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SIRT1/PGC-1α/Mfn2 pathway regulates mitochondrial homeostasis in VSMC to attenuate aging-related vascular calcification

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Aging-related vascular calcification is closely associated with the development and progression of arterial diseases in elderly patients. Studies have shown that mitochondrial fusion/fission imbalance plays a crucial role in the formation of aging-related vascular calcification, and upregulation of silent information regulator 1 (SIRT1) blocks mitochondrial damage thereby protecting vascular smooth muscle cells (VSMCs). Our study aimed to explore whether SIRT1 can inhibit the vascular calcification and its potential molecular mechanism. VSMCs were used to establish the vascular aging model, and confirmed by β-galactosidase staining. The overexpressed SIRT1 and si-PGC-1α were transfected into VSMCs and verified by RT-qPCR or Western Blot. Proteins relevant to vascular calcification were detected by ELISA, while the level of calcified nodules was stained by Alizarin Red S. The molecules relevant to mitochondrial fusion were detected by RT-qPCR and immunofluorescence. Mito Tracker staining was used to evaluate the mitochondrial membrane potential and morphology. The level of ROS and ATP in VSMCs and its supernatant were detected by reagent kits combined flow cytometry. The levels of β-galactosidase and calcification were higher in the senescent VSMCs compared to normal VSMCs, while mitochondrial membrane potential and fusion and the expression of the SIRT1/PGC-1α/Mfn2 pathway were reduced. Overexpression of SIRT1 promoted its downstream targets PGC-1α and Mfn2 expression and reversed these aging characteristics, inhibited VSMC apoptosis and ROS level while increasing the ATP secretion compared to senescence VSMCs. Knocking down PGC-1α inhibit the function of SIRT1 overexpression on senescence VSMCs. This study presents a novel mechanism to the mitochondria-mediated age-related vascular calcification, and provides the potential molecular targets for the prevention and treatment of arterial diseases in elderly patients.

Keywords Vascular calcification, Mitochondrial homeostasis, SIRT1/PGC-1α-ERRα/Mfn2 signaling, Aging, Vascular smooth muscle cells

Abbreviations

SIRT1	Silent information regulator 1
VSMCs	Vascular smooth muscle cells
VC	Vascular calcification
Mfn2	Mitochondrial membrane proteins -mitofusin2
PGC-1α	Proliferator-activated receptor gamma coactivator-1 alpha
ERRα	Estrogen-related receptor alpha
DMEM	Dulbecco's modified eagle medium
qPCR	Quantitative real-time PCR
ALP	Alkaline phosphatase
OPG	Osteoprotegerin
TNF-α	Tumor necrosis factor
OPA1	Optic atrophy 1
DRP1	Dynein-associated protein 1

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Vascular calcification (VC) is an increasing burden in aging societies worldwide, often found in patients with aging, diabetes, atherosclerosis, or chronic kidney disease. It is significantly associated with an increase in all-cause mortality and the rupture of atherosclerotic plaques[1]. However, the mechanisms underlying VC are not fully understood, and effective treatments for VC are currently lacking. Several therapeutic interventions have been shown to effectively inhibit the progression of VC in animal models and in vitro experiments; nevertheless, there are no specific therapies to reduce these calcifications in clinical practice, nor is there evidence that reducing calcification improves cardiovascular outcomes[2–3]. Therefore, a deeper understanding of the regulatory mechanisms of VC formation and therapeutic advancements is crucial for addressing this significant clinical problem.

The role of mitochondrial dysfunction in aging-related VC has garnered increasing attention from researchers. The maintenance of normal mitochondrial function relies on the homeostasis of mitochondrial fusion/fission. An imbalance in mitochondrial fusion/fission is a critical factor triggering mitochondrial dysfunction[4–5]. Recent studies discovered that mitochondrial fusion promotes the loss of the contractile phenotype of vascular smooth muscle cells, leading to their transformation into osteoblast-like and foam cell-like phenotypes, which promote vascular calcification[6–7]. Further research has demonstrated that weakened mitochondrial fusion is involved in the osteogenic transformation and calcification of aging VSMCs[8]. Moreover, the homeostasis of mitochondrial fusion/fission is tightly regulated by multiple molecules. In mammals, mitochondrial fusion is mainly regulated by outer mitochondrial membrane proteins -mitofusin1/2 (Mfn1/2)[9]. Among these, Mfn2 is a key molecule mediating mitochondrial fusion. In aging vascular smooth muscle cells, the capacity for mitochondrial fusion is diminished, accompanied by altered mitochondrial morphology, decreased membrane potential, and reduced mitochondrial DNA[10–11]. Silencing Mfn2 expression in VSMCs can block mitochondrial fusion, reduce mitochondrial membrane potential and mitochondrial DNA content, ultimately leading to osteogenic transformation and calcification. Therefore, Mfn2 may provide therapeutic targets to promote mitochondrial fusion, which prevents the calcification of aging VSMCs.

In addition, an increasing number of studies have shown that peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is involved in the process of mitochondrial fusion. As a transcriptional coactivator, it promotes mitochondrial fusion and biogenesis by regulating the activity of mitochondria-related proteins, ultimately modulating cellular energy metabolism[12]. The reduction of PGC-1α activity has been observed in various VSMCs injury models[13–15]. Reports showed that PGC-1α induces and coactivates estrogen-related receptor alpha (ERRα) expression, and the PGC-1α/ERRα complex acts on the androgen receptor response element (ERRE) in the promoter region of the Mfn2 gene, thereby upregulating Mfn2 expression, promoting mitochondrial fusion, and ultimately enhancing mitochondrial energy production[16]. This suggests that PGC-1α may target Mfn2 and regulate mitochondrial fusion.

