SIRT1-induced deacetylation of Akt expedites platelet phagocytosis and delays HEMEC aging

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Maintaining the health of the endothelium is of critical importance to prevention against cell aging. The current study was performed to clarify the role of sirtuin1 (SIRT1) in platelet phagocytosis in cell aging and identified its downstream molecular mechanism. Platelet phagocytosis by human endometrial microvascular endothelial cells (HEMECs) was characterized by transmission electron and fluorescence microscopy. Functional experiments were conducted to examine platelet phagocytosis and cell aging using the overexpression or knockdown plasmids of SIRT1 and G alpha-interacting, vesicle-associated protein (GIRDIN) as well as Akt inhibitor and activator. It was found that SIRT1 facilitated platelet phagocytosis by HEMECs, contributing to inhibition of cell aging. Akt activation facilitated platelet phagocytosis and repressed cell aging. GIRDIN overexpression accelerated platelet phagocytosis by HEMECs, leading to a delay in cell aging. GIRDIN phosphorylation at Ser1417 was induced by Akt activation, while activation of Akt was induced by SIRT1-mediated deacetylation, consequently augmenting platelet phagocytosis and delaying cell aging. Taken together, SIRT1 delayed aging of HEMECs by deacetylating Akt, phosphorylating GIRDIN, and inducing platelet phagocytosis. The study highlights a possible target for the prevention of HEMEC aging.

INTRODUCTION

Endothelium is a key inducer for vascular proliferation,[1] which is critical in the repair of blood vessels after injury.[2] Therefore, maintenance of endothelium health is of critical importance in several disorders such as hypertension, diabetes, and aging.[3,4] Endothelial cells, adjustable to their environment, are of great significance to vascular homeostasis.[5] In orthopedics, fully differentiated endothelial cells have been reported to be implicated in visceral tissue homeostasis, suggesting the potential application of endothelial cells to repair of soft tissues.[6] The aging of the endothelium has been studied, but the mechanism is not completely understood.[7] Of note, platelets have been highlighted to be phagocytosed by human umbilical vascular endothelial cells, and the phagocytosis accelerates endothelial survival and apoptotic resistance.[8] More interestingly, platelet phagocytosis by endothelial cells has been suggested to inhibit endothelial cell aging and

apoptosis.[9] Therefore, we attempted to further our understanding of the mechanism underlying the antiaging effects of platelet phagocytosis by human endometrial microvascular endothelial cells (HEMECs).

Sirtuin1 (SIRT1) is an enzyme that deacetylates proteins and therefore functions as a hub regulator of antiaging genes in cells.[10] Evidence exists indicating that SIRT1 activities diminish alone with advancing age, and, interestingly, augmentation in SIRT1 activities in tissues might serve as a promising therapeutic strategy for aging-induced metabolic dysfunction.[11] Kitada et al. have documented that oxidative stress, endothelial dysfunction, and senescence result in a significant decline in SIRT1 expression and further proposed the protection that SIRT1 confers against vascular aging.[12] Although SIRT1 has been reported to stimulate platelet aggregation,[13] whether SIRT1 is related to phagocytosis remains unknown. Therefore, we determined the implications of SIRT1 in platelet phagocytosis. On the other hand, SIRT1 has been shown to regulate Akt activity through deacetylation.[14] More importantly, activation of Akt signaling has been shown to enhance platelet phagocytosis by endothelial cells.[9] Based on previous studies showing that SIRT1 and Akt are both involved in aging and platelet phagocytosis,[10,15] we sought to determine if Akt activation would be responsible for platelet phagocytosis-mediated aging in HEMECs.

G alpha-interacting, vesicle-associated protein (GIRDIN, also known as CCDC88A) is a relatively new actin-binding protein. Dynamic reorganization of the actin cytoskeleton is crucial in cell morphogenesis and movement, especially in phagocytosis. As the direct downstream mediator of Akt signaling,[16,17] GIRDIN may be involved in Akt-mediated platelet phagocytosis.[9] Additionally, GIRDIN can augment epithelial polarity under energetic stress and hence affect aging, cancer, and dysbiosis.[18] Based on these previous findings, we
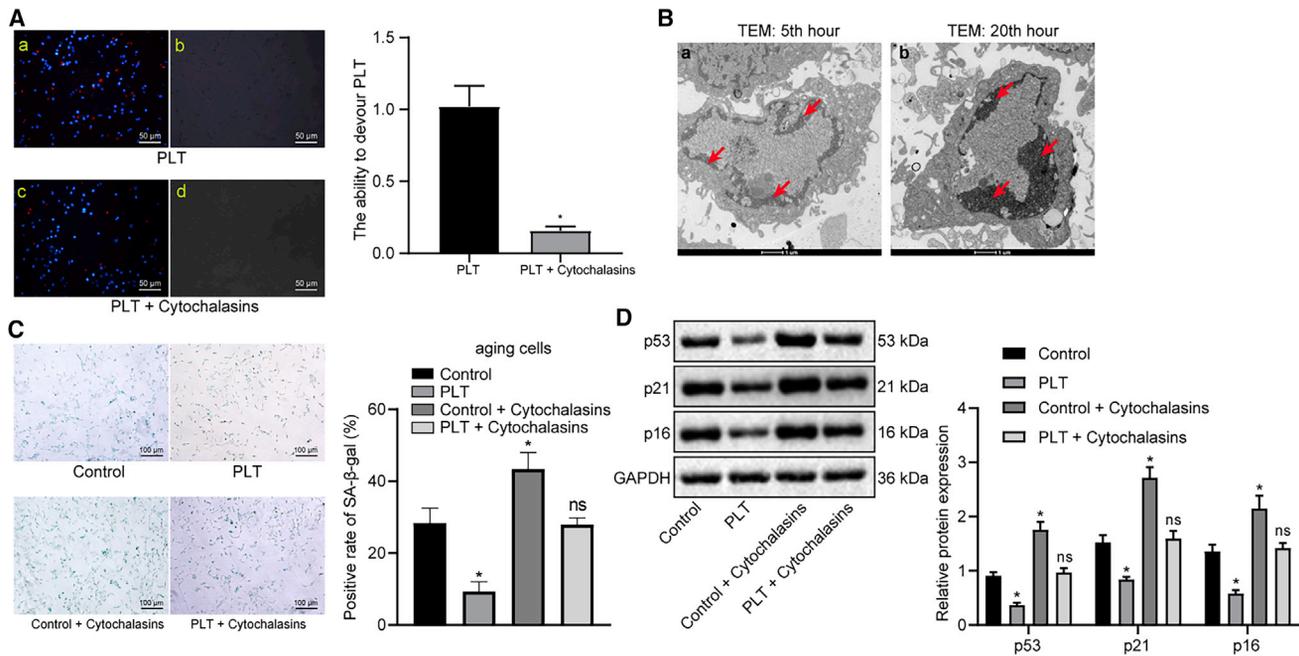
Figure 1. HEMECs induce platelet phagocytosis and restrain cell aging

(A) Platelet phagocytosis by HEMECs *in vitro* (200×). (B) Uptake of platelet visualized by transmission electron microscopy. HEMECs were incubated with platelets for 5 h (left) or 20 h (right) (10,000×). (C) Effects of platelet phagocytosis on the aging of HEMECs (100×). (D) Protein levels of cell aging-related proteins p53, p21, and p16 measured by western blot analysis. *p < 0.05 versus platelet coculture (HEMECs + platelets) or control (HEMECs only). ns, no significance. Data are expressed as mean ± standard deviation. Unpaired t test was utilized to compare data between two groups. One-way analysis of variance with Tukey's post hoc test was employed for comparison between multiple groups. The experiment was repeated 3 times independently.

examined the role of GIRDIN on Akt-mediated platelet phagocytosis and cell aging. In addition to cell apoptosis and viability, aging-related proteins p53, p21, and p16 were also measured in this study to accurately determine cell aging.

