SRT1720 Protects Against CSE-Induced Cellular Senescence via Accelerates of FOXO3-PINK1-mediated Mitophagy

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Research

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Abstract

Background

Chronic obstructive pulmonary disease (COPD) is often associate with cigarette smoke extract (CSE)-introduced bronchial epithelial cell senescence, mitochondrial fragmentation. Sirtuin-1(Sirt1) has been reported to play a crucial role in mitochondrial homeostasis and confers a protective role against the onset and development of CSE introduced bronchial epithelial cell senescence in COPD although the precise mechanism(s) remain elusive. Here we hypothesized that SRT1720, a pharmacological SIRT1720 activator, exerts protect against COPD by activating PINK1 mediated mitophagy, en route to preserved mitochondrial homeostasis.

Methods

COPD rats model was established by CS exposure. During 6 months of SRT1720 treatment, airway resistance, cellular senescence and mitochondrial injury, mitophagy in the lung tissues of model rats were examined by western blot(WB) and histochemical and immunofluorescence staining. Transmission electron microscopy was also carried to elucidate the effects of SRT1720.Human bronchial epithelial cells(HBEC) were used to clarify the underlying molecular mechanisms.

Result

During the introduction of CSE in cellular or rats, administration of SRT1720 improved airway resistance, cellular senescence and mitochondrial injury, accompanied with suppressed autophagy and mitophagy. Mitochondrial damage, cellular senescence and lung injury under contrast exposure were more severe in FOXO3 or Pink1 deficient cells and mice than in SRT1720 groups. Activation of Sirt1 by treating with SRT1720 induces autophagy enhanced. A Decrease in sirt1 expression caused by selisistat treatment promotes senescence.

Conclusions

Taken together, our data suggested that suppressed SIRT1/FOXO3/Pink1 signaling mediated mitophagy played a protective role in COPD by reducing mitochondrial reactive oxygen species (ROS).

Background

Chronic obstructive pulmonary disease (COPD) is one of the most common chronic and disabling diseases and a growing cause of morbidity and mortality[1]. It is currently the fourth leading cause of death worldwide, and the World Health

Organization (WHO) predicts that it will become the third leading cause by 2030. In general, COPD is classified as chronic inflammatory disease associated cell metabolism disorder resulting from cigarette smoke, oxidative stress and inflammatory injury[2-4]. Accelerated cellular senescence resulting from

cigarette smoke (CS) exposure with excessive reactive oxygen species (ROS) production has been implicated in the pathogenesis of COPD[5]. Nonetheless, the precise molecular mechanism(s) involved in the pathogenesis of COPD remains elusive.

Mitochondria, an organelle that maintains cell energy metabolism, is highly involved in cerebral ischemia/reperfusion injury[6]. The stability of its structure and function is considered an important therapeutic target. The process by which cells clear damaged or dysfunctional mitochondria and complete self-renewal to maintain mitochondrial quality control is called mitophagy[7, 8]. The autophagy and renewal of mitochondria are the prerequisites for repetitive operation and continuous supply of energy to cells[9]. Moderate mitophagy can protect mitochondrial function and help maintain cell energy metabolism and survival, excessive or insufficient autophagy will paralyze mitochondria and lead to cell death[10].

Sirtuin deacylase enzymes are important modulators of mitochondrial energy metabolism. Sirtuin 1(SIRT1) is the mammalian orthologue of the yeast silent information regulator 2 (Sir2), which is function as NAD+-dependent deacetylases as well as ADP ribosyltransferases[11-13]. SIRT1 can modulates the activity of deacetylates histone and transcription factors such as p53 and forkhead box O3 (FoxO3)[14, 15].Emerging evidences have shown sirt1, an anti-inflammatory and anti-aging protein, is reduced in lungs of patients with COPD[16]. Recently evidence has depicted the molecular mechanism of CS-induced cellular senescence via a SIRT1-FOXO3 axis protects against stress-induced premature senescence (SIPS) and various pathophysiological changes in COPD.[17]

SRT1720, a pharmacological Sirt1 activator, was found a positive regulator for emphysema via up-regulated the levels of foxo3, increased SIRT1 activity and inhibited AECII apoptosis[18-20]. Further studies also depicted a novel role for sirtuins in the pathogenesis and treatment of COPD. Moreover, the network of SIRT1-FOXO3-Pink1 was reported to govern mitophagy and mitochondrial balance between fusion and fission[21]. However, the role of Sirt1 in the regulation in COPD has not been fully elucidated.

In this context, this work was designed to determined (a) whether SIRT1 imposes any effect on the regulatory role of autophagy in cigarette smoke extract (CSE)-induced cell senescence of primary HBEC, if any;(b) whether the foxo3a-pink1 signaling pathway is involved in mitophagy regulation triggered by SIRT1.

Materials And Methods

Mice

We purchased 45 male C57B1/6 wild type mice(8 weeks,200±25g) from Shanghai Laboratory Animal Co., Ltd.(shanghai, China).We maintained the mouse at 21℃-25℃ and 40%-60% relative humidity in a 12-hour light/dark circle and provided them with water and food ad libitum in the Laboratory Animal Center of Zhejiang Industry University(Hangzhou, China).The mice were randomly sorted into 4 groups(15 mice/group):WT(group Ⅰ),CS exposure(group Ⅱ), WT +selisistat(group Ⅲ) and CS exposure

+selisistat(group Ⅳ).All animal protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University.

CS exposure

A custom-designed cigarette smoking chamber stimuli were used for generating the mice model of COPD. For studies involving 3 days of CS exposure, research grade cigarettes (Xiongshi; China Tobacco Zhejiang Industrial. Co., Ltd., China) were used to generate smoke. Briefly, cigarette smoke exposure were performed as follows：mouse in group Ⅲ and group Ⅳ were placed in the cigarette smoking chamber(60cm×50cm×40cm). After an interval of 10 minutes, the smoke of 5 new cigarettes was delivered into the chamber. CS concentration was set at a value of approximately 300 mg/m^3 total particulate matter(TPM) by adjusting the flow rate of the diluted medical air, and the level of carbon monoxide in the chamber was 350 ppm[22, 23], monitored by a real time aerosol monitor(MicroDust pro,Casella CEL, Bedford, UK). The mouse were exposed to smoke for 90 minutes daily for consecutive 24 weeks.

Administration of selisistat

Selisistat(30mg/kg body weight in 10%DMSO and 40%PEG300 and 5%Tween-80 and 45% saline,>95% pure by C-13 NMR and LCMS; synthesized by life chemicals) through oral gavage 1 hour prior to CS exposure daily. To study the therapeutic effect on emphysema, selisistat was orally administrated daily for 4 weeks after the development of CS induced emphysema.

Cell culture

Normal airways were obtained from 4st order bronchi from pneumonectomy and lobectomy specimens for primary lung cancer. Informed consent was obtained from all surgical participants as part of an approved ongoing research protocol by the ethics committee of Hangzhou medical college. HBEC were isolated with protease treatment and characterized as previously describe[24]. HBEC were serially passaged and used for experiments were performed with HBEC from non-COPD patients. HBEC was cultured in RPMI1640 with 10% fetal calf serum and penicillin-streptomycin(Gibo Life Technologies,15140-112).For in vitro study, HBEC were treated with CSE(1%) and SRT1720(4mM/L,>95% pure by C-13 NMR and LCMS; synthesized by life chemicals)for 48h.

