Downregulation of Dynamin-Related Protein 1 Contributes to Impaired Autophagic Flux and Angiogenic Function in Senescent Endothelial Cells

Jing-Rong Lin, Wei-Li Shen, Chen Yan, Ping-Jin Gao

**Objective**—Recent studies have shown that altered mitochondrial dynamics impairs the function in senescent endothelial cells (ECs). However, the underlying molecular mechanism remains to be elucidated. Herein, we investigated the role and underlying mechanism of mitochondrial fission protein dynamin-related protein 1 (DRP1) in vascular aging.

**Approach and Results**—We found that DRP1 expression is decreased in senescent ECs, accompanied with long interconnected mitochondria and impaired angiogenic function. In addition, there was marked increase of autophagosomes but not of autolysosomes (assessed as punctate dual fluorescent mCherry-GFP (green fluorescent protein) tandem-tagged light chain 3 expression) in senescent ECs, indicating impaired autophagic flux. DRP1 knockdown or pharmacological inhibition in young ECs resulted in elongated mitochondria, suppressed autophagic flux, premature senescence, and impaired angiogenic function. In contrast, adenoviral-mediated overexpression of DRP1 in senescent ECs restored autophagic flux and improved angiogenic function. EC senescence was associated with the increase of mitochondrial reactive oxygen species and antioxidant N-acetyl-cysteine restored autophagosome clearance and improved angiogenic function. Consistently, en face staining of old rat thoracic aorta revealed a decrease of DRP1 expression and increase of autophagosomes accumulation. Furthermore, in vivo knockdown of Drp1 in common carotid arteries significantly impaired the autophagosome clearance. Importantly, downregulation of Drp1 directly abrogated microvessels outgrowth from ex vivo aortic rings.

**Conclusions**—These results suggest that loss of DRP1 during senescence exacerbates ECs dysfunction by increasing mitochondrial reactive oxygen species and subsequently inhibiting autophagic flux. (Arterioscler Thromb Vasc Biol. 2015;35:1413-1422. DOI: 10.1161/ATVBHA.115.305706.)

**Key Words:** aging ■ autophagy ■ endothelial dysfunction ■ mitochondria ■ reactive oxygen species

Aging is a major risk factor for the development of cardiovascular disease. One of the characteristics of aging tissues is the impaired ability to regenerate, caused by the accumulation of senescent cells. Vascular endothelial cells (ECs) are situated at the interface between the blood and the vascular wall and critically involved in the maintenance of vascular homeostasis in health. Vascular EC with senescence militates against the integrity of the endothelium and impairs angiogenesis, which has been increasingly linked to the process of vascular aging and the development of cardiovascular pathologies. Thus, elucidating the mechanism underlying EC senescence may provide novel therapeutic strategies for vascular diseases.

Mitochondria are highly dynamic organelles whose morphology, distribution, and activity can be regulated by fusion and fission. Mitochondrial dynamics are regulated by the fusion proteins, mitofusin 1 and 2 and optic atrophy 1 (OPA1), and the fission proteins, dynamin-related protein (DRP) 1 and fission 1. The imbalance between mitochondrial fission and fusion affects cellular physiology and pathology. Mutations of Opal cause autosomal dominant OPA, whereas mutations of Drp1 lead to severe abnormal brain development. Interestingly, abnormally rounded mitochondria were found in many senescent postmitotic cell types and aged organisms, and maintenance of elongated mitochondria induces premature senescence. In addition, altered mitochondrial dynamics may impair EC function by increasing the mitochondrial reactive oxygen species (ROS) level in diabetes mellitus. Despite these insights, how mitochondria dynamics impact impaired EC function during senescence is not yet clear.

Dysfunctional mitochondria are selectively removed by autophagy, where the damaged organelle is engulfed by

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autophagosome and subsequently delivered to the lysosome for degradation.\textsuperscript{18} Reduced autophagy results in accumulation of dysfunctional mitochondria and has been linked to aging.\textsuperscript{19,20} Recent studies indicate that autophagy activation may contribute to longevity and inhibition of autophagic proteins significantly shortens life span in \textit{Caenorhabditis elegans}.\textsuperscript{21} More recently, it is implicated that mitochondrial dynamic proteins are involved in the initiation of autophagy in Parkinson disease and the formation of autophagosomes in cardiomyocytes.\textsuperscript{22–24} Nevertheless, little is known about the role of mitochondrial dynamics in regulation of autophagic process during cellular senescence.

In the present study, we sought to address whether endothelial dysfunction during senescence is determined by abnormal mitochondrial dynamics and autophagic process impairment. We investigated the significance of DRP1-mediated mitochondrial elongation in vascular aging and explored that autophagic flux may be a downstream target of DRP1. Our data showed that DRP1 deficiency during senescence inhibited the autophagic flux and eventually led to endothelial dysfunction.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Impaired Endothelial Function in Senescent Human Umbilical Cord Vein ECs

To achieve replicative senescence, freshly isolated human umbilical cord vein ECs (HUVECs) were serially passaged. We termed HUVECs in population-doubling levels <10 as young cells and HUVECs in population-doubling levels >40 as senescent cells. As shown in Figure 1A, senescent HUVECs demonstrated distinctive cellular morphological changes, including increased cell size and cytoplasmic granularity. Senescent HUVECs substantially increased the expression of senescence-associated-\(\beta\)-galactosidase (blue color; Figure 1A), a reliable biochemical marker for cellular senescence activity. Compared with young HUVECs, senescent HUVECs also dramatically upregulated the expressions of p16\(^{\text{INK4a}}\) and p21\(^{\text{WAF1/CIP1}}\) (Figure 1B), cyclin-dependent kinase inhibitors known to accumulate in aging cells and be responsible for cell cycle arrest.\textsuperscript{25,26} To further verify HUVECs as a model for replicative senescence, we measured the senescence-associated secretory phenotype of HUVECs by ELISA. Senescent HUVECs displayed higher levels of interleukin-6, plasminogen activator inhibitor 1, and monocyte chemotactic protein 1 secretion (Figure 1C). The ECs identity was confirmed by high expression of CD31 in both young and senescent HUVECs (Figure 1A in the online-only Data Supplement).

