

Metformin inhibits endothelial progenitor cell migration by decreasing matrix metalloproteinases, MMP-2 and MMP-9, via the AMPK/mTOR/autophagy pathway

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Abstract. The aim of the present study was to investigate the effect of metformin on endothelial progenitor cell (EPC) migration and to explore the possible mechanisms. EPCs were treated with metformin, and the migration of EPCs was evaluated by wound healing and Matrigel invasion assays. We also examined the expression levels of MMP-2 and MMP-9 in EPCs with or without metformin treatment via RT-PCR and western blot analysis, and activities of MMP-2 and MMP-9 in EPCs under different conditions was examined by zymography. Moreover, we also assessed the AMPK/mTOR/autophagy pathway to explore the possible mechanisms. Metformin treatment significantly down-regulated matrix metalloproteinase-2 (MMP-2) and MMP-9 expression, and subsequently decreased the migration of EPCs. Increased levels of phosphorylated (p)-AMPK and LC3II expression, as well as decreased levels of p-mTOR and p62 contributed to this phenomenon. The AMPK inhibitor compound C reversed the effect exerted by metformin. In conclusion, our results showed that metformin inhibited the migration of EPCs by decreasing MMP-2 and MMP-9. The AMPK/mTOR/autophagy pathway was demonstrated to be involved in the regulatory mechanisms.

Introduction

Endothelial progenitor cells (EPCs), which were firstly identified in adult human peripheral blood (PB) in 1997 (1), exhibit the capacity for endothelial differentiation (2) and angiogenic growth factor and cytokine secretion (3,4). As a novel thera-

peutic target for vascular diseases (5), they can incorporate into ischemic tissue and play an important role in vasculogenesis for physiological or pathological neovascularization (6-9). In addition, EPCs have been reported to play an important role in thrombosis resolution (10-13).

However, EPCs are present in very low numbers (0.1-0.01% of mononuclear cells). Moreover, some risk factors of vascular diseases, for example age, smoking, coronary artery disease (CAD), atherosclerosis and diabetes mellitus (DM) (14-16), may reduce the number of EPCs in PB (10). EPCs from CAD and DM patients exhibit decreased migration as well as mobilization capacity (16-18). Migration is essential for EPCs to mobilize to the sites of ischemic tissue. These factors decrease the numbers of EPCs in circulation and subsequently impair the capacity of vascular endothelium repair. Thus, how to promote EPCs to mobilize from the bone marrow (BM) niche and migrate into special sites remains a current issue.

Metformin, an activator of AMPK and an inhibitor of mTOR (19), is widely endorsed as initial therapy for type 2 diabetes because of its low cost, safety profile, and potential cardiovascular benefits (20). It has been demonstrated that metformin improves endothelial function and enhances the neovascularization of EPCs (21,22). At the same time, in our previous study, metformin promoted EPC differentiation (23). But, the exact effect of metformin on circulating EPC migration is still poorly understood. In this study, we investigated the effect of metformin on the migration of EPCs and explored the possible mechanisms.

Materials and methods

Antibodies and reagents. Antibodies for phospho(p)-AMPK (#4185), AMPK (#2532), p-Akt (#9018), Akt (#9272), mTOR (#2972), LC3B (#2775) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies for p62 (ab56416), matrix metalloproteinase-2 (MMP-2; ab92536) and MMP-9 (ab76003) were obtained from Abcam, Inc. (Cambridge, MA, USA). Antibody for p-mTOR (sc-293132) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Metformin, AMPK inhibitor compound C (CC), mitomycin C, and the antibody for β -actin (A3854) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Isolation and characterization of human EPCs. Mononuclear cells were isolated from PB using the Histopaque density centrifugation method. Fresh blood (50-100 ml) was collected from volunteer donors by venipuncture and anticoagulated with heparin. The Institutional Review Board at the Second Affiliated Hospital of Soochow University approved all protocols, and informed consent was obtained from all adult donors.

