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Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence.

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Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence

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Abstract

Autophagy has profound implications with aging. However, the true features of autophagy in aging development remain to be clarified. In the present study, we explored the status of autophagic flux during the development of cell senescence induced by oxidative stress. In this system, with increased autophagic structures though, the degradation of SQSTM1/p62 protein, the yellow puncta of mRFP-GFP-LC3 fluorescence and the activity of lysosomal proteolytic enzymes all decreased in senescent cells, indicating impaired autophagic flux with lysosomal dysfunction. The influence of autophagy activity on senescence development is confirmed by both positive and negative autophagy modulators and mTOR dependent autophagy activators, rapamycin and PP242, efficiently suppress cellular senescence through a mechanism relevant to restoring autophagic flux. Moreover, by time phased treatment of cells with antioxidant N-acetylcysteine (NAC), mitochondria uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) and ambroxol, a reagent with the effect of enhancing lysosomal enzyme maturation, we found that mitochondrial dysfunction plays a initiative role, while lysosomal dysfunction is more directly responsible for autophagy impairment as well as senescence. Interestingly, the effect of rapamycin on autophagy flux is linked to its role in functional revitalization of both lysosomal and mitochondrial functions. Together, this study demonstrates that autophagy impairment is crucial for oxidative stress-induced cell senescence, thus restoring autophagy activity should be a promising way for senescence retardation.

Key words: autophagy, senescence, oxidative stress, mitochondria, lysosomes, rapamycin

Abbreviations

PBS = Phosphate buffered saline

SA-β-gal = Senescence associated β-galactosidase

SAHF = Senescence-associated heterochromatin foci

qRT-PCR = Real-Time Quantitative Reverse Transcription PCR

MTT = Methyl thiazolyl tetrazolium

ROS = Reactive oxygen species

DAPI = 4',6-diamidino-2-phenylindole

DMEM = Dulbecco's modified eagle medium

H_2O_2 = Hydrogen peroxide

SIPS = Stress induced premature senescence

GFP = Green fluorescent protein

mRFP = Monomeric red fluorescence protein

LC3 = Microtubule-associated protein 1 light chain 3

mTOR = Mammalian target of rapamycin

TFEB = Transcription factor EB

HCQ = Hydroxychloroquine

3-MA = 3-Methyladenine

VPA = Valproic acid

COX = Cytochrome c oxidase

CHX = Cycloheximide

NAC = N-acetylcysteine

MMP = Mitochondrial membrane potential

PtdIns3K = Phosphoinositide 3-kinase

UCP = Uncoupling protein

IL = Interleukin

CCCP = Carbonyl cyanide m-chlorophenylhydrazine

EBSS = Earle's balanced salt solution

ULK = Unc-51-like kinase

UPR^{mt} = Mitochondrial unfold protein response

Introduction

Aging is a complicated process with its mechanism remains to be explored. Cellular senescence happens during aging development and is widely used as *in vitro* model for aging research. Stress-induced premature senescence (SIPS) develops faster than replicative senescence and has been established for studying the influence of extracellular or intracellular stress on aging process.[1] Known features of senescent cells include flat and enlarged cellular morphology, increased senescence associated β-galactosidase (SA-β-gal) activity, the appearance of senescence-associated heterochromatin foci (SAHF), cell growth arrest and senescence-associated secretory phenotype (SASP) such as IL-6 and IL-8 secretion.[2-6] Autophagy plays cytoprotective roles by turnover long lived proteins and scavenging damaged cellular components. Among different types of autophagy, macroautophagy is the most studied one and is composed of two stages: the early stage acting for the formation of autophagosome with capturing damaged cellular molecules and organelles; the late stage responsible for the digestion of entrapped components within autolysosome formed by the

fusion of autophagosome and lysosome.[7, 8] The updated consensus appeals that the real status of autophagy should be assessed not only by the number of autophagosomes and autolysosomes but also by evaluating the actual autophagic flux, such as monitoring the clearance of cell components in autolysosomes.[9, 10]

The relationship between autophagy and senescence is still inconclusive.[11] Some studies reported that autophagy is positively correlated with senescence, with increased autophagy activity in senescent cells.[12, 13] On the other hand, there are reports showing opposite view. For example, Kang *et al.* reported that autophagy impairment induces premature senescence in primary human fibroblasts.[14] Moreover, it is known that rapamycin, an mTOR inhibitor and an autophagy activator, could extend the lifespan of mice and be a potential anti-aging drug.[15]

The free radical theory of aging emphasizes the reactive oxygen species (ROS)-induced cellular damages for aging development.[16] Although ROS are physiologic signaling molecules, excessive produced ROS in cells under stressful situations, happened either naturally or artificially, are detrimental to cell components and homeostasis.[17] As mitochondria are primary source of ROS and primary target for ROS damage, their functional status is closely linked with aging development.[18, 19] In fact, age-related changes in cells are associated with declined mitochondrial function, accompanying with increased ROS and decreased ATP.[20]

To clarify the relationship between autophagy and senescence, the features of oxidative stress-induced senescent fibroblasts are characterized in the present study, with particular focusing on the status of autophagy flux and its role in senescence development. As an attempt for analyzing underlying mechanisms, we also closely concerned the influence of lysosomal and mitochondrial dysfunction on autophagy impairment, together with the effect of

autophagy modulators on autophagy flux restoration and cellular senescence attenuation.