To investigate the impact of cellular senescence on endothelial functions, we examined the in vitro angiogenic capacities...
of young and senescent HUVECs. As shown in Figure 1D, senescent HUVECs formed less developed tubule networks on matrigel, with significant reduction in both the number of tubule and the branch points. In addition, cell migration measured by transwell assay was also significantly attenuated in senescent HUVECs compared with young control HUVECs (Figure 1E).

We then examined endothelial nitric oxide synthase expression in young and senescent HUVECs. Senescent HUVECs showed modest reduction of phosphorylated endothelial nitric oxide synthase (at Ser 1177), with no significant change of total endothelial nitric oxide synthase expression (Figure 1B in the online-only Data Supplement).

**DRP1-Induced Changes of Mitochondrial Morphology in Senescent HUVECs**

To determine the role of mitochondrial elongation in senescence, mitochondrial morphology was analyzed with MitoTracker Mitochondrion Selective Probes. We confirmed that senescent HUVECs exhibited extensively elongated, thread-like mitochondria, whereas young HUVECs displayed smaller punctate mitochondria (Figure 2A). The maintenance of mitochondrial morphology and function depend on a balance between fusion and fission machineries. Next, the mRNA expression levels of fusion and fission genes (Opa1, Mfn1, Mfn2, Drp1, and Fis1) were analyzed by quantitative real-time polymerase chain reaction in young and senescent HUVECs. The transcript level of Drp1 strongly decreased in senescent HUVECs (≈54.9%), whereas the transcripts levels of Mfn1, Mfn2, Opa1, and Fis1 were not significantly altered (Figure 2B). Western blot analysis of DRP1, mitofusin 1, mitofusin 2, OPA1, and fission 1 also confirmed the reduction of DRP1 protein in senescent HUVECs (Figure 2C). Because the activity of DRP1 is important for mitochondrial fission and its activity is determined by its phosphorylation at Ser 616, we then examined the phosphorylation level of DRP1 at Ser 616. We did not observe the significant change of DRP1 phosphorylation between young and senescent cells (Figure 2C). These results together suggest that the elongation of mitochondria in aged HUVECs was mediated by decreasing DRP1 expression.

**Silencing of Drp1 Induces Senescence-Associated Phenotypic Changes and ECs Dysfunction**

To ascertain the role of decreased DRP1 expression and elongation of mitochondria in EC senescence, we first silenced this gene in young HUVECs, using adenoviruses expressing short hairpin RNA of Drp1 (shRNA-Drp1). Reduction of DRP1 protein levels in silenced cells was confirmed by Western blot (Figure 1B in the online-only Data Supplement). To control for off-target effects of shRNA, we re-expressed the Drp1 gene by adenoviral infection in the Drp1-shRNA-expressing cells. Re-expression of Drp1 rescued the depletion of DRP1 protein level and cell functional changes (Figure III in the online-only Data Supplement), confirming that the effect of Drp1 shRNA was specific. Quantitative polymerase chain reaction analysis confirmed that this treatment did not affect other mitochondrial dynamic-related genes (data not shown). Most cells treated with the Ad-NC (adenovirus-encoding non-template shRNA) showed a normal short tubular mitochondrial structure, whereas the Ad-shRNA-Drp1–treated cells had an elongated, net-like structure of mitochondria (Figure IVC in the online-only Data Supplement). After knockdown of Drp1 gene on the cell level, young HUVECs underwent premature senescence with increasing senescence-associated-β-galactosidase staining (Figure 3A), as well as p21 and p16 expression (Figure 3B; Figure 1B in the online-only Data Supplement), accompanied by the increase of interleukin-6, plasminogen activator inhibitor 1, and monocyte chemotactic protein 1 secretion (Figure 3C). We further assessed the effects of Drp1 downregulation on the ECs functions. We found that the ability of the cells to form tubule networks on matrigel and cell migration was largely attenuated in HUVECs with Drp1 knockdown (Figure 3D and 3E).

To further confirm the role of loss-of-DRP1 function in EC senescence, we treated young HUVECs with mdivi-1 (30 μmol/L; 24 hours), a specific inhibitor of DRP1. Mdivi-1 treatment resulted in significant elongation of the mitochondrial network in ECs and increased mitochondrial connectivity (Figure IVC in the online-only Data Supplement). Consistent with the effect of knockdown of Drp1 by Ad-shRNA-Drp1, mdivi-1 treatment induced the senescent phenotype (Figure 3A–3C) and resulted in endothelial dysfunction,
including impaired EC tube formation and cell migration (Figure 3D and 3E). Furthermore, CCK-8 assay shows that there is no significant toxicity observed after Ad-shRNA-Drp1 and mdivi-1 treatment (Figure IV A in the online-only Data Supplement). Taken together, these results indicate that prolonged depletion of Drp1 can induce premature senescence and loss of angiogenic capacities.

**Impairment of Autophagic Degradation Causes Endothelial Senescence and Dysfunction**

Turnover assays of autophagy-related proteins such as microtubule-associated protein light chain 3 (LC3) and p62 are used as indicator of autophagic flux. To address the role of autophagic process in cellular senescence, chloroquine (5 μmol/L; 24 hours), the lysosome acidification inhibitor, was introduced into young and senescent HUVECs to interrupt the degradation of autophagosomes. Cell viability assay revealed no cytotoxicity by chloroquine treatment (Figure V A in the online-only Data Supplement). Inhibition of lysosomes led to a significant accumulation of autophagosomes in young HUVECs, as indicated by the increased level of microtubule-associated protein light chain 3B (LC3-II) and p62 in the presence of chloroquine, suggesting intact autophagic flux (Figure 4A). However, there is no significant change in the level of LC3-II and p62 in senescent HUVECs, indicating that there is already-impairment of autophagosomes processing in senescent HUVECs (Figure 4A).