RESULTS
The platelet phagocytosis by HEMECs delays cell aging
Fluorescence microscopy showed a large portion of platelets colocalized with HEMECs (Figures 1Aa and 1Ab). When the phagocytosis inhibitor cytochalasin D (10 μg/mL) was mixed in the medium, the fluorescence signal diminished significantly (Figures 1Ac and 1Ad), indicating that the number of platelets colocalized with HEMECs was significantly reduced. In addition, platelets were observed to be phagocytosed by HEMECs. The phagocytosed platelets were round, without membrane extension (Figure 1Ba). Platelet internalization was increased with prolonged incubation time . Myelin-like substance was visible in HEMECs at 20 h, indicating that HEMECs can digest platelets (Figure 1Bb).

Aging cells produce SA-β-gal expression;[19] therefore, we characterized cell aging by determining the expression of SA-β-gal. SA-β-gal-positive cells were reduced after coculture with platelets (Figure 1C); however, cytochalasin D treatment partly normalized the diminished SA-β-gal-positive cells. In addition, aging-related proteins, including p53, p21, and p16,[20–22] were measured by western

blot assay. Those proteins were increased by treatment with cytochalasin D (Figure 1D). The data mentioned above suggest that HEMECs can phagocytose platelets and delay cell aging.

SIRT1 promotes platelet phagocytosis by HEMECs to suppress cell aging
Three short hairpin RNAs (shRNAs) targeting SIRT1 were constructed, and the efficiency was determined by qRT-PCR. shRNA2 was selected for further experiments because of its highest knockdown efficiency. Transfection with oe-SIRT1 plasmid, as expected, upregulated SIRT1 expression, while sh-SIRT1 knocked down SIRT1 in HEMECs (Figure 2A). SIRT1 overexpression inhibited cell aging, while SIRT1 knockdown enhanced cell aging (Figure 2B). In addition, PKH26-labeled platelets were cocultured with HEMECs transfected with oe-SIRT1/sh-SIRT1. Fluorescence microscopy displayed that SIRT1 overexpression enhanced the platelet phagocytosis in HEMECs, whereas SIRT1 knockdown reduced this ability (Figure 2C). Also, consistent results were obtained from transmission electron microscopic observation (Figure 2D). Flow cytometric data of fluorescent PKH26 label in HEMECs were in agreement with the results shown by fluorescence microscopy (Figure 2E).

Furthermore, the cell aging suppressed by platelet phagocytosis was further repressed by SIRT1 overexpression but rescued by SIRT1 knockdown (Figure 2F). In addition, SIRT1 overexpression reduced
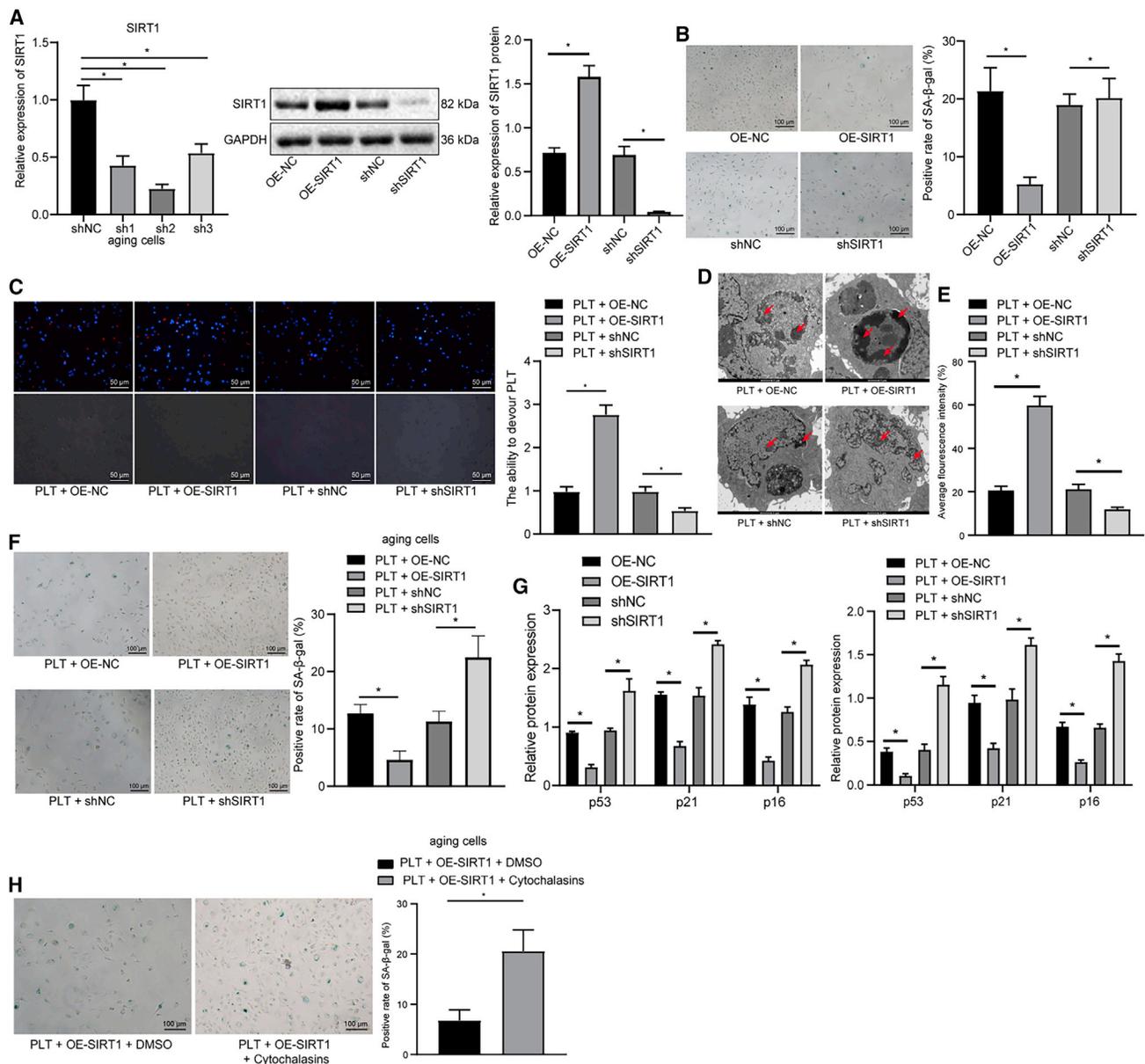
Figure 2. SIRT1 promotes platelet phagocytosis and suppresses cell aging

