Endothelial progenitor cells overexpressed with omentin-1 enhance the inhibition of neointimal hyperplasia via anti-inflammation

Yuan Xiang
Central South University
Zheng-Shi Zhou
Central South University
Chuan-Chang Li
Central South University
Ying Luo
Central South University
Ji Peng Zhou (zhoujipeng616@csu.edu.cn)
Central South University First Hospital: Xiangya Hospital Central South University

Research Article

Keywords: Coronary heart disease, Restenosis, Inflammation, Omentin-1, Endothelial progenitor cells

Posted Date: March 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-335234/v1

Abstract

Background

Endothelial progenitor cells (EPCs) play an important role in vascular repair. However, the functions of EPCs are obviously weakened in inflammatory microenvironment during restenosis. Therefore, we investigated whether omentin-1, an anti-inflammatory factor, can reduce neointima formation after carotid artery injury (CAI) in rats via improving EPCs functions damaged by inflammation and the underlying mechanisms.

Methods

Rats bone marrow-derived EPCs were isolated and cultured. EPCs were transfected with adenovirus vectors expressing human omentin-1 or green fluorescent protein (GFP). Rats received 2×10^6 EPCs with expressing omentin-1 or GFP by tail vein injection directly after CAI and again 24 h later. Hematoxylin-eosin staining and immunohistochemistry were used for analyzing neointimal hyperplasia. Besides, EPCs were treated with omentin-1 and tumor necrosis factor-α (TNF-α) in order to explore the underlying mechanism.

Results

Omentin-1 could significantly promote EPCs proliferation and tube formation, as well as inhibit apoptosis. EPCs overexpressed with omentin-1 using adenovirus vectors could extremely reduce the neointimal hyperplasia after CAI in rats. Besides, TNF-α could notably induce EPCs dysfunction including reduced proliferation, migration tube formation and increased apoptosis, which can be remarkably attenuated by omentin-1 via inhibiting of p38/CREB pathway. Besides, p38 agonist (anisomycin) could significantly reverse the protective effects of omentin-1 which attenuated the injury effects of TNF-α on EPCs.

Conclusions

EPCs overexpressed with omentin-1 can significantly reduce neointima formation after arterial injury by enhancing the functions of EPCs via inhibiting the p38/CREB pathway. Our results indicate that gene modified EPCs with omentin-1 may be an alternative strategy for the treatment of restenosis.

Background

Percutaneous coronary intervention (PCI) is the most widely recommended treatment to restore myocardial blood flow and reduce angina symptoms for coronary heart disease (CHD) patients. Nevertheless, an ineluctable clinical problem of this therapy is in-stent restenosis (ISR), which occurs in

approximately 20% of CHD patients with drug-eluting stents implantation [1]. It is generally believed that ISR can be briefly divided into 3 phases including a damage to the endothelium, the migration and proliferation of vascular smooth muscle cells (VSMCs) and the late remodeling phase [2]. Despite many approaches were used for clarifying the mechanism as well as the prevention of ISR, however, it is still a tricky problem that has not been completely solved [3].

Endothelial progenitor cells (EPCs) were identified as a subset of bone marrow-derived progenitor cells migrate into the peripheral blood circulation, which can quickly proliferate and differentiate into mature endothelial cells (ECs) with CD31, CD34, CD133 and VEGFR-2 antigens were commonly recognized as the surface markers [4–6]. EPCs can mobilize in response to vascular injury and home to the site of injury area, resulting in an enhanced angiogenesis and endothelial repair, therefore contributing to increased reendothelialization associated with decreased neointima formation [7, 8]. In view of these, cell therapy with EPCs may offer a promising strategy for accelerating reendothelialization and maintaining endothelial integrity in ISR [9]. Importantly, the microenvironment in ISR is essential for EPCs fate and function, especially inflammation, which is closely followed with endothelial injury, including platelet activation and recruitment of circulating leukocytes, releasing cytokines and growth factors [2]. Inflammatory factor, such as tumor necrosis factor-α (TNF-α) can dramatically result in EPCs dysfunction with reduced cell number, survival, migratory, and angiogenic activities [10, 11]. Therefore, much efforts are needed to improve the damage of inflammation on EPCs dysfunction, which may further ameliorate EPCs biological property and significantly enhance the therapeutic potentials [9].

Omentin-1, also referred to as intelectin-1, is a novel anti-inflammatory adipokine of 313 amino acids that is expressed in omental, epicardial and perivascular adipose tissue and primarily produced by adipose tissue vascular stromal cells [12]. Studies have shown that omentin-1 plays crucial roles in the maintenance of body metabolism and insulin sensitivity, anti-inflammatory, anti-atherosclerotic and cardiovascular protective effects via AMP-activated protein kinase/Akt/nuclear factor-κB/mitogen-activated protein kinase (ERK, JNK, and p38) signaling [13]. Besides, omentin-1 protects against high glucose-induced endothelial dysfunction through inhibiting endoplasmic reticulum stress and oxidative stress via activation of AMPK/PPARδ pathway [14]. However, whether omentin-1 improves EPCs functions especially damaged by inflammation is still unknow.

Therefore, the aims of this study were to investigate whether omentin-1 improves EPCs functions as well as gene modified EPCs with omentin-1 could inhibit neointima formation after carotid artery injury (CAI) in rats. Furthermore, we also explored whether omentin-1 ameliorates EPCs dysfunction damaged by inflammation was related with p38/CREB pathway. Our results may offer an alternative target for resisting EPCs dysfunction which may benefit for the prevention and treatment of ISR.

Methods

Isolation and cultivation of EPCs

Animals care and experimental procedures were approved by the Animal Research Committee of the Department of Laboratory Animals, Xiangya School of Medicine, Central South University. EPCs were isolated from the bone marrow of Sprague-Dawley (SD) rats (150-250 g) as previously described [6]. Briefly, total bone marrow-derived mononuclear cells (MNCs) were isolated from both femurs and tibias of male SD rats by density gradient centrifugation using Histopaque-1077 (Sigma Aldrich, St. Louis, MO, US). Isolated cells were subsequently maintained in complete EGM-2 medium supplemented with EGM-2-MV BulletKit (Lonza, Walkersville, MD, USA) and 10% fetal bovine serum (FBS) (ScienCell Research Laboratories, Inc. Carlsbad, CA, USA). Cells were maintained at 37 ℃ in a humidified atmosphere (5% CO_2). After 4 days, the unattached cells were removed by washing with phosphate buffer saline (PBS) for three times and replaced with fresh media and culture medium was changed every 2-3 days.

Identification of EPCs

After 7 days of culture, EPCs were determined by doubly positive for staining cells with FITC-labeled Ulex europaeus lectin I (UEA-1) and Dil-conjugated acetylated low-density lipoprotein (Dil-ac-LDL). More concretely, cells were incubated with 2.4 μg/ml Dil-labeled acetylated low-density lipoprotein (LDL) (Yiyuan biotechnology, Guangzhou, China) at 37 ℃ for 3 h and then fixed with 4% paraformaldehyde for 10 min. After washing with PBS, the cells were incubated with 15 μg/ml FITC-UEA-I (lectin) (Sigma Aldrich, St. Louis, MO, US) at 37 ℃ for 1 h. After staining, images were obtained by fluorescence microscope (DMi8, Leica). EPCs were also identified by flow cytometry. The surface markers investigated were FITC-conjugated CD34 (1:50, Bioss, Beijing, china), PE-conjugated CD133 (1:50, Novus Biologicals, Littleton, CO, USA), PE-conjugated CD31 (1:50, BD Biosciences, San Jose, CA, USA) and FITC-VEGFR-2 (1:30, Abcam, Cambridge, UK), blank served as negative control.