To further support the observation of impaired autophagosome processing with endothelial senescence, the relative abundance of autophagosomes and autolysosomes was assessed with adeno-virally transduced mCherry-green fluorescent protein (GFP) tandem-tagged LC3 as described previously. In the acidic environment of autolysosomes, red fluorescence of mCherry retains its fluorescence, whereas GFP loses its fluorescence. As a result, autophagosomes possess both red and green fluorescence, generating yellow puncta, whereas autolysosomes have only red fluorescence. Young HUVECs demonstrated constitutive (basal) autophagy with a preponderance of autolysosomes (red only dots) and a few autophagosomes (yellow dots; Figure 4B). In contrast, senescent HUVECs exhibited accumulation of autophagosomes and marked reduction of autolysosomes (Figure 4B). Taken together, these results indicate that autophagic degradation was defective in senescent HUVECs.

To determine whether impairment of autophagosome processing is responsible for HUVECs senescence and dysfunction, we pretreated young HUVECs with chloroquine. The effect of chloroquine on in vitro angiogenesis was then examined using matrigel assay and transwell assay. Results showed that young HUVECs treated with chloroquine formed much more incomplete and poorly connected tube networks than those of untreated HUVECs (Figure 4C). Consistently, the number of migration cells was significantly decreased after chloroquine treatment (Figure 4D). Moreover, we found significantly enhanced senescence-associated-β-galactosidase–positive cells and protein level of p21 and p16 after the chloroquine treatment (Figure VB and VC in the online-only Data Supplement). Induction of senescence was also verified by increased expression of other senescence marker interleukin-6, plasminogen activator inhibitor 1, and monocyte...
chemotactic protein 1 (Figure VD in the online-only Data Supplement).

Role of DRP1 in the Regulation of Autophagic Activity

The question raised here is whether the accumulation of autophagosomes is mainly because of the mitochondrial elongation by decline of DRP1 in senescent HUVECs. To address this question, we first assessed the inhibition of DRP1 by pharmacological (mdivi-1) or genetic (shRNA-mediated knockdown) approach in young HUVECs. We found that inhibition of DRP1 resulted in accumulation of the autophagosomes without an increase in autolysosomes (Figure 5A). Inhibition of DRP1 also provoked an increase in the protein level of LC3-II and p62 in young HUVECs, and however, there is no further increase in the levels of LC3-II and p62 after additional treatment of chloroquine (Figure 5B; Figure VIA and VIB in the online-only Data Supplement), suggesting the impaired autophagosome clearance. Next, we overexpressed Drp1 in senescent HUVECs, using adenovirus-encoding human Drp1. Overexpression efficiency of Drp1 in HUVECs was confirmed by Western blot (Figure IIIB in the online-only Data Supplement). Senescent HUVECs overexpression Drp1 showed fragmented mitochondrial morphology (Figure IVC in the online-only Data Supplement). We found that overexpressing Drp1 in senescent HUVECs decreased the protein levels of LC3-II and p62 and increased the autolysosome number (Figure 5A), but in the presence of chloroquine, the LC3-II and p62 levels were significantly increased (Figure 5B; Figure VIA and VIB in the online-only Data Supplement), indicating the restoration of autophagic flux. These data suggest that the DRP1-induced mitochondrial elongation during endothelial senescence provokes autophagosome formation but impairs their clearance.

DRP1 Regulates Autophagic Flux and Endothelial Function Through Modulating ROS Level

We sought to identify the signals involved in the effect of mitochondrial elongation followed by Drp1 downregulation on the autophagic process in senescent ECs. Cellular senescence can be induced by sublethal stresses, such as accumulation of ROS and DNA damage. Furthermore, the accumulation of mitochondrial damage is one of the most widely accepted causes of aging. Therefore, we examined the mitochondrial ROS levels with MitoSOX Red Mitochondrial Superoxide Indicator and found that excessive mitochondrial superoxide was accumulated in senescent HUVECs (Figure 6A). Treatment with N-acetyl-cysteine (NAC; 200 μmol/L; 24 hours), a glutathione precursor that acts as an ROS scavenger reduced the mitochondrial superoxide (Figure 6A). No significant toxicity was observed after NAC treatment compared with the control group (Figure VIIA in the online-only Data Supplement). NAC also increased number of autolysosomes and decreased number of autophagosomes (Figure 6B), indicating a restoration of autophagic flux by NAC treatment in senescent HUVECs. Moreover, antioxidant treatment improved the EC functions (Figure 6C and 6D), diminished the senescence-associated-β-galactosidase staining (Figure VIIB in the online-only Data Supplement) and suppressed the expression of vascular endothelial growth factor (VEGF) (Figure 6E).
of p21, p16, and senescence-associated secretory phenotypes in senescent HUVECs (Figure VIIC and VIID in the online-only Data Supplement), suggesting an important role for mitochondrial oxidative stress in the impaired endothelial functions associated with aging. More importantly, we found that overexpressing Drp1 via Ad-Drp1 strikingly reduced the mitochondrial superoxide and increased the autolysosome number in senescent HUVECs. Depletion or inhibition of DRP1 increased ROS level and impaired the autophagic process in young HUVECs (Figures 5 and 6A; Figure VI in the online-only Data Supplement). To determine whether the Drp1 regulation of autophagic flux was through modulating cellular ROS level, we examined autophagic flux and endothelial function after Drp1 overexpression in the presence of NAC treatment in senescent HUVECs. We observed that Drp1 overexpression and NAC treatment combined had no significant additive effect (Figure 6B–6D; Figure VIIA–VIID in the online-only Data Supplement) compared with only NAC treatment (Figure VI in the online-only Data Supplement). Immunoglobulin G negative controls demonstrated low red channel background (Figure 6B). Next, expression levels of autophagic marker LC3 and p62 were compared in the aortic ECs between young and old rat. LC3 puncta increased in the aortic ECs of old rat (Figure 7B; Figure VIIIA in the online-only Data Supplement), indicative of upregulated autophagosomes. The accumulation of autophagosomes might be resulted from a blockade of downstream autophagy clearance pathway. p62, a protein that