Preparation of cigarette smoke extract(CSE)

Cigarette smoke extract (CSE) was prepared as previously described with minor modification[10]. Forty milliliters of cigarette smoke were drawn into the syringe and slowly bubbled into sterile serum-free cell culture media in 15-ml BD falcon tubes. One cigarette was used for the preparation of 10 ml of solution. CSE solution was filtered(0.22μm;Merck Millipore,SLGS033SS) to remove insoluble particles and was designated as a 100%CSE solution.

Measurement of lung mechanics

Lung mechanical properties, including lung compliance and RL, were determined as described previously[25]. Briefly, the mouse was weighed, deeply anesthetized by i.p. injection of pentobarbital (90 mg/kg BW) and pancuronium (0.5 mg/kg BW), and tracheostomized. The trachea was cannulated, and the cannula was connected to a computer-controlled small animal ventilator (FlexiVent; SCIREQ).

Lung morphometry

Mouse lungs (which had not been lavaged) were inflated with 1% low-melt agarose at a pressure of 25 cm H2O, then fixed with 4% neutral buffered PFA [25, 26]. Fixed lung was dehydrated, embedded in paraffin, and sectioned into 4-μm sections using a rotary microtome (MICROM International GmbH). H&E staining was performed on the lung midsagittal sections to determine Lm of airspace using MetaMorph software (Molecular Devices) [25]. Ten randomly selected ×100 fields per slide were photographed in a blinded manner, and the images were manually thresholded. The airway and vascular structures were eliminated from the analysis.

Senescence-associated-β-galactosidase (SA-β-gal) activity assay

SA-β-gal activity was assessed using an *in situ* β-galactosidase staining kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Lung tissues were fixed in β-galactosidase stationary solution for 15 min, then washed 3 times for 10 min each in PBS. Sections were then incubated with 1 ml staining solution mixture (10 μl staining solution A, 10 μl staining solution B, 930 μl staining solution C and 50 μl X-gal solution) for 2 h at 37˚C. Following 3 washes with PBS, 5 fields of view from each of the 3 sections from each lung sample were examined using a light microscope (Olympus Corporation).

Transmission electron microscopy (TEM)

Murine lung tissues were fixed in 2 % glutaraldehyde for at least 24 h. Tissues were then immersed in 2 % osmium tetroxide and 1 % aqueous uranyl acetate, each for 1 h. After washed with a series of ethanol solutions (50 %, 70 %, 90 % and 100 %), tissues were transferred to propylene oxide, incubated in a 1:1 mixture of propylene oxide and EMbed 812 (Electron Microscopy Sciences) for 1 hour and then placed in a 70 °C oven to polymerize. Sections (75–80nm) were cut using a Leica ultramicrotome equipped with a Diatome diamond knife and collected on 200-mesh copper grids. After poststained in 5% uranyl acetate for 10 min and in Reynold’s lead citrate for 5

min, sections were observed using a 40–120 kV transmission electron microscope (FEI TECNAI G2 Spirit Biotwin, Hong Kong, China). For in vitro study, After treatment, HBEC were fixed with 2% glutaraldehyde/ 0.1 M phosphate buffer (pH7.4) and in 1% osmium tetroxide/0.1 M phosphate buffer (pH7.4), and dehydrated with a graded series of ethanol. The operations subsequent were performed as described above.

Immunofluorescence straining

Murine lung frozen sections were fixed with 4% paraformaldehyde. After washing with PBS for several times and incubating with goat serum for 1 h, they were incubated overnight with LC3B antibody(1:100,Cell Signaling Technology, USA)at 4 ℃. HBEC expressing EGFP-LC3B grown on 6-well culture slides were fixed with 4% paraformaldehyde for 15 min followed by permeabilization with 0.03% Triton X-100(Wako,16024751) for 60 min. After blocking with 0.1%BSA(Sigma Aldrich,A2153) for 60 min, the primary and secondary antibodies were applied according to the manufacture's instructions. Confocal laser scanning microscopy analysis of mitochondria was performed by TOMM20 staining, assessed with a confocal microscope(Carl Zeiss LSM510,Tokyo,Japan).Mito Tracker Red CMX Ros (MTR; Molecular Probes-Life Technologies,M-7512) staining (200nM, 30 min at 37℃) was also performed to evaluate the integrity of the mitochondrial membrane potential.

Western blotting

After treatments, murine lung tissues and HBEC were collected, digested and analyzed using a BCA assay(for protein concentration, Thermo Fisher Scientific,89900) with protease inhibitor cocktail(Roche Diagnostics, 11697498001). For each group, equal amounts of total protein were separated using SDS-PAGE gels, transferred to polyvinylidene difluoride membrane(PVDF,Millipore,ISEQ00010), and then incubated overnight at 4℃ with special primary antibody. Antibody used by mouse anti-Sirt1(Cell Signaling Technology,2947), rabbit anti-LC3B(Cell Signaling Technology,2577), rabbit anti-PINK1 (Abcam, ab23707), mouse anti-ACTB (Sigma-Aldrich, A5316), Foxo3A(Cell Signaling Technology, 2132), Ac-Foxo3a (Santa Cruz Biotechnology, sc-468). Then blots were incubated by anti-rabbit IgG, HRP-linked secondary antibody(1:5000,Cell Signaling Technology,7074) or anti-mouse IgG, HRP-linked secondary antibody(1:5000, Cell Signaling Technology, 7076) for 1 h. Finally, Blots were scanned and detected by the luminescence method. Band intensity was analyzed using the Image J software(Version 1.45).

Statistical analysis

All the experimental data are presented as the means ±SEM and analyzed by Prism version 6.0 (Graph-Pad Software, San Diego, USA). The *t*-test was performed to measure the differences between the two groups and one-way analysis of variance (ANOVA) followed by a Dunnett's test was performed to compare the differences among three or more groups. *P*-values<0.05 were considered statistical significance.

Results

Effect of Sirt1 inhibitor on CSE induced changs in lung function

Expression of Sirt1 in mouse with selisistat treatment was confirmed using western blot analysis. Our result showed that Sirt1 protein expression was drastically decreased in selisistat treatment mouse lung exposed to CS compare with their WT littermates (Fig.1A). Lung morphometry to examine alveolar mean linear intercept(L_m).CS exposure for 6 months induced a modest airspace enlargement in WT mice, whereas SIRT1 deficient mice exhibit airspace enlargement (Fig.1B). To evaluate the effect of Sirt1

inhibitor on CSE induced changes in lung function, Lung mechanical properties was employed to evaluate lung compliance and R_L at 3 cmH_2O positive end-expiratory pressure were obtained by fitting a model to each impedance spectrum. Our data showed that lung compliance was augmented in WT mice exposed to CS for 6 months, the effect of which was accentuated in selisistat treatment mice (Fig.1C and D). *Sirt1* deficiency decreased total lung resistance(R_L), although no significant change in R_L in WT mice was observed after 6 months of CS exposure.

