Pitavastatin activates mitophagy to protect EPC proliferation through a calcium-dependent CAMK1-PINK1 pathway in atherosclerotic mice

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Statins play a major role in reducing circulating cholesterol levels and are widely used to prevent coronary artery disease. Although they are recently confirmed to up-regulate mitophagy, little is known about the molecular mechanisms and its effect on endothelial progenitor cell (EPC). Here, we explore the role and mechanism underlying statin (pitavastatin, PTV)-activated mitophagy in EPC proliferation. ApoE−/− mice are fed a high-fat diet for 8 weeks to induce atherosclerosis. In these mice, EPC proliferation decreases and is accompanied by mitochondrial dysfunction and mitophagy impairment via the PINK1-PARK2 pathway. PTV reverses mitophagy and reduction in proliferation. Pink1 knockout or silencing Atg7 blocks PTV-induced proliferation improvement, suggesting that mitophagy contributes to the EPC proliferation increase. PTV elicits mitochondrial calcium release into the cytoplasm and further phosphorylates CAMK1. Phosphorylated CAMK1 contributes to PINK1 phosphorylation as well as mitophagy and mitochondrial function recover in EPCs. Together, our findings describe a molecular mechanism of mitophagy activation, where mitochondrial calcium release promotes CAMK1 phosphorylation of threonine177 before phosphorylation of PINK1 at serine228, which recruits PARK2 and phosphorylates its serine65 to activate mitophagy. Our results further account for the pleiotropic effects of statins on the cardiovascular system and provide a promising and potential therapeutic target for atherosclerosis.

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vascular endothelial injury contributes to the development of major cardiovascular diseases (CVDs), and promoting re-endothelialization after arterial injury is critical for their prevention. Circulating endothelial progenitor cells (EPCs) mobilize from the bone marrow and home to the sites of damaged vascular tissue to re-establish an intact endothelial layer. The level of circulating EPCs and measures of their function are used diagnostically to predict the prognosis of CVDs. Enhancing the proliferation and other functions of EPCs could, therefore, promote the recovery of endothelial integrity and ameliorate prognosis in CVDs.

Pharmacological agents that stimulate EPCs have aroused great interest. Among them, statins are considered the most effective and safe. Statins reduce cholesterol biosynthesis by inhibiting 3-hydroxyl-3-methyl coenzyme A (HMG CoA) reductase, are widely used to treat hyperlipidemia, and are primarily used for CVD prevention. Besides inhibiting cholesterol, statins decrease the production of non-steroidal isoprenoid compounds. Beyond lipid reduction, statins elicit pleiotropic effects across cell types, including anti-inflammatory, anti-oxidation, anti-apoptotic, and antithrombotic effects. Recently, atorvastatin was found to protect mesenchymal stem cells (MSCs) from hypoxia and serum deprivation by activating autophagy, however, it can cause side effects such as increased blood glucose levels. Statin-induced autophagy plays a critical role in diabetogenesis through hepatic gluconeogenesis. Our recent study revealed that store-operated calcium entry (SOCE)-induced autophagy protects EPC proliferation during ox-LDL exposure, providing potential evidence that statin-activated autophagy or mitophagy is related to EPC regulation. The mechanism of action of statins in autophagy or mitophagy induction remains unclear.

Atherosclerosis is a chronic progressive disease with the pathological changes in oxidative stress, immune response, and lipid metabolism, such as uncontrolled accumulation of lipids caused by desialylated lipoproteins. Increasing evidences reveals that mitochondrial dysfunction also contributes to atherosclerosis. In the early stage of atherosclerosis, increased production of reactive oxygen species (ROS) in mitochondria, accumulation of mitochondrial DNA (mtDNA) damage, and progressive respiratory chain dysfunction, resulted in endothelial cells (ECs) dysfunction and vascular smooth muscle cells (VSMCs) phenotypic conversion. After a long term, ECs apoptosis, VSMCs phenotypic conversion, and inflammatory cells infiltration further promoted the development of atherosclerosis and led to vulnerable plaque in the end. Mitophagy is the only mechanism to eliminate these mutant or damaged mitochondria and maintains mitochondrial homeostasis and energy metabolism. Recently, multiple mitophagy programs that operate independently or under crosstalk have been revealed, which function through modulated autophagy receptor activities at the mitochondrial outer membrane (OMM). OMM-localized receptors are ubiquitylated by the E3 ligase PARK2 to recruit ubiquitin-binding autophagy receptors such as NBR1, OPTN, and SQSTM1, which then can attach to autophagosomes via their LC3 interacting region (LIR) motif. OMM-anchor proteins that contain LIR motifs such as BNIP3L/Nix and FUNDC1, regulate mitophagy by phosphorylation. Mitochondrial membrane potential (MMP) dysfunction is observed in statin-induced myopathy, which contributes to pathogenesis. Statins might therefore activate mitophagy through OMM protein ubiquitylation, phosphorylation, and/or binding with autophagosomes, accounting for their pleiotropic effects.

Intracellular calcium concentration ([Ca^{2+}]) change an autophagic regulator is a controversial subject. Most evidence indicates that elevated [Ca^{2+}] levels increase autophagy, but other reports suggest that inositol 1,4,5-trisphosphate receptor (IP3R)-driven calcium signaling suppresses autophagy. Mitochondria are an important intracellular calcium store and actively participate in calcium signaling. Interestingly, statins influence skeletal muscle fibers and B lymphocyte calcium homeostasis through mitochondrial calcium extrusion. However, the action of mitochondrial calcium efflux channels is still under debate, as our laboratory has illustrated that calcium signaling regulates EPC autophagy. We proposed that statins might elicit mitochondrial calcium release in EPCs and be associated with mitophagy induction. Here, we investigated EPC mitophagy and proliferation in atherosclerotic mice, as well as the role of pitavastatin (PTV) in mitophagy induction and EPC proliferation.

Results

EPC proliferation inhibition and mitochondrial dysfunction in atherosclerotic mice. To establish atherosclerotic mice, we fed ApoE−/− mice with a high-fat diet (HFD, 21% fat, and 0.15% cholesterol) for 8 or 16 weeks (Supplementary Fig. 1a). We isolated aortas from mice fed HFD for 8 weeks (HFD8w), where atherosclerotic lesions appeared clearly compared to those on a normal diet (ND). More lesions were observed after 16 weeks of HFD (HFD16w) (Supplementary Fig. 1b). Immunostaining of the lesions showed CD68 positive cells, thus represented macrophage-rich in HFD8w mice atherosclerosis lesions and vascular wall (Supplementary Fig. 2). Additionally, we focused on the immunity state of the established atherosclerotic mouse. Atherosclerosis markedly increased levels of myeloid cell subsets with the highest proportion of neutrophils (Supplementary Fig. 1c). The elevated levels of circulating neutrophils pointed to enhanced myeloid cell supplied by the bone marrow. Likewise, we observed higher levels of the upstream progenitor cells. Compared to control, atherosclerotic mice have significantly more common myeloid progenitor cells (CMP, c-Kit+ CD41+), granulocyte macrophage progenitor cells (GMP, c-Kit+ CD64+ CD16/32+), common lymphoid progenitor cells (CLP, c-Kit+ CD127+ CD93+) and hematopoietic stem and progenitor cells (HSPC, c-Kit+ Sca+ lineage-) (Supplementary Fig. 1d).