**Figure 5.** Dynamin-related protein 1 (DRP1) regulates the autophagic flux. A, Representative immunofluorescence images demonstrating mCherry-green fluorescent protein (GFP)-LC3 localization in young and senescent human umbilical cord vein endothelial cells (HUVECs) infected with adenoviruses-encoding empty vector (Ad-EV), human Drp1 (Ad-Drp1), Ad-NC, and Ad-shRNA-Drp1 or treated with mdivi-1 (30 μmol/L; 24 hours). Quantitative analysis of autophagosomes (AL; yellow dots), autolysosomes (AL; red only dots) in merged images per cell (n=4 each). Scale bar, 20 μm. *P<0.05, **P<0.01, and ***P<0.005. Data are means±SEM. B, Western blot analysis of p62 and LC3-II in young and senescent HUVECs treated as in A. Quantitative analysis of p62 and LC3-II is shown in Figure VI in the online-only Data Supplement.

**Decreased DRP1 Expression and Impaired Autophagic Flux in Aged Rats**

To determine whether there are senescent vascular ECs in old rat, we examined senescence-associated-β-galactosidase activity in the thoracic aortas obtained from normal young (2 months) and old (22 months) Wistar–Kyoto rats. Strong β-galactosidase staining was observed throughout the surface of aorta endothelium from old rat, whereas aorta from young rat showed a little staining (Figure 7A). To elucidate whether DRP1 is involved in vascular aging in vivo, en face immunofluorescence staining of endothelial DRP1, mitofusin 1, mitofusin 2, OPA1, and fission 1 on rat aorta was performed. We found that only DRP1 was dramatically decreased in the aortic ECs of old rat compared with young rat (Figure 7B; Figure VIIIA in the online-only Data Supplement), whereas other fusion or fission proteins had no significant change (Figure VIIIB in the online-only Data Supplement). Immunoglobulin G negative controls demonstrated low red channel background (Figure 7B). Next, expression levels of autophagic marker LC3 and p62 were compared in the aortic ECs between young and old rat. LC3 puncta increased in the aortic ECs of old rat (Figure 7B; Figure VIIIA in the online-only Data Supplement), indicative of upregulated autophagosomes. The accumulation of autophagosomes might be resulted from a blockade of downstream autophagy clearance pathway. p62, a protein that

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links ubiquitinated aggregates for destruction within autophagosomes and is degraded on autophagosome processing, also increased in the aortic ECs of old rat (Figure 7B; Figure VIIIA in the online-only Data Supplement), suggesting impaired autophagic processing.

To further study the effect of DRP1 on angiogenesis in an ex vivo condition, the migration and proliferation of rat aortic ECs were examined using the aortic ring assay. Aortic rings were infected with Ad-NC or Ad-shRNA-Drp1 and cultured for 9 days in MCDB131 medium. Vasculogenesis was observed after 9 days of culture. Figure 7C shows microvessels outgrowth from the untreated or Ad-NC–infected aortic rings. On the contrary, aortic rings infected with Ad-shRNA-Drp1 significantly reduced outgrowth of capillary sprouts.

To determine whether DRP1 regulates the autophagy in vivo, rat carotid arteries were infected with Ad-NC or Ad-shRNA-Drp1 and cultured for 9 days in MCDB131 medium. Vasculogenesis was observed after 9 days of culture. Figure 7C shows microvessels outgrowth from the untreated or Ad-NC–infected aortic rings. On the contrary, aortic rings infected with Ad-shRNA-Drp1 significantly reduced outgrowth of capillary sprouts.

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Discussion

In this study, we demonstrated that loss of DRP1 during senescence impairs the endothelial function through suppressing the autophagic flux via upregulating mitochondrial ROS level. Several lines of evidence are provided to support our hypothesis. First, DRP1 expression is significantly decreased in senescent ECs, which is paralleled by a decreased activity of autophagy. Second, knockdown of Drp1 or inhibition of autophagic flux leads to the onset of senescence and endothelial dysfunction. Third, DRP1 regulates the autophagic flux by modulating the mitochondrial ROS level.

Mitochondrial fission is critical for the maintenance of mitochondrial function, and alterations of this process have been linked to many human diseases. In mammalian cells, DRP1 and fission 1 regulate mitochondrial fission, and deficiency of Drp1 or Fis1 leads to elongation of mitochondrial tubules, whereas overexpression of either restores mitochondrial morphology. A recent study reports that depletion of the Drp1 gene delays the transformation of filamentous to punctuate mitochondria and retards aging in fungal. In contrast, loss of mitochondrial division increases oxidative damage in mitochondria and causes cell death in mammalian neurons. The appearance of abnormally rounded mitochondria has long been recognized to increase during aging in mammals. Consistent with this notion, we found similar elongated and interconnected mitochondria in senescent HUVECs, which were caused by downregulation of DRP1 expression (Figure 2). Using pharmacological inhibition or
gene silencing, we demonstrated that loss of DRP1 was sufficient to endothelial replicative senescence. Senescence of ECs limiting the ability for angiogenesis and vascular healing has emerged as a contributor to endothelial dysfunction developing. Importantly, this study shows that inhibition of DRP1 induces EC senescence and promotes endothelial dysfunction (Figure 3). Taken together, these data indicate that DRP1 is involved in the endothelial dysfunction during senescence.