As a key upstream regulator of PGC-1α, Silent information regulator 2-related enzyme 1 (SIRT1) is an NAD+-dependent histone deacetylase[17] that can regulate various transcription factors[18–19], participating in DNA damage repair, cell cycle regulation, mitochondrial function protection, inflammation suppression, oxidative stress resistance, and lifespan extension[20–21]. SIRT1 enhances PGC-1α activity through deacetylation[22], activation of SIRT1 inhibits high glucose-induced osteogenic transformation of VSMCs by upregulating PGC-1α expression[23]. Hence, we speculate that SIRT1 targets PGC-1α to upregulate Mfn2, promoting the mitochondrial fusion in VSMCs (Graphical abstract). This may provide a novel mechanism and upstream targets for the treatment of aging-related vascular calcification.

Materials and methods

Cell culture and aging model

Vascular smooth muscle cells (VSMCs) (cat.no. CTCC-049HC; PH Biotechnology, Wuxi, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (cat. no. L110KJ; BasalMedia) supplemented with 10% fetal bovine serum (FBS) (cat. no. RL09136; Hyclone) and 1% penicillin-streptomycin (cat. no. B540732; Sangon Biotech) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were passaged at 80–90% confluency using trypsin-EDTA. To establish an aging model, VSMCs were cultured under prolonged replicative conditions by serial passaging until they reached senescence, and confirmed by senescence-associated β-galactosidase (SA-β-gal) staining (cat. no. G1580; Solarbio) according to the manufacturer's instructions. Cells were used for subsequent experiments at passages corresponding to early (passages 3–5) and late (passages 15–20) stages of senescence.

Western blotting

Total protein was extracted from cells using RIPA lysis buffe (cat. no. P0013B; Beyotime Biotechnology) and quantified using the BCA kit (cat. no. BL521A; Biosharp). The proteins were separated by SDS-PAGE on 12% gels and subsequently transferred to PVDF membranes (cat. no. IPVH00010; Millipore). After blocking with 5% BSA (cat. no. A9647; Biosharp Life Sciences) at room temperature for 1 h, the membranes were incubated with specific primary antibodies (SIRT1(cat. no. 60303-1-Ig; proteintech), PGC-1a (cat. no. 66369-1-Ig; Proteintech), Mfn2 (cat. no. 67487-1-Ig; PTM BIO), GAPDH (GB15004; Servicebio)) at 4˚C overnight. The membranes were subsequently incubated with the corresponding horseradish peroxidase-conjugated HRP-labeled sheep anti-mouse secondary antibody (cat. no. A0216; Beyotime Biotechnology) or HRP-labeled sheep anti-rabbit secondary antibody (cat. no. A0208; Beyotime Biotechnology) at room temperature for 1 h. Membranes were visualized using the ECL chemiluminescence reagent (cat. no. ECL-0011; Dingguo Prosperous). The gray values were analyzed by ImageJ 2X software (National Institutes of Health).

Mito tracker staining

To detect the level of mitochondrial membrane potential and morphology, VSMCs were incubated with 100 nM Mito Tracker Red CMXRos (cat. no. C1049B; Beyotime Biotechnology) or Mito Tracker Green (cat. No. C1048; Beyotime Biotechnology) respectively in serum-free medium at 37 °C for 30 min. After incubation, cells were washed with PBS and then added fresh pre-warmed culture medium at 37 °C, then observed under a laser confocal microscope, and images were captured for analysis.

To the Mito Tracker Red staining, the average optical density (AOD) was calculated by dividing the integrated density (sum of pixel values) by the area of the mitochondria. This parameter was used to assess the levels of mitochondrial membrane potential. Meanwhile, the mitochondrial morphology was observed by the Mito-Tracker Green staining.

Calcified nodule counting

Cells were fixed with 4% paraformaldehyde, and stained with Alizarin Red S solution (cat.no. CTCC-JD001; PH Biotechnology). To quantify calcified nodules, 1 mL of cetylpyridinium chloride (cat. no. 6004-24-6; Sigma-Aldrich) was added to dissolve the calcium deposits in the cells, and the optical density (OD) was measured at 560 nm using a microplate reader (cat. no. Multiskan MK3; Thermo) to assess the level of cellular calcification.

Plasmid transfection

The pcDNA3.1-SIRT1 for overexpressing SIRT1 and its control pcDNA3.1 were synthesized by Invitrogen (USA). Meanwhile, three siRNAs targeting PGC-1αwere constructed by OLIGOBIO (Beijing, China), and the sequence with the best inhibition effect was used for knock-down experiments, the sense sequence, 5'- GGACAGUGAU UUCAGUAAUTT-3', the antisense, 5'- AUUACUGAAAUCACUGUCCTT-3'. The siR-NC sense sequence, 5'-U UCUCCGAACGUGUCACGUTT-3', the antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. The siRNAs were transfected into VSMCs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RT-qPCR

Total RNA was extracted from VSMCs using TRIzol reagent (Invitrogen, USA), and reverse transcription was performed using the RNA and the Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). The harvested cDNAs were used as templates for quantitative real-time PCR (qPCR) analysis with SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA).The relative expression of mRNAs were analyzed using $2^{-\Delta\Delta Ct}$ method. The primers are shown as below: PGC-1α, F: 5'-GAGGCAGAAGCAGAAAGCAAT-3', R: 5'-GAGG CAGAAGCAGAAAGCAAT-3'; mtDNA, F: 5'-CGAAAACTCAACCCC-3', R: 5'- TGCCGAAAAGACACA-3'; Mfn2, F: 5'-GCCCCAAGAATAGCC-3', R: 5'- CCACAAGAATGCCCA-3'; Opa1, F: 5'-GGGTTGTTGTGGTT G-3', R: 5'- TTCACTGGAGAGCGT-3'; Drp1, F: 5'- ACACGATTGAAGGAA-3', R: 5'- GATGGCAGTCAGGA T-3'; GAPDH, F: 5'-GGTGAAGGTCGGTGTGAACG-3', R: 5'-CTCGCTCCTGGAAGATGGTG-3'.