Human mononuclear cells (MNCs) were isolated as previously described (24). Briefly, the anticoagulated blood was diluted 1:1 with phosphate-buffered saline (PBS). Mononuclear cells, isolated by density gradient centrifugation using Lymphocyte Separation Medium-LSM™ (MP Biomedicals, Santa Ana, CA, USA), were seeded onto collagen I-coated plates (Invitrogen, Carlsbad, CA, USA) at a density of 2.5×10^6 cells/cm² and cultured with Endothelial Cell Growth Medium-2 (EGM-2MV; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and maintained in a 37°C/5% CO₂ incubator. Medium was changed daily for 7 days and then every other day until the first passage. Cells within 10 passages were used for the following experiments.

EPC surface molecule analysis. EPC surface molecule analysis was performed as previous described by flow cytometry (25,26). Cells were detached with EDTA and labeled for 20 min at 4°C at manufacturer-recommended concentrations with fluorescent antibodies, including anti-VEGFR-2-PE (#560872), anti-CD45-FITC (#560976), anti-CD14-FITC (#561710), anti-CD133-PE (#130-080-801), and anti-CD31-PE (#566125) and anti-CD34-FITC (#560942). Fluorescent isotype-matched antibodies were used as negative controls. All antibodies were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA), except anti-CD133-PE (Miltenyi Biotec, Auburn, CA, USA). Cells were washed, paraformaldehyde-fixed (Tosoumis, Rockville, MD, USA), and analyzed on a FACSCalibur Instrument (Becton-Dickinson) with 10,000 events stored. Other commonly accepted criteria for identifying EPCs, for example, uptake of DiI-acLDL and FITC-UEA-I binding, were also performed.

To evaluate the effect of metformin, identified EPCs were incubated with 10 mM metformin for 24 h. In the setting of mechanism verification, AMPK inhibitor compound C was added at a concentration of 10 μ M.

Wound healing migration assay. A wound healing migration assay with EPCs was performed following previously published methods (27). Briefly, EPCs (4×10^6 cells) with 10 mM metformin were resuspended and seeded into a 6-well plate, and grew until ~100% confluence. Cells were treated with 10 μ g/ml mitomycin C for 3 h to block cell proliferation. A linear scratch was made by using a 10- μ l pipette tip. After washing with PBS twice, the cells were incubated with serum free EGM-2MV medium for specific times. Images were taken at 0 and 24 h at x40 magnification and the wound size was measured in 3 wells/group.

Matrigel invasion assay. EPC migration assay was performed in a Transwell system as described previously (28). A Transwell chamber was used (8- μ m, 24-well plate). In the invasion assay, the insert membranes were coated with diluted Matrigel. EPCs

(2×10^5 cells) were resuspended in 200 μ l serum-free EGM-2MV medium and seeded into the upper chamber after treatment with 10 μ g/ml mitomycin C for 3 h. The lower chamber was filled with EGM-2MV medium supplemented with 10% FBS, vehicle control or 10 mM metformin. After incubation at 37°C in 5% CO₂ for 24 h, the cells were stained with crystal violet and the cell number in each well was counted in 3 randomly picked fields (magnification, x200) under a light microscope. All the experiments were performed in triplicate.

Real-time reverse transcription polymerase chain reaction (RT-PCR). After EPCs were treated as described above, total cellular RNA was extracted using TRIzol reagent (Invitrogen). Real-time RT-PCR was carried out using a SYBR®-Green qPCR Mix (Thermo Scientific, MBI Fermentas, Waltham, MA, USA) and a Roche LightCycler 480 (Roche, Basel, Switzerland). Expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was assessed simultaneously in all samples as an internal control. Relative gene expression was determined by the $2^{-\Delta\Delta Cq}$ method (29). The following primers were used: MMP-2 sense, 5'-GGT TCC CCT GTT CAC TCT ACT TAG C-3' and antisense, 5'-CGG CTT GGT TTT CCT CCA T-3'; MMP-9 sense, 5'-CCC GGA GTG AGT TGA ACC A-3' and antisense, 5'-AGG GCA CTG CAG GAT GTC A-3'; GAPDH sense, 5'-GGT GGT CTC CTC TGA CTT CAA CA-3' and antisense, 5'-GTG GTC GTT GAG GGC AAT G-3'.