Autophagy is an intracellular bulk degradation system important for cell homeostasis, yet how mitochondrial dynamics regulate autophagy in vascular ECs is still an open question. This study provides evidence that DRP1 in HUVECs is profoundly involved in the regulation of autophagy. It should also be noted that in a recent publication Ikeda et al discussed their work on DRP1 in mitochondrial autophagy and cardiac protection of ischemia/reperfusion injury. Thus, our finding in senescent ECs and the finding of Ikeda et al in cardiac cells together further confirm the importance of DRP1 in regulating autophagy and also suggest that this might be a common mechanism in advanced aging and cardiovascular diseases. We used complementary approaches permitting increased accuracy in the assessment of autophagic flux. We used an LC3 construct tandem-tagged with mCherry and GFP probes, which have comparable intensities and photostability in the assessment of autophagic flux. In the present study, we found that inhibition of DRP1 suppressed the autophagosome and lysosome fusion with accumulation of autophagosome number. Moreover, restoration of autophagic degradation by the overexpression of Drp1 in senescent ECs further confirmed the critical role of DRP1 in regulating the autophagic process (Figure 5). Then, inhibiting autophagosome–lysosome fusion by chloroquine in young HUVECs increased the LC3-II and p62 protein to a level comparable with that of senescent HUVECs at basal levels, suggesting impaired autophagosome

Figure 7. Dynamin-related protein 1 (DRP1) regulates the autophagic flux and angiogenic function in vivo. A, Photographs and quantitative analysis of aortic endothelium from young and old rat stained with senescence-associated-β-galactosidase (SA-β-gal) staining. The dashed boxes indicate the area used in En face staining. Scale bar, 5 mm. B, Representative immunofluorescence images of DRP1, microtubule-associated protein light chain 3 (LC3), p62, and negative control (IgG) in young and old rat aortic endothelium. Endothelial cell morphology is shown by CD31 staining. Scale bar, 100 μm. C, Representative micrographs and quantitative analysis of microvessels sprouting in aortic ring infected with Ad-NC or Ad-shRNA-Drp1 (n=6 each). Scale bar, 1 mm. D, Representative immunofluorescence images of LC3, p62 in rat common carotid artery endothelium infected with Ad-NC or Ad-shDrp1. Scale bar, 100 μm. ***P<0.005. Data are mean±SEM.
clearance in senescent HUVECs. Several evidences reveal that autophagy is an essential in vivo process mediating accurate EC function. Pharmacological inhibition of autophagic flux or knockdown of the essential autophagy genes inhibits the maturation and secretion of endothelial von Willebrand factor. Under steady laminar shear stress, autophagy upregulates the endothelial nitric oxide synthase expression and inhibits endothelin-1 expression, whereas blocking autophagy restores the endothelin-1 expression and inhibits the endothelial nitric oxide synthase expression. Autophagy may directly influence the EC function by regulating endothelial cytokine expression. Similarly, our data demonstrated that impaired autophagic activity by chloroquine induced endothelial dysfunction in young HUVECs (Figure 4C and 4D).

Oxidative stress is crucially involved in the aging process in ECs, and we have reported that mitochondrial oxidative stress was significantly augmented in senescent HUVECs. It is noteworthy that ROS scavenger NAC treatment restored the autophagic flux and partly rescued the senescent phenotype of senescent HUVECs (Figure 6). Genetic manipulation of Drp1 demonstrated that the mitochondrial ROS was regulated by the Drp1 in HUVECs (Figure 6A). Furthermore, no significant additive effect on autophagic activity was observed by combining Drp1 overexpression and ROS inhibition (Figure 6B–6D), indicating that DRP1 is an upstream regulator of ROS in the regulation of autophagy. During senescence, loss of DRP1 induces endothelial dysfunction through inhibiting the autophagic process via upregulating ROS level. Impaired autophagy subsequently induces accumulation of damaged mitochondria, which in turn stimulates the produce of more ROS, leading to a perpetuating vicious cycle (Figure IX in the online-only Data Supplement).

Interestingly, we identified that DRP1 was decreased in aortic endothelium from old rat compared with young rat in association with accumulation of autophagosomes (Figure 7). Ex vivo and in vivo downregulation of DRP1 significantly increased the expression level of LC3-II and p62 and inhibited the angiogenesis of aortic rings. Although further medicine intervention experiments in vivo are needed, our results suggest that modulation of DRP1-Ros-autophagic processing signaling may represent a therapeutic approach to treat age-associated vascular pathologies.

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Disclosures
None.

References
Vascular endothelial cell with senescence militates against the integrity of endothelium and impairs angiogenesis, which is though to contribute to the process of vascular aging and the development of cardiovascular pathologies. Recent studies have shown that altered mitochondrial dynamics impairs the function in senescent endothelial cells. However, the underlying molecular mechanism remains unknown. Unveiling the mechanistic detail and identifying novel molecular targets is important for the development of novel therapeutic strategies. Our study demonstrates that mitochondrial fission protein dynamin-related protein 1 is decreased in senescent endothelial cell. Through loss-of- and gain-of-function of dynamin-related protein 1, we proved that dynamin-related protein 1 reduction causes mitochondria dysfunction, increased mitochondrial ROS, impaired autophagic flux, and endothelial cell senescence and dysfunction. Our findings suggest that dynamin-related protein 1 is a critical molecular target in maintaining endothelial homeostatic functions and preventing vascular aging.
Downregulation of Dynamin-Related Protein 1 Contributes to Impaired Autophagic Flux and Angiogenic Function in Senescent Endothelial Cells
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Materials and Methods
Isolation and in vitro culture of HUVECs