(A) Efficiency of SIRT1 overexpression and knockdown determined by qRT-PCR. (B) Effect of SIRT1 on aging of HEMECs (100×). (C) Effect of SIRT1 on platelet phagocytosis by HEMECs (200×). (D) Platelet uptake of HEMECs shown by transmission electron microscope. (E) Effect of SIRT1 on platelet phagocytosis determined by quantitative flow cytometric analysis. (F) Effect of SIRT1 on phagocytosis in HEMECs (100×). (G) Levels of cell aging-related proteins measured by western blot analysis. (H) Effect of cytochalasin D on the aging of HEMECs (100×). *$p < 0.05$. Data are expressed as mean ± standard deviation. Unpaired t test was utilized to compare data between two groups. One-way analysis of variance with Tukey's post hoc test was employed for comparison between multiple groups. The experiment was repeated 3 times independently.

the levels of aging-related proteins p53, p21, and p16, while SIRT1 knockdown increased their levels (Figure 2G). Cytochalasin D diminished the effects of SIRT1 overexpression on cell aging, showing enhanced cell aging after the inhibition of phagocytosis. These results showed that the antiaging effects of SIRT1 were closely related to the induction of platelet phagocytosis by HEMECs.

SIRT1 activates Akt to enhance platelet phagocytosis and impede cell aging

It was found that SIRT1 expression was increased in the presence of oe-SIRT1 while Akt expression did not change significantly and the extent of Akt phosphorylation was elevated (Figure 3A). Opposite results were observed in the presence of sh-SIRT1, indicating that
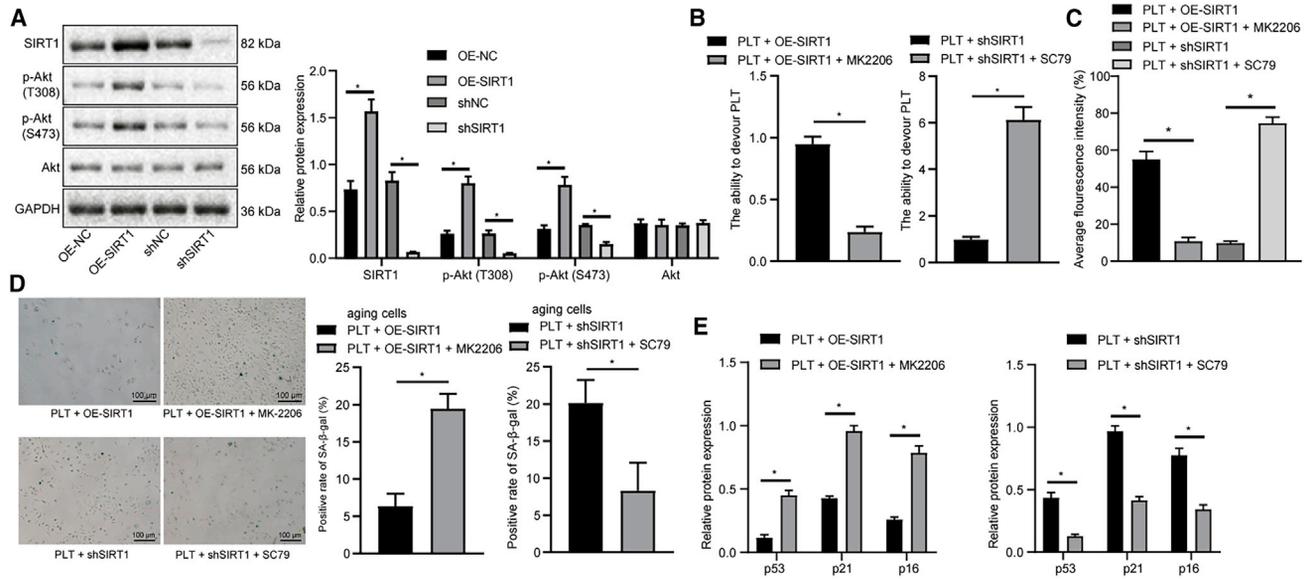
Figure 3. Akt activation is required for SIRT1-mediated platelet phagocytosis, and it suppresses cell aging

(A) Levels of SIRT1, Akt, and p-Akt proteins measured by western blot analysis. (B) Platelet phagocytosis in response to Akt activation or inhibition. (C) Platelet phagocytosis in response to Akt activation or inhibition determined by quantitative flow cytometric analysis. (D) The cell aging in response to Akt activation or inhibition (100×). (E) Expression of aging-related proteins p53, p21, and p16 in response to Akt activation or inhibition measured by western blot analysis. *p < 0.05. Data are expressed as mean ± standard deviation. Unpaired t test was conducted to compare data between two groups. The experiment was repeated 3 times independently.

SIRT1 overexpression resulted in activation of Akt, while SIRT1 knockdown inactivated Akt. Then, HEMECs overexpressing SIRT1 were treated with MK-2206 (Akt inhibitor), while SIRT1 knockdown HEMECs were treated with SC79 (Akt activator). In addition, MK-2206 treatment attenuated platelet phagocytosis induced by SIRT1 overexpression, while SC79 treatment restored the inhibition of platelet phagocytosis caused by SIRT1 knockdown (Figure 3B). Moreover, flow cytometric analysis of the fluorescent PKH26 label in HEMEC exhibited consistent results (Figure 3C). MK-2206 treatment counteracted the inhibitory effect of SIRT1 overexpression on cell aging, whereas cell aging promoted by SIRT1 knockdown was weakened after SC79 treatment (Figure 3D). Consistently, MK-2206 elevated the protein levels of p53, p21, and p16 in the presence of SIRT1 overexpression, and, on the contrary, SC79 diminished the level of these proteins in response to SIRT1 knockdown (Figure 3E). Together, these results indicated that Akt activation was induced in SIRT1-mediated platelet phagocytosis by HEMECs and stimulated cell aging.