Western blot analysis. EPCs (1×10^6 cells) were lysed in RIPA buffer, followed by high-speed centrifugation and bicinchoninic acid quantification. Cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. After blocking with 5% non-fat milk Tris-buffered saline-Tween-20 (TBS-T), the membranes were incubated with primary antibodies against AMPK, p-AMPK, Akt, p-Akt, p-mTOR, mTOR, MMP-2, MMP-9, LC-3B and p62. Appropriate horseradish peroxidase-conjugated secondary antibodies were applied. β -actin (Sigma-Aldrich) was used as the loading control. The protein bands were detected with Super Signal West PicoChemiluminescent Substrate (Pierce, Rockford, IL, USA) on X-ray film (Kodak, Tokyo, Japan).

SDS-PAGE gelatin zymography. The activities of MMP-2 and MMP-9 were determined by gelatin zymography as previously described (30,31). Ten micrograms of each sample was loaded onto a 8% SDS-polyacrylamide gel containing 1 mg/ml gelatin, electrophoresed, renatured, and developed. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 and destained in the same solution without dye. Gelatinase activity was visualized as clear bands against the blue-stained gelatin background and analyzed in a computer system. Three individual experiments were conducted with independent protein samples.

Statistical analyses. All statistical analyses were carried out using SPSS v21 (SPSS, Inc., Chicago, IL, USA). Data are presented as mean \pm standard deviation (SD). Student's t-test or one way ANOVA was utilized to examine differences between two groups or multiple group comparison. $P < 0.05$ was considered as statistically significant.