ELISA detection

To evaluate the level of angiosteosis, alkaline phosphatase (ALP), osteoprotegerin (OPG), and tumor necrosis factor-α (TNF-α) were detected using the corresponding reagents according to the manufacturer's protocol.

Immunofluorescent staining

The protein levels in VSMCs were detected by immunofluorescence. In brief, the VSMCs were collected and fixed with 4% paraformaldehyde, and then incubated with 10% goat serum for 1 h to avoid nonspecific binding. The primary antibodies including optic atrophy 1 (OPA1) (DF8587, Affinity), dynamin-related protein 1 (DRP1) (ab184247, abcam) and mitofusin 2(MFN2) (ab56889, Abcam) were added and incubated overnight at 4 °C. Fluorescently labeled secondary antibodies (FITC-labeled goat anti-rabbit; FITC-labeled goat anti-mouse; Cy3-labeled goat anti-rabbit; all obtained from Beyotime) were added and incubated for 1 h at room temperature. DAPI was used to label the nuclei of VSMCs and then observed under a fluorescence microscope (IX73, OLYMPUS).

Mitochondrial ROS detection

VSMCs were treated with the probe (cat.no. S0033S; Beyotime Biotechnology) at a concentration of 10 μmol/L. After removing the medium, DCFH-DA was added to the cells and incubated at 37 °C for 20 min. Mitochondrial ROS levels in cells were detected using a flow cytometer (cat. no. FACSVerse; BD Biosciences).

TUNEL analysis

Apoptosis of VSMCs was measured by TUNEL assay. In brief, TUNEL working solution (cat. no. C1089; Beyotime Biotechnology) was added and incubated at 37 °C for 1 h. Cells were then fixed with 4% paraformaldehyde and incubated with Hoechst stain at room temperature in the dark for 10 min. Cell apoptosis was visualized and photographed under a fluorescence microscope, and analyzed using ImageJ software.

ATP detection

The secretion of ATP in VSMCs was detected using an ATP assay kit (cat. no. E-BC-F002; Elabscience) following the protocol. Cells were lysed by thorough mixing with a pipette and centrifuged at 12,000 g for 5 min at 4 °C, and the supernatant was collected for subsequent analysis. The ATP standard solution was diluted with ATP detection lysis buffer to generate a concentration gradient for the standard curve. The ATP content was detected by adding the working solution and incubating at room temperature for 5 min. Then, the 20 μL of sample or standard was added to each well, and the relative light units (RLU) were measured using a luminometer. Finally, the concentration of ATP was converted to nM/mg protein.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0 and presented as mean ± SD. All experiments were repeated at least 3 times. Differences between two groups were analyzed by Student's t-test, while differences among three or more groups were analyzed by ANOVA for multiple comparisons. A *P* value less than 0.05 was considered as a statistically significant.

Results

Mitochondrial damage and calcification associated with senescent VSMCs

We first demonstrated that β-galactosidase expression is significantly elevated in aging VSMCs compared to the control group, indicating increased cellular senescence in aging VSMCs (Fig. 1A), and there is a notable trend toward increased calcification in aging VSMCs (Fig. 1B). Meanwhile, mitochondrial membrane potential was significantly decreased in senescent VSMCs (Fig. 1C), and the mitochondrial morphology has also changed (Fig. 1D). The expression of SIRT1, PGC-1α, and Mfn2 were significantly decreased in senescent compared to normal VSMCs (Fig. 1E). These indicating a significant damage in mitochondrial homeostasis, and support the link between aging, mitochondrial dysfunction, and altered cellular processes.

Impact of SIRT1-PGC-1α signaling on senescence VSMCs

The results of siRNAs targeting PGC-1α were verified by RT-qPCR, and we selected the siRNA-2 with the best inhibition effect for the subsequent experiment (Fig. 2A). WB results showed that the protein levels of SIRT1, PGC-1α, and Mfn2 were significantly elevated in the senescent VSMCs transfected with OE-SIRT1 compared to that senescent VSMCs without transfection. However, knocking down PGC-1α significantly decreased these levels (Fig. 2B-C). Meanwhile, compared to the senescent VSMCs, overexpression of SIRT1 significantly reduced the β-galactosidase-positive cells, whereas the number of stained cells were significantly increased in senescent VSMCs with both SIRT1 overexpression and siRNA-PGC-1α transfection (Fig. 2D). TUNEL staining showed that transfected overexpressing SIRT1 significantly reduced the apoptosis level of senescent VSMCs, whereas apoptosis was significantly increased in VSMCs with both SIRT1 overexpression and siRNA transfection (Fig. 2E). These results demonstrate the important role of the SIRT1-PGC-1α signaling in regulating the senescent VSMCs.

Effect of SIRT1-PGC-1α signaling on calcification in the senescence VSMCs

Regarding the level of calcific nodules, overexpression of SIRT1 significantly decreased the nodules and knocking down PGC-1α recovered the nodules (Fig. 3A). Furthermore, the levels of ALP and TNF-α were significantly reduced in supernatant of the OE-SIRT1 transfected aging VSMCs compared to the aging VSMCs, transfected with OE-SIRT1 and si-PGC-1α significantly increased their levels in aging VSMCs (Fig. 3B). Meanwhile, compared to the aging VSMCs, OE-SIRT1 significantly increased the OPG level, while decreased in the aging VSMCs transfected with OE-SIRT1 and si-PGC-1α (Fig. 3B). These indicate that the regulated function on vascular calcification in aging VSMCs by the SIRT1-PGC-1α signaling.