Carotid artery injury

Carotid artery injury (CAI) was induced as described previously [15]. Briefly, 350-400 g male SD rats were anesthetized by an intraperitoneal injection of 10% chloralic hydras (4 mL/kg), and the surgical procedures were performed under sterile conditions. Using a dissecting microscope, the bifurcation of the left carotid artery was exposed via a midline incision of the ventral side of the neck. Two ligatures were placed proximally and distally around the external carotid artery. The distal ligature was then tied off. After temporary occlusion of the internal and common carotid artery by the vascular clamp, a V-shaped arteriotomy was performed in the ligatures of the external carotid artery, to introduce a 2F arterial embolectomy catheter (Edwards Lifesciences, California, USA) that was slightly pumped 1.5 kPa gas into the balloon and completely fills out the vessel. The catheter was passed along the common carotid artery in a rotating manner for three times. After removal of the catheter, the proximal ligature of the external carotid artery was tied off. Normal blood flow was reassured, and the skin was closed with single sutures using 6-0 silk. After induction of arterial injury and 24 h later, each rat received 2×10^6 EPCs by intravenous tail vein injection which have transfected with adenovirus vectors expressing human omentin-1 (Ad-omentin-1) or green fluorescent protein (Ad-GFP) for 72 h.

Construction and infection of Adenoviral Vector

Adenovirus vector expressing human omentin-1 (Ad-omentin-1) and adenovirus-carrying green fluorescent protein (Ad-GFP) used as a control were both constructed by Vigene Biosciences (Jinan, China) using pAd-EF1a-GFP adenovirus vector. Adenovirus was added to the cells at 70%–80% confluence with a multiplicity of infection (MOI) of 100, and then incubated in EGM-2 medium without FBS and antibiotics for 12 h. After the transfection, the medium was removed, and the cells were maintained in the complete medium for 48 h before further treatment.

Immunohistochemistry

For immunohistochemistry, we used antibodies to TNF-α (1:200, Santa Cruz, Texas, USA) and Ki-67 (1:200, Abcam, Cambridge, UK). Paraffin section was rehydrated and endogenous peroxidase activity was blocked for 30 minutes by endogenous peroxidase blocking buffer (Beyotime Biotechnology, Shanghai, China). Primary antibody was incubated at 4 °C overnight, followed by 30 minutes for biotinylated secondary antibody (1:500, Abcam, Cambridge, UK). All specimens were counterstained with hematoxylin-eosin (HE) staining solution (Beyotime Biotechnology), then neutral gum sealed piece for storage. Images were obtained after scanning the section through Pannoramic MIDI (3D-Histech, Budapest, Hungary) and analyzed with ImageJ software.

Cell proliferation assay

Cell proliferation was assessed with the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) and 5-ethynyl-2’-deoxyuridine (EdU) proliferation assay (RiboBio Co., Guangzhou, China). For the CCK-8 test, cells were plated onto 96-well plates (3×10^3 cells/well) in a triplicate pattern. After treating with recombinant human omentin-1 protein (Novus Biologicals) with 0-500 ng/ml. For combined treatments with omentin-1 and TNF-α, omentin-1 (300 ng/ml) was added to the medium for 6 h before TNF-α (10 ng/ml) treatment and then continuously incubated with 37 °C for 24 h. Cells were added with 100 μl of fresh medium supplemented with 10 μl of CCK-8 solution for another 2 h at 37 °C. The optical density (OD) at 450 nm was measured. For the proliferation of EdU assay, cells were plated with 4×10^3 cells/well in a triplicate pattern, and then the proliferation was assessed by the EdU assay kit according to the manufacturer's instructions. The EdU positive cells were viewed under fluorescence microscopy (DMi8, Leica) and the number calculated by counting at least three random separate fields. These images were analyzed by ImageJ software.

Cell apoptosis assay

We used low FBS medium to induce EPCs apoptosis. In brief, cells were washed with PBS, the culture medium was replaced with EGM-2 added with omentin-1 (0-500 ng/ml) and then the cells were placed at 37 °C for 24 h. For reversal of apoptosis by omentin-1, omentin-1 (300 ng/ml) was added to the EPCs medium for 6 h before TNF-α (10 ng/ml) treatment and then continuously incubated with for 37 °C 24 h. The apoptosis cells were assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences)

according to the manufacturer's instructions. After treatment, cells were harvested and washed in ice-cold PBS, then resuspended in 100 μl of binding buffer, 5 μl Annexin V-FITC solution was added to the cells and incubated for 15 min at room temperature in the dark. This was followed by further incubation with 3 μl PI solution for 10 min, and then they were analyzed immediately by bivariate flow cytometry using a Cytek Dxp Athena flow cytometer with Flowjo CE software and analyzed using FlowJo software (Ver X.0.7; FlowJoLLC, Eugene, OR). Approximately 3-4×10^5 cells were analyzed in each sample.

In vitro tube formation assay

EPCs were incubated in 6-well plates and cells were treated as described above. After treatment, EPCs (4×10^3 cells/well) were seeded in 96-well plates that had been pre-coated with Matrigel Matrix (70 μl/well) (CORNING Life Sciences, Tewksbury, MA, USA). After incubation for 6 h in serum-containing media, images of tube morphology were captured by an inverted microscope and analyzed using ImageJ software.

Western blot analysis

EPCs were lysed for 30 minutes on ice in RIPA lysis buffer (Beyotime Biotechnology), and the protein concentration was determined by BCA Protein Assay Kit (Beyotime Biotechnology). Total protein (50 to 100 μg) was resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane (Immobilon-P, Millipore, Bredford, MA, USA), and subjected to immunoblot analysis. The primary antibodies for p38 MAPK (1:1000, Cell Signaling Technology, MA, USA), P-p38 MAPK (1:1000, Cell Signaling Technology), CREB (1:1000, Cell Signaling Technology), P-CREB (1:1000, Cell Signaling Technology) and horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Texas, USA) were used. The proteins were detected using ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, California, USA) with enhanced chemiluminescence reagents (Millipore), and bands were analysed with Image J software normalized by GAPDH (Abcam).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparisons between two groups were measured using Student's t test, and comparisons of multiple groups were performed with one-way analysis of variance (ANOVA). All statistical analyses were performed using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA), and a two-tailed *P* value <0.05 was considered to be statistically significant.

Results

Characteristics of EPCs

When the bone marrow-derived EPCs were cultured for 4-5 days in EGM-2 medium, they appeared to form foci or cord-like structures in the same field. The typical spindle-shape cells had begun to sprout from the

foci after 6-7 days. At this time, they showed obvious characteristics for Dil-ac-LDL uptake (Fig. 1A, red) and lectin binding (Fig. 1B, green). The phenotype of EPCs was characterized by double positive (96.21 ±6.50 %) (Fig. 1C) of Dil-ac-LDL and lectin. Furthermore, flow cytometry also was used to identify EPCs. First, the CD 34 and VEGFR-2 antibodies were used to the identification of MNCs (Fig. 1D). Other studies have shown that those cells which in the form of foci-shape during the early stage significantly expressed CD133 (endothelial progenitor marker), while negatively expressed CD31 [6]. We also found that EPCs expressed much CD133 (73.5%) at the stage of spindle-shape cells after 7 days (Fig. 1E, F). With the differentiation of EPCs, the stem cell phenotype CD133 was gradually decreased (6.68%), accompanied with gradually increased endothelial phenotype CD31 (98.4%), indicating that EPCs gradually differentiate into ECs. After two weeks of culture, cells remarkably expressed CD31 but barely expressed CD133 (Fig. 1E, F).

Omentin-1 improved EPCs function

Evidences have showed that omentin-1 could significantly improve ECs functions, however, whether omentin-1 can improve the function of EPCs is still unknown [16]. To explore the effects of omentin-1 on the EPCs, we firstly investigated the proliferation ability of EPCs using EdU and CCK-8 assay, respectively. When EPCs were treated with different concentrations of omentin-1 (0, 100, 300, 500 ng/ml), we found that omentin-1 (300 ng/ml) could significantly promote EPCs proliferation determined by CCK-8 assay (Fig. 2A) which was also confirmed by EdU assay (Fig. 2B, C). Furthermore, we also performed flow cytometry to investigate whether omentin-1 influenced EPCs apoptosis and found that 300 ng/ml omentin-1 could significantly inhibit EPCs apoptosis. Interestingly, 100 ng/ml omentin-1 also showed the obvious inhibitory effect (Fig. 2D, E). Besides, in vitro tube formation assay was used to estimate the angiogenic ability of EPCs and the results revealed that omentin-1 (300 ng/ml) could significantly promote tube formation ability of the EPCs (Fig. 2F, G). Taken together, all these results demonstrated that omentin-1 could enhance the functions of EPCs.