GIRDIN inhibits cell aging by accelerating platelet phagocytosis

Three shRNAs targeting GIRDIN were designed and tested for knockdown efficiency. shRNA2 was selected for further experiments because of its highest knockdown efficiency. Transfection with oe-GIRDIN plasmid, as expected, increased GIRDIN expression, while sh-GIRDIN reduced the expression of GIRDIN (Figure 4A). GIRDIN overexpression enhanced platelet phagocytosis, while GIRDIN knockdown suppressed this process (Figure 4B). Transmission electron microscopy also showed that GIRDIN overexpression promoted, while GIRDIN knockdown inhibited, the phagocytosis of platelets by

HEMECs (Figure 4C). Fluorescent PKH26 labeling in HEMECs by flow cytometry also exhibited consistent results (Figure 4D). GIRDIN overexpression inhibited cell aging, whereas GIRDIN knockdown promoted this process (Figure 4E). In addition, GIRDIN overexpression reduced the protein levels of p53, p21, and p16, but GIRDIN knockdown enhanced their levels (Figure 4F). Cytochalasin D treatment restored the aging of HEMECs overexpressing GIRDIN (Figure 4G). Hence, we could conclude that GIRDIN inhibited cell aging by accelerating platelet phagocytosis.

Akt promotes GIRDIN Ser1417 phosphorylation to inhibit platelet phagocytosis-mediated cell aging

Since SIRT1 activated Akt to repress cell aging while GIRDIN inhibited cell aging, the focus was shifted to identify the potential regulatory relationships among SIRT1, Akt, and GIRDIN. For the purpose of verification, GIRDIN phosphorylation level was tested when the cells were treated with Akt activator or inhibitor, the results of which exhibited that Akt activator SC79 stimulated GIRDIN phosphorylation while Akt inhibitor MK-2206 disrupted GIRDIN phosphorylation (Figure 5A). GIRDIN-knockout HEMECs were developed using CRISPR-Cas9, and the most efficient one was selected for subsequent experiments (Figure 5B). After that, HEMECs were transfected with wild-type GIRDIN and mutant GIRDIN (cannot be phosphorylated since Ser1417 was mutated). We found that GIRDIN was not phosphorylated in the presence of mutant GIRDIN while GIRDIN phosphorylation could be induced in the presence of wild-type GIRDIN (Figure 5C). Additionally, cells were treated with MK-2206 or SC79 in the presence of wild-type GIRDIN or mutant GIRDIN. In the presence of GIRDIN,
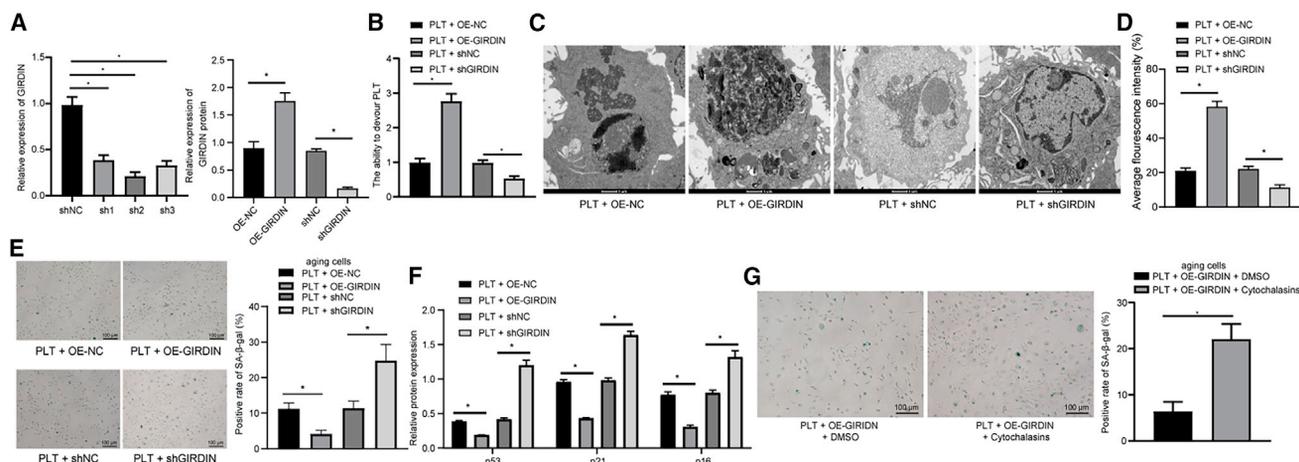
Figure 4. GIRDIN inhibits cell aging by promoting platelet phagocytosis

(A) Efficiency of GIRDIN knockdown or overexpression. (B) Platelet phagocytosis after GIRDIN overexpression or knockdown. (C) Platelet uptake by HEMECs after GIRDIN overexpression or knockdown shown by transmission electron microscopy. (D) Platelet phagocytosis after GIRDIN overexpression or knockdown determined by quantitative flow cytometric analysis. (E) Cell aging after GIRDIN overexpression or knockdown (100×). (F) Expression of cell aging-related proteins after GIRDIN overexpression or knockdown measured by western blot analysis. (G) Effect of cytochalasin D on cell aging in the presence of GIRDIN (100×). *p < 0.05. Data are expressed as mean ± standard deviation. Unpaired t test was utilized to compare data between two groups. One-way analysis of variance with Tukey's post hoc test was employed for comparison between multiple groups. The experiment was repeated 3 times independently.

platelet phagocytosis was stimulated, while MK-2206 weakened this effect. However, platelet phagocytosis was not changed with or without SC79 treatment in the presence of mutant GIRDIN (Figure 5D). Flow cytometric data on PKH26 labeling in HEMEC displayed consistent results (Figure 5E). In addition, Akt activation stimulated platelet phagocytosis-mediated inhibition of cell aging by inducing the phosphorylation of GIRDIN, whereas this inhibition was not observed in the cells transfected with mutant GIRDIN (Figure 5F). Moreover, MK-2206 elevated the expression of aging-related proteins p53, p21, and p16 in the presence of wild-type GIRDIN in the coculture system with platelets. However, SC79 did not affect the expression of these proteins in the presence of mutant GIRDIN (Figure 5G). Taken together, Akt activation induced GIRDIN phosphorylation at Ser1417, thereby accelerating platelet phagocytosis-mediated inhibition of cell aging.

SIRT1 deacetylates Akt to phosphorylate GIRDIN and promote platelet phagocytosis

Akt was suggested to stimulate GIRDIN phosphorylation based on the aforementioned data. For the purpose of verification, we further conducted immunoprecipitation assay to confirm the regulatory relationships among SIRT1, Akt, and GIRDIN in HEMECs. The results showed that silenced SIRT1 significantly reduced the enrichment of GIRDIN in HEMECs, indicating a potential regulatory relationship between SIRT1 and GIRDIN in HEMECs (Figure 6A). Furthermore, the western blot assay suggested that SIRT1 had no effect on total GIRDIN expression but affected GIRDIN phosphorylation (Figure 6B). Additionally, SIRT1 knockdown was witnessed to reduce the phosphorylation of Akt and GIRDIN (Figure 6C). This regulatory relationship in the process of platelet phagocytosis was further analyzed. The results suggested that SIRT1 knockdown reversed GIRDIN-mediated induction

of platelet phagocytosis (Figures 6D and 6E). Besides, SIRT1 knockdown enhanced cell aging (Figure 6F) and increased the expression of aging-related proteins p53, p21, and p16 in the presence of oe-GIRDIN (Figure 6G). The above results demonstrated that SIRT1 deacetylated Akt to activate Akt, hence repressing GIRDIN phosphorylation and accelerating platelet phagocytosis.