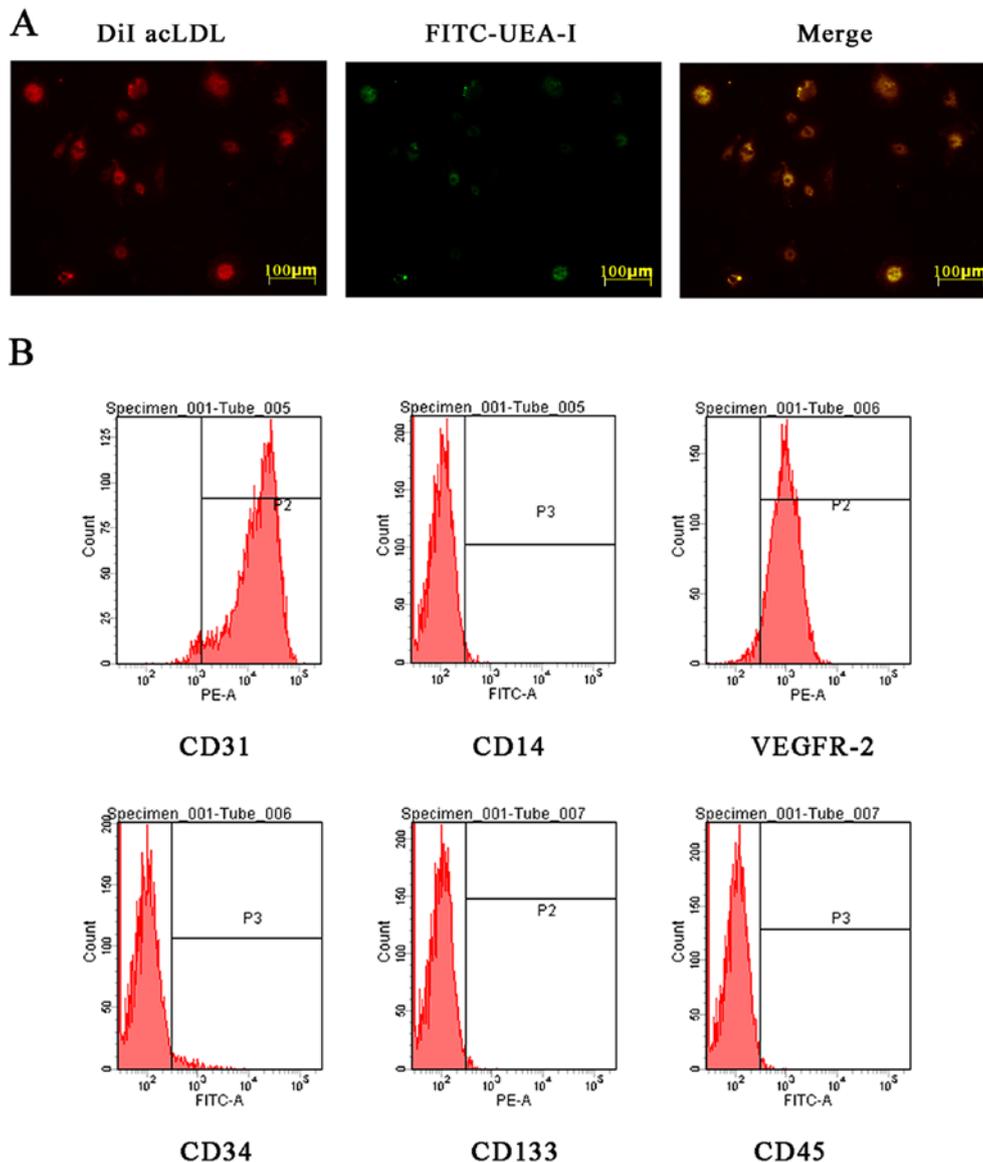


Figure 1. Characterization of endothelial progenitor cells (EPCs). (A) DiI-Ac-LDL uptake and FITC-UEA-I binding of isolated EPCs were determined by fluorescence microscopy. (B) Flow cytometric analysis of EPCs. Cells were labelled with antibodies to CD31, CD14, VEGFR-2, CD34, CD133 and CD45. Histograms represent cell number (y-axis) vs. fluorescence intensity (x-axis, log scale). Cells (10,000) were acquired and gates were set on living cells.

Results

EPC characterization. Isolated MNCs were cultured in EGM-2MV medium supplemented with 10% FBS and growth factors to be induced into EPCs. EPCs were identified by morphology, fluorescence double-staining and flow cytometry. Most adherent cells were double stained by DiI-AcLDL and FITC-UEA-I (Fig. 1A). The flow cytometric analysis matched with the previously described EPC phenotype (25,26) (Fig. 1B). The results of identification of these cells were consistent with the characterization of late-outgrowth EPCs.

Metformin attenuates EPC migration. Wound healing migration and Matrigel invasion assays were employed. The results showed that metformin treatment significantly decreased EPC migration (Fig. 2).

Metformin regulates EPC migration by influencing MMP-2 and MMP-9. Moreover, we examined the expression levels of MMP-2 and MMP-9 in EPCs with or without metformin treatment via RT-PCR and western blot analysis. The results revealed decreased expression of MMP-2 and MMP-9 in EPCs treated with metformin (Fig. 3A and B), indicating that a decreased activity of gelatinase and fibrinolysis, may contribute to this phenomenon.

The expression of MMP-2 and MMP-9 in EPCs at different conditions was also examined by zymography. Three bands were found in the gel, representing pro-MMP-9, active MMP-9 and active MMP-2. The expression levels of both MMP-2 and MMP-9 were significantly decreased in the EPCs after incubation with metformin (Fig. 3C).