Primary human umbilical cord vein endothelial cells (HUVECs) were isolated using collagenase digestion as described previously. Cells were cultured in M199 medium with 20% fetal bovine serum (FBS, Gibco, 16000-044), 50 mcg/ml Endothelial Cell Growth Supplement (ECGS, Biomedical Technologies, BT-203), 100 U/ml Penicillin-Streptomycin (Gibco, 15140-148) and 100 mcg/ml Heparin (Sigma-Aldrich, H4784) in 0.1% gelatin-coated culture dish. When subcultures reached 80–90% confluence, serial passaging was performed by trypsinization, and the number of population doubling level (PDLs) was monitored for further experiments. PDLs were calculated as described previously; briefly, the number of population doublings (PD) that occurred between passages was calculated according to the equation PD=log₂(CH/CS), where CH is the number of viable cells at harvest and CS is the number of cells seeded. We termed HUVECs in PDLs < 10 as “young cells” and HUVECs in PDLs > 40 as “senescent cells”.

Animal Model

The studies were performed in 2 groups of male Wistar Kyoto (WKY) rats: old rats (22 months old, 382 ± 31g body wt) and young rats (2 months old, 172 ± 17g body wt). The temperature was held constant at 24 °C in the animal housing. All animal procedures were approved in accordance with the institutional guidelines established by the Committee of Ethics on Animal Experiments at the Chinese Academy of Sciences.

Determination of SA-β-gal activity

Cytochemical staining for senescence-associated-β-galactosidase (SA-β-gal) was detected as described previously, using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, 9860). Cells with cytosolic blue staining were counted from random fields, and the percentage of SA-β-Gal-positive cells was calculated.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected at the end of treatment and stored at −20 °C until analyzed by ELISA for IL-6, MCP-1, and PAI-1. Samples were assayed according to the manufacturer’s instructions (R&D Systems).

In vitro angiogenesis analysis using matrigel

The matrigel assay was used to assess the spontaneous formation of capillary like structures in vitro, was performed as previously described. HUVECs were plated at a density of 5 × 10⁴ cells/well in 48-well plates previously coated with growth factor-reduced matrigel matrix (BD Bioscience, 356234). After 6 h of incubation, tube formation was observed with a computer-assisted microscope (Nikon). Loops number and branch number were manually counted in random microscopic fields from each well.
**Endothelial migration assay**  
Cell migration assays were performed using 6.5 mm diameter and 8.0 μm pore size Transwells (Costar) coated with 0.5% gelatin as previously described. The lower chamber contained 50 ng/ml VEGF as a chemoattractant. Endothelial cells were prepared in serum-free medium, and 4 x 10^4 cells were added to the upper chamber in migration buffer (M199 containing 0.1% BSA). After 4 h of incubation at 37 °C, cells were removed from the upper surface of the membranes with a cotton swab, and cells that migrated to the lower surface were fixed with 4% paraformaldehyde for 30 minutes and then stained with 0.1% crystal violet (Sigma, C6158) for 10 minutes. Migrated cells were then counted under a microscope.

**Cytotoxicity tests**  
Cell viability was assessed using Cell Counting Kit-8 assay (CCK-8) (Dojindo Laboratories, CK04). In total, 2 x 10^4 cells (100 μl) were seeded on 96-well plates. 24h after the treatment of drugs, 10 μl CCK-8 solution was added to each well followed by 4h incubation at 37 °C. Next, the OD value for each well was read at a wavelength of 450 nm to determine cell viability using a microplate reader. The wells containing only medium were used as a control.

**Quantitative PCR with reverse transcription**  
Total RNA was extracted from different samples using an RNaseasy Plus Mini Kit (QIAGEN, 74134) following the manufacturer's instructions and treated with DNase I (Promega, Z358B-C) for 15 min to eliminate the potential contamination of genomic DNA. cDNA was generated by reverse-transcribed total RNA (1 μg) using oligo (dT) primer and ReverTra Ace reverse transcriptase (Toyobo, FSQ-101). Q-PCR was performed and analyzed by kinetic real-time PCR using the ABI PRISM 7900 system (Applied Biosystems, Foster City, CA, USA) with SYBR Green Realtime PCR Master Mix plus (Toyobo, QPK-212) for relative quantification of the indicated genes. The sequence of primers used in the experiments was shown in Supplemental Table I . The transcript of GAPDH was used for internal normalization.

**Western blot analysis**  
Briefly, Cells were lysed in RIPA lysis buffer (Millipore, 20-188) with phosphatase inhibitor (1 mM sodium vanadate, 1 mM sodium fluoride) and protease inhibitor (1 mM PMSF, 1 ug/ml leupeptin , 1 ug/ml pepstatin) for 30 min on ice. After centrifugation for 15 min at 12,000 g (4 °C), the protein concentration of the samples was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, 23225). The proteins were subjected to SDS-PAGE and immunoblotted with primary antibodies: DRP1 (32898), CD31 (376764), and GAPDH (365062) were purchased from Santa Cruz Biotechnology, p21^{Waf1/Cip1} (AP0713) and p16^{Ink4a} (BS6431) were purchased from Bioworld, phospho-eNOS (Ser1177) (9571), eNOS (9572) and LC3A/B (4108) were purchased from Cell Signaling Technology, p62 (P0067) was purchased from Sigma-Aldrich (1:1000 dilutions). The specific reactive bands were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, 2313 & 2314).
Bands were visualized using the Western Lightning® Plus-ECL (PerkinElmer, NEL104001EA) in an Odyssey Imager and quantified with Image J.

**Mitochondrial morphology staining**
Mitochondrial morphology was detected by MitoTracker Mitochondrion Selective Probes (Molecular Probes, M7514). Living cells were incubated with 50 nmol/L Mito-tracker green for 30 minutes at 37 °C. After the incubation, the cells were washed three times with phosphate-buffered saline (PBS). Fluorescence at excitation/emission wavelength of 590/516 nm was observed using Zeiss inverted fluorescence microscope.