Effect of Sirt1 inhibitor on CSE-induced airspace enlargement, cellular senescence and mitochondrial morphology

To evaluate the effect of Sirt1 inhibitor on CSE induced lung injury, SA-β-gal activity assay and transmission electron microscopy were used to examine cellular senescence and mitochondrial morphology, respectively. Our finding revealed that CS exposure for 6 months induced a modest airspace enlargement in WT mice, which was further augmented in selisistat treated mice. As expected, CS exposure significantly the level of SAβgal activity in lungs of selisistat treated mice versus WT littermates. Transmission electron microscopy also revealed more swollen an damaged mitochondria in WT mice exposed to CS, the effect of which was augmented by Sirt1 inhibitor treated mice. The mitochodria were slightly changed in selisistat treated mice compared with WT mice. (Fig2)

Effect of Sirt1 inhibitor on CSE-induced autophagy regulation

To determine the effect of Sirt1 inhibitor on CES induced autophagy regulation, immunofluorescence and western blotting were used to examine LC3 puncta in frozen section of lungs and LC3B protein expression, respectively. Our data revealed that the number of LC3B puncta was significant decreased in WT mice exposed to CS, with a more pronounced response in Sirt1 inhibitor mice(Fig3A).Meanwhile western blot analysis revealed that the LC3Ⅱ protein level were overtly downregulated in WT mice exposed to CS, the effect of which was accentuated by Sirt1 inhibitor(Fig3B).Sirt1 deficiency itself elicited a decrease in LC3 puncta and LC3Ⅱ level.

Signaling mechanism(s) in Sirt1 inhibitor induced autophagy regulation in emphysema

To explore the possible mechanism(s) in Sirt1 inhibitor accentuation of CSE induces changes in autophagy, western blot analysis was employed to examine the level of Ac-Foxo3A and Pink1. our result revealed that the ratio of Ac-Foxo3a to Foxo3A was greatly increased following CSE exposure, the effect of which was exacerbated by more upregulated by Sirt1 inhibitor. Sirt1 deficiency also increased the ratio of Ac-Foxo3a to Foxo3a. PINK1 expression was reduced in WT mice exposed to CSE, the effect of which was unaffected by Sirt1 inhibitor.Sirt1 deficiency also decreased the level of PINK1.(Fig.4.)

Alteration of levels of Sirt1 and LC3B by CSE in primary human bronchial epithelial cells

HBEC were treated with 10% fetal calf serum and CSE (1%) in the presence or absence of SRT1720 for 48h,and the control cells were cultured with 10% fetal calf serum. Western blotting revealed that LC3Ⅱ

protein level began to decline and p62 expression began to rise at CSE(1%) and was further reduced at CSE(2.5%),consistent with the previous report[14].We next examined the time course of autophagy downregulation. HBEC were exposed to CS (2.5%) for 6-48 hour, while 10% fetal calf serum were used as control. A significant reduction of LC3Ⅱ protein levels and increased p62 expression was observed from 24h,the effect of which was present throughout the entire examination period. The expression of Sirt1 began to decrease at 12h and remained consistent thereafter(Fig5).

Effect of Sirt1 overexpression on CSE induced ROS production, apoptosis, and mitophagy in HBEC

To determine the effect of Sirt1 overexpression on CSE induced the reduction of mitochondrial ROS production, DCFH-DA assays and MitoSOX Red staining were applied to evaluate the association between mitophagy and ROS production in HBEC. Our data showed that CSE treatment results in a rise of damaged mitochondria, total and mitochondrial ROS production, which was significantly reduced by SRT1720(Fig6A,B). JC-1 staining revealed that mitochondrial membrane potential of CSE group was significantly decreased, compared with normal control group, the effect of which was largely counteracted by SRT1720(Fig6C). Result from transmission electron microscopy showed that more obviously swollen and damaged mitochondria were observed in cells with CSE treatment, with were reserved by SRT1720(Fig6D).

Effect of Sirt1 overexpression on CSE induced autophagy regulation

To examine the effect of Sirt1 overexpression on CSE induced autophagy regulation, confocal microscopy was performed in HBEC stably experssion EGFP-LC3. Colocalization of TOMM20-stained mitochondria and EGFP-LC3B dots was used to determine autophagosome formation,and western blotting determining LC3B and p62 protein level was used.Our results showed that although CSE induced EGFP-LC3B dot formation,colocalization with mitochondria was barely detected in the absence of SRT1720. SRT1720 treatment alone induced EGFG-LC3B dot formation accompanied by limited colocalization with TOMM20-stained mitochondria(yellow dots),CSE treatment markedly enhanced EGFP-LC3B dot formation concomitant colocalization with TOMM20-stained mitochondria in the presence of SRT1720(Fig).For cellular exposed to CSE,the level of LC3Ⅱ were with significantly difference after overexpressing of Sirt1. The upregulated expression of p62 in response to CSE challenge was reversed by SRT1720.

Effect of Sirt1 overexpression on the mechanism(s) involved in CSE induced autophagy regulation

To determine the effect of Sirt1 overexpression on the mechanism(s) involved in CSE induced autophagy regulation,western blotting analysis was applied to detect the expression of Ac-Foxo3A and Pink1. The results revealed that Sirt1 level was increased after SRT1720 treatment. In CSE condition ,Sirt1 and Pink1 were both reduced,while the ratio of Ac-Foxo3A to Foxo3a was increased.The effect of CSE treatment was obviously reversed by overexpressing Sirt1 and this effect was also noted in normal HBEC with SRT1720.

Discussion

Our study finding that CSE induced HBEC and COPD downregulates anti-aging sirt1 and suppressed the Sirt1-Foxo3A signaling cascade. It is the protein levels demonstrated decreased Pink1 in CSE group compared with control group. Concomitantly, CSE induced fragmented mitochondria accumulated and slightly mitophagy activation[27, 28]. Activation of Sirt1 mediated Foxo3 deacetylation, resulting in an increase of Pink1 in HBEC treated with CSE, restoration of HBEC autophagy and protection against HBEC apoptosis[29, 30]. SIRT1 inhibitor lead to suppressed Sirt1-Foxo3 signaling and Pink1 mediated mitophagy may participate in CSE induced downregulation of HBEC autophagy and increased HBEC apoptosis[31]. SRT1720 and selisistat experiments clarify that both Sirt1 and Foxo3 are indicate a likely role for mitophagy in the pathogenesis of CSE exposure[32-34]. Moreover, recent studies observed modestly upregulation of PINK1 in lung homogenates from COPD patients[35, 36]. Therefore, the exact role of PINK1 in mitophagy has not been well done defined because of the existing contradistinction.