The mechanism of SIRT1-PGC-1α signaling regulating senescence VSMCs

To explore the mechanism of SIRT1-PGC-1α regulating senescent VSMCs, we found that the mtDNA and molecules related to the mitochondrial fusion, including OPA1, DRP1 and MFN2, are regulated by the SIRT1 and PGC-1α. In detail, OE-SIRT1 significantly elevated the expression of mtDNA, OPA1, and MFN2, while it reduced DRP1; Conversely, si-PGC-1α inhibited the function of OE-SIRT1 on these expression in VSMCs (Fig. 4A-B). These results indicated that the regulated function of SIRT1-PGC-1α signaling on aging VSMCs were associated with the process of mitochondrial fusion.

Therefore, we further study the regulated function of the SIRT1-PGC-1α signaling in mitochondria of aging VSMCs. We found that the mitochondrial membrane potential and fusion was significantly increased in senescent VSMCs with OE-SIRT1 transfection, and the secretion of ATP also increased while the ROS level was decreased, while the function of OE-SIRT1 was reversed by the si-PGC-1α in the senescence VSMCs (Fig. 5A-D). These results indicated that mitochondrial homeostasis in aging VSMCs is affected by the SIRT1-PGC-1α-Mfn2 signaling axis.

Discussion

We have known that mitochondrial function is pivotal for maintaining cellular homeostasis, contractility, and response to vascular stress in VSMCs[24]. Mitochondrial fusion is vital for maintaining mitochondrial function by promoting the exchange of mitochondrial DNA, proteins, and lipids. It also helps mitigating oxidative stress and cellular damage by diluting damaged components across an expanded mitochondrial network. In VSMCs, mitochondrial homeostasis supports energy production and cellular responses to both physiological and pathological stimuli. Disruptions in mitochondrial homeostasis can lead to impaired energy production, increased oxidative stress, and contribute to the pathogenesis of vascular diseases, including vascular calcification[25]. In this study, we demonstrated the important role of the SIRT1-PGC-1α-Mfn2 signaling on regulating of mitochondrial function, through which reduced the calcification level in senescent VSMCs.

SIRT1 influences mitochondrial function through its deacetylase activity, which modulates the activity of various transcription factors and coactivators involved in mitochondrial biogenesis and function[26]. In VSMCs, SIRT1 helps maintain mitochondrial integrity and function by promoting mitochondrial biogenesis and enhancing mitochondrial fusion processes, and its activity is essential for counteracting oxidative stress and cellular aging[27,28]. PGC-1α drives the production of mitochondrial proteins and supports mitochondrial fusion by regulating the expression of fusion-related proteins[29]. The reduction in SIRT1 activity can affect PGC-