Omentin-1 reduced neointima formation of carotid artery after balloon injury in rats

As we have proved that omentin-1 could dramatically enhance EPCs functions, so we speculated that omentin-1-modified EPCs may improve vascular repair. To detect the effect of omentin-1 on neointima formation in response to vascular injury, rats were treated with Ad-omentin-1 or Ad-GFP (as control) EPCs via tail intravenous injection after subjecting to CAI. The transfection efficiency of adenovirus in EPCs was assessed by GFP using fluorescence microscopy and western blot (Supplementary Fig.1). As shown in Fig. 3A, we performed HE-stained sections from carotid arteries of Ad-omentin-1 or Ad-GFP-treated rats at 14 days after surgery. The results revealed that Ad-omentin-1 EPCs treated rats showed a significant reduction of the neointimal hyperplasia (35.46 ± 16.36% vs 100 ± 25.83%, $P < 0.01$, Fig. 3B) and I/M ratio (74.35 ± 36.94% vs 249.6 ± 64.46%, $P < 0.01$, Fig. 3C) compared with control rats, but no hyperplasia of intima was found in the sham groups (Fig. 3A-C). To further investigate the effect of omentin-1 on vascular cell proliferation in the intima-media area, the proliferation marker Ki67 was assessed by immunohistochemistry in the injured vessel at 14 days after surgery. Quantitative analysis of Ki67

indicated that Ad-omentin-1 EPCs significantly decreased the number of Ki67-positive cells in the intima-media area compared with the control group (19.17 ± 6.85% VS 43.33 ± 9.75 %, $P < 0.01$Fig. 3D, E). In contrast, little Ki67-labeled cells were observed in the sham groups (Fig. 3D, E). It is therefore conceivable that EPCs overexpressed with omentin-1 can lead to the reduction of neointimal hyperplasia after mechanical vascular injury.

Omentin-1 improved EPCs dysfunction damaged by TNF-α

It is well known that arterial injury after stent implantation can activate acute inflammatory response in the injured site especially for TNF-α, which could reduce the number of EPCs [11, 17, 18]. To further determine whether omentin-1 protect EPCs functions damaged by TNF-α, we treated EPCs with TNF-α with or without omentin-1. When EPCs treated with TNF-α, the proliferation of EPCs was markedly decreased which was resumed by omentin-1 (Fig. 4A). The same effect of omentin-1 was also illustrated by EdU assay (Fig. 4B, C). Considering that apoptosis caused by inflammation reduces the tissue repair capacity of EPCs, we examined the protective effect of omentin-1 on EPCs apoptosis induced by TNF-α. As a result, we observed that TNF-α mainly induced late phase apoptosis (Annexin V^+ / PI^+), had part effect on the early apoptosis (Annexin V^+ / PI^-), and omentin-1 could significantly reverse the stimulative apoptosis of EPCs induced by TNF-α (Fig. 4D, E). Besides, in vitro tube formation of EPCs was markedly weakened incubated with TNF-α whereas omentin-1 could obviously reverse the damage (Fig. 4F, G). All these results suggested omentin-1 could reverse EPCs dysfunction under the inflammatory environment.

Omentin-1 protects EPCs function under inflammation through p38/CREB pathway

Previous study has demonstrated p38 MAPK and its downstream target CREB participated in the impairment of EPCs induced by TNF-α, so we further investigated whether omentin-1 could protect EPCs from TNF-α through p38/CREB pathway [11]. As confirmed previously, we also found that TNF-α could induce the phosphorylation of p38 [11]. however, omentin-1 could significantly abolish p38 phosphorylation induced by TNF-α (Fig. 5A, B). Meanwhile, the phosphorylation of downstream molecule CREB, a transcription factor, was also activated by TNF-α, which was also inhibited by omentin-1 (Fig. 5A, C). Therefore, these results demonstrated that omentin-1 improved EPCs functions damaged by TNF-α may through inhibition of p38/CREB pathway.

Anisomycin could reverse the protective effects of omentin-1 on EPCs

In the above experiments, we have proved that omentin-1 protected EPCs functions by inhibiting p38/CREB pathway. To further verify these results, we incubated EPCs with anisomycin (a p38 agonist) in the presence of TNF-α and omentin-1, and found it significantly decreased the the proliferation as well as tube formation of EPCs. (Fig. 6A, B and E, F). Meanwhile, we also found that when p38 was activated, that the apoptosis was remarkably increased as indicated by flow cytometry (Fig. 6C, D). Consistently, we further examined the expressions of p38/CREB pathway under the treatment of anisomycin. It is obviously that p38 phosphorylation was significantly activated, and its downstream phosphorylated

CREB also showed the same trend (Fig. 6G-I). All these results clearly stated that p38 agonist anisomycin could offset the protection of omentin-1 on EPC when damaged by TNF-α.

Discussion

In this study, we investigated the role of omentin-1 in improving EPCs functions during vascular repair in rats and the underlying mechanism. We found that omentin-1 could significantly promote EPCs proliferation, tube formation, as well as inhibit apoptosis. Based on these results, we also found that EPCs overexpressed with omentin-1 using adenovirus vectors could significantly reduce the neointimal hyperplasia after CAI in rats. Furthermore, we expounded the underlying mechanism involved in this process in vitro. We demonstrated that omentin-1 could ameliorate TNF-α-induced impairment in proliferation, tube formation, and apoptosis of EPCs via p38/CREB pathway. Collectively, these findings mainly suggested that omentin-1 attenuated the formation of neointima after arterial injury by enhancing the functions of EPCs under inflammatory responses.

Based on the preclinical and clinical data, EPCs therapy has been suggested as a potential therapeutic strategy for arterial injury [8, 18, 19]. Furthermore, accumulating evidence suggested that EPCs repair the damaged vascular mainly by reendothelialization [20]. However, the success of EPCs therapy still has its limitations. One of them is that the number of EPCs has decreased significantly when long-term exposure to the condition of hyperlipidemia and hyperglycemia [21]. The other is that EPCs therapy is compromised by the CD34-positive cells differentiation into smooth muscle progenitor cells (SMPC) and decreased function of EPCs mainly due to inflammatory cascade in the harsh environment, which in turn inhibits vascular repair and reendothelialization [22–25]. Thus, to circumvent these problems, many approaches have been attempted to enhance EPCs functions.

Omentin-1 is a recently discovered adipokine which is preferentially produced by visceral adipose tissue and acts as an anti-inflammatory mediator [25]. Although omentin-1 has been reported to exert proproliferation, inhibiting apoptosis, increasing secretion of angiogenic cytokines, and enhancing the ability for tube formation of MSCs [26, 27], whose specific role and underlying mechanism in EPCs still unclear. In the present study, we illustrated that omentin-1 could promote the functions of EPCs. by employing different parallel assays including CCK-8, EdU, tube formation and apoptosis to validate the functions of omentin-1. Taken together, our results as well as other studies demonstrated that omentin-1 exhibits beneficial effects for EPCs, ECs and MSCs [16, 26].

As omentin-1 was beneficial for EPCs, therefore we investigated whether EPCs overexpression with omentin-1 could reduce the neointimal hyperplasia after CAI in rats. Actually, we observed that Ad-omentin-1 EPCs could significantly reduce the neointimal hyperplasia after balloon injury. Furthermore, we also detected the proliferation marker Ki67 using immunohistochemistry which was significantly lower in Ad-omentin-1 EPCs group than in the control group. Consequently, these findings suggested that omentin-1 could strengthen the ability of EPCs to reduce the development of balloon injury-induced neointimal hyperplasia in rats. However, the underlying mechanism was remain not completely clarified. It

is known that arterial injury after stent implantation can activate acute inflammatory response, as a result, a large number of inflammatory factors will be increased rapidly, such as TNF-α, interleukins 6 (IL-6), interleukins 8 (IL-8), interleukins 11 (IL-11) [17]. Most notably, TNF-α is a master acute immunoinflammatory response factor expressed in the injured site, which markedly promotes the development of restenosis [17, 18, 28, 29]. Based on these, we speculated that omentin-1 modified EPCs reduced intimal hyperplasia after arterial injury may through anti-inflammation.