**Construction of recombinant adenoviruses**
Recombinant adenoviruses encoding human Drp1 (Ad-Drp1), empty vector (Ad-EV), short hairpin RNA of Drp1 (Ad-shRNA-Drp1), and nontemplate shRNA (Ad-NC) were prepared as described previously using the pAdEasyTM vector system (Qbiogene). Briefly, human Drp1 was cloned into pShuttle-CMV (Qbiogene, USA), the short heparin Drp1 sequence (forward: AATTCGGACATCATCCAGCTGCCTCAGTGTGCT TCTGAGGCAGCTGGATGATGTCTTTTT; reverse: GATCCTTTCCGAACGTGCAGTAATTCAAGAGATGACACGTTCGGAGAAG) and nontemplate shRNA sequence (forward: GATCCTTTCCGAACGTGCAGTAATTCAAGAGATGACACGTTCGGAGAAG) was cloned into reconstituted pShuttle-U6 (Qbiogene, USA) and homologously recombined in bacteria BJ5183 with pAdeasy-1. The recombinant plasmid was transfected to 293A cells, and recombinant adenoviral plaques were isolated and further purified by two rounds of plaque assays as described previously. The titers of stocks measured by TCID50 assay on HEK293 were 2 × 10^{10} pfu/mL for Ad-EV and Ad-NC, 6 × 10^{9} pfu/mL for Ad-Drp1, and 1 × 10^{10} pfu/mL for Ad-shRNA-Drp1.

**Immunofluorescence imaging**
To monitor the various stages of autophagy, the tandem mCherry-GFP-LC3 (tf-LC3) adenovirus construct was used in this study. GFP fluorescence is quenched in the acidic pH of the lysosomal compartment, thereby limiting the use of GFP-LC3 to the identification of autophagosomes. However, mCherry continues to fluoresce, and mCherry-LC3 can be used to identify both autophagosomes and autolysosomes. By using tf-LC3 and determining the number of red dots that overlay green dots and appear yellow in merged images, the number of autophagosomes can be evaluated. The red dots that do not overlay green dots and appear red in merged images indicate autolysosome formation. In brief, to perform image-based analysis for autophagy, simply infected HUVECs with the tf-LC3 adenovirus for 24 hours, and then the cells were treated and imaged for GFP and RFP by using confocal fluorescence microscopy. Images were acquired and analyzed using Zeiss Axiovision software. Punctate fluorescent tagged LC3 dots were counted and expressed as number per cell.
HUVECs were identified by the expression of endothelial specific-surface markers CD31. Briefly, after washing with PBS, cells were fixed with 4% paraformaldehyde for 30 minutes, blocked for 30 minutes with 5% BSA in PBS and then incubated with primary antibodies against CD31 (1:200 dilution) and fluorescence-conjugated secondary antibody (1:200 dilution, Invitrogen, A-11062). Nuclei were stained with Hoechst. Images were acquired and analyzed by Zeiss inverted microscope.

**En face immunofluorescence staining**

Immunofluorescence staining of rat aortic ECs were performed as described previously. Briefly, rats were anesthetized with pentobarbital sodium (30 mg/kg body weight). Subsequently, the whole thoracic aorta was dissected, stripped of adventitial tissue carefully, cut open longitudinally, permeabilized with 0.4% Triton X-100 in PBS for 10 min and blocked with 10% Normal Goat Serum in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature. Next, aortas were incubated rabbit anti-DRP1 or rabbit anti-LC3 or rabbit anti-p62 and mouse anti-CD31 in the blocking buffer overnight at 4 °C. After rinsing with washing solution 3 times, fluorescence-conjugated secondary antibodies (1:500 dilutions, Alexa Fluor 555 labeled anti-rabbit IgG, Invitrogen, A-31572; Alexa Fluor 488 labeled anti-mouse IgG, Invitrogen, A-10680) were applied for 1 h at room temperature. Finally, after another 3 rinses in the washing solution, aortas were mounted in the Fluorescence Mounting Medium (Dako, S302380). Aortas were examined by a laser-scanning confocal microscope (Zeiss) with 40x lens.

**Ex vivo Aortic ring assay**

To examine the migration and proliferation of rat aortic endothelial cells in an ex vivo condition, an aortic ring assay was performed using type I Collagen (BD Bioscience, 354236) as described previously. The whole thoracic aorta was dissected from rats, stripped of adventitial tissue, and cut into 1-mm ring segments. The recombinant adenovirus was isolated from 293 cells and purified by CsCl centrifugation. The titers of stocks were 1 \times 10^{11} pfu/mL for Ad-NC and Ad-shRNA-Drp1. Pieces of aortas were immediately exposed or not to recombinant adenoviruses at 5 \times 10^9 plaque-forming units (pfu) in 0.5ml DMEM in 24-well plate at 37 °C in 5% CO₂. After one day of infection, aortic rings were extensively rinsed with DMEM, placed in 150ul type I collagen coated plates (48-well), and cultured with MCDB131 (Gibco, 10372019) medium containing 2.5% FBS and 30ng/ml VEGF (Peprotech, 40031). Quantify microvessel growth at day 9 by Zeiss inverted microscope.

**In vivo adenoviral gene transfer**

The in vivo adenoviral gene transfer in rat common carotid artery was performed as described previously. Wistar Kyoto rats weighing 250g to 350g were anesthetized with pentobarbital sodium. The right common carotid artery was isolated through vascular clamps to expose a 1.5-cm-segment of the artery from the bifurcation. Adenoviral solution containing Ad-NC or Ad-shRNA-Drp1 (2X10^9 pfu) in PBS was introduced into the common carotid through a syringe placed in the external carotid
artery and incubated for 10 minutes. After the viral solutions were withdrawn, the external carotid artery was ligated, and blood flow was restored. The common carotid arteries were excised at day 3 for further study.