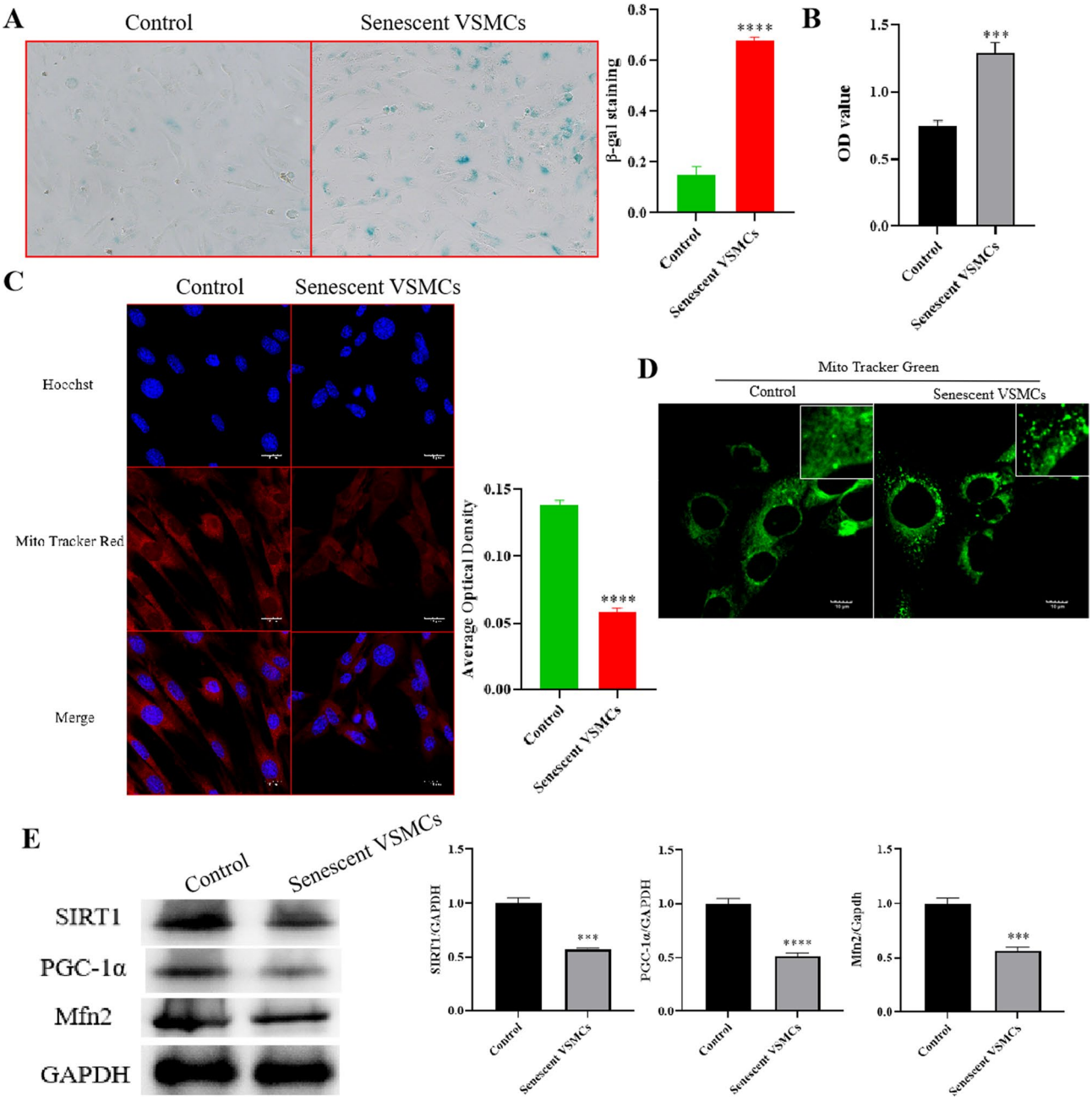


Fig. 1. Mitochondrial homeostasis and Calcification on the Aging Process of Vascular Smooth Muscle. (**A**). SA-β-gal staining results demonstrate increased β-galactosidase activity in aged vascular smooth muscle cells compared to controls. (**B**). Calcium nodule detection shows a trend towards increased calcification in aged VSMCs. (**C**). Mito Tracker Red staining reveals reduced mitochondrial membrane potential in aged VSMCs. (**D**). Mito Tracker Green staining reveals mitochondrial morphological changes in aged VSMCs. (**E**). Western blot analysis shows decreased expression levels of SIRT1, PGC-1α, and Mfn2 in aged VSMCs compared to controls. $^{***}P < 0.001$, $^{****}P < 0.0001$, indicated a significant difference between groups.

1α function and, consequently, mitochondrial biogenesis and fusion. The decline in mitochondrial function is associated with increased oxidative stress, apoptosis, and the development of age-related vascular diseases such as vascular calcification[30]. Therefore, the regulatory role of SIRT1 in PGC-1α are potential targets for the mitochondrial function. We first confirmed the association between the calcification of aged VSMCs and mitochondrial homeostasis as well as the signaling axis (Fig. 1). Meanwhile, our finding showed that knock-out PGC-1α also reduces SIRT1 expression (Fig. 2B-C). Although there is currently no direct evidence for the regulation of SIRT1 by PGC-1α, previous studies demonstrated the network between PGC-1α, SIRT1 and AMP-activated protein kinase (AMPK)[31]– [32], hence suggesting a feedback mechanism was existed between SIRT1 and PGC-1α.

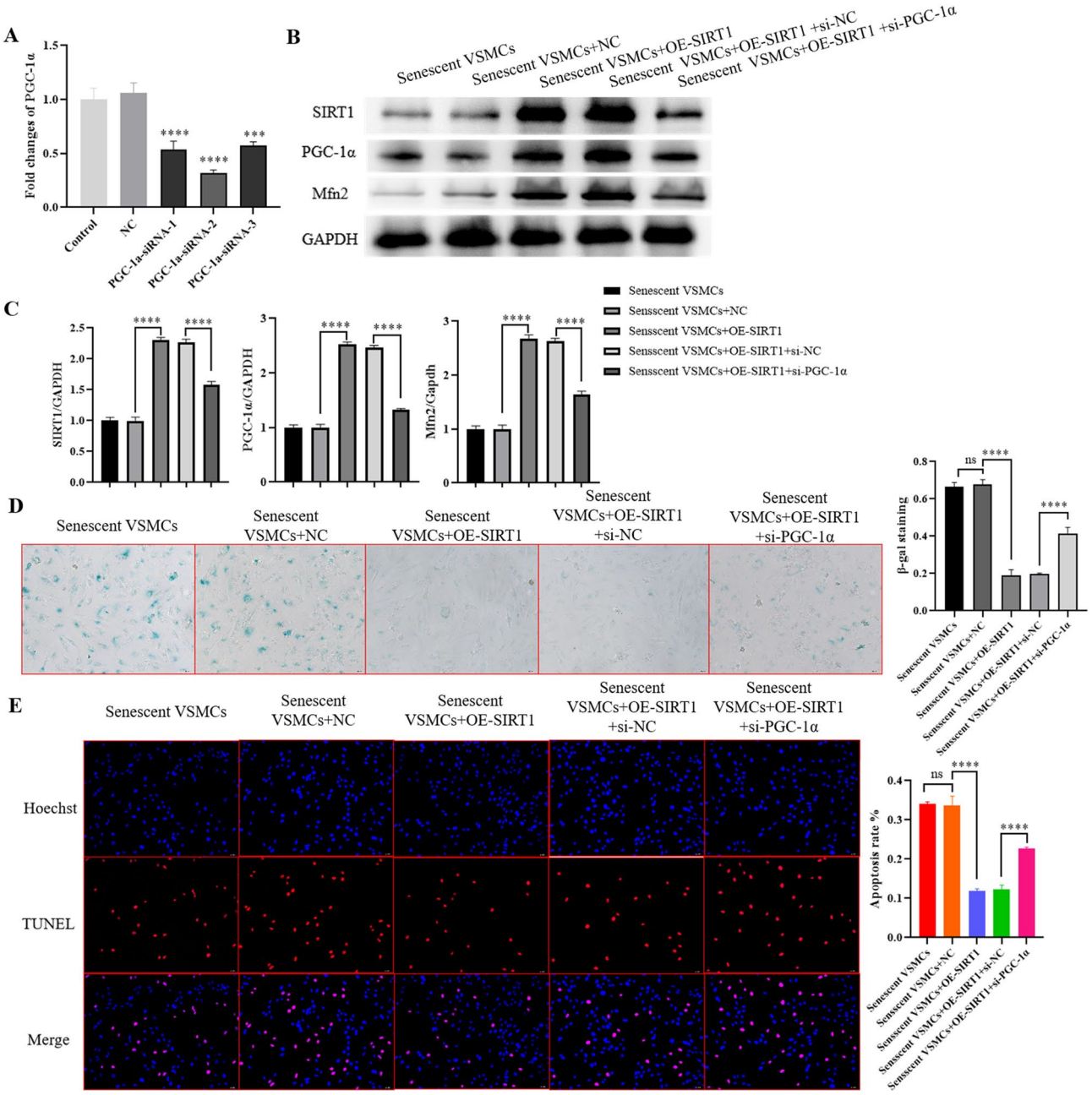


Fig. 2. Effects of SIRT1-PGC-1α signaling on Cellular Senescence in Aging VSMCs. (**A**) Verification of siRNAs of PGC-1α in VSMCs by RT-qPCR. (**B**) The expression of the SIRT1, PGC-1α, and Mfn2 were detected by Western blotting. (**C**) Gray analysis of the western blot results of SIRT1, PGC-1α, and Mfn2. (**D**) Detection of the level of cellular senescence by SA-β-gal staining in each group. (**E**) The apoptosis of VSMCs in each group were detected by Tunel staining. ns: no significance; ***$p < 0.001$, ****$P < 0.0001$, indicated a significant differences between groups.