Results

H_2O_2 treatment induces cellular senescence with intracellular ROS elevation

First, we utilized a method involving 45 min incubation of NIH3T3 cells in suspension with 400 μM of H_2O_2 treatment to establish SIPS cell model and monitored the progress of cellular senescence up to 7 days after H_2O_2 treatment. As shown in Fig. 1A, H_2O_2-treated cells gradually became enlarged, flattened and most of these cells were SA-β-gal staining positive after 3 days. SAHFs were also evident in H_2O_2-treated cells as large nuclei and irregularly shaped puncta appeared (Fig. 1B). In addition, we observed apparent cell growth arrest (Fig. 1C), as well as elevated expressions of p53 protein and *Cdkn1a* (P21), *Il6* mRNAs in H_2O_2-treated cells (Fig. 1D-F). H_2O_2 treatment also triggered subsequent intracellular ROS elevation (Fig. 1G). Besides, in MRC-5, a human lung fibroblast cells, we also successfully induced cellular senescence by using the same method (Fig. S1). These results collectively demonstrate that in fibroblasts, the H_2O_2 treatment protocol we used could reliably induce cellular senescence in 3 to 5 days and the following experiments were mostly done in 3 or 5 days after H_2O_2 treatment.

Autophagic structures increase but autophagic flux impairs in senescent cells

The biological relevance of autophagy and senescence has been under investigation.[21, 22] To elucidate the status of autophagy during SIPS, autophagic structures were observed by transmission electronic microscope and an apparent increase in the number of vacuole or

vesicular-like structures in the cytoplasm of senescent cells was found (Fig. 2A). To characterize the increased vacuole or vesicular-like structures, LysoTracker Red was used to stain lysosomes or autolysosomes, leading to the observation that lysosomal structures greatly increased in H_2O_2-treated cells (Fig. 2B). Moreover, in a NIH3T3 cell line stably expressing mRFP-LC3, the punctate LC3 distribution was observed from day 1 after H_2O_2 exposure, with an apparent increase at day 3 and day 5. These results show the increase of autophagic structures in H_2O_2-induced senescent cells.

Given the accumulation of autophagic structures can be resulted from either increased autophagic induction or decreased autophagic degradation,[9, 10] we measured autophagic flux in our model. First, endogenous SQSTM1/p62 protein was examined as it mainly degraded through autophagic pathway.[23] The SQSTM1 protein in H_2O_2-treated cells increased apparently at day 3 and day 5 compared to control cells (Fig. 2D). Then, to justify the increase of SQSTM1 protein is due to increased synthesis or decreased degradation, a degradation-blocking experiment was conducted using hydroxychloroquine (HCQ), a reagent which elevates lysosomal pH and inhibits lysosomal degradation. As shown in Fig. 2E, different from the apparent increase of SQSTM1 protein in control cells caused by HCQ treatment, the level of SQSTM1 protein kept almost unchanged in H_2O_2-treated cells, suggesting that lysosomal degradation capacity in H_2O_2-treated cells was low enough to compare to that caused by HCQ treatment. Next, we tested the half-life of SQSTM1 protein by inhibiting protein synthesis with cycloheximide (CHX), and found that SQSTM1 protein in H_2O_2-treated cells degraded much slower than that in control cells, with the half-life being around 12 h and 7 h in H_2O_2-treated and control cells, respectively (Fig. 2F). Furthermore,

using NIH3T3 stably expressing tandem mRFP-GFP-LC3,[24] we found that while EBSS starvation induced puncta like distribution of LC3 protein accompanied with the quenching of green fluorescence, H_2O_2 treatment did not (Fig. 2G). These results collectively indicate that impairment of autophagic flux happened in H_2O_2 induce senescent cells.

Chronic blockage of autophagic flux is sufficient to induce cellular senescence

To verify the relationship between autophagy and cellular senescence, we tried to assess whether the impairment of autophagic flux could induce cellular senescence. When normal NIH3T3 cells were treated with sublethal dose of HCQ and leupeptin, two lysosome inhibitors capable of blocking autophagic degradation,[10] the cells exhibited increased intracellular ROS (Fig. 3A). In parallel, SA-β-gal positive cells and *Il6* mRNA expression also increased apparently after 7 days (Fig. 3A & 3B). Moreover, we used shRNA-mediated *Atg5* knockdown that inhibited autophagy in NIH3T3 (Fig. 3C & 3D). We found that SA-β-gal positive cells are conspicuous in *Atg5* gene silenced cells rather than in control shRNA cells that both experienced a 15 days culturing without H_2O_2 treatment (Fig. 3E). As another senescence marker, *Il6* mRNA expression also increased apparently in *Atg5* knockdown cells (Fig. 3F). These results indicate that chronic autophagy impairment could induce cellular senescence.

mTOR dependent autophagy activators attenuate senescence in a manner related to autophagy flux restoration

We further investigated how autophagy regulators affect cell senescence. Seven different autophagy regulators, including 4 activators and 3 inhibitors were tested for their influences on