By mitophagic eliminate of damaged mitochondria and damaged organelles within lysosome, autophagy maintains cellular homeostasis[37]. Improper mitophagic has been implicated in the pathogenesis to different types of pulmonary diseases, and recently papers finding insufficient autophagy associated with complications of pulmonary including acute lung injury and idiopathic pulmonary fibrosis[38]. The depressed autophagy was proved to have a detrimental role in lung function of CES induced COPD although contrary findings have also been reported[39]. To elucidate the function role of mitophagy that using activation and inhibitor experiments in vitro and in vivo models. The CSE induced cellular damage was attenuated with Sirt1 overexpression, while the cellular damage was exacerbated in emphysema mice deficient in Sirt1, suggesting an insufficient role for mitophagy in COPD/emphysema[14, 40].

In the line with the recent findings CSE-induced emphysema lung dysfunction, the Sirt1-Foxo3-Pink1 pathway is involved in regulating the mitophagic elimination of CSE induced damaged mitochondria that modulates cellular senescence[41]. In this research, EM shows autophagy and mitophagy activation induced by Sirt1 result in attenuated mitochondrial numbers and enhanced degradation of mitochondrial debris(Fig 2).It has been reported that Pink1 levels in COPD smokers tend to be lower than those in control lung, and Pink1 overexpression increase mitophagy activity, indication that Pink1 is not only a pivotal predisposition to COPD development but also a crucial role in mitochondrial quality control in terms of regulation of senescence associated disorders[42, 43]. In this work, we found suppressed Pink1 in CSE induced HBEC or deficiency of Sirt1 condition, in concordance with other papers. Some studies demonstrated an inhibited autophagic flux in COPD lungs. In our vivo experiment, autophagy and Pink1 mediated mitophagy both reduced 6 months after CSE exposure, which can be regard as advanced stages of COPD.

Sirt1, as one of the intracellular deacetylases, is an important role in many processes of mitochondrial function[13]. Intriguingly, Studies demonstrated that Sirt1 may act as a tumor suppressor in several types of cancers. In response to CSE stimulus, Sirt1 was deficiency is increased apoptotic and mitochondrial dysfunction in bronchial epithelial cell. Airspace were enlarged obviously and the amount of alveoli was

significantly decrease in selisistat treat mice than control group mice, along with more depressed mitophagy. Sirt1 protects against emphysema through Foxo3-mediated maintain mitochondrial homeostasis and reduction of cellular senescence. Overexpression of Sirt1 inverted Sirt1-Foxo3a-Pink1 signaling mediated mitophagy and senescence of epithelial cell, indicating that Sirt1-Foxo3a-Pink1 signaling was a crucial pathway involved in overexpression Sirt1 promotion of alveoli survival under CSE exposure[11, 44, 45]. Recent advances demonstrated inflammatory reactions involved in Sirt1 induced regulation of mitophagic degradation[46]. It was reported that Sirt1 activated mitophagy by inducing TLR9(toll-like receptor 9) recognition and activation of IL1B/interleukin 1β,via deacetylation Foxo3 with the subsequent downregulation of NALP3 inflammasome[47, 48].

Conclusion

In summary, We demonstrated that the CSE induced mitophagy and autophagy by Sirt1-Foxo3-Pink1 path in HBEC. Suppressed Sirt1-Foxo3-Pink1 signaling and reduced Pink1 expression levels in COPD lung that downregulation of mitophagy and acceleration of cell senescence. Although the precise mechanism through other positive regulators regulated epithelial cell mitophagy in the setting of CSE exposure is still unclear. Our current study further supported the notion that the effect of Sirt1-Foxo3 signaling and Pink1-association mitophagy may explain the mechanism of insufficient mitophagy in COPD lung.

Abbreviations

ANOVA: One-way analysis of variance; COPD: Chronic obstructive pulmonary disease; CS: Cigarette smoke; CSE: Cigarette smoke extract; Foxo3: Forkhead-box class O3; HBEC: Human bronchial epithelial cells; PINK1: phosphatase/tensin homolog on chromosome 10 (PTEN)-induced putative kinase 1; ROS: Reactive oxygen species; TMP: Total particulate matter; TEM: Transmission electron microscopy; WB: Western blot;

Declarations

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Authors' contributions

DY and YJ conceived of the study. HJ and YL participated in design of the study and performed the experiments. HJ carried out the statistical analysis. DY helped in the interpretation of the data and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The software and all relevant raw data are freely available to scientists.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Experimental Animal Ethics Committee of Hangzhou medical college and performed in accordance with the guidelines for the care and use of laboratory animals set by Zhejiang Industry University (Hangzhou,China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