Metformin regulates migration via the AMPK/mTOR/autophagy pathway. After metformin treatment, an increased level

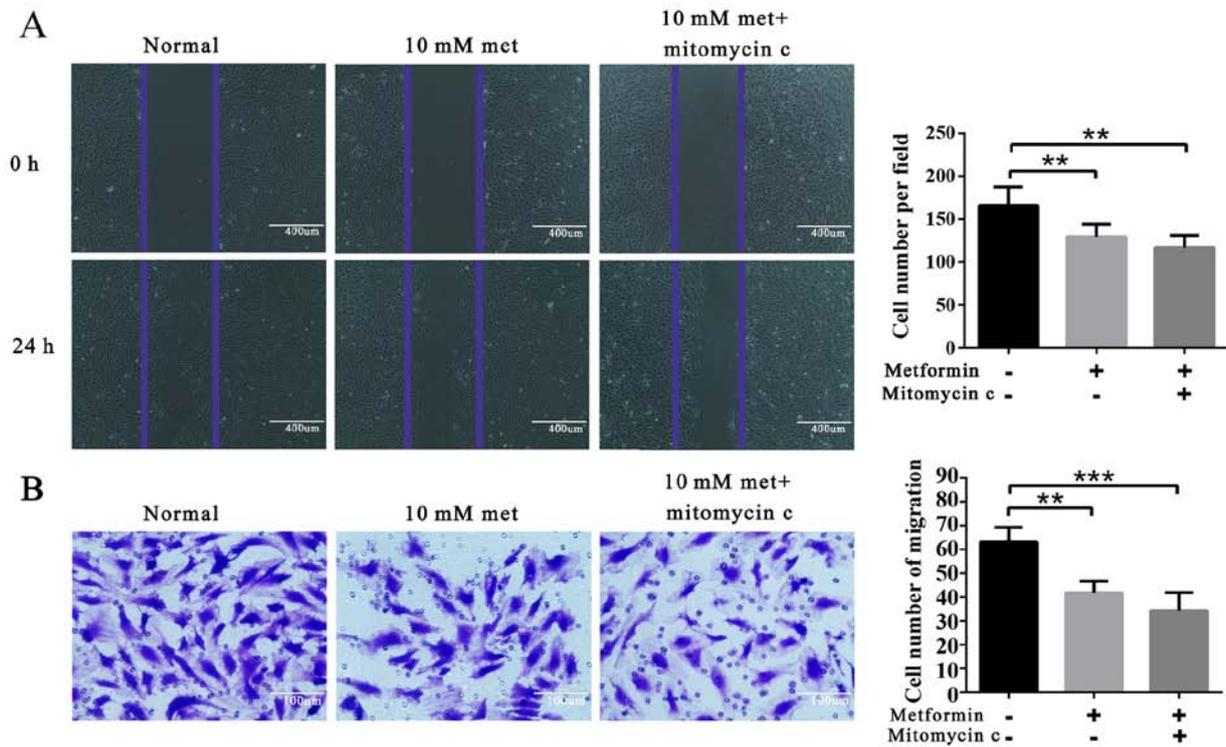


Figure 2. Effect of metformin on endothelial progenitor cell (EPC) migration. Metformin significantly decreases EPC migration. (A) Wound healing assay. Representative images of migrated EPCs in normal, metformin (met) and metformin combined with mitomycin C (met + mitomycin C) groups (magnification, x40). Wound closure was monitored by microscopy at 24 h. (B) Transwell assay. Representative images of migrated EPCs in normal, met and met + mitomycin C groups (magnification, x200). Cell migration was monitored by microscopy at 24 h following the cell inoculation in the chamber. **P<0.01, ***P<0.001 when comparison was performed between groups.