SIPS. The concentrations of these chemical compounds were determined by preliminary experiments. As shown in Fig. 4A, rapamycin and PP242, two mTOR-dependent autophagy activators, showed a potent anti-senescent effect as they dramatically reduced the percentage of SA-β-gal positive cells; on the other hand, two mTOR-independent autophagy activators, valproic acid (VPA) and LiCl, showed minor influence on the senescent state of H_2O_2-treated cells (Fig. 4A). As to autophagy inhibitors, bafilomycin A1 and HCQ, two reagents blocking lysosomal function, increased SA-β-gal positive cells in H_2O_2-treated cells (Fig. 4B), while 3-methyladenine (3-MA), an autophagy inhibitor targeting to phosphoinositide 3-kinase (PtdIns3K) which influences autophagy at early stage,[25] did not (Fig. 4B). Further investigations were performed using two representative reagents, rapamycin and bafilomycin A1. As shown, their regulatory role in senescence tested by the *Il6* mRNA level was in accordance with SA-β-gal staining (Fig. 4C). To confirm if the role of these reagents in SIPS development is relevant to autophagy regulation, we tested whether autophagy flux is influenced by these two reagents in our model. Using NIH3T3 cells stably expressing tandem mRFP-GFP-LC3, we found that rapamycin increased while bafilomycin A1 decreased the ratio of red/yellow puncta in H_2O_2-treated cells, showing the restoration and aggravation of autophagic flux, respectively (Fig. 4D). In addition, the abundance of SQSTM1 decreased in rapamycin treated cells and increased in bafilomycin A1 treated cells at day 3 after H_2O_2 treatment (Fig. 4E). The effects of rapamycin and bafilomycin A1 on SIPS were similar in human fetal lung fibroblast cell line MRC-5 as in NIH3T3 cells (Fig. S1).These results demonstrate that rapamycin is an efficient anti-senescence reagent that can act through up-regulating autophagic flux in our system.

Lysosomal and mitochondrial dysfunction happens during SIPS development

Since the capacity of lysosomal degradation is a rate-limiting factor for autophagic flux,[26, 27] we assessed the function of lysosomes in cells. We found that the activity of lysosomal acid phosphatase decreased in senescent cells (Fig. 5A) and the size of lysosomes increased in these cells (Fig. 5B).[28] Moreover, the abundance of cathepsin B, a representative protease in lysosome, reduced in senescent cells (Fig. 5C), concurrently with a decline of cathepsin B activity measured by Magic Red Cathepsin B kit (Fig. 5D). Taken together, these results reveal that the oxidative stress-induced impairment of autophagic flux in our senescent cells is closely associated with reduced degradation ability of autolysosomes and lysosomes.

As damaged or dysfunctional mitochondria have been connected to senescence,[18, 20] we functionally assayed mitochondria in our system. We found that mitochondrial DNA actually increased after H_2O_2 treatment. This increase also happens to mitochondrial DNA integrity, although H_2O_2 indeed decreased its integrity right after treatment (Fig. S2). These results indicate that mitochondria biogenesis is activated during the development of senescence. However, the function of mitochondria is not activated, since mitochondrial depolarization was detected in H_2O_2-treated cells (Fig. 5E), with a decrease of both cellular ATP content (Fig. 5F) and mitochondrial cytochrome c oxidase (COX) activity (Fig. 5G). To clarify the role of mitochondria in SIPS, a mitochondrial uncoupling reagent carbonyl cyanide m-chlorophenylhydrazine (CCCP) was used in the experiment.[19] As shown, when cells were pretreated with CCCP, the mitochondrial content remains stable (Fig. S3). However, the intracellular ROS generation after H_2O_2 treatment significantly reduced (Fig. 5H), coinciding

with attenuated cellular senescence revealed by SA-β-gal staining (Fig. 5I) and *Il6* mRNA expression (Fig. 5J). These results indicate the important role of dysfunctional mitochondria derived ROS in SIPS development.

The importance of intracellular ROS on the dysfunction of lysosomes and autophagy in our senescent cells was further proved by another SIPS model established by treatment of pyocyanin, a reagent inducing intracellular ROS production.[29] We found that both ROS and cellular senescence were induced by pyocyanin in a dosage dependent manner (Fig. S4). Under this kind of oxidative stress, the decrease of lysosomal acid phosphatase activity and the increase of SQSTM1 protein were also prominent (Fig. S5). Consistently, the treatment of cells with antioxidant NAC attenuated the senescence induced by pyocyanin (Fig. S6).

Asynchronous dysfunction of mitochondria and lysosomes during SIPS development

To explore the mechanistic relationship between lysosomal and mitochondrial dysfunction during SIPS development, the time sequenced functional assays for these two organelles were conducted. First, we tested the effect of antioxidant NAC on the development of SIPS. Although SIPS did not be rescued from co-treatment of NAC and H_2O_2, it was weakened when NAC was included in culture medium after H_2O_2 treatment (Fig. S7), indicating that intracellular ROS induction after H_2O_2 treatment might be a crucial event for SIPS development. This possibility was confirmed by time phased detection for cellular ROS and we found an increase in ROS level following H_2O_2 treatment, peaking at 4 to 8 h (Fig. 6A), accompanying with mitochondrial depolarization (Fig. S8). On the other hand, different from this implication of mitochondria dysfunction in the early phase of SIPS development, the sign

