by Western blot as in panel A. \*\* $P < 0.01$  vs. the normal-glucose group. # $P < 0.05$ , ## $P < 0.01$  vs. the high-glucose group without adiponectin treatment ( $n = 4-5$ ).

Hyperglycemia is a primary contributor to the reduced number and impaired functions of EPCs in diabetes (33). In humans, the number of circulating EPCs correlates inversely with the fasting blood glucose concentration as well as the A1C level (4,34). In vitro studies demonstrate that high glucose decreases the EPC number by reducing cell proliferation (35), increasing apoptosis (36), and accelerating premature senescence (37–39). However, it is noteworthy that high glucose–induced apoptosis of EPCs was observed only when the cells were cultured in the presence of 50 mmol/l glucose (36). In the present study, we found that prolonged exposure of human EPCs to 25 mmol/l glucose for 3 days does not induce obvious apoptosis but leads to a marked elevation of premature senescence, as determined by senescence-associated  $\beta$ -galactosidase assay. These results are in line with several recent reports that high glucose ranging from 10 to 25 mmol/l reduces the self-renewal capacity and induces senescence of human EPCs but not their apoptosis (27,39).

Furthermore, stress-induced premature senescence, but not apoptosis, is attributed to tumor necrosis factor- $\alpha$ –induced decrease in the number of human EPCs (28).

The present results demonstrate that adiponectin prevents high glucose–induced EPC senescence by downregulating the expression of p16<sup>INK4A</sup>, a well-established senescent marker that has recently been shown to be a key mediator of the aging process in stem cells (29). In hematopoietic stem cells, ROS induces the expression of p16<sup>INK4A</sup> through activation of p38 MAPK, and this in turn limits the lifespan of the cells by inducing senescence (30). Hyperactivation of the ROS/p38 MAPK pathway appears to be a primary contributor to the reduced number and dysfunction of EPCs in diabetic patients and elderly individuals (21,27). EPCs from diabetic patients contain a substantially higher ROS level compared with those of healthy individuals, and suppression of high glucose–induced ROS accumulation by either pharmacological or genetic intervention reverses the reduced number and the impaired reendo-

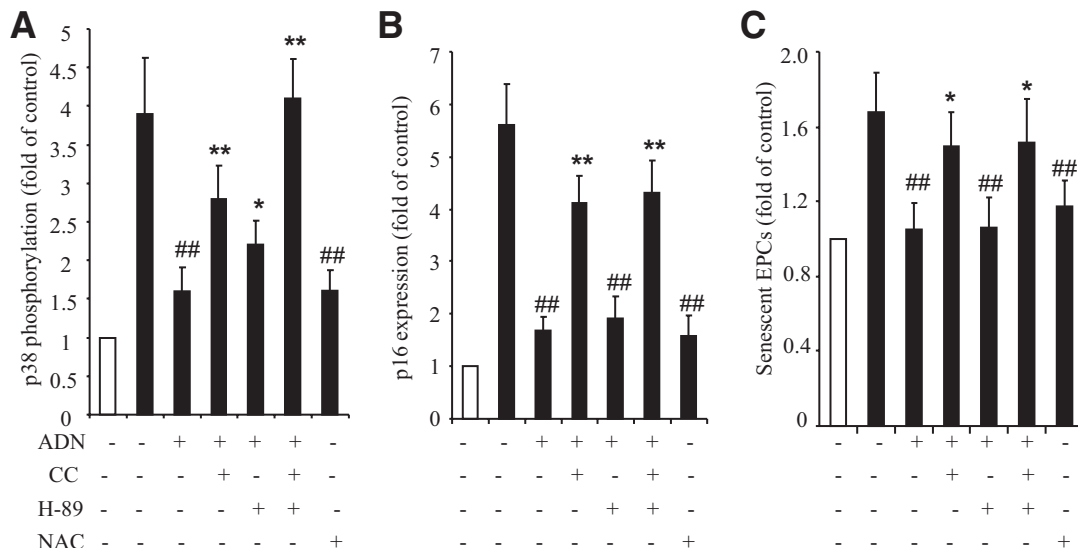


**FIG. 6.** Adiponectin prevents high glucose-induced elevation of ROS levels through both AMPK and cAMP signaling pathways. Human EPCs isolated from peripheral blood were grown under normal glucose (5 mmol/l) (open bar), normal glucose plus 20 mmol/l mannitol as an osmotic control (gray bar), or high glucose (25 mmol/l) (black bar) and were treated with different concentrations of recombinant adiponectin (ADN), 5 μmol/l compound C (CC), and/or 5 μmol/l H-89 for 72 h as indicated. Intracellular DCF-sensitive ROS generation is expressed as fold over normal glucose control. \**P* < 0.05, \*\**P* < 0.01 (*n* = 5–6).