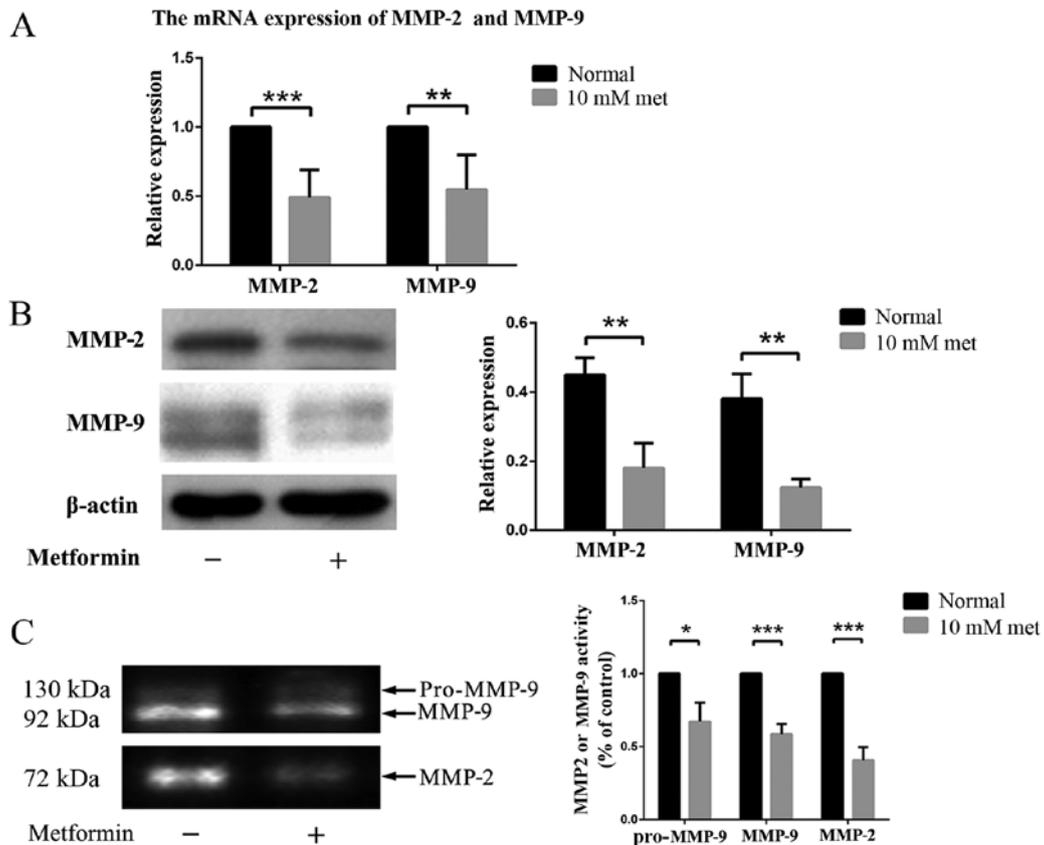


Figure 3. Metformin regulates endothelial progenitor cells (EPCs) via matrix metalloproteinase-2 (MMP-2) and MMP-9. (A) Quantitative polymerase chain reaction analyses of the expression of MMP-2 and MMP-9. (B) Western blot analyses of MMP-2 and MMP-9. (C) Gelatin zymogram of active MMP-2 and MMP-9. *P<0.05, **P<0.01, ***P<0.001 when comparison was performed between groups.