The deacetylation process can be impaired when SIRT1 levels decrease, leading to dysfunctional mitochondrial fusion, reduced mitochondrial biogenesis, and diminished antioxidant responses[33]. The resulting mitochondrial dysfunction exacerbates oxidative stress, which in turn leads to the accumulation of mitochondrial damage. Low SIRT1 expression directly correlates with increased levels of mitochondrial reactive oxygen species (ROS), as the compromised antioxidant defense fails to neutralize excess ROS. While high ROS levels contribute to mitochondrial DNA damage, lipid peroxidation, and protein oxidation, all of which further impair mitochondrial function[34]. This dysfunction also triggers apoptosis, as the loss of mitochondrial membrane potential and the release of pro-apoptotic factors from damaged mitochondria initiate the cell death cascade[35]. Additionally, impaired mitochondrial fusion and biogenesis lead to a decrease in ATP production, and reducing the energy available for essential cellular processes, thereby exacerbating cellular aging and dysfunction[36]. Our study demonstrated that overexpression of SIRT1 reduced the cellular aging and apoptosis (Fig. 2D-E), which is

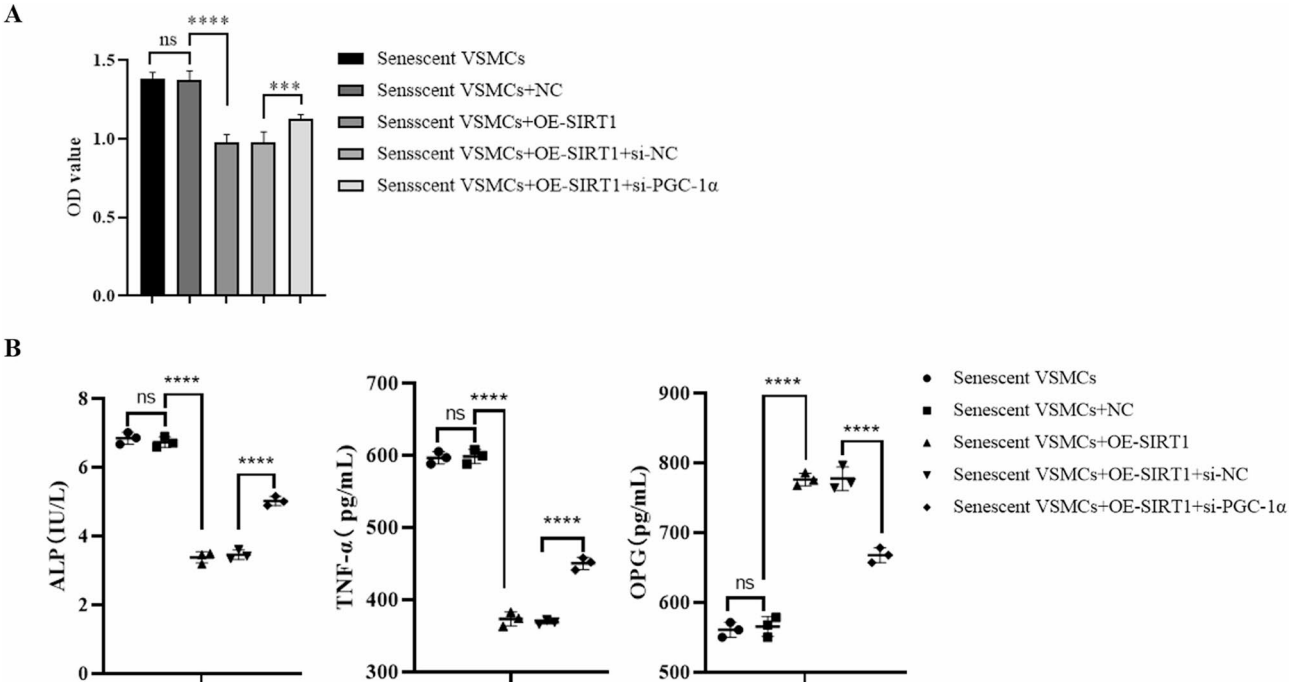


Fig. 3. Effects of SIRT1-PGC-1α signaling on vascular calcification in Aging VSMCs. (**A**) Calcium nodule detection in each group by Alizarin Red S staining. (**B**) Factors associated with vascular calcification were detected by ELISA. ns: no significance; $^{***}p < 0.001$, $^{****}P < 0.0001$, indicated a significant differences between groups.

consistence with these reports. In VSMCs, impaired mitochondrial homeostasis resulting from reduced SIRT1 expression leads to a cascade of detrimental effects. The increase in mitochondrial ROS and subsequent oxidative stress disrupt cellular signaling pathways, impair VSMC contractility and promote cellular senescence[37]. This dysfunction manifests as a loss of the contractile phenotype in VSMCs and a shift towards a synthetic, pro-inflammatory phenotype, which is linked to vascular diseases[37]. The shift in VSMC phenotype, coupled with increased oxidative stress and apoptosis, creates a pro-calcific environment[38]. Oxidative stress-induced damage to cellular components, along with apoptotic bodies released from dying VSMCs acts as nucleation sites for calcium phosphate deposition, thereby promoting vascular calcification. The decline in ATP production further impairs the cells' ability to maintain calcium homeostasis, thereby exacerbating calcification. Consequently, the aging-induced reduction in SIRT1 expression induces the pathological calcification of the vascular wall, a hallmark of vascular aging and a significant risk factor for cardiovascular diseases, which is consistent with our findings (Fig. 3).