SIRT1 phosphorylates GIRDIN to promote viability and migration of HEMECs

In addition to platelet phagocytosis, we further evaluated whether other functions of HEMECs such as growth, migration, and apoptosis could be affected by SIRT1 and GIRDIN (Figures 7A–7C). After platelet phagocytosis, the viability and migration of HEMECs were strengthened, while their apoptosis was restrained. In addition, GIRDIN overexpression enhanced viability and reduced apoptosis of HEMECs, both of which were reversed by SIRT1 knockdown. Hence, SIRT1 enhanced viability and migration of HEMECs by inducing GIRDIN phosphorylation.

DISCUSSION

There are a few important findings in this study. First of all, HEMECs were capable of phagocytosing platelets. The second important finding was that SIRT1 or GIRDIN overexpression increased platelet phagocytosis, leading to delayed aging in HEMECs, while Akt knockdown reduced platelet phagocytosis, leading to increased cell aging. Of great importance, Akt stimulated GIRDIN phosphorylation at Ser1417, while SIRT1 induced deacetylation of Akt to promote GIRDIN phosphorylation. Collectively, the activation of receptor tyrosine kinase activates SIRT1, causing deacetylation of Akt (and PDK1) (Figure 8). PDK1 activates Akt by phosphorylation at 308
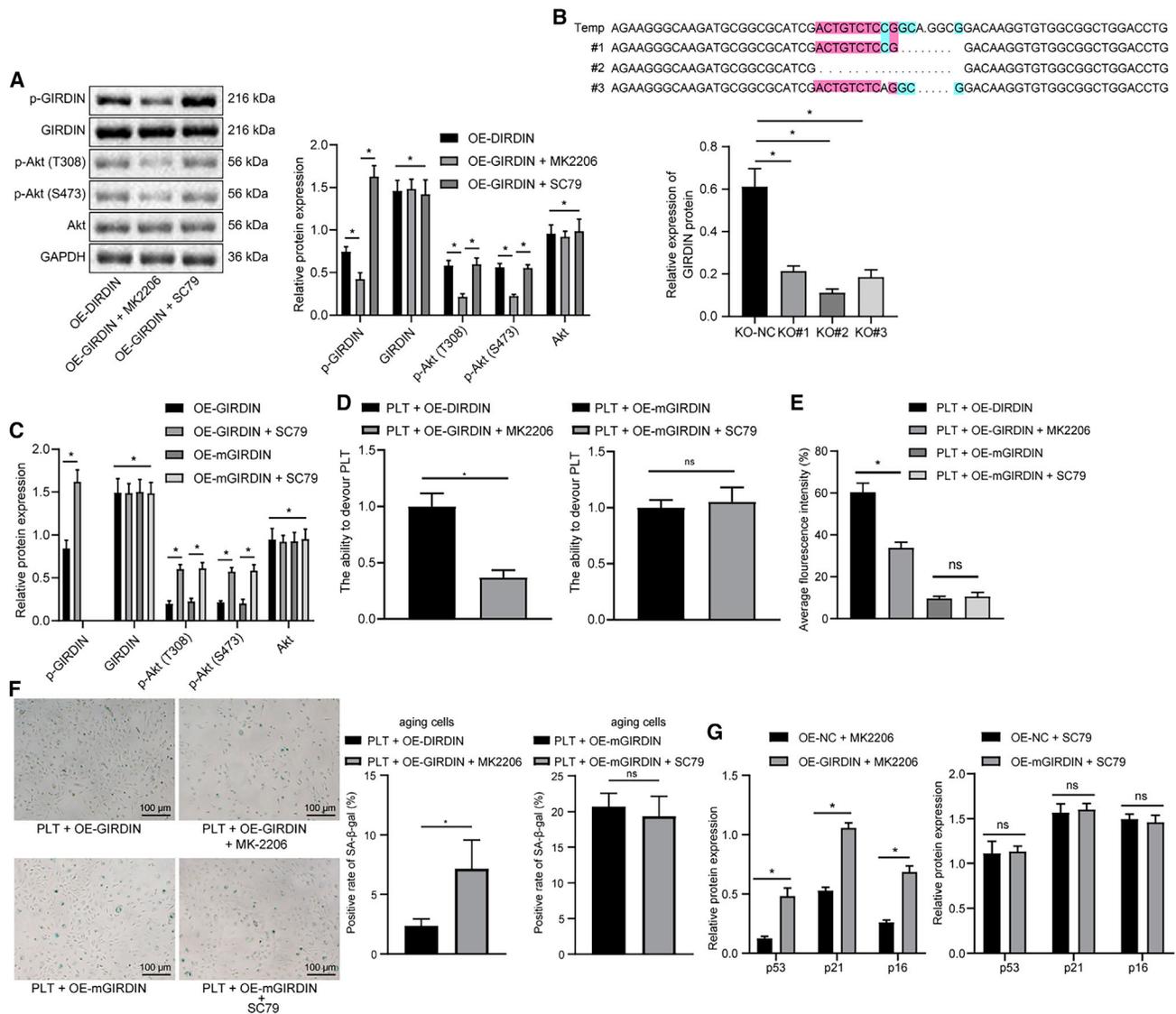
Figure 5. Akt promotes GIRDIN Ser1417 phosphorylation to induce platelet phagocytosis-mediated inhibition of cell aging

(A) GIRDIN phosphorylation after treatment with Akt inhibitor or activator determined by western blot analysis. (B) GIRDIN knockout efficiency measured by western blot analysis. (C) Phosphorylation of GIRDIN in the presence of wild-type or mutant GIRDIN after treatment with Akt inhibitor or activator. (D) Platelet phagocytosis by HEMECs in the presence of wild-type or mutant GIRDIN after treatment with Akt inhibitor or activator. (E) Flow cytometric analysis of platelet phagocytosis in the presence of wild-type or mutant GIRDIN after treatment with Akt inhibitor or activator. (F) HEMEC aging in the presence of wild-type or mutant GIRDIN after treatment with Akt inhibitor or activator (100×). (G) Expression of cell aging-related proteins p53, p21, and p16 in the presence of wild-type or mutant GIRDIN after treatment with Akt inhibitor or activator. $*p < 0.05$. ns, no significance. Data are expressed as mean ± standard deviation. Unpaired t test was employed to compare data between two groups. The experiment was repeated 3 times independently.

and 473, leading to increased phosphorylated GIRDIN at Ser1417. Activated GIRDIN increases platelet phagocytosis and leads to delayed aging in HEMECs. These signaling mediators, including SIRT1, Akt, and GIRDIN, may be potential therapeutic targets for treating HEMEC aging that deserve future studies.