Next, we used TNF-α to simulate the inflammatory environment to explore the effect of omentin-1 on EPCs in vitro. Although TNF-α can promote the proliferation of smooth muscle cells and part tumor cells, it also induces apoptosis of aortic ECs [30, 31]. In our experiments, stimulation of EPCs with TNF-α leading decreased proliferation and tube formation function, but the apoptotic cells were also significantly increased at the same time. We further demonstrated that omentin-1 pretreatment markedly reversed TNF-α induced the impairment of EPCs proliferation and the increase of apoptosis. Collectively, these findings suggested that omentin-1 may exert protective property on EPCs in inflammatory environment. We therefore reasoned that the activation of inflammation in restenosis may inhibit the endothelial repair partly through inhibiting the functions of EPCs, which can be reversed by omentin-1.

Further, we explored the underlying mechanism of omentin-1 regulating the functions of EPCs. According to previous study, the classic pathway of p38 MAPK mainly activated by TNF-α [11, 32]. More concretely, the p38 MAPK is mediated by regulation of TNF-α mRNA stability and translation initiation [28]. Compelling evidence has indicated that the activation of p38 MAPK/CREB mainly impaired the proliferation and differentiation of EPCs [11]. In our study, we also confirmed these results that TNF-α could activate p38/CREB in EPCs. Therefore, the activation of p38/CREB pathway in the microenvironment of inflammation results in a major dysfunction on EPCs. Previous study also showed that omentin can inhibited TNF-α induced vascular cell adhesion molecule 1 (VCAM-1) expression via preventing the activation of p38 in VSMCs [33]. Similarly, our results illustrated that omentin-1 could rescue the dysfunction of EPCs damaged by TNF-α, accompanying with the decreased activation of p38 MAPK/CREB. To further confirm the effect of omentin-1 on p38/CREB pathway, we used p38 MAPK agonist anisomycin, which could significantly reverse the protective effect of omentin-1 on EPCs. All the above results strongly demonstrated that omentin-1 reverses the dysfunction of EPCs damaged by TNF-α through p38/CREB pathway. In addition, we didn't investigate the receptor of omentin-1 as well as the direct evidence for omentin-1 to interact with p38 MAPK in EPCs, which need further studies.

Conclusions

In summary, our results demonstrated that omentin-1 could improve the dysfunction of EPCs damaged by TNF-α, as well as EPCs overexpressed with omentin-1 can reduce neointimal hyperplasia in rats with CAI, and the underlying mechanism may be related with the inhibition of p38/CREB pathway. Therefore, targeting omentin-1 in EPCs may be considered as an alternative strategy in the treatment of restenosis.

Abbreviations

EPCs: Endothelial progenitor cells; CAI: Carotid artery injury; GFP: Green fluorescent protein; TNF-α: Tumor necrosis factor-α; PCI: Percutaneous coronary intervention; CHD: Coronary heart disease; VSMCs: Vascular smooth muscle cells; ECs: Endothelial cells; ISR: In-stent restenosis; SD: Sprague-Dawley; MNCs: Mononuclear cells; UEA-1: Ulex europaeus lectin I; LDL: Low-density lipoprotein; Ad-omentin-1: Adenovirus vectors expressing human omentin-1; Ad-GFP: Green fluorescent protein; MOI: Multiplicity of infection; HE: Hematoxylin-eosin; CCK-8: Cell counting kit-8; EdU: 5-ethynyl-2'-deoxyuridine; OD: Optical density; SMPC: Smooth muscle progenitor cells; IL: Interleukins; VCAM-1: Vascular cell adhesion molecule 1

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YL, JPZ and CCL conceived the project, funded this study and revised the manuscript. YX and JPZ designed and performed most experiments, performed statistical analysis and wrote the manuscript. ZSZ partly participated in the animal experiment.

Funding

This study was supported by the National Natural Science Foundation of China (No. 81800277, No. 81670453 and No. 81873509) and Natural Science Foundation of Hunan Province (No. 2018JJ6057).

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

Animals care and experimental procedures were approved by the Animal Research Committee of the Department of Laboratory Animals, Xiangya School of Medicine, Central South University.

Consent for publication

The authors have approved the content and agree to submit it for publication.

Competing interests

The authors declare no conflicts of interest.

Author details

[1]Department of Geriatric Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China. [2]Department of Laboratory Animal, Xiangya School of Medicine, Central South University, Changsha, Hunan 410013, China. [3]National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.

References

1. Mehilli J, Kastrati A, Byrne R A, Bruskina O, Iijima R, Schulz S, Pache J, Seyfarth M, Massberg S, Laugwitz K L, Dirschinger J, Schomig A. Paclitaxel- versus sirolimus-eluting stents for unprotected left main coronary artery disease. J Am Coll Cardiol. 2009; 53(19): 1760-8.
2. Varela N, Lanas F, Salazar L A, Zambrano T. The Current State of MicroRNAs as Restenosis Biomarkers. Front Genet. 2019; 10(10): 1247.
3. Jukema J W, Ahmed T A, Verschuren J J, Quax P H. Restenosis after PCI. Part 2: prevention and therapy. Nat Rev Cardiol. 2011; 9(2): 79-90.
4. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner J M. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997; 275(5302): 964-7.
5. Zhao X, Liu H Q, Li J, Liu X L. Endothelial progenitor cells promote tumor growth and progression by enhancing new vessel formation. Oncol Lett. 2016; 12(2): 793-99.
6. Wei S, Huang J, Li Y, Zhao J, Luo Y, Meng X, Sun H, Zhou X, Zhang M, Zhang W. Novel zinc finger transcription factor ZFP580 promotes differentiation of bone marrow-derived endothelial progenitor cells into endothelial cells via eNOS/NO pathway. J Mol Cell Cardiol. 2015; 87(17-26.
7. Wils J, Favre J, Bellien J. Modulating putative endothelial progenitor cells for the treatment of endothelial dysfunction and cardiovascular complications in diabetes. Pharmacol Ther. 2017; 170(98-115.
8. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, Nickenig G. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res. 2003; 93(2): e17-24.
9. Xu B Y, Xiang M X, Wang J A. Endothelial Progenitor Cells and In-stent Restenosis. Curr Stem Cell Res Ther. 2015; 10(4): 364-71.
10. Yue Y, Wang C, Benedict C, Huang G, Truongcao M, Roy R, Cimini M, Garikipati V N S, Cheng Z, Koch W J, Kishore R. Interleukin-10 Deficiency Alters Endothelial Progenitor Cell-Derived Exosome Reparative Effect on Myocardial Repair via Integrin-Linked Kinase Enrichment. Circ Res. 2020; 126(3): 315-29.
11. Seeger F H, Haendeler J, Walter D H, Rochwalsky U, Reinhold J, Urbich C, Rossig L, Corbaz A, Chvatchko Y, Zeiher A M, Dimmeler S. p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. Circulation. 2005; 111(9): 1184-91.