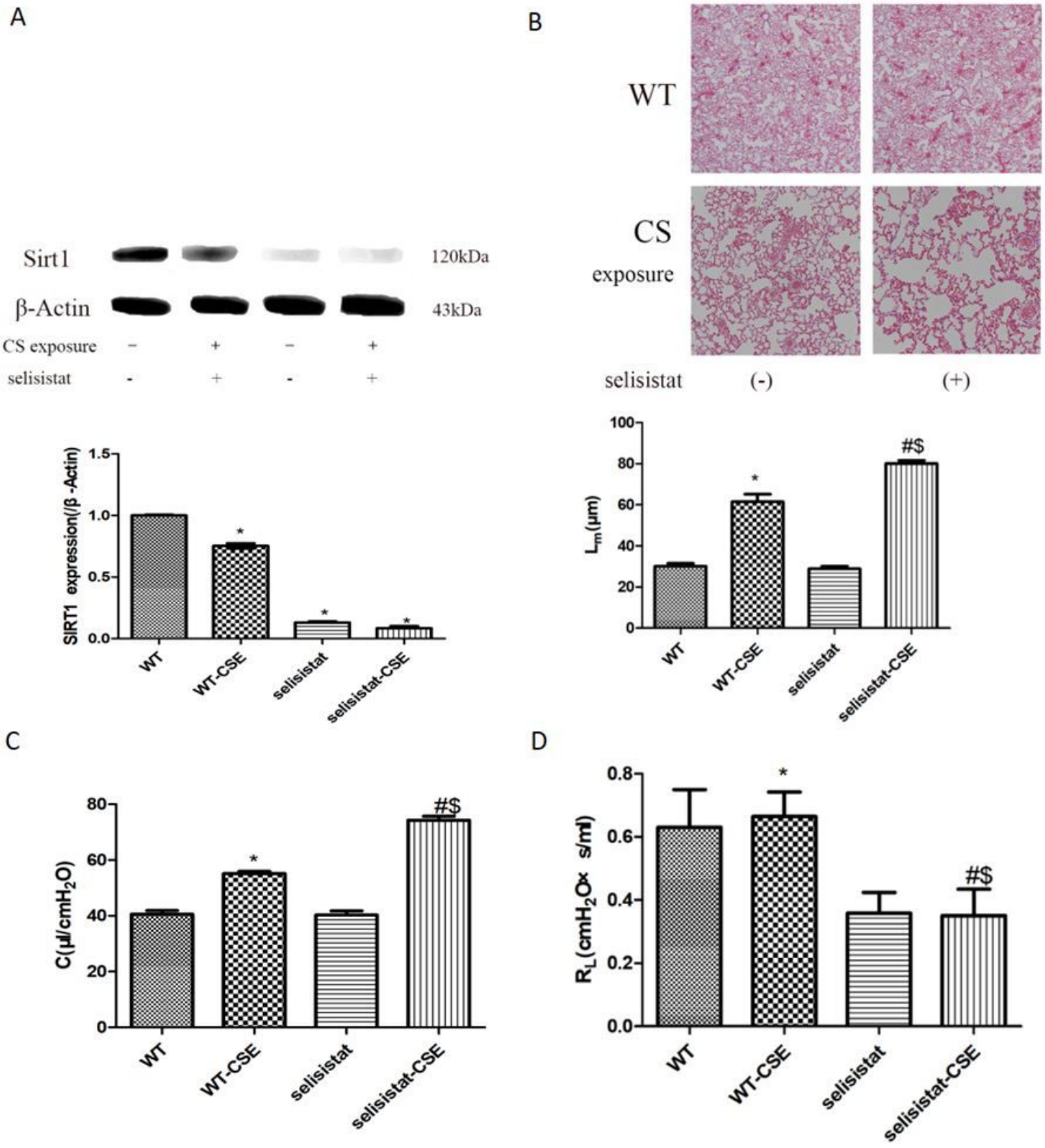

Figure 1

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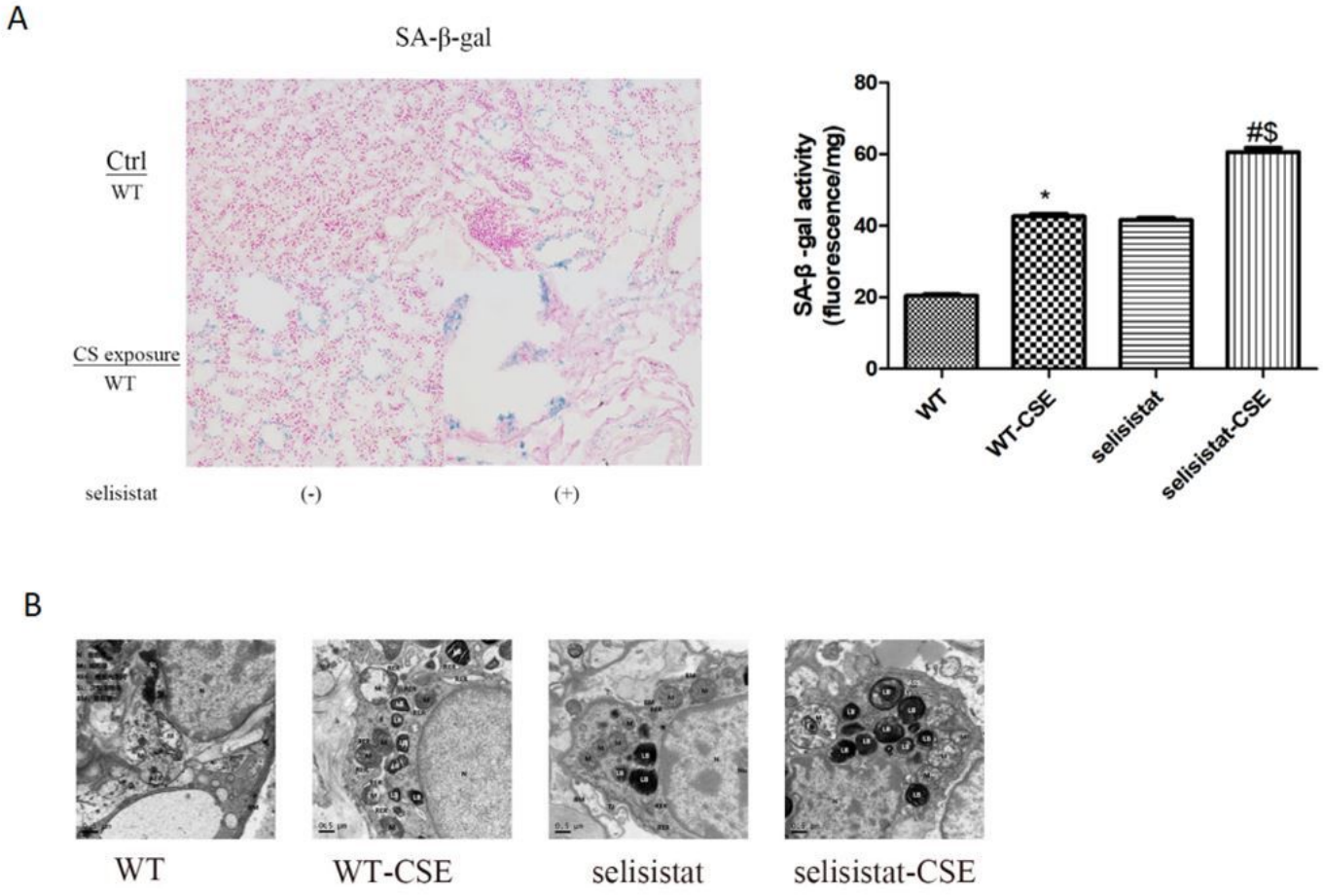

Figure 2

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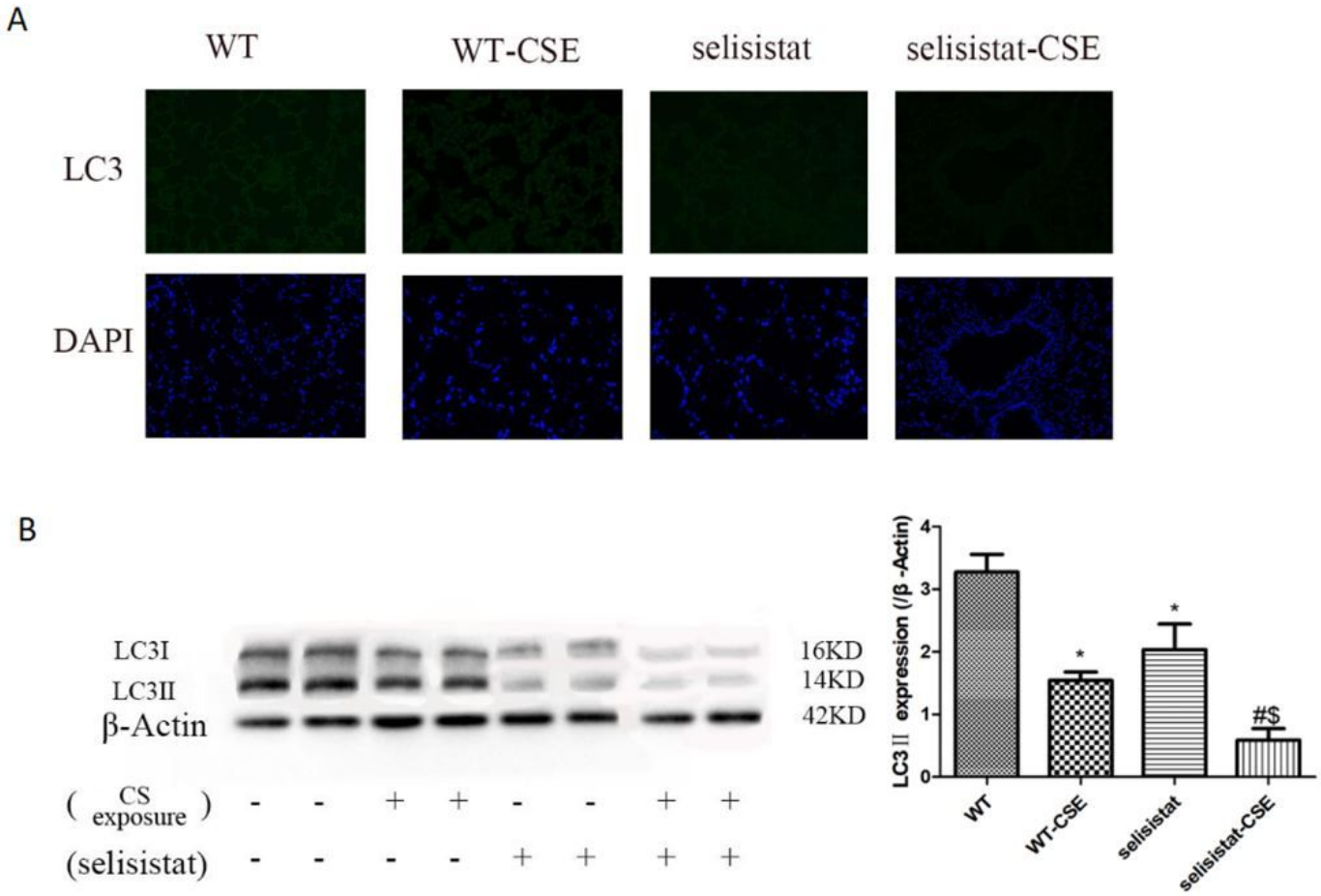