EPC proliferation from mice on different diets was evaluated by a new approach of real-time cell analyzer (RTCA) and a traditional method of cell counting kit-8 (CCK-8). RTCA results showed that the normalized cell index of EPCs significantly decreased in HFD8w and HFD16w mice (Fig. 1a). CCK-8 assays revealed that the absorbance intensities decreased by 25.24% in HFD8w mice compared to that in ND mice, and this effect was pronounced in HFD16w mice with a 47.31% reduction (Fig. 1b). MMP in HFD EPCs decreased significantly (Fig. 1c), as indicated by a decrease in the intensity ratio of red to green JC-1 fluorescence compared with that in ND EPCs (Fig. 1e). To further explore the mitochondrial function of EPCs in atherosclerosis, we applied a mitochondrial-targeted fluorescent superoxide sensor. HDF EPCs displayed increased mitochondrial superoxide generation, measured by MitoSOX red fluorescence, indicating ROS accumulation in atherosclerotic mice EPCs (Fig. 1d). Moreover, we observed swollen mitochondria with distorted cristae in HFD EPCs than in ND EPCs using TEM (Fig. 1g). Together, this confirmed that EPC proliferation decreased with mitochondrial dysfunction in atherosclerotic mice.

Mitophagy is impaired in EPCs from atherosclerotic mice. Mitophagy serves as a safeguard in maintaining mitochondrial homeostasis and dynamic. Mitophagic defect or inhibition has been confirmed to accelerate disease progression and worsen the outcome in multiple animal models of CVD. Mitophagy impairing is usually accompanied by mitochondrial...
accumulation, dysfunction, and morphology disorder. Therefore, we examined the mitophagic markers of EPCs from atherosclerotic mice. As shown in Fig. 2a–c, HFD8w significantly decreased the turnover of MAP1LC3B-II and increased SQSTM1 accumulation in EPCs compared with those in control group. These effects were more pronounced in EPCs from HFD16w mice. In addition, we screened the major mitophagic proteins on the mitochondrial membrane in EPCs of atherosclerotic mice (Fig. 2e), where both the accumulation of PINK1 and recruitment of PARK2 were decreased (Fig. 2d, f). However, neither BNIP3L/NIX or MFN2 levels were significantly different compared to those in control mice (Fig. 2g, h).

To further corroborate these findings, we utilized a pH-sensitive tandem GFP-mRFP-LC3 adenovirus to infect EPCs for
24 h before observation using laser scanning confocal microscopy (LSCM). Yellow puncta, which were a combination of RFP and GFP fluorescence, represented autophagosomes, whereas free red puncta represented autolysosomes, where acidic pH quenches GFP fluorescence. The results showed that both the yellow and free red puncta decreased significantly in EPCs of HFD8w and HFD16w mice compared with those in ND mice (Fig. 2i), suggesting that the numbers of both autophagosomes and autolysosomes decreased. In addition, we as well employed mtKeima, a useful tool in the assessment of mitochondrial level. As shown in Fig. 2j, EPCs from HFD8w and HFD16w mice showed a significant lower mitophagy index in comparison to those from ND mice. Both the LSCM and western blots data confirmed that mitophagy was impaired in EPCs of atherosclerotic mouse and that the PINK1/PARK2 pathway might be associated with this process.

PTV reverses mitophagy and improves proliferation of atherosclerotic mouse EPCs. RTCA and CCK-8 were both used to evaluate the proliferation of EPCs from atherosclerotic mice after exposure to 0, 0.1, 0.5, and 1.0 μM PTV. RTCA results showed that the normalized cell index of EPCs significantly increased with the increase of PTV concentration, indicating that PTV increased proliferative ability in a dose-dependent manner. In accordance with RTCA results, CCK-8 assay revealed similar results after different time intervals and doses of PTV treatments (Fig. 3a, b). To check whether mitophagy was associated with this effect, we measured the ratio of red related to green fluorescence intensity in different groups, scale bar: 50 μm. Mitochondrial superoxide levels were labeled with MitoSOX fluorescence indicator for 20 min. Fluorescence was visualized via LCSM. Representative images showed the fluorescence intensity in different groups, scale bar: 50 μm. Mitochondrial morphology was captured by transmission electron microscope. Representative images showed the mitochondrial morphology in different groups, scale bar: 1 μm. (Cells were isolated from 3 mice for 1 experiment and 5 independent experiments were performed, mean ± SD, **P < 0.01).

MAP1LC3B with immunofluorescence. Pearson’s coefficient analysis revealed that the number of mitochondria colocalized with endogenous MAP1LC3B increased after 0.5 μM PTV treatment for 24 h (Fig. 3g), which represented mitophagosome accumulation. Both experiments indicated that PTV improves atherosclerotic EPCs proliferation along with activating mitophagy.

Mitophagy inhibition blocks PTV-induced EPC proliferation improvement in atherosclerotic mice. To address the effects of mitophagy on PTV-induced EPC proliferation amelioration, we utilized gene-silencing as well as pharmacological techniques to inhibit mitophagy activity. Atg7 was significantly knocked-down 72 h after lentiviral infection (Fig. 4a). Reduction in MAP1LC3B-II transformation and ATG12-ATG5 conjugation, as well as accumulation of SQSTM1 in the Atg7-silenced group, further confirmed that mitophagy was effectively inhibited (Fig. 4a). Next, we applied 0.5 μM PTV to Atg7-silenced EPCs for 24 h. The results suggested that inhibition attenuated PTV-induced EPC proliferation improvement compared to that in the PTV alone group (Fig. 4b). A similar pattern emerged after application of 3-methyladenine (3-MA), an autophagy pharmacological inhibitor, before PTV treatment in EPCs of atherosclerotic mice (Fig. 4c). Both datasets demonstrated that PTV-induced mitophagy contributes to EPCs proliferation improvement.

PTV induces mitophagy in EPCs from atherosclerotic mice via PINK1-PARK2 pathway. We detected reduced expression of PINK1 and PARK2 in EPCs from atherosclerotic mice. Thus, to examine whether this was related to PTV-activated mitophagy, we analyzed PINK1 accumulation and PARK2 recruitment in the mitochondria of EPCs. Western blots results suggested that PTV increased PINK1 accumulation and PARK2 recruitment in mitochondrial membrane in a dose-dependent manner (Fig. 4d–f). This was confirmed by immunofluorescence analysis under LSCM. PARK2 was translocated from the cytoplasm to localize at the mitochondrial membrane after 0.5 μM PTV treatment for 24 h (Fig. 4g). Moreover, autophagic marker MAP1LC3B dots were extensively colocalized with PARK2 under PTV treatment, indicating that PINK1–PARK2-mediated mitophagy served as the major effect in EPCs (Fig. 4h).