The vascular endothelium has many important functions, including angiogenesis,[23] maintaining vascular tone,[24] mechano-sensor,[25] and

vascular proliferation.[1] Therefore, maintaining the health of the endothelium against aging is very important. In the current study, we found that platelet phagocytosis delayed aging in HEMECs, as characterized by increased cell viability and reduced apoptosis. These results are consistent with previous studies showing that platelet phagocytosis by endothelial cells has a variety of cellular functions, such as inhibiting cell aging and apoptosis and accelerating cell viability.[8,9] Likewise, erythrocyte phagocytosis by ECs has been
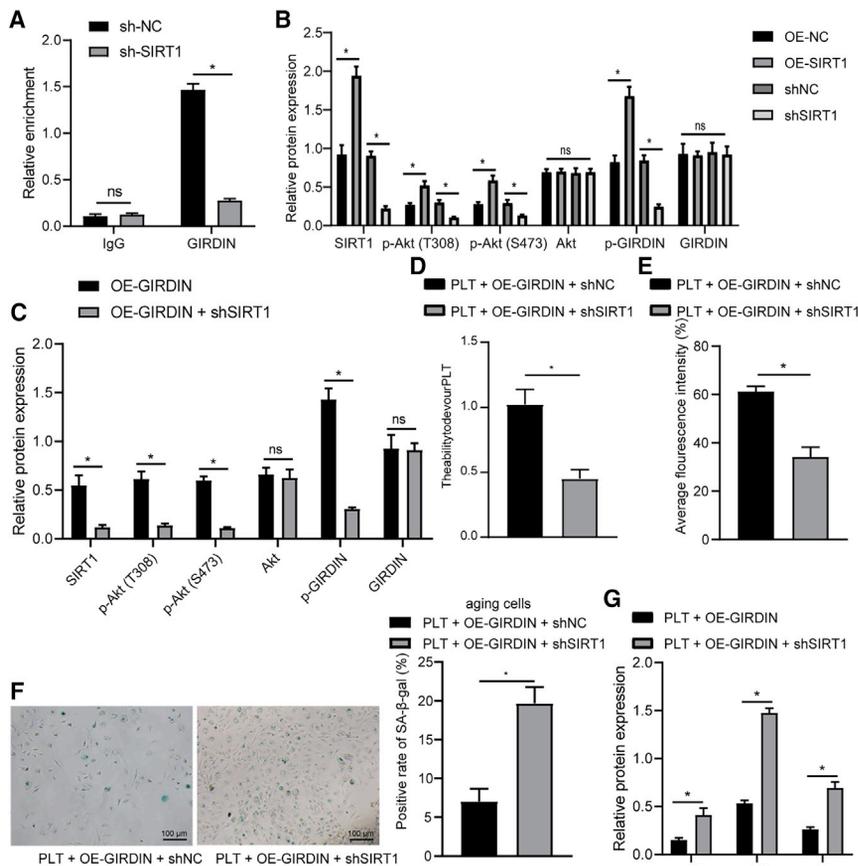
Figure 6. SIRT1 deacetylates Akt and induces GIRDIN phosphorylation to enhance platelet phagocytosis

(A) Regulatory relationship between GIRDIN and SIRT1 determined by immunoprecipitation. (B) Expression of Akt, p-Akt, endogenous GIRDIN, and p-GIRDIN proteins in the presence of oe-SIRT1 or sh-SIRT1 determined by western blot analysis. (C) Expression of Akt, p-Akt, OE-GIRDIN, and p-GIRDIN proteins after SIRT1 knockdown. (D) Platelet phagocytosis after SIRT1 knockdown in the presence of GIRDIN. (E) Platelet phagocytosis after SIRT1 knockdown in the presence of GIRDIN determined by quantitative flow cytometric analysis. (F) Cell aging after SIRT1 knockdown in the presence of GIRDIN (100×). (G) Expression of cell aging-related proteins p53, p21, and p16 after SIRT1 knockdown in the presence of GIRDIN. *p < 0.05. ns, no significance. Data are expressed as mean ± standard deviation. Unpaired t test was performed to compare data between two groups. The experiment was repeated 3 times independently.

demonstrated to be facilitated by aging and glycation,[26] suggesting the implication of phagocytosis in cell aging. More importantly, we found that SIRT1 was responsible for diminished cell aging. This result is also comparable to previous findings showing that SIRT1 may be an important regulator of antiaging genes in cells.[10,12] SIRT1 is an enzyme that deacetylates proteins. Thus, we found that SIRT1 deacetylated Akt, a result that has been shown previously.[14] Akt is an important signaling molecule that is involved in many cell functions, including proliferation and apoptosis.[27,28] Akt signaling pathway has also been shown to be related to phagocytosis.[8] Other previous studies also showed that SIRT1 and Akt were both involved in aging and platelet phagocytosis,[9,15] and our results provide additional evidence showing the critical roles of SIRT1 and Akt in HEMEC aging.

Another important finding of the current study was that GIRDIN phosphorylation was enhanced by Akt, leading to increased platelet phagocytosis and reduced cell aging. The signaling relationship between GIRDIN and Akt was also shown in previous studies.[16,17] Moreover, Akt has also been shown to regulate the phosphorylation of GIRDIN, which is linked to aging.[18,29] GIRDIN has been shown to increase cell proliferation and reduce apoptosis in cancer cells.[30] In fact, Akt and GIRDIN have been implicated as targets of oncogenes.[31] Our results provided evidence that GIRDIN has effects on other cell types.

Aging-related proteins p53, p21, and p16 were also measured in this study. Our results indicated that p53 is diminished by platelet phagocytosis. Among them, p53 is well studied in aging. It has been shown that p53 is related to MDM2 and DNA damage in aging.[20,32] Moreover, p53 is also associated with Arf.[33,34] Therefore, the downstream mechanism of the antiaging effect of platelet phagocytosis may be related to diminished MDM2, DNA damage, and/or Arf in HEMECs, which deserves further study. On the other hand, the downstream mechanisms of p21 and p16 in aging in the context of this study also warrant further investigation.

There are a few notable limitations of this study. First, all studies have been performed in cell culture. Results from this study should be further substantiated in *in vivo* studies. Second, although this study showed reduced HEMEC aging, further studies should focus on the functional improvement of the antiaging effects of platelet phagocytosis, such as improved vasodilation. Third, other potential signaling molecules, including the effects of insulin growth factor (IGF)-1, PDK1, and phosphatidylinositol 3-kinase (PI3K), should also be determined. Fourth, the possibility that a substance secreted by platelets plays the role of suppressing HEMEC aging should be excluded to provide more reliable experimental data for clinical application.