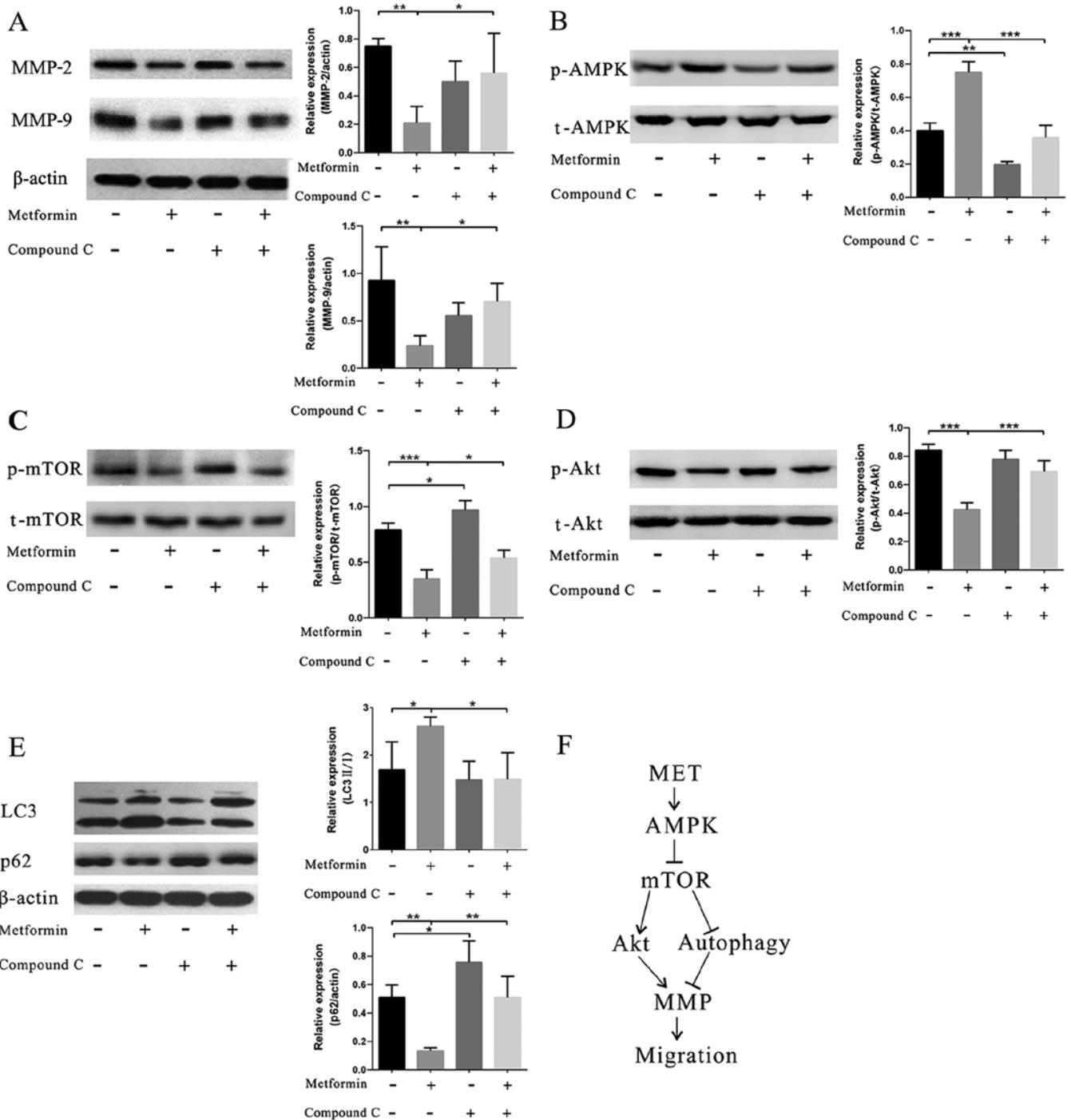


Figure 4. Metformin regulates endothelial progenitor cell (EPC) migration via the AMPK/mTOR/autophagy pathway. (A) Western blot analysis of matrix metalloproteinase-2 (MMP-2) and MMP-9 expression after 10 mM metformin and/or 10 μ M AMPK inhibitor compound C treatment (n=3). (B-D) Western blot analysis of p-AMPK/t-AMPK, p-mTOR/t-mTOR and p-Akt/t-Akt expression respectively after 10 mM metformin and/or 10 μ M AMPK inhibitor Compound C treatment (n=3). (E) Western blot analysis of LC3 and p62 expression after 10 mM metformin and/or 10 μ M AMPK inhibitor Compound C treatment (n=3). (F) A proposed model of the regulation of EPC migration by metformin. *P<0.05, **P<0.01, ***P<0.001.

in AMPK phosphorylation was observed (Fig. 4B), whereas the addition of AMPK inhibitor CC into the system reversed the effect of metformin. Correspondingly, MMP-2 and MMP-9 exhibited reversed changes compared with the levels of AMPK phosphorylation (Fig. 4A). Moreover, we also found decreased levels of mTOR and Akt phosphorylation (Fig. 4C and D). Furthermore, the expression of autophagy components in the EPCs was tested. An increased level of LC3B and a decreased

level of p62 showed that the autophagy pathway was involved in the effect of metformin (Fig. 4E).