To further corroborate these findings, we used shRNA to knock down Pink1 and Park2, respectively (Fig. 5a). Silencing Pink1 or Park2 reduced the PTV-mediated increase in MAP1LC3B-II levels (Fig. 5b–e). Additionally, silencing Pink1 or Park2 reversed the proliferation improvement of PTV in CCK-8 assays (Fig. 5f, g). A similar pattern was shown in the RTCA experiment (Fig. 5h, i). For removing the interference of residual PINK1 on mitophagy completely, we established Pink1 KO mice.
As is shown in Fig. 5j, negative signal of PINK1 and less expression of PARK2 in Pink1 KO mice represented the stable CRISPR technique for removing sequence. As expected, EPCs isolated from Pink1 KO mice showed less MAP1LC3B (Supplementary Fig. 5a) and decreased co-localization with mitochondria as compared to the WT mice (Supplementary Fig. 5b, c). Consistent with shPink1 KO mice showed less MAP1LC3B (Supplementary Fig. 5a) and decreased co-localization with mitochondria as compared to the WT mice (Supplementary Fig. 5b, c). Both impairment of mitophagy and EPC proliferative ability were also observed in Pink1 KO mice (Fig. 5k, l). Both impairment of mitophagy and EPC proliferative ability caused by PINK1 ablation can be reversed by PTV treatment. That all strongly indicated that the presence of PINK1 and downstream PINK1-PARK2 pathway are essential for PTV-activated mitophagy, which contributes to maintain the normal EPC proliferation.

PTV elicits mitochondrial calcium release. As it is still unclear whether PTV is capable of activating the calcium signal pathway in EPCs and whether the change in $[Ca^{2+}]$, or $[Ca^{2+}]_m$ is associated with mitophagy induction, we treated EPCs with various concentrations of PTV for 24 h and the calcium probe fluo3-AM. PTV increased fluorescence intensity in EPCs under an LSCM.
The higher the PTV concentration, the stronger the fluorescence intensity was. $F_{\text{min}}$ was detected by measuring fluorescence intensity in the presence of EGTA (extracellular Ca$^{2+}$ chelator) and BAPTA-AM (cell-permeable intracellular Ca$^{2+}$ chelator). $F_{\text{max}}$ was obtained by saturating intracellular Ca$^{2+}$ (ionomycin plus Ca$^{2+}$). Calculation of $[\text{Ca}^{2+}]_i$ (Methods) revealed that PTV significantly increased $[\text{Ca}^{2+}]_i$ (Fig. 6a, b). To further elucidate where the $[\text{Ca}^{2+}]_i$ derived from after PTV treatment, we marked $[\text{Ca}^{2+}]_i$ with fluo3-AM and $[\text{Ca}^{2+}]_m$ with Rhod2-AM in Ca$^{2+}$ free medium, separately. Then we added various concentrations of PTV to Ca$^{2+}$ free medium under LSCM. PTV induced a $[\text{Ca}^{2+}]_i$ transient increase especially 0.5 $\mu$M (Fig. 6c, green line) and 1.0 $\mu$M (Fig. 6c, red line). In rhod2-AM marked Ca$^{2+}$ free medium, we detected a transient $[\text{Ca}^{2+}]_m$ dose-dependent decrease (Fig. 6d, particularly at PTV 0.5 $\mu$M [green line] and 1.0 $\mu$M [red line]). These results strongly suggest that $[\text{Ca}^{2+}]_i$ increase is in part derived from mitochondrial calcium release.
CAMK1 activation contributes to PTV-induced mitophagy.

Intracellular calcium was the major regulator of mitophagy and the downstream protein kinases were reported to induce mitophagy or autophagy. Whether PTV-induced [Ca\(^{2+}\)] \(_i\) increase is associated with mitophagy activation as well is unknown. Therefore, we accordingly measured the activity of calcium-dependent protein kinases. We measured the activity of calcium-dependent protein kinases by screening the phosphorylation status of the CAMK family after PTV treatment. The data indicated that PTV increased phosphorylation of CAMK1 at the Thr\(^{177}\) site in EPCs in a dose-dependent manner (Fig. 6e).

To address the impact of activation of CAMK1 on mitophagy induction, we applied BAPTA-AM (20 \(\mu\)M), a cell-permeable intracellular Ca\(^{2+}\) chelator, to pretreat EPCs before PTV exposure. BAPTA-AM effectively inhibited phosphorylation of CAMK1 at Thr\(^{177}\) site (Fig. 7a, c), meanwhile reversed PTV-induced mitophagy (Fig. 7b, d). We also utilized gene-silencing techniques to knockdown Camk1 (Fig. 7e), which reduced the turnover of MAP1LC3B-II (Fig. 7f, g), the number of autophagosomes (Fig. 7h) and mitophagy index (Fig. 7i) increased by PTV. Furthermore, knockdown Camk1 decreased the colocalization of MAP1LC3B with mitochondria in PTV treatment (Fig. 7j), implicating that CAMK1 was related to PTV-activated mitophagy.

CAMK1 contributes to phosphorylation of PINK1 to induce mitophagy.

In this study, we have confirmed that both the CAMK1 and the canonical PINK1-PARK2 pathway are associated with mitophagy activation after PTV treatment. Consequently, we explored whether CAMK1 was associated with mitophagy activation after PTV treatment. Consistently, we investigated whether CAMK1 was associated with mitophagy activation after PTV treatment. We next employed an in vitro kinase assay to observe whether CAMK1 is associated with PINK1 phosphorylation independent of other proteins. As shown in Supplementary Fig. 6b, in vitro kinase assay further demonstrated that CAMK1 contributed to PINK1 phosphorylation in atherosclerotic EPCs. Thus, our above data supported that CAMK1 is closely associated with PINK1 phosphorylation and PTV-induced mitophagy. However, whether CAMK1 directly and/or indirectly phosphorylates PINK1 still needs further explore.

Mitophagy-dependent homeostasis maintenance and ROS clearance contribute to PTV beneficial effects on EPCs.

PTV treatment alleviated ROS accumulation in the mitochondria and reversed mitochondrial swelling and cristae fracture in atherosclerotic EPCs (Fig. 8d). When mitophagy was inhibited by knockdown of Atg7 or Pink1, the effects of PTV on ROS clearance were decreased (Fig. 8e, g). In addition, both in Atg7\(^{-\text{gt}+}\) EPCs and Pink1\(^{-\text{gt}+}\) EPCs, impairment mitochondria accumulated, mitochondria swelled and cristae fractured in EPCs (Fig. 8d), indicating that PTV-induced mitophagy contributed to mitochondrial homeostasis maintenance. To confirm CAMK1 was associated with this process, we silenced Camk1 by LV transfection before PTV application. We found that the beneficial effects of PTV on mitochondria in atherosclerotic EPCs were reversed in Camk1 silenced groups. Unsurprisingly, compared with the PTV treatment EPCs, decreased MMP (Fig. 8e, g) and accumulation of ROS (Fig. 8f, h) was shown in the shPink1 \(+\) PTV, shCamk1 \(+\) PTV and Atg7\(^{-\text{gt}+}\) PTV groups. These results suggested that CAMK1-PINK1-mediated mitophagy was associated with PTV-involved ROS clearance and mitochondrial homeostasis maintenance in atherosclerotic EPCs.