The decrease in SIRT1 levels leads to hyperacetylation and inactivation of PGC-1α, diminishing its ability to drive mitochondrial biogenesis and homeostasis. This reduction in PGC-1α activity further impairs mitochondrial homeostasis, creating a feedback loop in which declining SIRT1 activity exacerbates mitochondrial dysfunction through the loss of PGC-1α activity. In scenarios where SIRT1 activity is compromised, PGC-1α can partially compensate by promoting mitochondrial biogenesis and maintaining mitochondrial dynamics[39]. However, when PGC-1α is also knocked down, this compensatory mechanism is abolished, resulting in severe mitochondrial dysfunction (Fig. 4). Mitochondrial fusion is essential for maintaining mitochondrial function and integrity, particularly in VSMCs, where it helps preserve cellular energy production and reduce oxidative stress[40]. This explains our findings that knocking down PGC-1α reverses the effect of SIRT1 on mitochondrial homeostasis (Figs. 4 and 5A). Furthermore, the reduction in PGC-1α regulated Mfn2 also results in impaired mitochondrial fusion, leading to fragmented mitochondria, increased mitochondrial ROS production, and a decline in ATP synthesis[17,40]. This disruption in mitochondrial dynamics can exacerbate the dysfunction of VSMCs, thereby contributing to vascular aging and calcification.

Therefore, aging leads to decreased SIRT1 expression in VSMCs, which inhibits the effect of SIRT1 on PGC-1α deacetylation. The reduced deacetylation of PGC-1α decrease its activity, and further downregulates the Mfn2 expression, all these effects inhibit the mitochondrial fusion, subsequently inducing mitochondrial dysfunction, as well as osteogenic transformation and calcification of VSMCs.

Conclusions

Although limitations exist in this study, including studies on single VSMC cells and the lack of in vivo validation data. We found that the aging related VSMCs calcification were associated with mitochondrial homeostasis and damage and regulated by the SIRT1-PGC-1α signaling. This study provides a new insight into the mechanisms of age-related vascular calcification. Moreover, these findings offers therapeutic targets, biomarker potential for

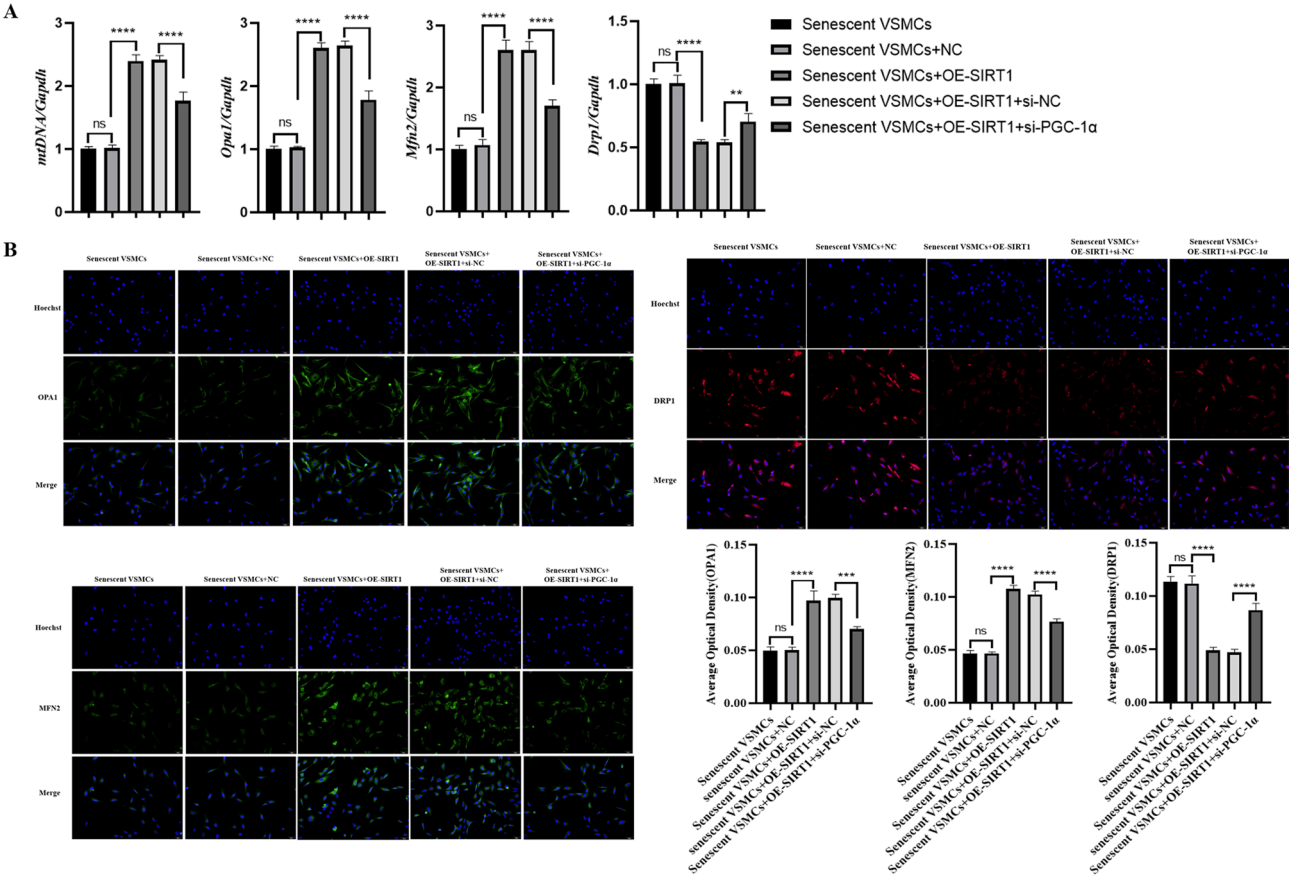


Fig. 4. Effects of SIRT1-PGC-1α signaling on the level of Mitochondrial mtDNA and fusion-related molecules in Aging VSMCs. (**A**) The mRNA levels of mtDNA and fusion-related molecules were detected by RT-qPCR. (**B**) The expression of fusion-related molecules were detected by immunofluorescence. ***$p < 0.001$, ****$P < 0.0001$, indicatd a significant differences between groups. ns: no significance; ***$p < 0.001$, ****$P < 0.0001$, indicated a significant differences between groups.

the severity of vascular calcification, combination therapy possibilities, which will lay the groundwork for the treatment of age-related arterial disease in the future.