Taken conjointly, SIRT1 enhances platelet phagocytosis and delays cell aging by a mechanism that involves deacetylation of Akt and phosphorylation of GIRDIN. Those findings may aid in the comprehensive understanding of molecular mechanisms involved in cell aging and the development of potential protective targets against dysfunction in the endothelium.
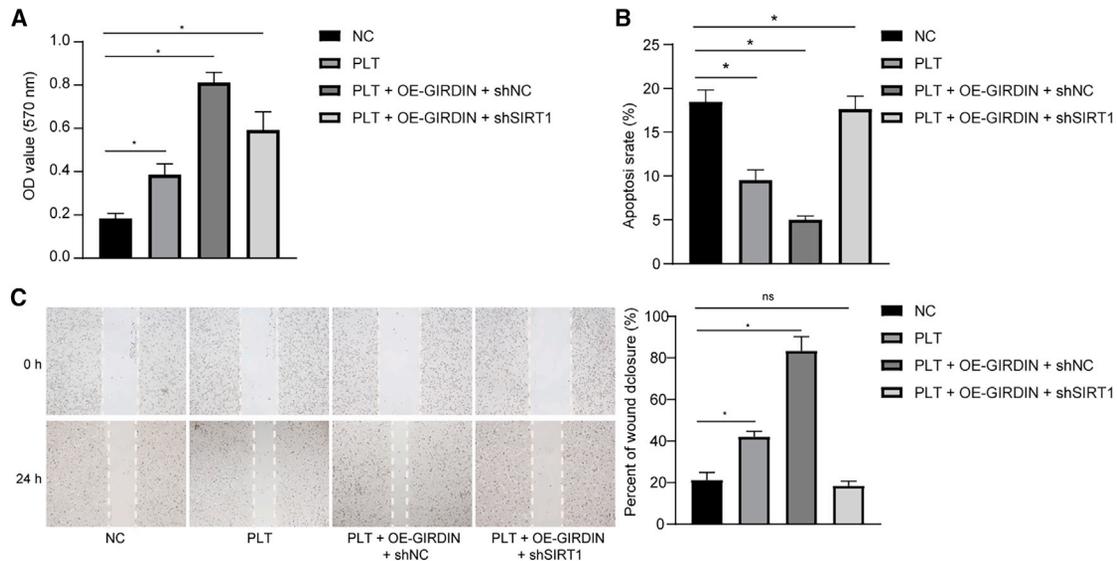
Figure 7. SIRT1 induces GIRDIN phosphorylation and facilitates viability and migration of HEMECs

HEMECs were transfected with oe-GIRDIN /or cotransfected with sh-SIRT1 after induction of platelet phagocytosis. (A) Cell viability determined by MTT assay. OD, optical density. (B) Cell apoptosis determined by quantitative flow cytometric analysis. (C) Cell migration determined by scratch test. *$p < 0.05$. ns, no significance. Data are expressed as mean ± standard deviation. One-way analysis of variance with Tukey's post hoc test was conducted for comparison between multiple groups.

MATERIALS AND METHODS

Cell culture and transfection

HEMECs (ATCC, Rockville, MD, USA) were cultured in endothelial cell culture medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco), 10 μg/mL streptomycin, and 100 U/mL penicillin in a 37°C, 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). When cells were in the logarithmic growth phase, cells were trypsinized and seeded in 6-well plates at 1×10^5 cells/well. Cells were transfected (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) after 24 h of culture and when cell fusion reached ∼75%. shRNA was cloned into PLKO.1 vector (Sigma, St. Louis, MO, USA). The shRNA sequences are shown in Table 1.

Platelet isolation

Platelets were isolated from 3-mL blood samples from a healthy blood donor. Blood was anticoagulated with 0.01 M sodium citrate. Platelet-rich plasma was prepared by centrifugation at 500 × g for 15 min at room temperature. Platelets were precipitated by centrifugation at 2,000 × g for 6 min and washed with 37°C HEPES buffer (0.1 mM
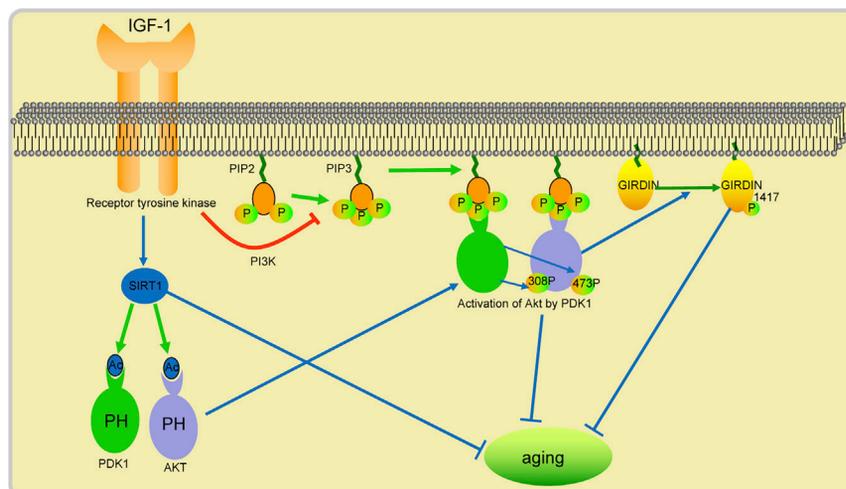

Figure 8. The mechanism graph of the regulatory network and function of SIRT1 in aging of HEMECs

SIRT1 restrains cell aging through GIRDIN phosphorylation via Akt signaling pathway.

Table 1. Primer sequences for shRNAs

Gene	Primer sequence
shNC	SHC007 (Sigma)
shSIRT1#1	5′-CCGGGATGATCAAGAGGCAATTAATCTCGAGATTAATTGCCTCTTGATCATCTTTTTG-3′
shSIRT1#2	5′-GTACCGGCATGAAGTGCCTCAGATATTACTCGAGTAATATCTGAGGCACTTCATGTTTTTTG-3′
shSIRT1#3	5′-CCGGGAGACTGTGATGTCATAATTACTCGAGTAATTATGACATCACAGTCTCTTTTTG-3′
shGIRDIN#1	5′-CCGGCCGCACATATTCAAGAGGTAACTCGAGTTACCTCTTGAATATGTGCGGTTTTTTG-3′
shGIRDIN#2	5′-CCGGGCAAGCTAAGCAAGATTGAATCTCGAGATTCAATCTTGCTTAGCTTGCTTTTTTG-3′
shGIRDIN#3	5′-CCGGGCTTTCATTACCAGCTCTGAACTCGAGTTCAGAGCTGGTAATGAAAGCTTTTTTG-3′

sh, short hairpin; shRNA, short hairpin RNA.

NaCl, 2.68 mM KCl, 1 mmol $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 0.1% glucose, pH 6.8). Platelet concentration was adjusted to 1×10^7–1×10^8/mL in the same buffer.

Platelet phagocytosis test *in vitro*
Platelet phagocytosis was detected by platelet-specific membrane-linked PKH26 red fluorescent cell-linking reagent (Sigma). Experiments were performed under a transmission electron microscope (TEM) and a fluorescence microscope.

Cell senescence assessment by β-galactosidase staining
Cell culture medium in 6-well plates was removed, and cells were washed with PBS. SA-β-gal staining fixation solution (1 mL) was added to each well for 20 min at room temperature. Staining fixation solution was removed, and cells were washed 3 times with PBS for 3 min each. PBS was removed, 1 mL of staining working solution was added to each well, and they were covered with Parafilm and incubated at 37°C overnight. Cells were observed and counted under a microscope. Three random fields were observed, and 50 cells were counted in each field.