Disrupting of CAMK1-mediated mitophagy prevents EPC-mediated repairment of vascular endothelium.

To investigate whether CAMK1-mediated EPC mitophagy contributed to repairment of damaged vessels, we established a model of carotid artery intima injury in mice. AcLDL-DiI-labeled pEGFP-N2-EPCs were pre-treated by 0.5 \(\mu\)M PTV for 24h. All these mice have received EPC transplantation treatment. After 7 days of transplantation, labeled cells were traced to home and showed red (AcLDL-DiI) and green fluorescence in line in carotid artery intima (Fig. 9a, white arrow). However, silencing Atg7 or Camk1 before transplantation markedly reduced EPC proliferation and

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**Fig. 3** PTV improved EPC proliferation and reversed impaired mitophagy in atherosclerotic mice. a CCK-8 results showed that PTV improved atherosclerotic EPCs proliferation activity in dose- and time-dependent manners after 0, 12, 24, or 48h PTV exposure at a series of concentrations (0, 0.1, 0.5 or 1.0 \(\mu\)M). b After seeding on E-plates for 24h, EPCs were treated with different concentrations of PTV (0, 0.1, 0.5, or 1.0 \(\mu\)M) respectively (black arrow) and monitored by RTCA. The normalized cell index indicated PTV dose-dependently decreased EPC proliferation. c HFD8w Apoe\(^{-\text{gt}1/2}\) mice EPCs were incubated with PTV (0, 0.1, 0.5 or 1.0 \(\mu\)M) for 24h. Western blots revealed that PTV markedly increased the turnover of MAP1LC3B-II and reversed expression of SQSTM1 in dose-dependent manners. d HFD16w Apoe\(^{-\text{gt}1/2}\) mice EPCs were incubated with PTV (0, 0.1, 0.5 or 1.0 \(\mu\)M) for 24h. Western blots revealed that PTV markedly increased the turnover of MAP1LC3B-II and reversed expression of SQSTM1 in dose-dependent manners. e EPCs from atherosclerotic mice were infected by tandem GFP-mRFP-LC3 adenovirus for 24h before exposure to PTV (0.5 \(\mu\)M) 24h, BAF1 (10 nM) 6h alone. Representative images and quantitative analysis of yellow and free red puncta formation in different groups. Scale bar: 10 \(\mu\)m. f EPCs from atherosclerotic mice were transfected by mtKeima plasmid for 12h before exposure to PTV (0.5 \(\mu\)M) for 24h. Mitotracker Deep Red was used to mark mitochondria and immunofluorescent staining was applied to mark MAP1LC3B in atherosclerotic EPCs. Representative merged images and Pearson’s overlap coefficient analysis showed that the yellow area increased in PTV treatment group compared with HFD group, scale bar: 10 \(\mu\)m. (n = 10 cells per group, EPCs were isolated from Apoe\(^{-\text{gt}1/2}\) mice fed with high-fat diet for 8 weeks, cells were isolated from 3 mice for 1 experiment and 3 independent experiments were performed, mean ± SD, *P < 0.05, **P < 0.01).
home to carotid artery intima in comparison to PTV and PTV + VC groups (Fig. 9a, white arrow). Furthermore, Evans Blue staining was performed to evaluate the reendothelialized area after EPC transplantation in mice. As shown in Fig. 9b, the reendothelialized area in PTV group was significantly larger than in the PTV + shAtg7 and PTV + shCamk1 groups. These results demonstrated that either knockdown Atg7 or Camk1 significantly reduced EPC-mediated reendothelialization of injured carotid arteries, indicating CAMK1-mediated mitophagy is required for EPC function and repairment of damaged vessels.
Fig. 4 PTV activated PINK1-PARK2 dependent mitophagy. a Representative western blots for the detection of ATG7, ATG12-ATG5 conjugate, MAP1LC3B-II, and SQSTM1 after infection showed that Atg7 was successfully knocked down and mitophagy was effectively inhibited in Atg7 silencing group. b Silencing Atg7 before PTV treatment significantly reduced proliferative activity compared with PTV alone group. c 3-MA (2 mM) was added to inhibit mitophagy before 0.5 μM PTV treatment. CCK-8 assay showed that the proliferative activity in 3-MA + PTV group reduced significantly compared with PTV alone group. (d-f) PTV treatment (0, 0.1, 0.5, or 1.0 μM) for 24 h increased PINK1 accumulation and PARK2 recruitment in mitochondrial membrane in dose-dependent manner. g Mitotracker Deep Red was used to mark mitochondria and immunostain was employed to mark PARK2 in atherosclerotic mice EPCs. Merged images and Pearson’s overlap coefficient analysis indicated that PARK2 mostly localized on mitochondria in PTV treatment EPCs related to HFD group, scale bar: 10 μm. h Atherosclerotic mice EPCs were co-immunostained for MAP1LC3B and PARK2. Overlap of PARK2 with MAP1LC3B were observed in PTV treatment EPCs in comparison to HFD group and Pearson’s overlap coefficient was employed to analyze the co-localization, scale bar: 10 μm. (n = 10 cells per group, EPCs were isolated from ApoE−/− mice fed with high-fat diet for 8 weeks, cells were isolated from 3 mice for 1 experiment and 3 independent experiments were performed, mean ± SD, *P < 0.05, **P < 0.01).

Discussion

In the current study, we revealed both proliferation inhibition and mitophagy impairment in atherosclerotic EPCs. We showed that PTV elicits calcium release from mitochondria to activate CAM1K, which increases the level of phosphorylated PINK1 and PINK1 further recruits PARK2; PARK2 then localizes to the mitochondrial membrane and was phosphorylated to activate mitophagy (Fig. 9c). Moreover, CAM1K-PINK1-mediated mitophagy maintains EPC proliferation under atherosclerotic conditions, depending on ROS clearance and maintenance of mitochondrial homeostasis.