of lysosomes dysfunction appeared much later, showing as the activity of cathepsin B did not decrease, but slightly increased, within the first 24 h after H_2O_2 treatment (Fig. 6A). Prolonged experiments showed that cathepsin B activity began to decrease from day 3 after H_2O_2 treatment, together with a further elevation of ROS production (Fig. 6B). Importantly, NAC application did increase lysosomal cathepsin B activity in our system (Fig. 6C), suggesting a possible causal relationship between dysfunctional mitochondria derived ROS overproduction and lysosomal dysfunction. This temporal sequenced dysfunction of lysosomes and mitochondria in our system was further revealed by using NAC, CCCP and ambroxol, the last one acts as an enhancer of lysosome enzyme maturation.[30,31] After the suppressive effect of CCCP on ROS overproduction and the promoting effect of ambroxol on lysosomal cathepsin B activity were confirmed (Fig 6D and 6E), we applied these reagents at 24 h duration after H_2O_2 treatment. As the results, NAC showed an obvious anti-senescence effect when applied in the first 48 h after H_2O_2 treatment (Fig. 6F), while the effect of CCCP was only seen when applied within the first 24 h (Fig. 6G). Interestingly, the anti-senescence time window of ambroxol was the longest, prolonging up to 48-72 h (Fig. 6H). These results suggest that, mitochondria dysfunction precedes lysosomes dysfunction during SIPS development.

Autophagy modulators regulate the function of mitochondria and lysosomes in senescent cells

Then, we asked if the influence of autophagy modulators on cellular senescence is relevant to the function of mitochondria and lysosomes, which might be helpful to provide the ground for developing new interventions against aging. With respect to mitochondrial function, we found

that mitochondrial membrane potential (MMP) was elevated by rapamycin and lowered by bafilomycin A1 (Fig. 7A). Accordingly, rapamycin substantially decreased intracellular ROS and increased intracellular ATP while bafilomycin A1 did the opposite (Fig. 7B & 7C). As to lysosomal function, we found that rapamycin apparently increased the expression of cathepsin B (Fig. 7D), as well as the activity of this enzyme in senescent cells (Fig. 7E). Collectively, our results provide novel evidence for the influence of autophagy modulators on senescence, specifically revealing the effect of autophagy activator rapamycin on protecting mitochondria and on activating lysosome related degradation.

Discussion

In this study, we addressed the functional role of autophagic flux in the development of oxidative stress-induced cellular senescence. The main findings are: 1. autophagic flux impaires in our SIPS cells; 2. mitochondrial dysfunction and related ROS production, are initiative while subsequently lysosome dysfunction is directly responsible for autophagy impairment and senescence development; 3. the effect of mTOR-dependent autophagy activator rapamycin on senescence prevention associates with its role in the restoration of autophagic flux as well as mitochondrial and lysosomal function. Our findings provide novel evidence to demonstrate that autophagy activation is a promising strategy to counteract cellular senescence or even aging, and are supportive for the idea that the interplay between mitochondria and lysosomes plays an important role in the maintenance of cell health, herein should be a reasonable intervention point for anti-aging intervention.

In recent years, the relationship between autophagy and senescence is a hot topic with

283 contradictory conclusions existed in previous reports.[11] Although many factors, such as cell
284 type, the kind of inducer, stress intensity and measuring time point, can impact the conclusion
285 about autophagy status, we consider the methods and indexes used for evaluating autophagy
286 activity should be carefully considered. For this reason, instead of emphasizing the increase in
287 the quantity and size of autophagic structures, which can be caused by the alterations either in
288 autophagic structure formation or in autophagic degradation,[10] we particularly evaluated the
289 status of autophagic flux during SIPS development. By utilizing functional measurements,
290 particularly those working on the assessment of autolysosomal protein degradation, we get
291 chance to ensure that autophagy impairment is positively correlated with senescence
292 development. This result is in accordance with some other reports.[14, 32, 33] We also noticed that
293 opposite correlation is demonstrated in some researches. For example, it is reported that
294 inhibition of autophagy by knocking down Atg5 or Atg7 in a tumor cell line suppressed the
295 induction of senescence.[12] We consider that the reasonable interpretation for these
296 inconsistent might lie on the distinct metabolic characteristics of cells and senescence
297 induction approaches used by different studies.
298 The restoration of autophagic flux is an attractive concept for anti-aging therapeutics.
299 Consistent with previous studies,[34, 35] our study confirmed the effectiveness of autophagy
300 activation on senescence prevention. Interestingly, we found that different autophagy
301 activators and inhibitors have different influences on SIPS. Two mTOR-dependent autophagy
302 activators, rapamycin and PP242, exhibited obvious anti-senescence effect, while
303 mTOR-independent/PtdIns3K-dependent ones, VPA and LiCl, showed minor influence. These
304 results not only confirmed the crucial role of autophagic flux in SIPS development but also

305 revealed the effectiveness of mTOR inhibition on SIPS repression.