12. Leandro A, Queiroz M, Azul L, Seica R, Sena C M. Omentin: A novel therapeutic approach for the treatment of endothelial dysfunction in type 2 diabetes. Free Radic Biol Med. 2020;
13. Watanabe T, Watanabe-Kominato K, Takahashi Y, Kojima M, Watanabe R. Adipose Tissue-Derived Omentin-1 Function and Regulation. Compr Physiol. 2017; 7(3): 765-81.
14. Liu F, Fang S, Liu X, Li J, Wang X, Cui J, Chen T, Li Z, Yang F, Tian J, Li H, Yin L, Yu B. Omentin-1 protects against high glucose-induced endothelial dysfunction via the AMPK/PPARdelta signaling pathway. Biochem Pharmacol. 2020; 174(113830.
15. Zhang W, Trebak M. Vascular balloon injury and intraluminal administration in rat carotid artery. J Vis Exp. 2014; 94):
16. Maruyama S, Shibata R, Kikuchi R, Izumiya Y, Rokutanda T, Araki S, Kataoka Y, Ohashi K, Daida H, Kihara S, Ogawa H, Murohara T, Ouchi N. Fat-derived factor omentin stimulates endothelial cell function and ischemia-induced revascularization via endothelial nitric oxide synthase-dependent mechanism. J Biol Chem. 2012; 287(1): 408-17.
17. Joviliano E E, Piccinato C E, Dellalibera-Joviliano R, Moriya T, Evora P R. Inflammatory markers and restenosis in peripheral percutaneous angioplasty with intravascular stenting: current concepts. Ann Vasc Surg. 2011; 25(6): 846-55.
18. Inoue T, Croce K, Morooka T, Sakuma M, Node K, Simon D I. Vascular inflammation and repair: implications for re-endothelialization, restenosis, and stent thrombosis. JACC Cardiovasc Interv. 2011; 4(10): 1057-66.
19. Padfield G J, Newby D E, Mills N L. Understanding the role of endothelial progenitor cells in percutaneous coronary intervention. J Am Coll Cardiol. 2010; 55(15): 1553-65.
20. Werner N, Priller J, Laufs U, Endres M, Bohm M, Dirnagl U, Nickenig G. Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. Arterioscler Thromb Vasc Biol. 2002; 22(10): 1567-72.
21. Liao Y F, Feng Y, Chen L L, Zeng T S, Yu F, Hu L J. Coronary heart disease risk equivalence in diabetes and arterial diseases characterized by endothelial function and endothelial progenitor cell. J Diabetes Complications. 2014; 28(2): 214-8.
22. Jeong H S, Hong S J, Cho S A, Kim J H, Cho J Y, Lee S H, Joo H J, Park J H, Yu C W, Lim D S. Comparison of Ticagrelor Versus Prasugrel for Inflammation, Vascular Function, and Circulating Endothelial Progenitor Cells in Diabetic Patients With Non-ST-Segment Elevation Acute Coronary Syndrome Requiring Coronary Stenting: A Prospective, Randomized, Crossover Trial. JACC Cardiovasc Interv. 2017; 10(16): 1646-58.
23. Valgimigli M, Rigolin G M, Fucili A, Porta M D, Soukhomovskaia O, Malagutti P, Bugli A M, Bragotti L Z, Francolini G, Mauro E, Castoldi G, Ferrari R. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation. 2004; 110(10): 1209-12.
24. Chao J, Guo Y, Chao L. Protective Role of Endogenous Kallistatin in Vascular Injury and Senescence by Inhibiting Oxidative Stress and Inflammation. Oxid Med Cell Longev. 2018; 2018(4138560.

25. Wan W, Li Q, Zhang F, Zheng G, Lv Y, Wan G, Jin X. Serum and Vitreous Concentrations of Omentin-1 in Diabetic Retinopathy. Dis Markers. 2015; 2015(754312.
26. Yin L, Huang D, Liu X, Wang Y, Liu J, Liu F, Yu B. Omentin-1 effects on mesenchymal stem cells: proliferation, apoptosis, and angiogenesis in vitro. Stem Cell Res Ther. 2017; 8(1): 224.
27. Gnecchi M, He H, Noiseux N, Liang O D, Zhang L, Morello F, Mu H, Melo L G, Pratt R E, Ingwall J S, Dzau V J. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 2006; 20(6): 661-9.
28. Sabio G, Davis R J. TNF and MAP kinase signalling pathways. Semin Immunol. 2014; 26(3): 237-45.
29. Zhang Y, Yang X, Bian F, Wu P, Xing S, Xu G, Li W, Chi J, Ouyang C, Zheng T, Wu D, Zhang Y, Li Y, Jin S. TNF-alpha promotes early atherosclerosis by increasing transcytosis of LDL across endothelial cells: crosstalk between NF-kappaB and PPAR-gamma. J Mol Cell Cardiol. 2014; 72(85-94.
30. Lei Q, Gu H, Li L, Wu T, Xie W, Li M, Zhao N. TNIP1-mediated TNF-alpha/NF-kappaB signalling cascade sustains glioma cell proliferation. J Cell Mol Med. 2020; 24(1): 530-38.
31. Rastogi S, Rizwani W, Joshi B, Kunigal S, Chellappan S P. TNF-alpha response of vascular endothelial and vascular smooth muscle cells involve differential utilization of ASK1 kinase and p73. Cell Death Differ. 2012; 19(2): 274-83.
32. Kondreddy V, Wang J, Keshava S, Esmon C T, Rao L V M, Pendurthi U R. Factor VIIa induces anti-inflammatory signaling via EPCR and PAR1. Blood. 2018; 131(21): 2379-92.
33. Kazama K, Usui T, Okada M, Hara Y, Yamawaki H. Omentin plays an anti-inflammatory role through inhibition of TNF-alpha-induced superoxide production in vascular smooth muscle cells. Eur J Pharmacol. 2012; 686(1-3): 116-23.

Figures

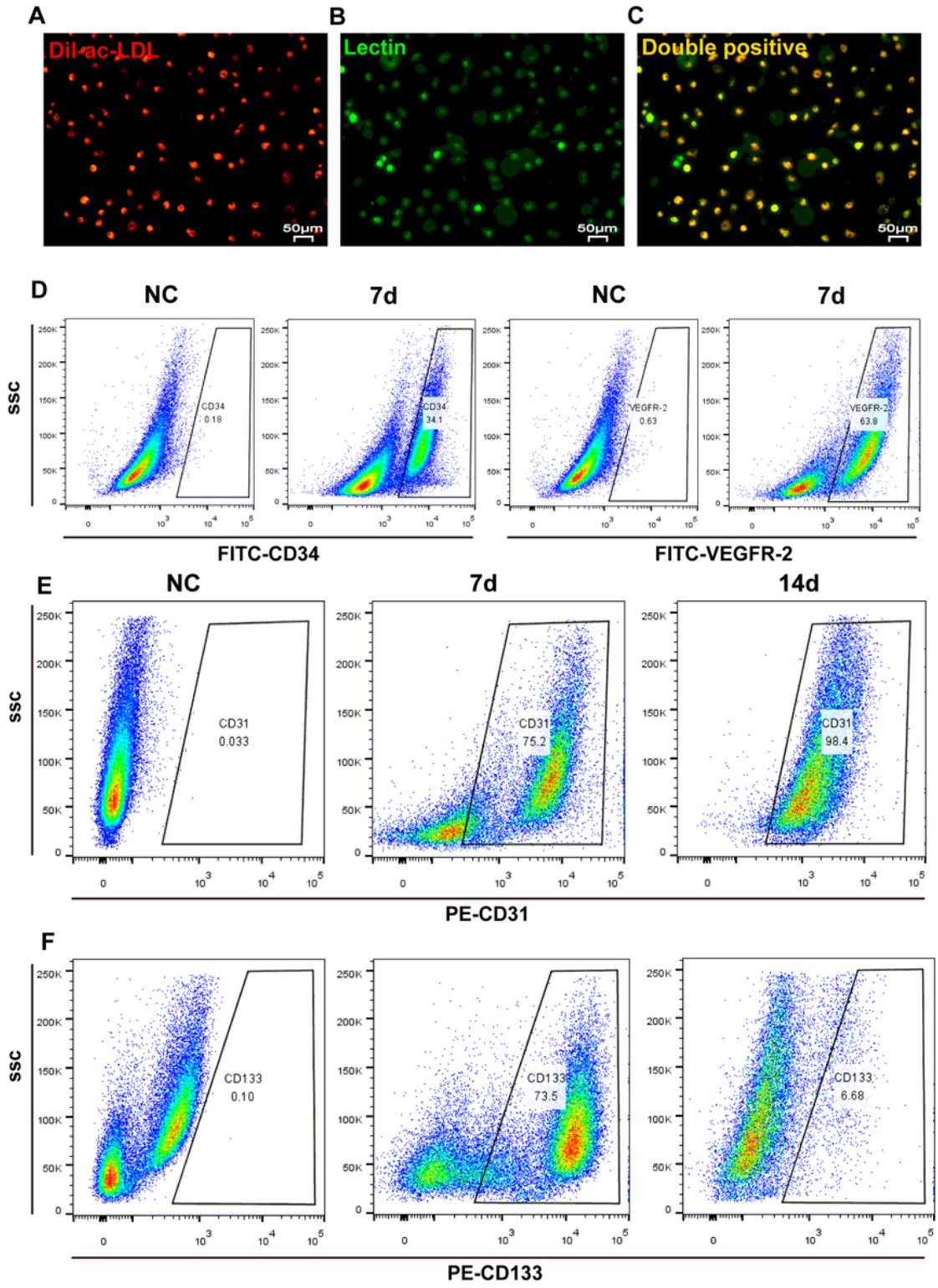

Figure 1

Identification of EPCs derived from rats bone marrow. EPCs was identified at 6-7 days. A-C Representative images of Dil-ac-LDL uptake (red, ×200) and lectin binding (green, ×200), double positive cells were characterized as EPCs, scale bar=50 μm. D FITC-conjugated CD34 and FITC-conjugated VEGFR-2 antibodies were used for the identification of mononuclear cells by flow cytometry. E, F The EPCs

differentiation was stained with antibodies against PE-conjugated CD133 and PE-conjugated CD31 at 7 and 14 days, respectively. NC, as negative controls.