Mitochondria are an abundant source of energy in most cell types. Mitochondrial homeostasis depends on mitochondrial dynamics, biogenesis, and the timely removal of worn-out portions. Mitophagy specifically eliminates damaged and DNA mutant mitochondria, and exerts a major role in homeostatic
quality control, as well as contributing to physiological processes, such as erythrocyte maturation and α-synuclein degradation in neurons. MtDNA damage and deletion lead to reduced complex I activity and decreased oxidative phosphorylation, which further increased mitochondrial dysfunction. The accumulation of damaged mitochondria further results in ROS overproduction, cellular disorder, and even apoptosis. In the present study, EPC mitophagy was impaired in the atherosclerotic environment, which resulted in ROS accumulation and EPC proliferation inhibition. On the contrary, the activation of mitophagy ameliorates mitochondrial dysfunction and cell toxicity in disease. In Alzheimer’s disease, mitophagy induction degrades amyloid-beta (Aβ) and reduces plaque formation and memory deficits in animal models. In diabetes mellitus (DM), mitochondrial dysfunction results from advanced glycation end (AGE) product accumulation aggravated by hyperglycemia-induced glycoxidative stress; mitophagy degrades AGEs and clears damaged mitochondria. Mitophagy activation in platelets alleviates thrombotic injuries in DM. Hence, mitophagy safeguards against disease and represents a promising therapeutic target.

Since mitophagy is a form of selective macroautophagy, they share partial molecular processes and pathways, such as both the formation of autophagosomes and mitophagosomes needs MAP1LC3 cleavage and ubiquitination in phagophore and envelops the substrate to lysosomes. In this study, we employed MAP1LC3B and SQSTM1 to evaluate mitophagy, in addition, we found that mitophagy-related PINK1 and PARK2 were decreased in EPC from atherosclerosis mice and mitophagy flux data confirmed mitophagy inhibition, which suggested that mitophagy was the major mechanism instead of macroautophagy. Importantly, the vast majority of MAP1LC3B colocalized with mitochondria, and MAP1LC3B colocalized with PARK2. It is now generally believed that PINK1 was canonical mitophagy protein, and PINK1, PARK2, and ubiquitin have pivotal roles in priming mitophagy. However, the entire regulatory landscape and the precise control mechanisms of mitophagy remain to be elucidated. We used Pink1 KO mice and PINK1 silencing method to disturb mitophagy to confirm that the benefit effect of PTV on EPC contributed to mitophagy reversion. Therefore, we considered that PTV protect EPC dependent on PINK1-PARK2 pathway-mediated mitophagy, rather than macroautophagy.

To date, several mitophagy receptor systems have been mechanistically elucidated. The well-characterized PINK1-PARK2 program targets mitophagy receptors to depolarize mitochondria via ubiquitylation of proteins on the OMM. PINK1 senses the mitochondrial depolarization state, accumulates on the OMM, and recruits PARK2 from the cytoplasm. After recruitment, E3 ligase PARK2 ubiquitylates numerous LIR-containing autophagy receptor proteins in OMM, including SQSTM1, OPTN, and NBR1. These ubiquitylated proteins bind with MAP1LC3 through the LIR motif, which promotes mitochondria engagement and sequestration by autophagosomes. Another mitophagy receptor system is the transmembrane

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**Fig. 6 PTV elicited mitochondrial calcium release and activated CAMK1.** a EPCs were incubated with different concentrations of PTV (0, 0.1, 0.5 or 1.0 μM) for 24 h. Then cells were incubated with calcium probe fluo3-AM. The representative images showed the fluorescence intensity in different groups. EGTA + BAPTA-AM was used to obtain the minimum fluorescence intensity (F<sub>min</sub>) while ionomycin + calcium was used to obtain the maximum fluorescence intensity (F<sub>max</sub>), scale bar: 50 μm. b Quantitative analysis of fluorescence intensity showed that PTV treatment markedly increased intracellular calcium concentration. c Mitochondrial calcium was marked by Rhod2-AM probe. Different concentrations of PTV (0, 0.1, 0.5 or 1.0 μM) were added to the free calcium medium. PTV elicited mitochondrial calcium release in dose-dependent manner. d Intracellular calcium was marked by fluo3-AM probe. Different concentrations of PTV (0, 0.1, 0.5 or 1.0 μM) were added to the free calcium medium. PTV elicited intracellular calcium increase in dose-dependent manner. e Representative western blots for phosphorylation and total CAMK1 after 0.5 μM PTV treatment for 24 h indicated that PTV dose-dependently increased phosphorylation of CAMK1 at Thr<sup>177</sup> site. (EPCs were isolated from ApoE<sup>−/−</sup> mice fed with high-fat diet for 8 weeks, cells were isolated from 3 mice for 1 experiment and 3 independent experiments were performed, mean ± SD, *P < 0.05, **P < 0.01).
Fig. 7 CAMK1 activation contributes to PTV-induced mitophagy. (a–d) EPCs were pretreated with BAPTA-AM (20 mM) for 20 min followed by PTV (0.5 μM) for 24 h. Representative western blots (a) and quantitative analysis (c) indicated that BAPTA-AM significantly decreased PTV-induced CAMK1 phosphorylation at Thr177 site. Representative western blots (b) and quantitative analysis (d) indicated that BAPTA-AM significantly decreased the expression of MAP1LC3B-II induced by PTV. e Lentiviral vector carrying Camk1 shRNA was employed to knockdown Camk1 in EPCs. Representative western blots showed that Camk1 was effectively knocked down. Camk1 was knocked down by shRNA for 72 h before PTV treatment. Representative western blots (f) and quantitative analysis (g) showed that Camk1 knockdown significantly decreased the expression of MAP1LC3B-II induced by PTV. h EPCs from atherosclerotic mice were transfected by lentivirus to knock down Camk1. Camk1 knockdown group, control group, and vector control group EPCs were infected by tandem GFP-mRFP-LC3 adenovirus for 24 h before exposure to PTV (0.5 μM) 24 h. Representative LSCM images showed puncta formation in different groups. Scale bar: 10 μm. Quantitative analysis of yellow and free red puncta. PTV increased the number of yellow puncta compared with control. Camk1 knockdown significantly decreased the number of yellow puncta increased by PTV. i EPCs from atherosclerotic mice were transfected by lentivirus to knock down Camk1. All the groups were infected by mtKeima plasmid for 12 h before treatment by PTV. Representative images showed puncta formation in different groups. Scale bar: 25 μm. PTV treatment increased the red fluorescence intensity turnover, indicating that more mitochondria were transferred to lysosomes. This effect was blocked by silencing Camk1. Quantitative analysis of the fluorescent area showed that PTV increased mitophagy index, but knock down Camk1 blocked this effect. j Merged images revealed that Camk1 knockdown reduced the co-localization of MAP1LC3B and mitochondria in EPCs according to Pearson’s overlap coefficient analysis. Scale bar: 10 μm. (n = 10 cells per group, EPCs were isolated from 3 ApoE−/− mice fed with high fat diet for 8 weeks and 3 independent experiments were performed, mean ± SD, *P < 0.05, **P < 0.01).
Fig. 8 CAMK1 contributes to phosphorylation of PINK1 and PARK2 to PTV-induced mitophagy. EPCs were stably transfected with Camk1 knockdown lentiviral vector 72 h before 0.5 μm PTV treatment for 24 h. a Immunoblot was employed to detect phosphorylation of PINK1 Ser228 and PARK2 Ser65 in each group. We normalized the expression of total PINK1 or PARK2 to compare the phosphorylation level. b Quantitative analysis revealed that PTV significantly up-regulated Ser228 phosphorylation in PINK1 but Camk1 knockdown remarkably reduced this effect in PECs from atherosclerotic mice. c Quantitative analysis revealed that PTV significantly increased PARK2 Ser65 phosphorylation but Camk1 knockdown reversed this effect in PECs from atherosclerotic mice. d PTV treatment reversed HFD8w-induced mitochondrial swelling and rupture of mitochondrial cristae; either PINK1 or ATG7 silencing blocked the effect of PTV on mitochondria of EPCs. e PTV treatment reversed HFD8w-induced ROS production, scale bar: 10 μm. f PTV treatment reversed HFD 8w-induced MMP decreased, scale bar: 10 μm. Quantitative analysis indicated the effect of PTV on ROS (g) and MMP (h) of EPCs can be inhibited by silencing ATG7, PINK1 blocked, and 3-MA pretreatment. (EPCs were isolated from 3 ApoE−/− mice fed with high fat diet for 8 weeks and 3 independent experiments were performed, mean ± SD, *P < 0.05, **P < 0.01).
protein family on OMM, a group of mitophagy receptors that are transcriptionally regulated and engage in mitophagy receptor activity through the phosphorylation status of their LIR motif. This protein group contains BNIP3L/NIX45–47 and FUNDC148. In our study, we have screened the expression of both PINK1, PARK2, and BNIP3L. As PINK1-PARK2 rather than BNIP3L showed significant difference in atherosclerotic conditions as well as after PTV treatment, we infer that the PINK1-PARK2 pathway plays a major role in mitophagy induction in EPCs. As a mitochondrial serine/threonine kinase, PINK1 acts as a sensor and initiates the clearance of the damaged mitochondria under stress, particularly in Parkinson’s disease49 and ischemic acute kidney injury50. PINK1 has been reported to exert both autophosphorylation and phosphorylation activity, affecting cell damage by mitophagy regulation and signal transduction51. The role of phosphorylation at Ser228 of PINK1 has been well-studied during MMP decrease, and this has been considered as the classical mitophagy activation pathway32. Notably, our research provided a finding that increased intracellular calcium concentration could activate PINK1. Together with the fact that both the phosphorylated form of PINK1 and PARK2 were decreased when shCamk1, implying that the activation of CAMK1 may relate to the phosphorylation of PINK1. Though in our study, we employed an in vitro kinase assay to exclude the possibilities of PINK1 phosphorylated by other proteins, there is still lack of sufficient evidence to support that CAMK1 could directly phosphorylate PINK1. Therefore, further study is needed to explore how CAMK1 activates PINK1 phosphorylation.