Figure 3

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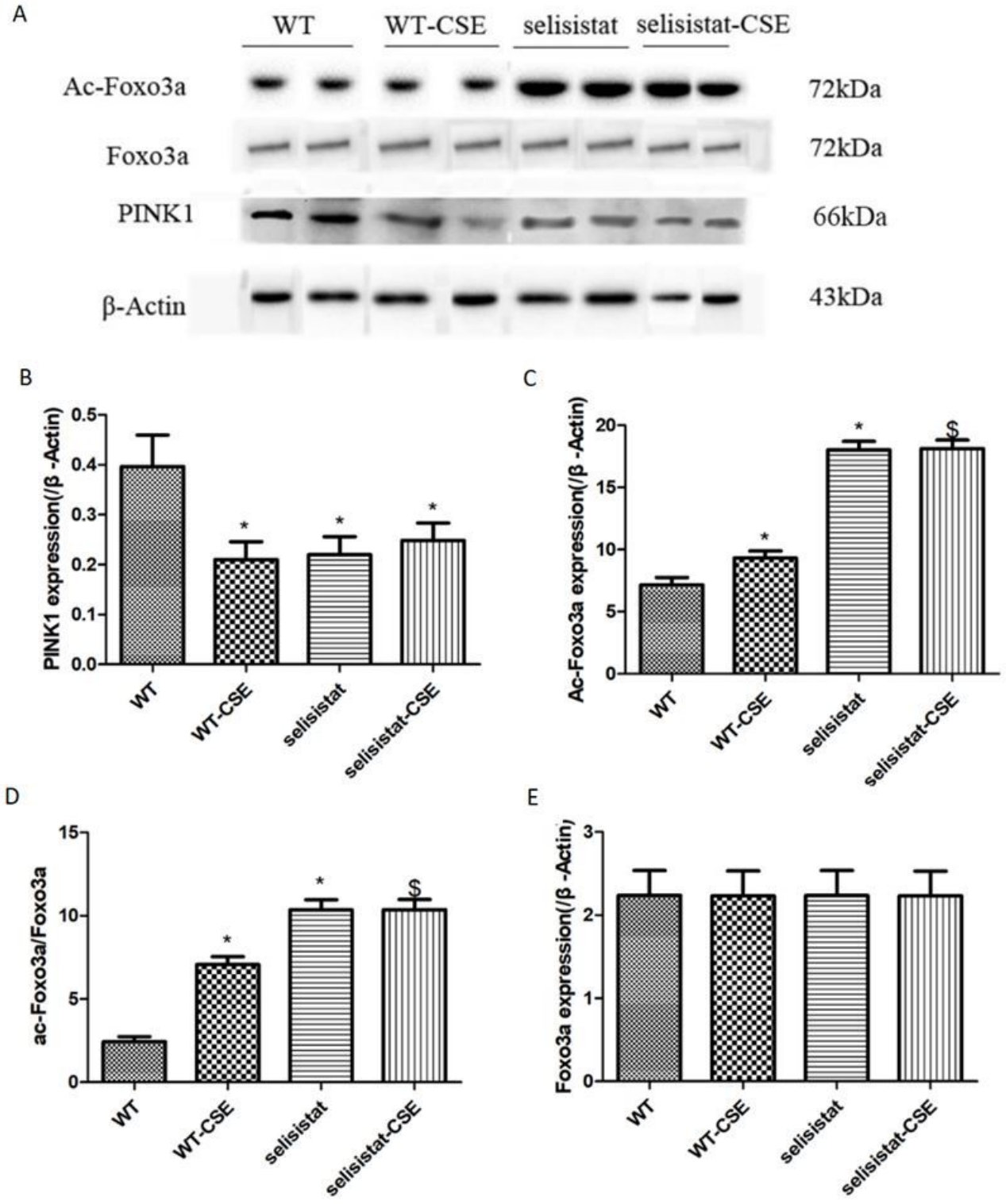