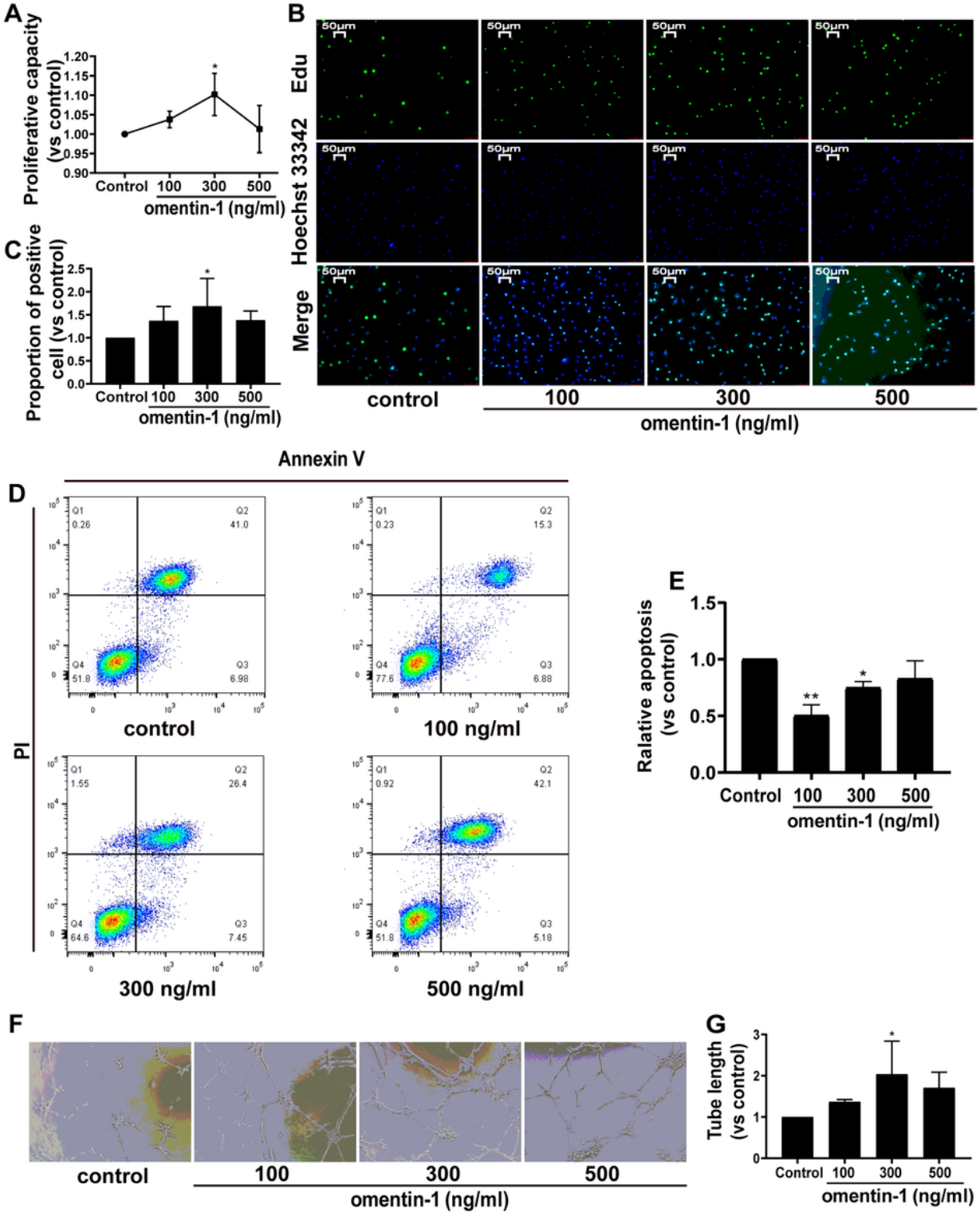

Figure 2

Omentin-1 promotes the functions of EPCs. A-C EPCs were treated with omentin-1 (0, 100, 300 and 500 ng/ml) for 24 h, and then the proliferation of EPCs were examined by CCK-8 and EdU assay, respectively. D, E EPCs were induced to apoptosis with 0.1% FBS in the medium, then continuously incubated with

omentin-1 (0-500 ng/ml) for 24 h. Cells apoptosis were confirmed using Annexin V/propidium iodide (PI) by flow cytometry. Annexin V-/PI- represented viable cells, Annexin V+/PI- cells represented early apoptotic cells, and Annexin V+/PI+ cells represented apoptotic cells in terminal stages. F, G EPCs were incubated in complete medium for 6 h in Matrigel (70 μl) after treated with different concentrations of omentin-1 for 24 h, and the total length of the tube network per field was quantified (magnification ×200). Data were presented as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, vs the control group.

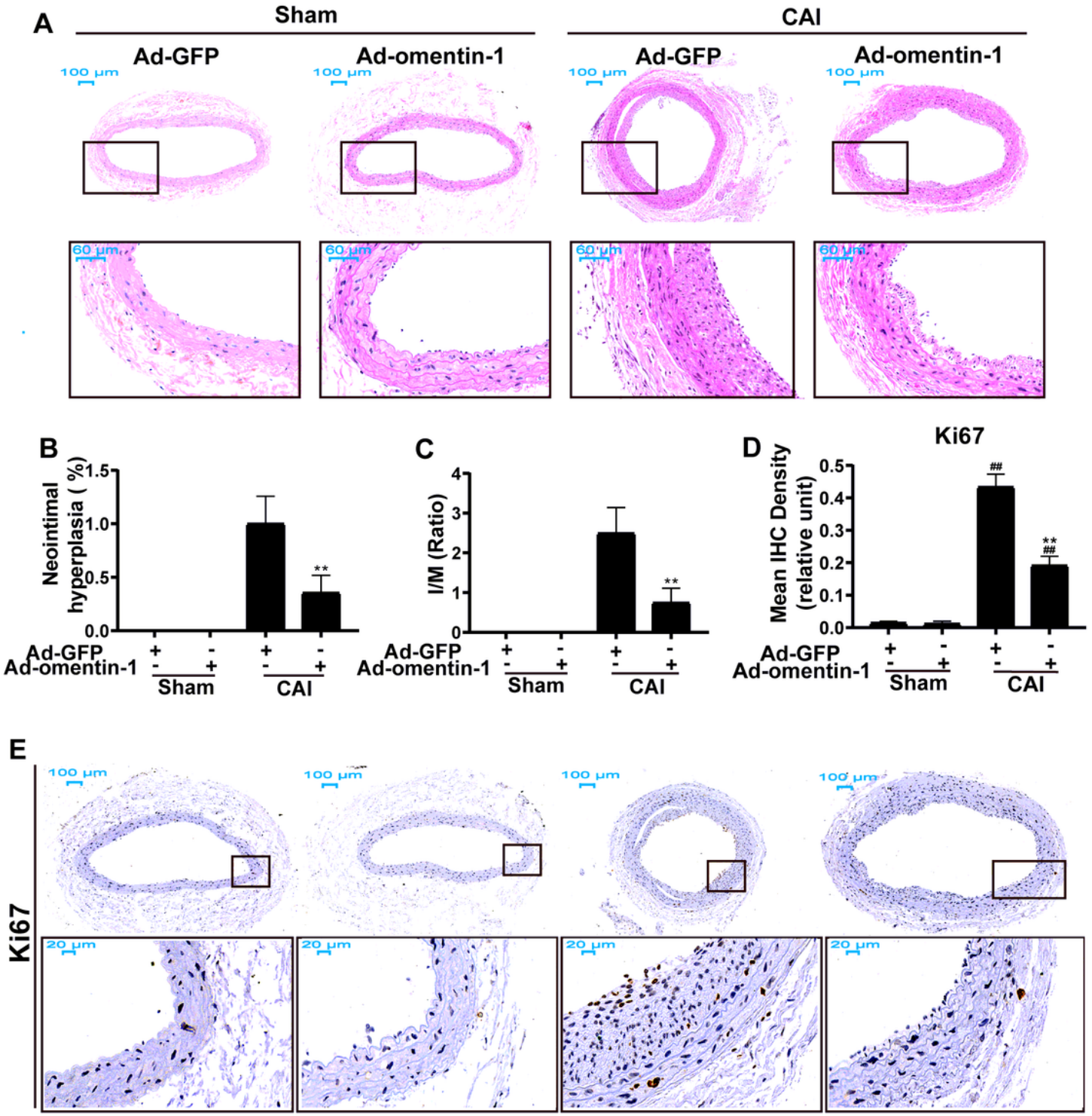

Figure 3

Omentin-1 can reduce intimal hyperplasia after CAI in rats. Balloon-injured rat carotid arteries were harvested 14 days after injury. A Representative images of HE staining in sham and CAI group. B, C Quantification of neointimal growth index and intima-to-media (I/M) ratio. D, E Sections of the left carotid artery were immunostained for ki67. Quantification of IHC intensity of ki67 in the intima-media area. *P < 0.05, **P < 0.01, vs Ad-GFP in CAI group; ##P < 0.01, vs the corresponding control group. n=5-6. Scale bar=100 μm.