In the past few decades, CAMKs have been confirmed to play roles in synaptic plasticity, circuit development, and neuronal transmission. The best-characterized CAMKs are CAMK1, CAMK2 and CAMK4 through their neuronal functions52. CAMK1 is highly expressed in many human tissues and is central to the calmodulin-dependent protein kinase cascade and intracellular signal transduction such as endosome formation, Golgi to lysosome transport, ion transport, protein transmembrane transport as well as phosphorylation, and ubiquitin ligase activation53. Thus, we speculated that CAMK1 might also be associated with mitochondria to lysosome transport, despite little information regarding CAMK1 in autophagy or mitophagy regulation. This study reveals that CAMK1 contributed to PINK1 phosphorylation to induce mitophagy. Coupled with our recent research that shows that [Ca2+]i accumulation results in targeting of CAMKK2 to activate autophagy in EPCs15, this study further
The role of statins as vasculoprotective agents is mostly attributed to their circulating cholesterol-lowering effect. However, over the decade, many clinical trials indicated statins can also act as anti-inflammatory, anti-oxidants, immunomodulators, and inhibitors of platelet aggregation[9-12]. This pleiotropy results from the inhibition of the synthesis of essential isoprenoid intermediates and inactivation of related signaling proteins. Statins were also reported to cause epigenetic modifications by promoting histone H3 and H4 acetylation or demethylation by DNMTs inhibition[57,58]. A recent study also found that simvastatin changed the expression of more than 400 miRNAs in different cell types. As individual miRNAs can potentially bind to several miRNAs, potential downstream effects are innumerable[9]. Our findings suggest that PTV reverses protective mitophagy through the PARK2-PARK2 pathway in atherosclerotic mice EPCs. Similarly, Allen et.al found that simvastatin-mediated cardioprotection was dependent on the upregulation of mitochondria through AKT-MTOR suppression in cardiomyocytes[11]. This effect was abolished by supplementation of mevalonate suggesting that it, or downstream isoprenoid intermediates, contributed to statin-induced autophagy or mitophagy in cardiomyocytes. Other reports have indicated that statins inhibit oxidative stress by CYP4, which enhances autophagy and improves muscle health and function in muscular dystrophy[60]. However, in this study, we demonstrate that PTV elicits calcium release and phosphorylates CAMK1, which further regulates the phosphorylation of PINK1 and PARK2 to induce mitophagy. PTV-induced mitophagy was successfully blocked by calcium chelators and Camk1 silencing. The different signaling pathways may be related to the variety of calcium channels and the calcium sensitivity between excited and non-excited cells.

Previous studies have demonstrated that statins improve EPC function in vivo and in vitro. Although not fully elucidated, the mechanisms may involve increased expression of NOS3[61] or mechanism remains elusive. Many reports consider calcium as an intracellular organelle involved in calcium homeostasis in ECs too[67]. In addition, simvastatin triggers a weak mitochondrial Ca2+ efflux through NCLX in skeletal muscle[68]. Another possible mechanism depends on the ubiquitous hydron-calcium exchanger (HCX), the identity of which is still controversial[69]. In addition, the mitochondrial permeability transition pore (mPTP) has non-specific channel property that also contributes to mitochondrial calcium extrusion, but its molecular composition is not yet clear[69]. EPCs work as non-excited cells, it is likely that NCLX is responsible for PTV-elicted mitochondrial calcium release in EPCs. Of course, this needs to be clarified in future studies.

In conclusion, as shown in the schematic drawing (Fig. 9c), EPC proliferation and mitophagy are inhibited in atherosclerotic mice. PTV promotes mitochondrial calcium release and reverses EPC mitophagy, contributing to an improvement in proliferation. The activated mitophagy relied on the mitochondrial calcium release activated by CAMK1, as well as CAMK1-dependent phosphorylation of PINK1 and PARK2.