Figure 4

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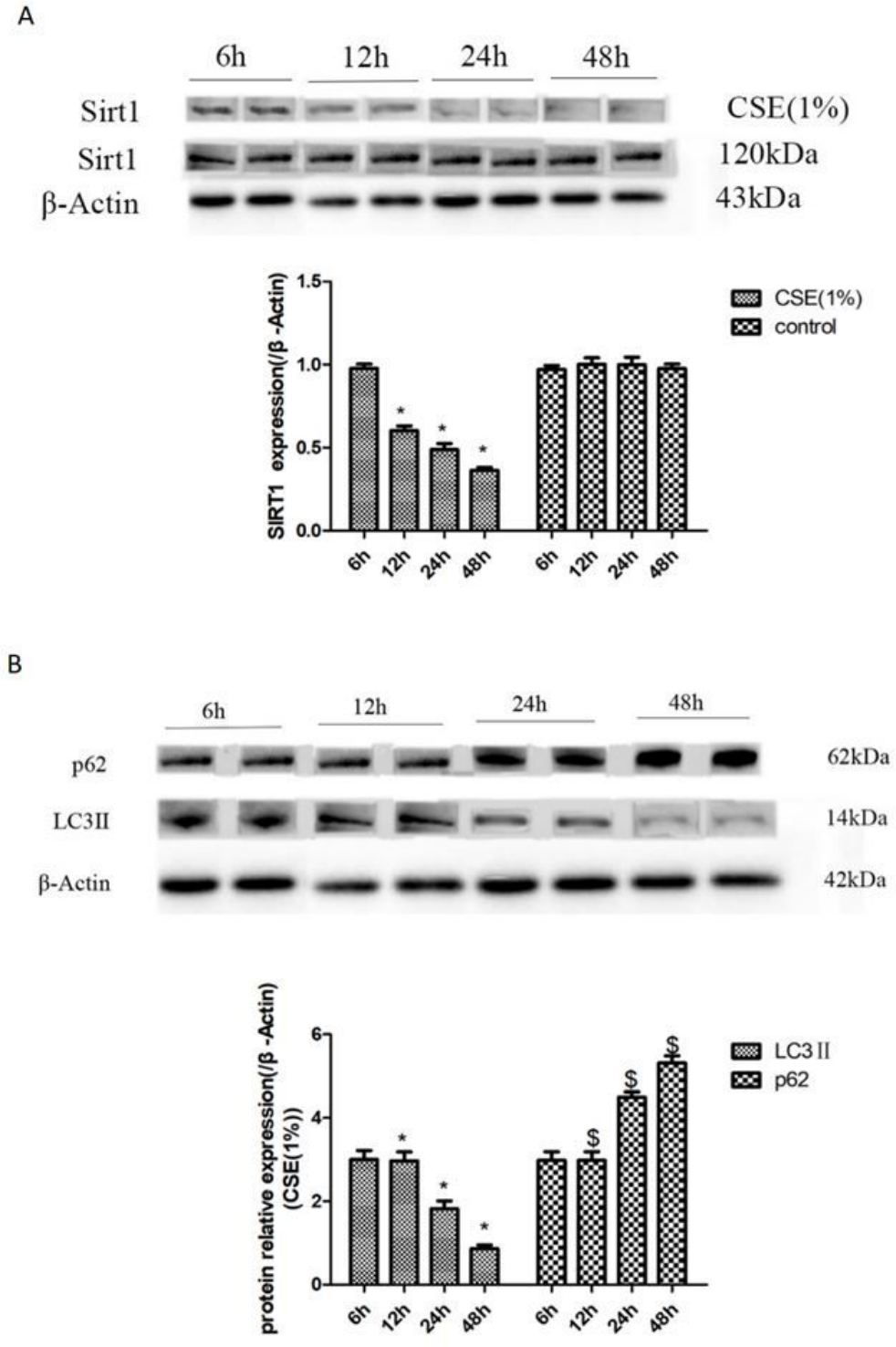

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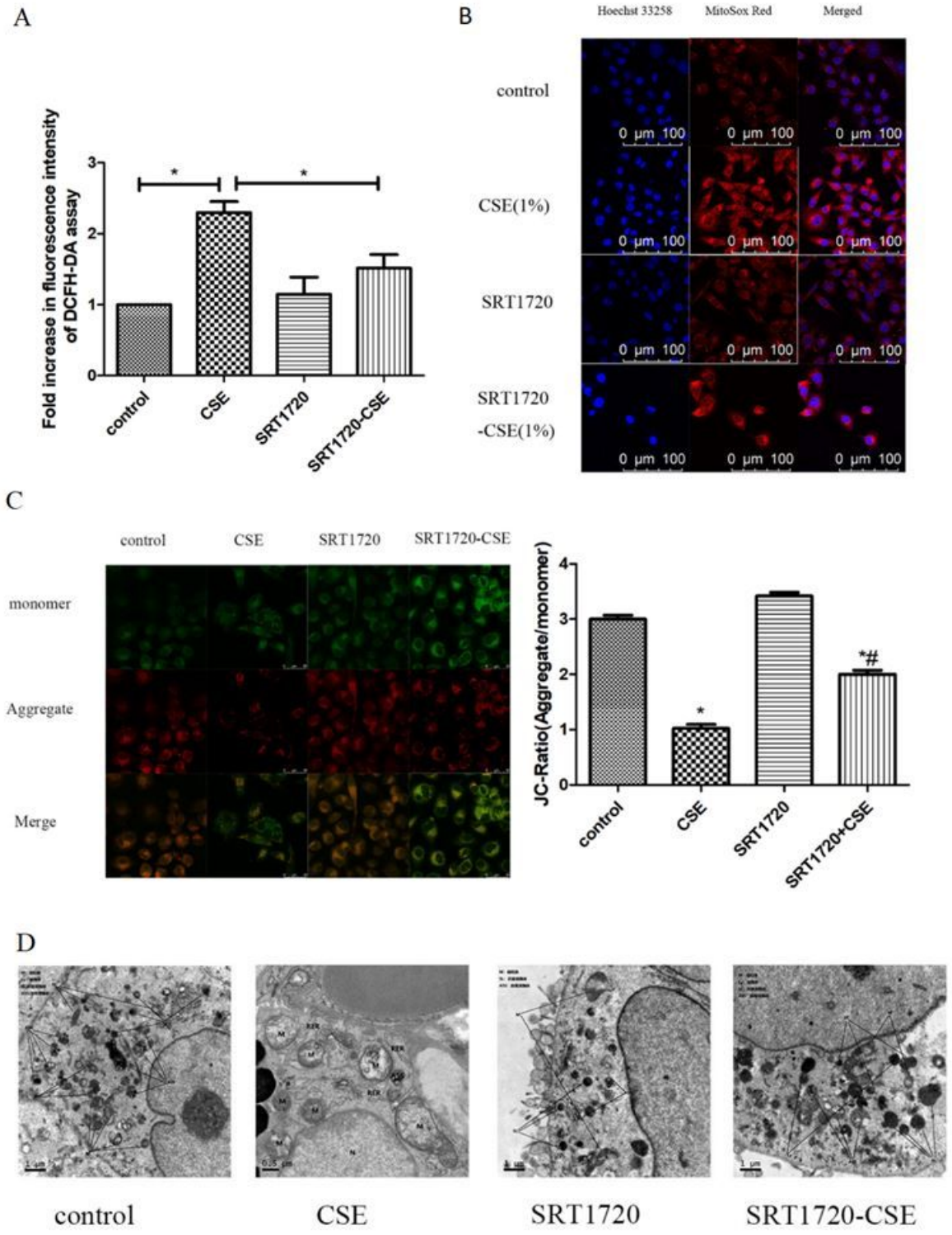