306 The connection between mTOR inhibition and autophagy activation has not been established
307 completely. Previous interpretation emphases the role of mTOR inhibition in the restoration of
308 ULK activity which influences the early stage of autophagy.[36] Recent evidence show that
309 mTOR also regulates autophagic flux by influencing the late stage of autophagy.[28, 37] However,
310 the role of mTOR in autophagic flux regulation has not yet been studied in senescent cells. In
311 the present study, the effect of rapamycin-mediated mTOR inhibition on autolysosomal activity
312 is proved as rapamycin increased the expression and revived the activity of cathepsin B during
313 SIPS (Fig. 7D & 7E). This is inconsistent with a previous report which states that mTOR
314 suppression by PP242/Torin induced lysosomes activation but not rapamycin.[38] In our
315 consideration, this inconsistency might result from the different concentrations of rapamycin
316 used in two experiments: that they used is 10 folds higher (1 μM) than we used (100 nM). It
317 has been reported that high concentration of rapamycin could elevate the pH of lysosomes,
318 thus covering up its role in lysosomal activation through mTOR suppression.[39] The situation
319 about mTOR downstream protein TFEB, an essential transcription factor for
320 autophagy-related genes, is under pursuing in our system. We surprisingly found that, TFEB
321 localized in nucleus of H_2O_2 treatment-induced senescent cells, either rapamycin was added
322 or not; however, the transcription of TFEB-targeted lysosomal genes, such as *Gns* and *Lamp1*,
323 was not activated.[51] These data provide two open questions: If mTOR could regulate the
324 function of autolysosomes through a way independent of TFEB? If TFEB could be functionally
325 regulated in a manner independent of mTOR? Intensive investigations are needed to clear
326 these questions.

Aspect of the importance of mitochondria dysfunction on SIPS induction, evidence obtained in the present study, showing as elevated ROS generation, the protective role of NAC and pyocyanin-promoted SIPS development. Importantly, the pretreatment of cells with CCCP alleviated ROS production along with attenuated development of SIPS. In fact, the imperative role of mitochondria in cellular senescence was i evidenced by Clara Correia-Melo *et al.* recently when this manuscript in preparing, They found that mitochondria depletion reduced a spectrum of senescence effectors and phenotypes while preserving ATP production and cell survival via enhanced glycolysis. [40] Their work is inspiring because it affirmed the concept that mitochondria should be a major putative therapeutic target for interventions impacting on the senescent phenotypes because of their undesired role in cellular senescence. Our time course experiments are supportive for this concept, by having the data about enhanced ROS generation with the increased mtDNA content during SIPS development (supplementary data), The increase in mtDNA indicates that although experienced an injury and became dysfunctional within hours after H_2O_2 treatment, mitochondria content in cells actually increased afterwards. For the mechanism about the biogenesis of mitochondria upon stress loading, a recent published article concerned the role of mitochondrial unfolded protein response (UPR^{mt}).[52] Whether UPR^{mt} is responsible for the increase of mitochondrial biogenesis during SIPS needs to be investigated in future studies. It is worthy of noting here that using CCCP weakened ROS generation and SIPS development in our system. This result matches the protective role of UCP protein under stress.[53] Our results are also consistent with the “uncoupling to survive” theory,[41] which suggests that partially uncoupling of mitochondria under stressed condition may be beneficial for SIPS prevention.

The interplay between mitochondria and lysosomes in cellular senescence is attractive but yet proved. As the attempt to clarify this issue, time-dependent alterations of the function of these two organelles were investigated in this study. What we understand upon this investigation are: 1. The biogenesis of both mitochondria and lysosomes is activated during the development of SIPS, but the function of newly generated organelles is compromised comparing to that in proliferating cells; 2. the dysfunction of mitochondria happens within hours after H_2O_2 treatment, whereas that of lysosomes happens days later, so that the former seems to be an initiative event for senescence development and to play as a trigger of autophagy impairment; 3. although the dysfunction of lysosomes happening subsequently after the dysfunction of mitochondria, the consistency of its dysfunction with autophagy impairment indicates its importance role for SIPS development; 4. the influence of lysosomes on mitochondria should not be neglected, as the inhibitors of lysosomes induced intracellular ROS generation (Fig. 3A), and rapamycin alleviated the dysfunction of mitochondria (Fig. 7A-C). Our results are supportive for the mitochondrial-lysosomal axis theory of aging proposed by Brunk and Terman,[43] as the drugs ameliorate the function of each of these organelles exhibit potent anti-senescence effect. Upon this finding, we believe that a combined treatment targeting both mitochondrial and lysosomes should be beneficial for aging-related diseases, such as neurodegenerative and cardiovascular diseases.

In conclusion, this study provided functional evidence showing the occurrence of autophagy impairment in H_2O_2-induced senescent cells and emphasized the characteristics of the dysfunction of autolysosomes/lysosomes for SIPS development. By exploring the initiative role of intracellular ROS and mitochondria malfunction during SIPS development, interplay

between mitochondria and autophagic flux was discovered. Furthermore, this study revealed the efficiency of mTOR inhibition-mediated autophagy restoration on SIPS prevention, expanded our understanding on the relationship between autophagy and senescence as well as the possible mechanisms about the role of rapamycin in aging prevention. Further study is needed to understand the specific underlying mechanism that how impaired autophagy relates to senescence and to open new way for developing safe and effective strategy against aging.