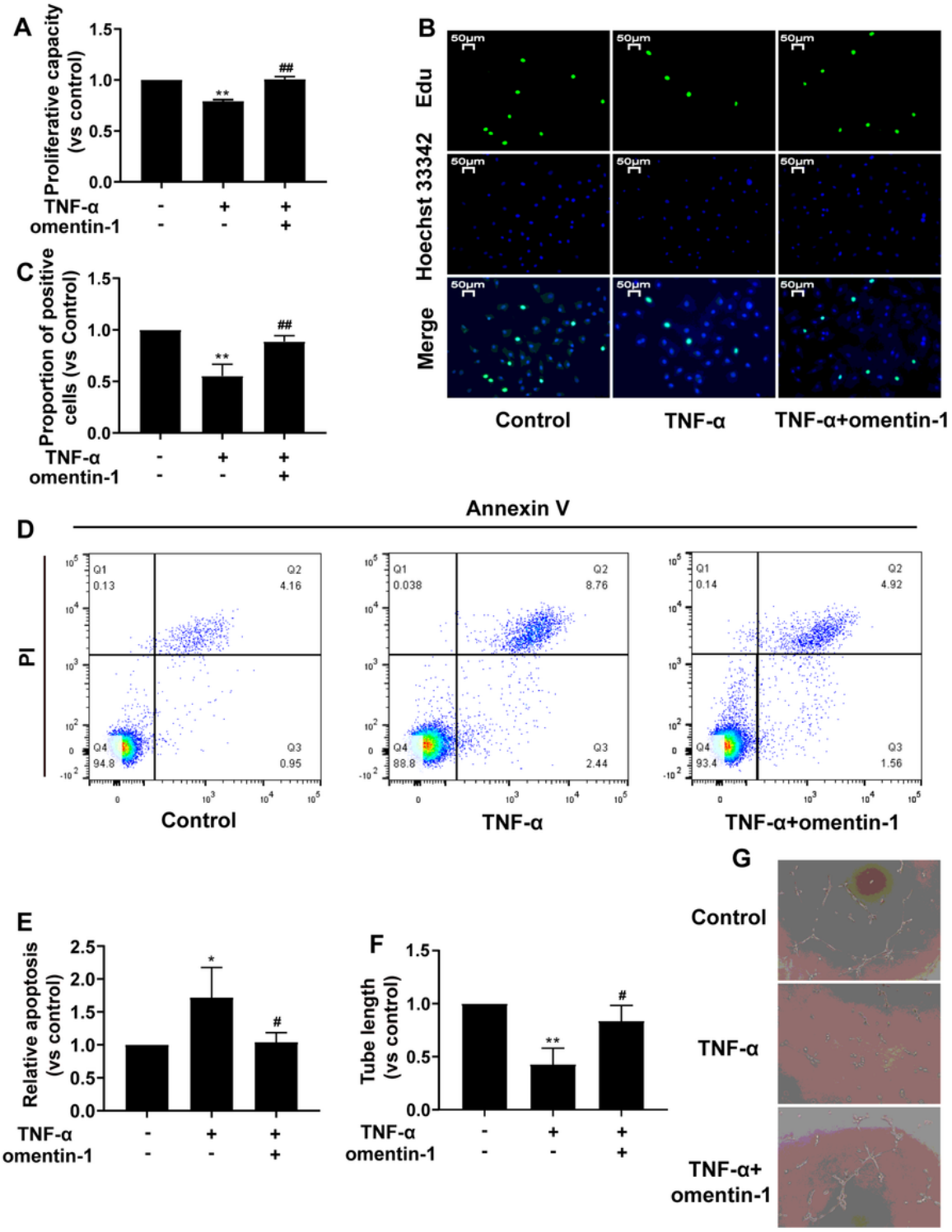

Figure 4

Omentin-1 protects EPCs functions through anti-inflammation. EPCs were preincubated with omentin-1 (300 ng/ml) for 6 h before TNF-α (10 ng/ml) treated with 24 h. A CCK-8 assay was used to determine the proliferation, the optical density (OD) at 450 nm was measured. B, C The proliferation function was also tested by EdU assay, and Hoechst 33342 was used to assess the total number of EPCs (×200). Scale bar=50 μm. D, E The Annexin V/ PI was used to confirm the apoptotic cells by flow cytometry. F, G EPCs were incubated in complete medium for 6 h in Matrigel (70 μl), and the total length of the tube network per field was quantified (magnification ×200). Data were presented as mean ± SD. *P < 0.05, **P < 0.01, for vs control group; #P < 0.05, ##P < 0.01, for vs TNF-α. Each experiment was repeated three times.

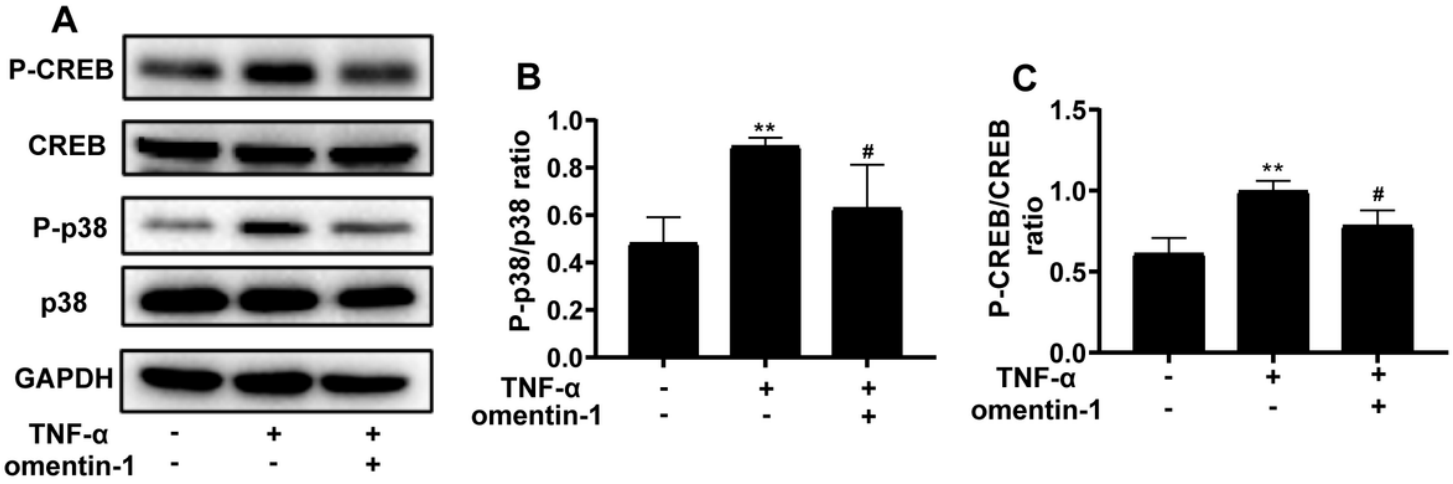

Figure 5

Omentin-1 protect EPCs from TNF-α through inhibition of p38/CREB pathway. The EPCs were preincubated with omentin-1 (300 ng/ml) for 6 h, subsequently treated with TNF-α (10 ng/ml) for 24 h. A-C Representative of western blot images and quantification of P-p38 and P-CREB protein expressions in EPCs. Data are presented as the mean ± SD from three independent experiments. **P < 0.01, for vs control group; #P < 0.05, for vs TNF-α.