Our results represent evidence for mechanism for the effect of statins on EPCs through protective mitophagy activation. Enhancing protective mitophagy improves EPC survival rate, protects proliferation, and promotes vascular endothelialization to slow down the progress of atherosclerosis. Furthermore, modification of [Ca2+]2, through mitochondrial calcium release and the activation of CAMK1 may contribute to a therapeutic breakthrough promoting protective mitophagy regulation. Our findings may provide a proliferation-promoting and mitophagy-activating mechanism as well as a therapeutic target in the EPCs of atherosclerotic patients.

Methods

Antibodies and media. Antibodies against mouse MAP1LC3B (2775S), SQSTM1 (5114S), PINK1 (6946S), Mfn2 (9482S), ATG7 (8558S), BNIP3L/NIX (12396) were purchased from Cell Signal Technology. Antibody for PINK1 (ab23707), PARK2 (ab77924), TOMM20 (ab186734), phospho-Thr172-PARK2 (ab62215), CAMK1 (ab68234), AMPK (ab32047) and phospho-Thr172-AMPK (ab133448) were obtained from Abcam. Antibody for phospho-Ser65-PARK2 (bs-19882R) was purchased from Bioss. Antibody for phospho-Ser228-PINK1 (AF7081) was obtained from Affinity Biosciences. Antibody for ATG5 (AP1812A) was from Abgent and the antibody could detect both ATG12-ATG5 conjugate (molecular mass was 55 kDa) as well as free ATG5 (molecular mass was 32 kDa). Antibodies for flow cytometry test were as follows: APC anti-mouse CD64 (139305, Biolegend), BV421 anti-mouse CD16/32 (101332, Biolegend), PE anti-mouse c-Kit (12-1171-82, ebioscience), PE-Cy7 anti-mouse CD41 (25-0411-80, ebioscience), FITC anti-mouse CD127 (12-1171-81, ebioscience), FITC anti-mouse CD93 (12-5892-81, ebioscience).

FEG-2MV BulletKit medium from Lonza included endothelial basal medium (EBM-2, CC-3156) and 10% fetal bovine serum (CC-4101A), recombinant Homo sapiens (Hs) IGF1 (CC-4115A), HsVEGF (CC-4414A), HsEGF (CC-4317A), HsFGF2/FGF-B (CC-4414A), heparin (CC-4396A) and ascorbic acid (CC-4116A). Lympyoprep (1.083, 10381), iodonucin (407950), bafloymycin A1 (BAFA1, B1793), FITC-UEA-1 (L9096) and 3-methyladenine (3-MA, M9281) were purchased from Sigma. Pigvatavastin (PTV, B1124) was obtained from ApexBio Technology.

Isolation and characterization of EPCs. All animal procedures were purchased from the Animal Center of Third Military Medical University (Army Medical University) and approved by the Experimental Animal Ethics Committee of the Third Military Medical University before performing the study and confirmed to the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, USA, 2011). Isolation and characterization of EPCs were performed as previously reported[15]. In brief, male ApoE−/− mice (8 or...
16 weeks age) were anesthetized with an intramuscular injection of 5 mg/kg xylazine and 100 mg/kg ketamine, then sacrificed by cervical dislocation. Bone marrow was harvested from the tibias as well as femurs of mice. Bone marrow-derived mononuclear cells (BMNCs) were isolated by density-gradient centrifugation. At last, the BMNCs were cultivated in EGM-2MV BulletKit medium. As described in our recent work, briefly, characteristic phenotypes of EPCs were identified by immunostained with Dil-acLDL and UEA-1 lectin. The treble-positive cells were then analyzed for the expression of CD313, CD34, VEGF-R2, and CD31 by flow cytometry.

**Blood routine test.** To assess circulating immune cell content in ath erosclerotic mice, peripheral blood was obtained from the orbital sinuses, and 20 µl of whole blood was mixed with 200 µl diluent immediately. The blood cells of the mice were analyzed using Mindray BC-5300 Vet animal automatic hematology analyzer.

**Bone marrow cells isolation and flow cytometric analysis.** Bone marrow was harvested by flushing the femurs and filtered through 40 µm filters. BMNCs were isolated using density-gradient centrifugation followed by washing 3 times in PBS. Next, collected cells were stained with the mixed antibodies for flow cytometry. After staining with the listed markers for 45 min in the dark at 4 °C, samples were washed twice, and transferred to a cell strainer to obtain a single-cell suspension. Finally, cells were analyzed using a flow cytometer (Beckman Gallios).

**Immunohistochemistry.** Aortic root was snap-frozen in optimal cutting temperature compound, and 4 µm frozen sections were cut and the endogenous peroxidase activity blocked with Hematoxylin staining solution (P1000A, Beyotime Biotechnology) for 10 min. Antigen retrieval was performed with quick antigen retrieval solution (Beyotime Biotechnology), then sections were blocked with 5% normal serum, and incubated with the primary antibody (1:100, ab125212, Abcam) for 24 h at 4 °C, followed by biotinylated secondary antibody (P00101, Beyotime Biotechnology) diluted 1:100, and counterstain with hematoxylin (CO107, Beyotime Biotechnology) for 1 min. Clear the tissue slides in 3 times of xylene and coverslip using mounting solution, then sections were observed under microscopy.

**Cell proliferation assays.** Cell proliferation was checked by the xCelligence Real-Time Cell Analyzer instrument (RTCA, ACEA Biosciences, San Diego, CA, USA). Measurement of cell proliferation was described in detail in our previous study.

In addition, we applied traditional cell counting-kit-8 (CCK8, Beyotime Biotechnology, C0038) to evaluate EPC proliferation. EPCs were plated on the 96-well culture plate and underwent different treatments, before addition of WST-8 dye (M0582, Beyotime Biotechnology). At last, the BMNCs were cultivated in EGM-2MV BulletKit medium. As described in our recent works, briefly, characteristic phenotypes of EPCs were identified by Immunofluorescence detection and quantification using the BCA assay (P00102, Beyotime Biotechnology). Total protein was separated by SDS-PAGE and transferred to PVDF membranes. The membrane was blocked at 37 °C for 1 h with 5% non-fat milk and 0.5% Tween-20 (T8220, Solarbio). The membranes were continuously incubated with primary antibodies overnight at 4 °C. The membranes were incubated with HRP-conjugated secondary antibodies at 37 °C for 2 h. Bands of proteins were visualized by chemiluminescence detection and quantified by Image Quant TL software (GE Healthcare, Sweden).

**Immunofluorescence.** EPCs were stained with MitoTracker Deep Red FM (M36008, Molecular Probes, Eugene, OR, USA). EPCs were each group were incubated for 15 min at 37 °C in 0.5 ml measurement buffer containing 5 mM MitoSOX Red. Cells were then washed twice with PBS and observed under LSCM.