Discussion

EPCs, as promising therapeutic targets for vascular diseases, have been verified to improve vascular function in substantial animal and preliminary human studies (32,33). Migration is

essential for EPCs to mobilize to the sites of ischemic tissue. For example, in mice, EPCs were increased 24 h after DVT induction, peaking 48 h thereafter (12). However, the number of EPCs in DM patients was decreased, which may result from attenuated migration as well as mobilization capacity (34,35). Metformin is widely endorsed as initial therapy for type 2 diabetes. In a study by Chen *et al*, metformin enhanced the number of circulating EPCs (36). Yet, in another study, Esposito *et al* (37) found no effects on the number of circulating EPCs when newly diagnosed type 2 diabetes patients were treated with metformin. Thus, the exact role of metformin on EPC migration has not been fully elaborated. In addition, there are no studies *in vitro* to explore the effect of metformin on EPC migration. Thus, in the present study, we aimed to investigate the effect of metformin on the migration of EPCs and explore the possible mechanisms. We found that metformin treatment attenuated EPC migration via regulating the expression of MMP-2 and MMP-9. In addition, the AMPK/mTOR/autophagy pathway was found to be involved in the effect of metformin.

It has been demonstrated that metformin is an agonist of AMPK (19), which is a member of a metabolite-sensing protein kinase family and has the capacity for the regulation of cell differentiation and migration (38,39). Augmented AMPK activity inhibits cell migration (39). This may be related to the inhibition of MMPs regulated by AMPK (40,41). MMPs are reported to play an important role in extracellular matrix degradation thereby facilitating the migration of leukocytes, monocytes and other types of cells in the setting of vascular diseases (42). In previous studies, it was found that metformin could regulate endothelial cell and tumor cell migration by inhibiting MMP expression. For example, metformin inhibited the migration by attenuating the expression of MMP-2 and MMP-9 in human umbilical vein endothelial cells partially through an AMPK-dependent pathway (43). Hwang *et al* (44) found that metformin blocked the migration and invasion of tumor cells by inhibition of MMP-9 activation. Fang *et al* (45) also reported that metformin reduced A498 cell migration and invasion *in vitro* by decreasing MMP-2. In the present study, we found that metformin inhibited EPC migration by reducing the expression and impairing the activity of MMP-2 and MMP-9, which was consistent with previous studies in other cells. We also detected the expression of AMPK, mTOR as well as Akt and found that metformin promoted AMPK phosphorylation but attenuated mTOR and Akt phosphorylation. The latter two protein kinases are involved in migration and MMP expression (46,47). Meanwhile, these changes in protein kinases could result in an increased level of autophagy (Fig. 4F).

Metformin could induce autophagy by simultaneously activating AMPK and inhibiting mTORC1 in both AMPK-independent and AMPK-dependent approaches (48,49). According to previous research, autophagy plays an important role in cell migration regulation but in a controversial manner. On the one hand, autophagy enhances production of various cytokines and thereby facilitates migration and invasion of lung cancer cells (50). However, on the other hand, autophagy inhibits cell migration and invasion by slowing down the lysosomal degradation of certain molecules or proteinases, such as MMPs and integrins, which are critical for cell migration (28,51). In our previous study (28), we found that the different roles of autophagy in regulating cell migration may be associated with

the fundamental level of autophagy and the extent of autophagy regulation.

There are a few limitations in the present study. First, we only collected EPCs from normal healthy subjects to examine the effect of metformin. The effect of metformin on the EPCs derived from patients with DM or CAD has not yet been examined. Second, the role of metformin in EPC migration under hyperglycemia should be performed in the future. Appropriate animal model studies should also be carried out to verify the functional change of EPCs *in vivo*. Therefore, further studies are needed to address these issues.

In conclusion, we here showed that metformin treatment regulated the migration of EPCs. The mechanisms may be associated with the AMPK/mTOR/autophagy-related pathway. The latter attenuates the expression of MMP-2 and MMP-9 (Fig. 4F).

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