Protein expression measurement by immunoblotting
Cells were harvested after trypsinization and lysed with an enhanced RIPA lysis buffer (Boster, Wuhan, China) containing a protease in-

Table 2. Antibody catalog number and company

Antibody	Company	Catalog number
SIRT1	Abcam	ab32441
GAPDH	Abcam	ab9484
p-Akt (T308)	Abcam	ab38449
p-Akt (S473)	Abcam	ab81283
t-Akt	Cell Signaling Technology	9272
GIRDIN	Abcam	ab179481
p-GIRDIN	Abcam	ab111440
Goat anti-rabbit IgG H&L	Abcam	ab205718

SIRT1, Sirtuin1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p, phosphorylated; t, total; Akt, protein kinase B; GIRDIN, G alpha-interacting, vesicle-associated protein; IgG, immunoglobulin G.

hibitor. Protein concentration was determined by a bicinchoninic acid (BCA) protein quantification kit (Boster). Proteins were separated by 10% SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% BSA at room temperature for 2 h. Membranes were incubated with primary antibodies (1:5,000) at 4°C overnight (Table 2). Membranes were washed, and horseradish peroxidase (HRP)-labeled secondary antibody (1:5,000–10,000) was added and incubated for 1 h at room temperature. Enhanced chemiluminescence solution (EMD Millipore, Burlington, MA, USA) was added and incubated for 1 min at room temperature. The membranes were sealed, exposed to X-ray for 5–10 min, and developed. Gray intensity of each protein band was determined by ImageJ software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Flow cytometric platelet phagocytosis evaluation
Flow cytometry was employed to detect fluorescent PKH26-labeled platelets phagocytized by endothelial cells. Cells were incubated in HEPES buffer (134 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.40) and anti-platelet antibody (AIp21; ab112238; Abcam, Cambridge, UK) at 4°C for 30 min. Cells were washed with $1 \times$ PBS buffer for 15 min. Fluorescence intensity was determined by flow cytometer (BD FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometric apoptosis detection
Apoptosis was determined with the Annexin V/PI Apoptosis Kit (MultiSciences Biotech, Hangzhou, China). Cells were harvested and centrifuged at $2,000 \times g$ for 5 min at 4°C. Cells were resuspended in 0.5 mL of $1 \times$ annexin binding buffer at 5×10^5 cells/mL. The cells were stained with Annexin V-FITC and propidium iodide (PI) for 10 min at room temperature in the dark. Signals were immediately analyzed by the flow cytometer.

RNA quantitation and gene expression quantitation
Total RNA was extracted using TRIzol reagent (15596026, Invitrogen). RNA was reverse-transcribed to cDNA with the PrimeScript RT Reagent Kit (RR047A, Takara, Kusatsu, Japan). Quantitative

Table 3. Primer sequences for qPCR

Gene	Forward sequence	Reverse sequence
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′
SIRT1	5′-ACAGGTTGCGGGAATCCAAA-3′	5′-GTTCATCAGCTGGGCACCTA-3′

qPCR, quantitative polymerase chain reaction.

real-time PCR was performed with the Fast SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 7300 RT-PCR system (Applied Biosystems). Each sample was tested in triplicates. GAPDH was used as an internal reference. Relative expression of target genes normalized to GAPDH (primer sequence shown in Table 3) was calculated by the $2^{-\Delta\Delta Ct}$ method.

CRISPR-Cas9
Designed primer sequence (Thermo) was constructed to the lenti CRISPRv2 vector. Plasmid expression vector was transferred into Stbl3 *Escherichia coli* for amplification, culture, and extraction. CRISPR-Cas plasmid was transfected to prepared HEMECs with Lipofectamine 2000. Resistant cells were selected by antibiotics. Cells were seeded into 96-well plates for continuing drug screening when cells were 50% confluent. Genomic DNA was extracted, and mutations were compared by sequencing. Single guide RNA (sgRNA) sequences are shown in Table 4.

Immunoprecipitation
After transfection with negative control for short hairpin RNA (sh-NC) and sh-SIRT1, the protein concentration of the supernatant in HEMECs was measured with a BCA kit (Bio-Rad Laboratories, Hercules, CA, USA) and then adjusted using cell lysis to be equal. Then, 700 μL of supernatant was incubated with 2 μg of antibodies to immunoglobulin G (IgG) (ab205718, 1:50, Abcam), GIRDIN (ab250102, 1:50, Abcam), and Akt (ab8805, 1:50, Abcam) and 150 μL of agarose microbeads at 4°C overnight with shaking. The immunoprecipitate was centrifuged at 3,000 rpm for 5 min to remove the supernatant and then washed with cell lysis 5 times, followed by western blot analysis.

Site-directed mutagenesis
GIRDIN mutation at its Ser1417 or antiphosphorylated alanine was performed with a QuickMutation gene site-directed mutagenesis kit (Beyotime, Beijing, China). Generation of PCR-based mutant plasmids, digestion of DpnI-based template plasmids, and subsequent cleavage or sequencing were all based on the instructions of this kit.

Table 4. Primer sequences for sgRNA

Gene	Sequences
sgGIRDIN	5′-GTCATGAACTGCTCCAGAAG-3′
sgControl	Syno negative control sgRNA1

sgRNA, single guide RNA.

Viability measurement by MTT-based assay
Cell suspension (200 μL/well) was seeded into a 96-well culture plate and cultured in a 37°C, 5% CO_2 incubator. After 3 days of culture, cells were incubated with 5 mg/mL MTT solution (20 μL/well, C0009, Beyotime) for 4 h. Supernatant was discarded after incubation, and 200 μL of MTT lysis buffer was added to each well, followed by shaking for 10 min. Optical density was determined at 570 nm with an automatic microplate reader. Each sample was tested in triplicate.

Scratch test
Even horizontal lines were drawn at intervals of 0.5–1 cm on the bottom surface of a 6-well plate with at least 5 lines/well. Cells (5×10^5 cells/well) were added to a 6-well plate. Cells were incubated overnight in medium containing 10% fetal bovine serum. A sterile 10-μL pipette was utilized to make scratches perpendicular to the bottom line of the culture plate. The migration distance of scratches was measured and recorded under an optical microscope at 0 and 24 h of incubation. Images were taken under an inverted microscope to observe cell migration.

Statistical analysis
SPSS 21.0 (IBM, Armonk, NY, USA) was employed for statistical analysis. Data are expressed as mean ± standard deviation. Unpaired t test was conducted to compare data between two groups. One-way analysis of variance with Tukey's post hoc test was utilized for comparison between multiple groups. Differences were considered significant when $p < 0.05$.

AUTHOR CONTRIBUTIONS
Y. Lan: study design, data collection; M.D.: data interpretation; Yangfang Li: data analysis; Yongjun Li: figures, writing; Y.D. and Z.C.: polishing, revising. All authors read and approved final version of manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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