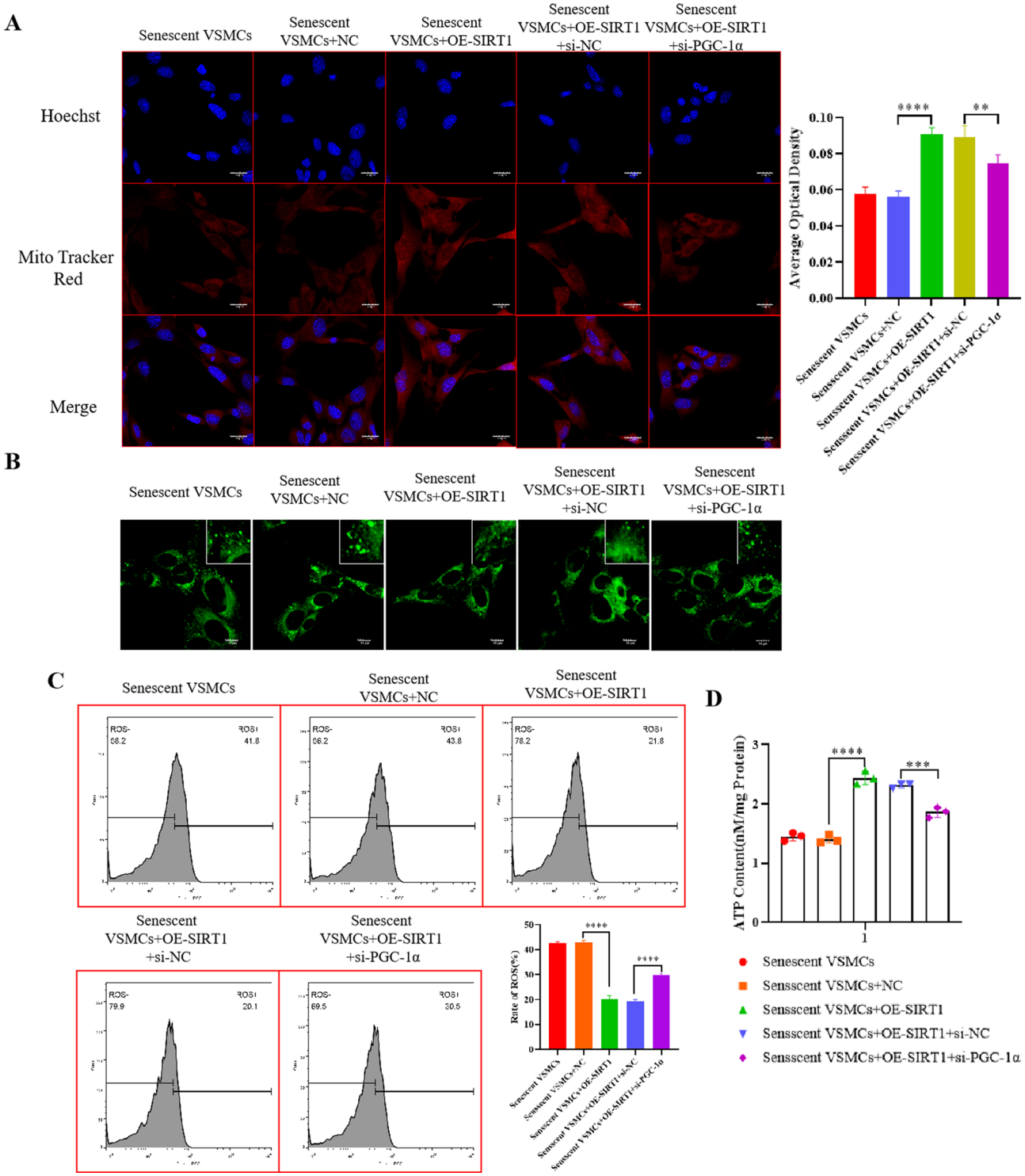


Fig. 5. Effects of SIRT1-PGC-1α signaling on the Mitochondrial functions in Aging VSMCs. (**A**). Mitochondrial membrane potential were detected by Mito Tracker Red staining. (**B**). Mitochondrial morphology were observed using Mito Tracker Green staining. (**C**) The ROS level in the VSMCs were detected by the ROS detection reagent. (**D**) The ATP secreted level from VSMCs were detected by the ATP detection kit. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}P < 0.0001$, all indicated significant differences between groups.

Data availability

The data used to support the findings of this study can be obtained from the corresponding author upon reasonable request.

Received: 20 May 2025; Accepted: 24 September 2025
Published online: 30 October 2025

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Author contributions
CM.F designed the study and revised the manuscript. C.W performed the experiments and analyzed the data, as well as the manuscript writing. ZF.W performed the cell culture and part of the technical detection. L.T and XJ.H provided the support of the experiments and data analysis. all authors reviewed the manuscript.

Funding
This study was funded by the Teaching Hospital Research Special Program of Wannan Medical College (Grant Number: WK2023JXYY147).

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-21905-7.

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