Materials and Methods

Reagents and antibodies

Rapamycin (R0395), VPA (P4543), LiCl (203637), bafilomycin A1 (B1793), HCQ (H0915), 3-MA (M9281), NAC (A7250) CHX (C7698) and pyocyanin (P0046) are from Sigma. PP242 is from Cayman Chemical Company (13643). Leupeptin is from Amresco (J580). 2',7'-dichlor-fluorescein-diacetate (DCFH-DA) is from Applygen (C1300). JC-1 and CCCP are from Beyotime (C2006). Ambroxol hydrochloride is from Boehringer Ingelheim (Mucosolvan®, 2 ml : 15 mg). Anti-ACTB (sc-47778), anti-SQSTM1 (sc-28359) and anti-CSTB antibodies (sc-6493) are from Santa Cruz Biotechnologies. Anti-TP53 antibody is from Cell Signaling Technology (#9282).

Cell culture and treatments

The murine fibroblast NIH3T3 and human fetal lung fibroblast MRC-5 cell line were obtained from Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences and

cultured in a complete medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum) in a humidified incubator at 37 °C and 5% CO_2.

For monitoring cell proliferation status, normal, H_2O_2-treated and starved cells were inoculated in 6-well plates in 3 replicates for each time point and harvested by trypsinization at 24 h intervals for 7 days. The numbers of cells were counted under microscope.

For senescence induction, growing cells at about 80% confluence were collected into an Eppendorf tube after trypsinization, suspended in PBS with 1×10^6 cells/ml density and exposed to 400 μM H_2O_2 at 37 °C. During H_2O_2 exposure, the tube was turned upside down gently 5-10 times with a 5 minutes interval. After 45 min, cells were washed with PBS once and resuspended with complete medium. The control cells are treated with PBS at the same time. Then both control cells and H_2O_2-treated cells were split into individual wells in culture plate with a density about 2×10^4 cells/cm^2 and cultured with complete medium for various durations as indicated in individual experiments. After treatment, the media of both control and H_2O_2-treated cells were changed every 3 days.

To evaluate the effect of activators and inhibitors of autophagy activity on SIPS processing, different chemical reagents were added into culture medium after H_2O_2 treatment, with their concentrations indicated as in the figure legends. These concentrations were determined by preliminary experiments. The stock solutions of rapamycin and bafilomycin A1 were dissolved in DMSO, those of PP242, VPA, LiCl, HCQ and 3-MA in PBS, respectively.

SA-β-gal staining

SA-β-gal activity was determined using SA-β-gal staining kit (Beyotime, C0602) according to

414 standard protocol.[3] Senescent cells were identified as bluish green stained cells by
415 microscopy. More than 500 cells in 6 random fields were counted to determine the percentage
416 of SA-β-gal-positive cells in total cells.
417
418 ## SAHF detection
419 Cells were fixed *in situ* with 4% paraformaldehyde and washed by PBS. DAPI (Beyotime,
420 C1006) at 300 nM concentration in PBS was added for 5 min incubation. The cells were then
421 washed 3 times by PBS, drained and mounted. DAPI-stained nuclei with blue fluorescence
422 were viewed by fluorescence microscope.
423
424 ## Western blot analysis
425 Whole cell lysates were prepared by directly denaturing cell pellets in 2×SDS loading buffer,
426 and then boiling for 10 min. Western blotting assays were done as described previously.[44]
427 Anti-TP53, anti-SQSTM1, anti-CSTB antibodies were used as primary antibodies. Every
428 experiment was repeated 3 times, and representative data were shown.
429
430 ## Real-time quantitative reverse transcription PCR (qRT-PCR)
431 Total RNA extraction, reverse transcription and real-time PCR amplification were performed
432 as described previously.[45] PCR primers for *Cdkn1a* gene are 5'-GTGGCCTTGTCGCTGTC
433 TT-3' (forward) and 5'-GCGCTTGGAGTGA-3' (reverse), for *Il6* gene are 5'-ACTCACCTCT
434 TCAGAACGAATTG-3' (forward) and 5'-CCATCTTTGGAAGGTTCAGGTTG-3' (reverse), for
435 *18s* rRNA are 5'-TTGACGGAAGGGCACCACCAG-3' (forward) and 5'-GCACCACCACCCAC

GGAATCG-3' (reverse). The experiments were triplicated, and the data about the *Cdkn1a* and *Il6* genes were adjusted by the values for *18s* gene and shown as relative fold changes against control.

Electron microscopy

PBS washed cells were collected to Eppendorf tubes by cell scrapers. After centrifuging, 4% paraformaldehyde was loaded carefully on cell pellets for an overnight fixation at 4 °C. Fixed cells were further treated and sliced, and then the images of their ultrastructure were recorded under a transmission electron microscope.

Lysosome labeling with LysoTracker Red DND-99 and mitochondrion labeling with MitoTracker Green

Lysosomes were labeled by LysoTracker Red (Life technologies, L7528) according to manufacturer's protocol. Briefly, LysoTracker Red was added into cultural cells with the concentration of 1:20000 dilution from stock solution and incubated at 37 °C for 30 min. Images were taken by fluorescent microscope and the size of lysosomes were measured by using Image J software to analyze the pixels a single lysosome occupying. We counted at least 50 cells of random area in each group and the size in control cells was normalized to 1. Mitochondria were labeled by MitoTracker Green FM (Life technologies, M7514) according to manufacturer's protocol. Briefly, MitoTracker Green FM was added to the cultured cells with the concentration of 1:10000 dilution from stock solution and incubated at 37 °C for 30 min. Images were taken by fluorescent microscope

Stable transfected Cells expressing fluorescent LC3 protein

mRFP-LC3 expression construct (pmRFP-LC3) and mRFP-GFP-LC3 expression construct (ptf-LC3) were provided by Dr. Yoshimori.[24] DNA was transfected into NIH3T3 using Lipofectamine 2000 (Invitrogen, 11668-019) according to the manufacturer's protocol. 500 μg/ml G418 (Invitrogen, 11811) was used for selecting stable expression cell clones. More than 50 clones were pooled, expanded and used for experiments. These two pooled stable cell populations were named as mRFP-LC3 cells and mRFP-GFP-LC3 cells, respectively.