thelialization capacity of EPCs associated with diabetes (21,40). Likewise, pharmacological inhibition of p38 MAPK alone is sufficient to increase the number of EPCs in patients with cardiovascular disease and to prevent premature EPC senescence induced by high glucose or TNF-α (27,28,37). Consistent with these findings, the present results show that high glucose-induced p16<sup>INK4A</sup> expression and senescence of EPCs is largely mitigated by either the antioxidants (N-acetylcysteine [NAC]) or by the p38 MAPK selective inhibitor SB203508 but not by the ERK1/2 inhibitor PD98059 or the JNK inhibitor SP600125. Furthermore, the inhibition of high glucose-induced premature senescence by adiponectin is accompanied by a marked reduction in intracellular accumulation of ROS and activation of p38 MAPK. It is

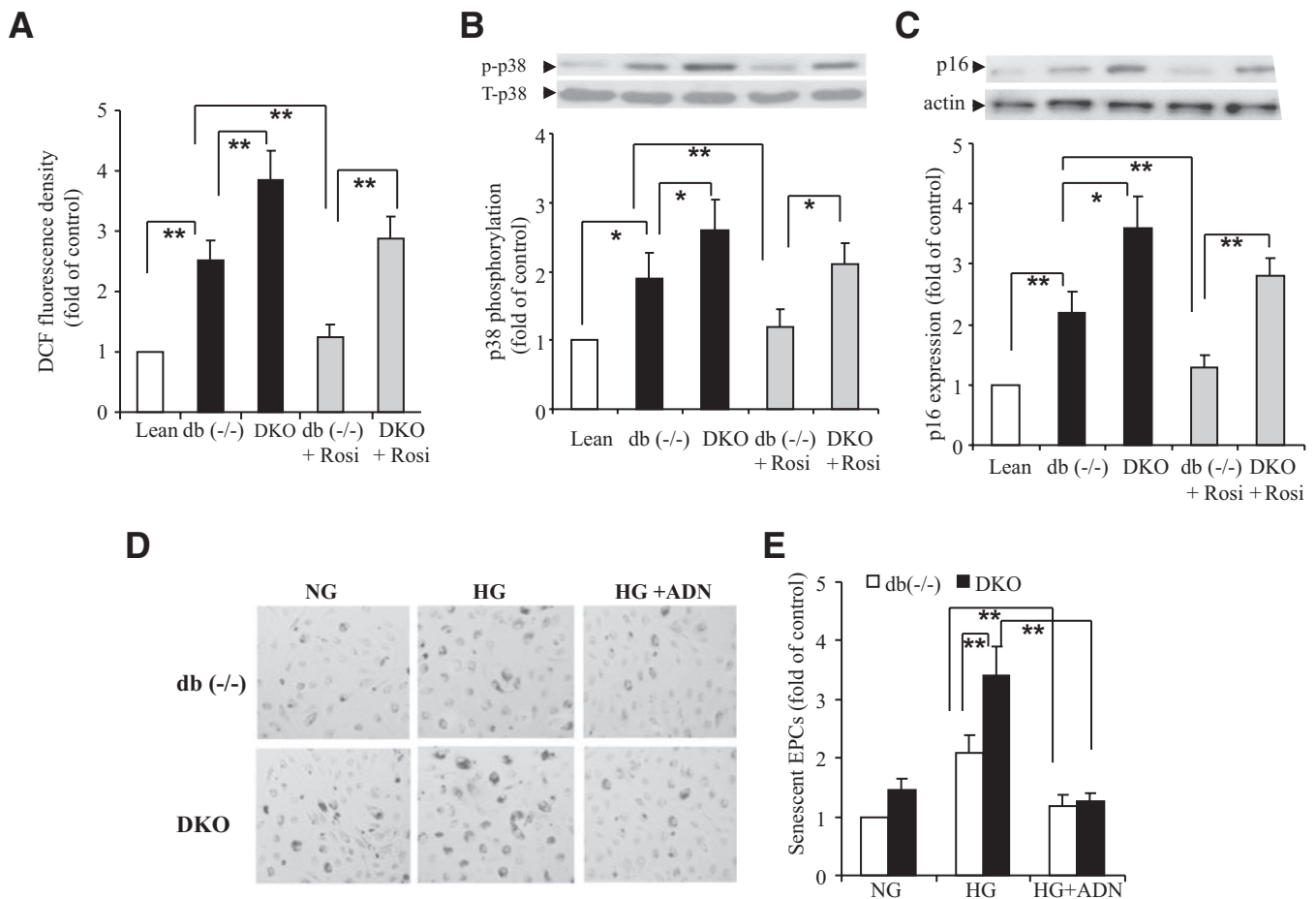
noteworthy that the suppression of p38 MAPK by adiponectin was observed only when it was activated by high glucose and not under normal glucose conditions, suggesting that the effect of adiponectin on p38 MAPK is indirect, perhaps secondary, to the decrease of intracellular ROS.