Figure 6

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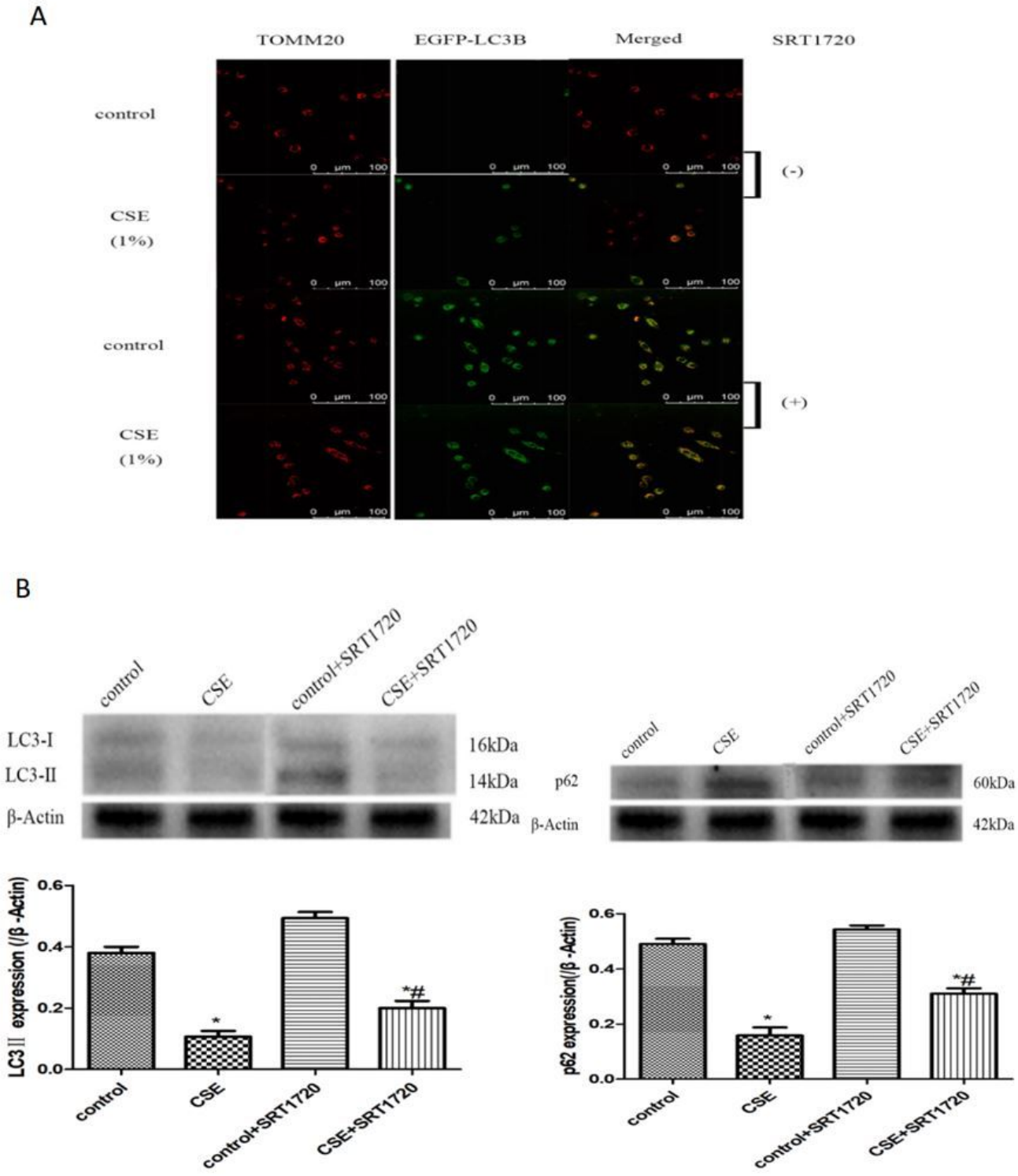

Figure 7

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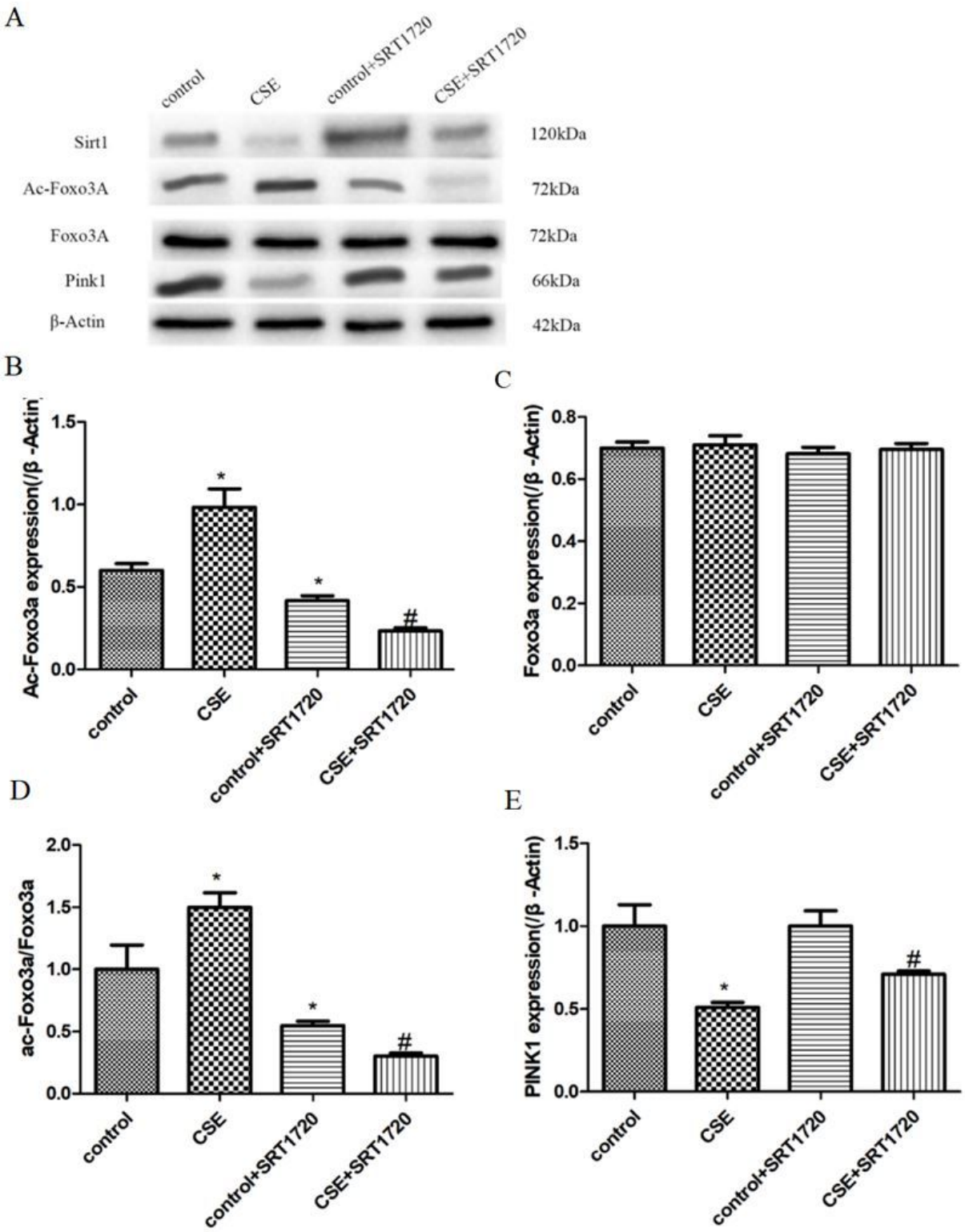

Figure 8

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