**Mitochondrial membrane potential analysis.** Mitochondrial membrane potential (MMP) was measured by MOMP assay kit and JC-1, a marker of mitochondrial activity (C2006, Beyotime Biotechnology). JC-1 accumulates and aggregates in polarized mitochondria (red), and becomes monomeric (green) and retained in cytosol when MMP is lost, so a decrease of red/green fluorescence intensity ratio represents depolarization. Briefly, EPCs were collected and incubated with 0.5 ml JC-1 working solution for 25 min at 37 °C (in the dark) and then washed in cold JC-1 staining buffer twice. Cells were then resuspended in medium. Red fluorescence intensity was measured at 525 nm (ex) and 590 nm (em) and green fluorescence excitation at 490 nm (ex) and 530 nm (em) using LSCM.

**Mitochondrial ROS measurement.** Mitochondrial ROS was measured with Hoechst33258 (5 µM) and Rhod2-AM at room temperature is 570 nM. For detection of mitochondrial ROS, cells were incubated for 24 h. Western blots were used to measure the efficiency of infection Atg7, Pink1, and Park2 in EPCs.

**Gene silencing.** EPCs were cultured on a glass-bottomed dish for 5 days, before addition of lentivirus vector (LV) carrying different shRNA to the medium at a multiplicity of infection of 100. LVs contained shRNA targeted to Atg7 (Hanbio Technology, Shanghai, China), Camk1, Pink1, or Park2 (Gene Pharma, Shanghai, China). After 48 h, transfection media was replaced with fresh media and incubated for 24 h. Western blots were used to measure the efficiency of infection Atg7, Pink1, and Park2 in EPCs.

**Pink1 knockout mouse model.** The Pink1 knockout (KO) mouse model (C57BL/6) was created by CRISPR/Cas-mediated genome engineering (Cygain Biociences). Cas9 and gRNA were co-injected into fertilized eggs and thus disrupting the main kinase domain due to the reading frame shift. The established 8-week Pink1 KO mice have exons 4–7 deleted as target site, which covers 0.92% (~2382 bp) of the coding region.

**Mitochondrial ROS measurement.** Mitochondrial ROS was measured with MitoSOX red (M36008, Molecular Probes, Eugene, OR, USA). Briefly, cells in each group were incubated for 15 min at 37 °C in 0.5 ml measurement buffer containing 5 mM MitoSOX Red. Cells were then washed twice with PBS and observed under LSCM.

**Mitochondrial morphology by transmission electron microscopy.** EPCs were collected, fixed in 2.5% glutaraldehyde at 4 °C for 2 h, and immersed in 1% osmium tetroxide for 2 h. Fixed cells were washed in PBS, dehydrated in acetone, and embedded in Epon 812 (SPI Supplies, West Chester, PA, USA). After slicing the samples were observed under a JEM-1400PLUS TEM (JEOL, Tokyo, Japan) operating at 100 kV.

**Immunoblotting.** EPCs were harvested and rinsed 3 times in ice-cold PBS. Then, cells were lysed by cell lysis buffer (89090, Pierce) containing 2 mM sodium orthovanadate and 0.5 mM PMSF. EPCs were centrifuged in 14000 g for 15 min and protein concentrations measured using the BCA assay (P00102, Beyotime Biotechnology). Total protein was separated by SDS-PAGE and transferred to PVDF membranes. The membrane was blocked at 37 °C for 1 h with 5% non-fat milk and incubated with primary antibody diluted 1:100 overnight at 4 °C. Then, we washed the membrane 3 times with TBS (AR0031, Boster Biological Technology) with 0.5% Tween-20 (T8220, Solarbio). The membranes were continuously incubated with HRP-conjugated secondary antibodies at 37 °C for 2 h. Bands of proteins were visualized by chemiluminescence detection and quantified by Image Quant TL software (GE Healthcare, Sweden).

**Immunofluorescence.** EPCs were cultured on a glass-bottomed dish for 5 days, before addition of lentivirus vector (LV) carrying different shRNA to the medium at a multiplicity of infection of 100. LVs contained shRNA targeted to Atg7 (Hanbio Technology, Shanghai, China), Camk1, Pink1, or Park2 (Gene Pharma, Shanghai, China). After 48 h, transfection media was replaced with fresh media and incubated for 24 h. Western blots were used to measure the efficiency of infection Atg7, Pink1, and Park2 in EPCs.

**In vitro kinase assay.** An in vitro kinase assay was performed as previous published. In brief, 1 μg recombinant PK1 protein (denatured) (ab116177) was...
incubated in the presence or absence of 25 ng CAMK1 for 10 min at 30 °C with the following additions: 10 mM MgCl₂, 0.2 mM ATP, 1 mM CaCl₂, and 1 μM CaM in 50 μL reaction system. Reactions were terminated by boiling in SDS–2-ME dissociation solution and analyzed by immunoblot.

**EPC transplantation and tracing in vivo.** To observe whether the pre-treated EPCs were capable of homing to the site of injury and showing better proliferation capacity, enhanced green fluorescent protein (EGFP)-labeled EPCs were marked by acLDL-Dil (Invitrogen, CA, USA) for 1 h. Then, 200 μL EGFP and acLDL-Dil labeled EPCs (1 × 10⁵) were injected to mice via tail vein. 7 days later, EPC tracking and immunohistochemistry were performed. Images of the stained cells were obtained by a fluorescence microscope (Leica TCS-SP5).

**Measurement of reendothelialization.** Endothelial regeneration was evaluated by staining the denuded areas by injecting 200 μL of 3 % Evans Blue dye with saline via the tail vein into the heart. The left common carotid artery was then harvested 5 mm away from the carotid bifurcation. The reendothelialized area appeared white in color (unstained), whereas the non-endothelialized lesions appeared blue (stained). The unstained areas (in white) and the total carotid artery areas were measured. The ratio of reendothelialized areas (unstained area) versus the total carotid artery area was calculated.

**Statistics and reproducibility.** All analyses were performed using SPSS 19.0 software. The measurement variables are presented as the mean ± standard deviation (SD). Significance was determined using t-test corrected for multiple comparisons (Least-Significant Difference). Nonparametric ANOVA (Kruskal–Wallis) followed by the Dunn multiple comparison post-hoc test was used when one or more datasets showed non-normal distribution. Number of biological replicates and observations were described in the figure legends. Statistical significance was considered at P < 0.05, with *P < 0.01. For graphs, all data were analyzed using GraphPad Prism software (version 5.0 or 8.4.0).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability
All data support the main and supplementary figures are either available online, or available from the corresponding authors upon reasonable request. Source data behind the graphs can be found in Supplementary Data 1.

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Author contributions
L.H. and Y.J. conceived the approach. L.H. provided the overall supervision of the project. The development of methodology was performed by J.Y., M.J.S., and H.T. X.B.G. contributed to the validation of the overall replication. Y.Q.Y. and R.C. specifically performed the experiments. Statistical analysis and generation of figures were carried out by R.Z.C., C.L., and J.H.Z. All authors interpreted the results. J.Y., M.J.S, and H.T. wrote the first manuscript. L.H. and J.Y. wrote the final manuscript on which all authors commented and RC contributed to the process of manuscript revision.

Competing interests
The authors declare no competing interests.

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