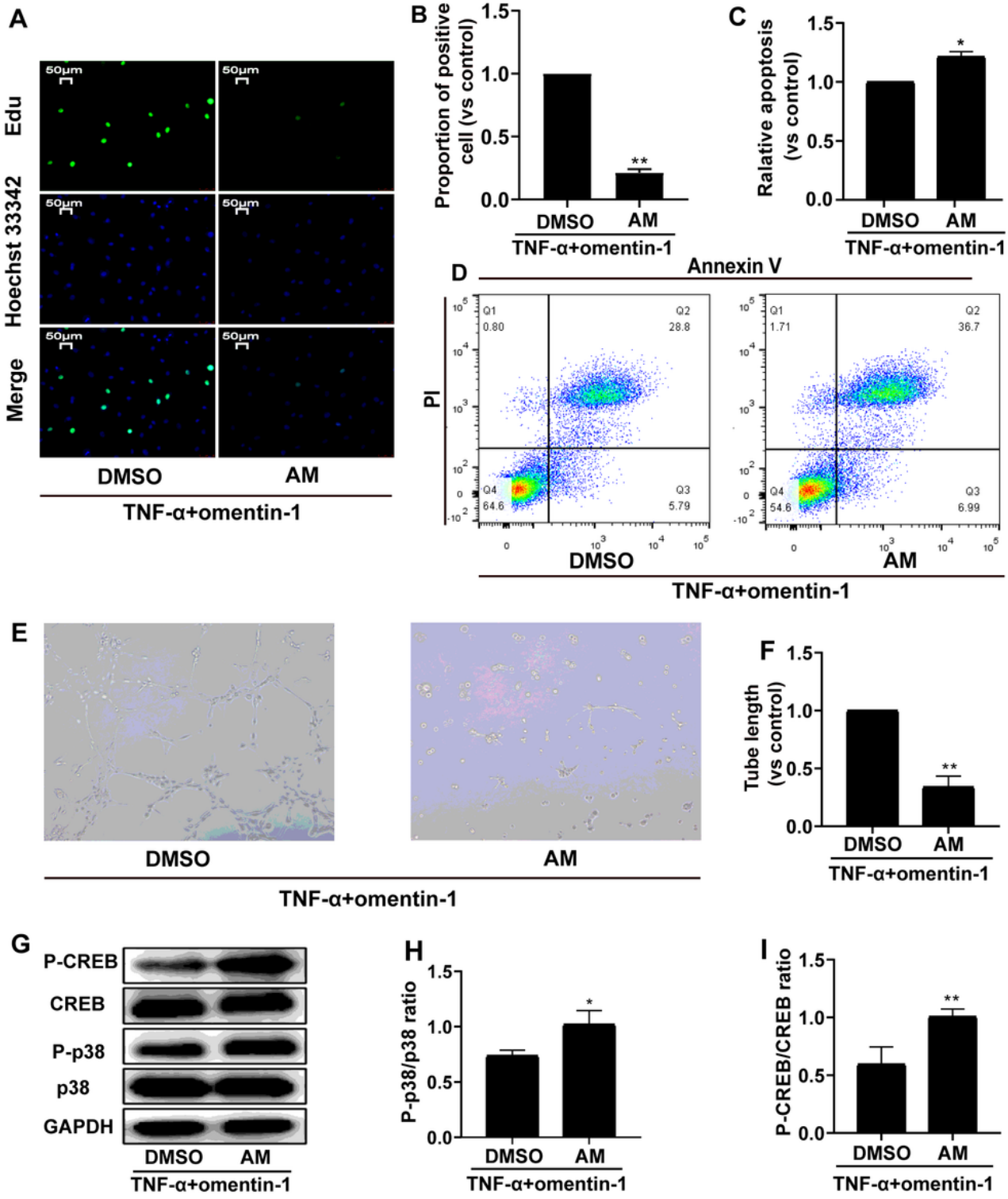

Figure 6

Anisomycin reverses the protection of omentin-1 on EPCs. Preincubation with omentin-1 (300 ng/ml) for 6 h before TNF-α (10 ng/ml) treatment with 30 min, and then together with DMSO or anisomycin (500 ng/ml) for 24 h. A, B EdU assay was performed to examine the proliferation of EPCs, and Hoechst 33342 was used to assess the total cells number. Merge images were used to calculate the proportion of EdU-positive cells. C, D The Annexin V+/ PI+ was used to confirm the apoptotic cells of late stage. E, F

Matrigel was used to investigate the function of tube formation in vitro. EPCs were incubated in complete medium for 6 h in Matrigel (70 μl), and the total length of the tube network per field was quantified (magnification ×200). G-I Representative of western blot images and quantification of phosphorylated p38 and CREB protein expressions. Data were presented as the mean ± SD. *P < 0.05, **P < 0.01, for vs +DMSO group. Each experiment was repeated three times.

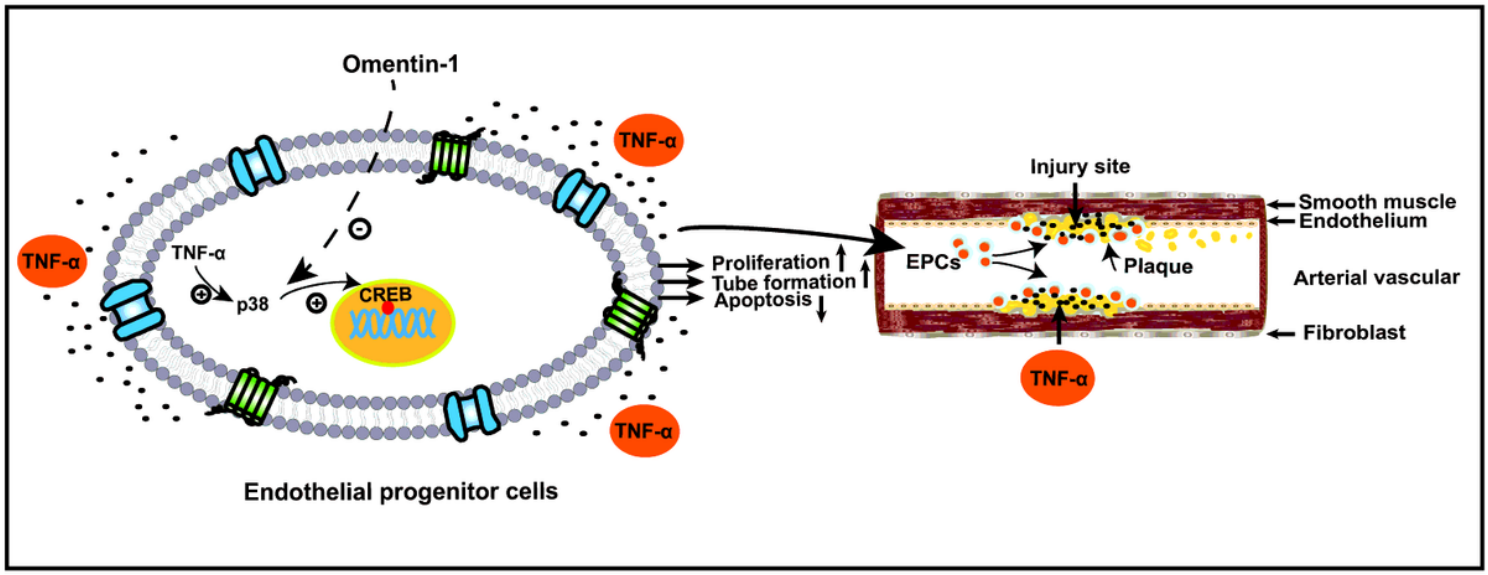

Figure 7

Schematic diagram illustrating the procedure for omentin-1 promotes EPCs function and reduces intimal thickness of injured arteries. In the inflammatory microenvironment, the function of proliferation, tube formation of EPCs was significantly inhibited and apoptosis also strengthened. however, omentin-1 can reverse this effect through p38/CREB pathway. Enhanced function of EPCs promotes endothelial repair and reduces intimal thickness of injured arteries.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.tif