Intracellular ROS detection

Reactive oxygen species (ROS) production was detected by ROS detecting probe DCFH-DA (Applygen, C1300) combined with flow cytometry detection.[46] DCFH-DA turns to green fluorescent molecule called DCF when oxidized by ROS, so that intracellular ROS level can be reflected by fluorescent intensity produced by DCF. Three independent experiments were conducted.

Knockdown of Atg5 by shRNA

ShRNA plasmid targeting mouse *Atg5* (AGAACCATACTATTTGCTT) were synthesized by Genechem (25978). NIH3T3 cells were transfected with control or *Atg5* shRNA and a polyclonal pool of NIH3T3 cells were selected by adding 2 μg/ml puromycin (Sigma, P8833).

Lysosomal acid phosphatase assay

Lysosomal acid phosphatase activity was assayed by a commercially available kit (Beyotime, P0326) according to manufacturer's instructions. The activity of lysosomal acid phosphatase was normalized by average lysosome content which was measured via a process including Lyso-tracker staining of living cells, PBS washing, cell splitting with 1% Triton X-100 and fluorescence intensity reading by a fluorescence microplate reader.

Cathepsin B activity assay

Cathepsin B activity was measured by Magic Red cathepsin B detection kit (ImmunoChemistry Technologies, #937). Control or H_2O_2 treated NIH3T3 cells were cultured in 24-well plates with different treatment for indicated time. Then cells were loaded with Magic Red Cathepsin B reagent for 1 h and washed with PBS twice. More than 10 fluorescent images were taken and representative images were shown. For quantification of cathepsin B activity, the fluorescence of Magic Red cathepsin B probe was measured by fluorescent microplate reader.

Mitochondrial membrane potential assay (JC-1)

Mitochondrial membrane potential was monitored by a mitochondrial-specific dual fluorescence probe, JC-1.[47] Briefly, JC-1 (Beyotime, C2006) was added to reach a final concentration of 5 μg/ml and incubated for 20 min, and then the cells were washed twice with medium and imaged under a fluorescent microscope.

Measurement of mitochondrial DNA content and integrity using qPCR

Mitochondrial DNA integrity was measured according to a qPCR method described

previously.[50] The primers used for qPCR are TGCCCCTCTTCTCGCTCCGG (forward), GGCGATAACGCATTTGATGGCC (reverse) for amplifying short fragment in D-loop and TGGGGGCCAACCAGTAGAACA (forward), TGCGTCTAGACTGTGTGCTGTCC (reverse) for amplifying semi long fragment in D-loop. The mitochondrial DNA content was calculated according to the ratio of short D-loop/genomic fragment. The mitochondrial DNA integrity was calculated according to the ratio of semi long/short fragment.

Intracellular ATP level

Intracellular ATP level was measured by a commercially available intracellular ATP measurement kit (Nanjing Jiancheng, A095) according to manufacturer's instructions.

Cytochrome c oxidase (COX) activity

Determination of COX activity by spectrophotometry in protein extracts from the cells was performed using a commercially available COX activity kit (GenMed Scientifics, GMS10014.3.1) according to manufacturer's instructions.

Statistical analysis

Data are expressed as means ± SD from at least three biological replicates. The difference between control and treated was examined by Student's t-test. The difference between multiple groups was examined by one-way ANOVA with Bonferroni post-hoc. $p < 0.05$ is considered to be significant and $p < 0.01$ was considered highly significant.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Short term of H_2O_2 treatment is sufficient to induce cellular senescence.

NIH3T3 cells were treated with PBS (Ctrl) or with 400 μM H_2O_2 in PBS as described in Materials and Methods, and shifted to culture in a complete medium for indicated days.