Measurement of mitochondrial oxidative stress
Mitochondrial superoxide was detected by MitoSOX™ Red Mitochondrial Superoxide Indicator (Molecular Probes, M36008). Living cells were incubated with 100 nmol/L MitoSOX Red for 30 minutes at 37 °C. After the incubation, the cells were washed three times with PBS. Fluorescence at excitation/emission wavelength of 510/580 nm was observed using Zeiss inverted fluorescence microscope. Fluorescence intensity was analyzed in independent fields with Image J.

Statistical analysis
Data was expressed as means± standard error of the mean (SEM). Significant differences between the two groups were estimated using Student’s t-test. A P value < 0.05 was considered statistically significant.

References for the supplement
## Table I

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Figure 1. A, Immunofluorescence (red) and western blot analysis of CD31 in both young (Y) and senescent (S) HUVECs. Scale bar: 10 µm. B, Western blot and quantitative analysis of phospho-eNOS and eNOS in young and senescent HUVECs (n = 3 each). *P<0.05 vs. young HUVECs. Data are mean ± SEM.
Figure II. A, Schematic representation of adenoviruses encoding Drp1 (Ad-Drp1) and shRNA-Drp1 (Ad-shRNA-Drp1). B, Immunoblotting analysis for Drp1 protein level in HUVECs after the adenoviral infection (n = 3 each). GAPDH was used as a control. ***P<0.005 vs. Ad-NC (adenoviruses encoding nontemplate shRNA), ##P<0.01 vs. Ad-EV (adenoviruses encoding empty vector). Data are mean±SEM.
Figure III. A, Western blot and quantitative analysis of DRP1 in young HUVECs infected with Ad-NC, Ad-shRNA-Drp1 or Ad-Drp1 (n = 3 each). B, Representative micrographs and statistical summary of SA-β-gal staining of HUVECs treated as in A (n = 4 each). Scale bar: 200 µm C, Photomicrographs and quantification of tube formation on matrigel of HUVECs treated as in A (n = 3 each). Scale bar: 200 µm. D, Quantitative analysis of cell migration with transwell assay in young HUVECs treated as in A (n = 5 each). \*P<0.05, \**P<0.01, \***P<0.005 vs. Ad-shDrp1.
Figure IV. A, Cell viability of HUVECs after 24 h incubation with mdivi-1 (30 µmol/L), or infected with Ad-NC, Ad-shRNA-Drp1 as determined by a CCK8 cell viability assay (n = 3 each). ns, no significant. B, Quantitative analysis of p21 and p16 in HUVECs treated as in A (n = 3 each). C, Representative micrographs of mitochondrial morphology by mito-tracker green staining of HUVECs infected with adenoviruses encoding Ad-NC, Ad-shRNA-Drp1, Ad-EV, Ad-Drp1 or treated with mdivi-1.Dil, saline-diluent control. Scale bar: 20 µm. *P<0.05, **P<0.01 vs. Dil. ##P<0.01 vs. Ad-NC. Data are mean±SEM.
Figure V. A, Cell viability of HUVECs after 24 h incubation with chloroquine (CQ, 5 µmol/L), as determined by a CCK8 cell viability assay (n = 3 each). B, Representative micrographs and quantitative analysis of SA-β-gal staining of young HUVECs treated with CQ (n = 4 each). Scale bar: 200 µm. C, Western blot and quantitative analysis of p21 and p16 protein levels in young HUVECs treated with CQ (n = 3 each). D, Expression level of IL-6, PAI-1, MCP-1 in young HUVECs treated with CQ by ELISA assay (n = 3 each). **P<0.01, ***P<0.005 vs. saline-diluent control (Dil). Data are mean ± SEM.
Figure VI. A, Quantitative analysis of p62 in young and senescent HUVECs infected with Ad-EV, Ad-Drp1, Ad-NC and Ad-shRNA-Drp1 or treated with mdivi-1 (30 μmol/L, 24 h) (n = 3 each). B, Quantitative analysis of LC3-II in young and senescent HUVECs treated as in A (n = 3 each). *P<0.05, **P<0.01 vs. saline-diluent control. Data are mean ± SEM.
Figure VII. A, Cell viability of HUVECs after 24 h incubation with NAC (200 µmol/L), or infected with Ad-Drp1 as determined by a CCK8 cell viability assay (n = 3 each). B, Representative micrographs and quantitative analysis of SA-β-gal staining of senescent HUVECs treated as in A (n = 4 each). Scale bar: 200 µm. C, Western blot and quantitative analysis of p21 and p16 protein levels in senescent HUVECs treated as in A (n = 3 each). D, Expression level of IL-6, PAI-1, MCP-1 in senescent HUVECs treated as in A (n = 3 each). *P<0.05, **P<0.01 vs. saline-diluent control (Dil). Data are mean ± SEM.
Figure Ⅷ. A, Fluorescence intensity analysis of DRP1, LC3 and p62 in young and old rat aortic endothelium (n = 3 each). B, Representative immunofluorescence images and fluorescence intensity analysis of MFN1, MFN2, OPA1, FIS1 in young and old rat aortic endothelium (n = 3 each). Scale bar: 100 µm. C, Western blot and quantitative analysis of DRP1 in rat common carotid artery infected with Ad-NC or Ad-shRNA-Drp1 (n = 3 each). D, Fluorescence intensity analysis of LC3 and p62 in rat common carotid artery endothelium treated as in C (n = 3 each). *P<0.05, **P<0.01, ***P<0.005. Data are mean±SEM.
Figure IX. Scheme illustrating loss of Drp1 during the senescence exacerbates the impaired ECs function by inhibiting the autophagic flux via upregulating mitochondrial ROS.