The antioxidant property of adiponectin has been implicated previously in both clinical and animal studies (15,20,41,42). In humans, plasma levels of adiponectin correlate inversely with the markers of oxidative stress (such as 8-epi-prostaglandin F2α) (41). Adiponectin has been shown to decrease oxidative stress in cardiomyocytes (42), mature endothelial cells (15), and liver cells (20,43), although the underlying mechanism is still a matter of debate. In human umbilical vein endothelial cells, the suppressive effect of adiponectin on high glucose-induced ROS production is mediated predominantly by the cAMP/PKA pathway but not AMPK (15). On the other hand, the antioxidant activity of adiponectin in hepatocytes and hepatic stellate cells is primarily a downstream event of AMPK activation, which in turn induces expression of uncoupling protein-2 that inhibits mitochondria ROS production (20,44). In this study, we showed that both cAMP/PKA and AMPK potentially contribute to adiponectin-mediated suppression of high glucose-evoked ROS production in human EPCs. This conclusion is supported by the fact that a complete abrogation in the antioxidant activity of adiponectin was achieved only when both PKA and AMPK were inhibited. Noticeably, pharmacological or genetic inhibition of PKA had little effect on adiponectin-induced suppression of high glucose-induced p16<sup>INK4A</sup> expression and senescence of EPCs. This finding suggests that cAMP/PKA only plays a minor role in mediating the suppressive effect of adiponectin on high glucose-induced ROS production and senescence of EPCs. On the other hand, pharmacological or genetic inhibition of AMPK alone was sufficient to attenuate the suppressive effect of adiponectin on high glucose-induced ROS accumulation, p38 MAPK, or senescence of EPCs, suggesting that AMPK is likely to be an key mediator of adiponectin actions in EPCs.



**FIG. 7.** Effects of compound C, H-89, and antioxidants on adiponectin-mediated suppression of p38 MAPK phosphorylation, p16<sup>INK4A</sup> expression, and cellular senescence in human EPCs. Cells were grown in normal (open bar) or high (black bar) glucose and were treated with or without 15 μg/ml adiponectin (ADN), 5 μmol/l compound C (CC), 5 μmol/l H-89, or 2 mmol/l of the antioxidant NAC as indicated. \**P* < 0.05, \*\**P* < 0.01 vs. the high-glucose plus adiponectin group. ##*P* < 0.01 vs. the high-glucose group. *n* = 4–6.





**FIG. 8.** Protective effects of adiponectin against high glucose-induced activation of the ROS/p38 MAPK/p16<sup>INK4A</sup> signaling cascade and cellular senescence in bone marrow-derived EPCs in mice. Lin<sup>-</sup>c-Kit<sup>+</sup>Flk-1<sup>+</sup> cells were isolated from 12-week-old male lean mice, db<sup>-/-</sup> mice, or DKO mice treated with vehicle control or rosiglitazone (Rosi) for 4 weeks using the magnetic sorting method. Intracellular ROS levels (A), phosphorylation of p38 MAPK (B), and p16<sup>INK4A</sup> expression (C) were measured immediately after the cell isolation. D: Representative image of β-gal staining to detect senescence of Lin<sup>-</sup>c-Kit<sup>+</sup>Flk-1<sup>+</sup> cells isolated from db<sup>-/-</sup> mice or DKO mice grown in normal glucose (NG) or high glucose (HG) (25 mmol/l) for 3 days in the presence or absence of recombinant adiponectin (ADN) (10 μg/ml). E: Quantitative analysis of senescent cells (expressed as fold of normal glucose control). \**P* < 0.05, \*\**P* < 0.01 (*n* = 6).

In line with our findings, a number of recent studies have documented the suppressive effect of AMPK on high glucose-induced oxidative stress in endothelial cells by promoting mitochondrial biogenesis (45) and inhibiting NADPH oxidase activity (46) or by inducing the expression of uncoupling protein-2 (20,44). Although the role of AMPK in EPCs remains poorly understood, pharmacological activation of this kinase promotes adhesion and differentiation of EPCs in vitro (47). Therefore, besides protection against premature senescence, AMPK activation by adiponectin might exert additional benefits on enhancing the reendothelialization capacity of EPCs in vivo.

In summary, the present study provides both in vivo and in vitro evidence supporting the protective effects of adiponectin against diabetes-induced decrease in the number of EPCs and impairment in endothelial repair. These findings suggest that pharmacological intervention to increase the production of adiponectin by adipocytes and/or to enhance adiponectin signaling pathways might represent an effective therapeutic strategy to improve endothelial repair and neovascularization in patients with diabetes and cardiovascular disease. However, the findings in this study were mainly based on rodent models and in vitro experiments. The clinical relevance of these findings remains to be established. Because both subtypes of adi-

ponectin receptors (adipoR1 and adipoR2) are expressed in human early EPCs and mouse bone marrow-derived EPCs, whether the favorable effects of adiponectin in these cells are mediated by these two receptors requires further investigation.

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J.C. researched data and wrote the manuscript. Y.L. researched data. Y.H. designed experiments and edited the manuscript. K.S.L.L. supervised the study, contributed to the discussion, and edited the manuscript. R.L.C.H. researched data. W.T.W. researched data. K.K.Y.C. researched data. Y.W. researched data. P.M.V. advised the study, contributed to the discussion, and edited the manuscript. A.X. designed the study, analyzed data, and wrote the manuscript.

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