673 Representative control cells were cultured to the longest time point in the experiment. (A)
674 Images showed the cellular morphology and SA-β-gal staining of control and H_2O_2-treated
675 NIH3T3 cells. (B) H_2O_2-treated cells were stained with DAPI at indicated days to show SAHFs.
676 Circles indicate typical SAHFs. Apoptosis was induced by 1.5 mM H_2O_2 for comparison. (C)
677 Cell counting was conducted for 7 days and growth curves of control, H_2O_2-treated and serum
678 starved NIH3T3 cells are shown. (D) TP53 protein was measured by Western blot. (E) (F)
679 Relative mRNA levels of *Cdkn1a* and *Il6* genes were analyzed by qRT-PCR. (G) ROS
680 generation in NIH3T3 cells was labeled by DCFH-DA probe and quantified by flow cytometry.
681 Data are presented as the means ± SD from 3 independent experiments. *p <0.05 and **p
682 <0.01 compared to control.
683
684 **Figure 2. Autophagic structures increase but autophagic flux impairs in senescent cells.**
685 NIH3T3 cells were treated with or without H_2O_2 as described in Fig. 1. (A) Transmission
686 electronic microscopy of control or H_2O_2-treated NIH3T3 cells at day 5. Images on the right of
687 each group are enlarged areas in rectangles. Arrows show the vesicle-like structures. (B) The
688 lysosome content of control or H_2O_2-treated NIH3T3 cells was probed by Lyso-tracker Red
689 DND-99 and images were taken under a fluorescent microscope by using the same exposure
690 parameters. (C) Fluorescent images of mRFP-LC3 NIH3T3 cells treated with or without H_2O_2
691 at indicated time points. (D) Samples of control or H_2O_2-treated NIH3T3 were collected at
692 indicated time points and Western blots were performed by using SQSTM1 antibody. (E)
693 SQSTM1 protein expression at day 3 in control or H_2O_2-treated NIH3T3 cells with or without 5
694 μg/ml HCQ for 12 h. (F) Control or H_2O_2-treated NIH3T3 cells were cultured for 3 days before

adding CHX (100 μg/ml). Samples were collected at indicated time points after the addition of CHX and SQSTM1 protein was determined by Western blot. Statistics show the time phased degradation of SQSTM1 protein from 3 independent experiments. (G) Fluorescent images of EBSS starved or H_2O_2-treated NIH3T3 cells stably expressing mRFP-GFP-LC3.

Figure 3. Blockage of autophagy flux is sufficient to induce cellular senescence.

(A) NIH3T3 cells were treated with HCQ (3 μg/ml) or leupeptin (5 μg/ml) for 7 days. DCFH-DA fluorescence and SA-β-gal staining was used to detect intracellular ROS and the senescent state of these cells respectively. Statistics show the percentages of SA-β-gal positive cells. (B) Relative *Il6* mRNA expressions of the cells in (A) were quantified by qRT-PCR. (C) NIH3T3 cells were transfected with control shRNA or Atg5 shRNA and positive cells were selected by 2 μg/ml puromycin for 7 days. Western blots of ATG5 in control and ATG5 knockdown cells. (D) Western blot of SQSTM1 in control and ATG5 knockdown cells. (E) The same cells in (C) were cultured for another 5 days before treated with or without H_2O_2 and incubated for 3 days. Images show SA-β-gal staining of control and ATG5 knockdown cells. Statistics showed the percentages of SA-β-gal positive cells. (F) Relative *Il6* mRNA expression of control and Atg5 knockdown cells cultured for 15 days. The data are presented as means ± SD from 3 independent experiments and **p <0.01.

Figure 4. Autophagy restoration attenuates SIPS development.

(A) (B) Different autophagy regulators including rapamycin (100 nM), PP242 (500 nM), VPA (1 mM), LiCl (10 mM), bafilomycin A1 (50 nM), HCQ (3 μg/ml) and 3-MA (500 μM) were added to

717 H_2O_2-treated NIH3T3 cells and incubated for 3 or 5 days. Images of SA-β-gal staining are
718 shown. Statistics show the percentages of SA-β-gal positive cells at day 5. (C) *Il6* mRNA level
719 in H_2O_2-treated cells cultured with rapamycin or bafilomycin A1. (D) Confocal images of
720 H_2O_2-treated tandem mRFP-GFP-LC3 cells with rapamycin or bafilomycin A1. (E) SQSTM1
721 proteins in H_2O_2-treated NIH3T3 cells with rapamycin or bafilomycin A1 were examined by
722 Western blots. The data are presented as means ± SD from 3 independent experiments. *p
723 <0.05 and **p <0.01 when compared to DMSO.
724
725 **Figure 5. Autophagy impairment couples with lysosomal and mitochondrial dysfunction.**
726 NIH3T3 cells treated with or without H_2O_2 as described in Fig. 1. (A) *In vitro* acid phosphatase
727 activity assay was performed at day 3 after H_2O_2 treatment. (B) Lysosomes of control or
728 H_2O_2-treated NIH3T3 cells were loaded with LysoTracker Red DND-99 at day 3 and images
729 were taken by confocal microscope. The images in rectangles are 1.5 fold enlarged. Statistics
730 show the relative lysosome size in control and H_2O_2-treated cells. (C) Endogenous cathepsin
731 B protein in control and H_2O_2-treated cells were detected by Western blot. (D) Cellular
732 cathepsin B activity was visualized by using Magic Red Cathepsin B detection kit at indicated
733 days. Cells treated with bafilomycin A1 (50 nM) for 12 h were shown as negative control. (E)
734 Mitochondrial membrane potential in cells was measured by JC-1 staining. Fluorescent
735 images were taken at indicated days. (F) Cellular ATP level of control (5d) or H_2O_2-treated (1d,
736 3d, 5d) NIH3T3 was measured. (G)Mitochondrial COX activity of control (5d) or H_2O_2-treated
737 (1d, 3d, 5d) NIH3T3 was assayed. (H) NIH3T3 cells pretreated with DMSO or 10 μM CCCP for
738 6 h accepted H_2O_2 treatment as described in Fig. 1. Images of DCFH-DA fluorescence was

taken at indicated days. (I) SA-β-gal staining was performed at day 5 of the cells in (H). (J) Relative *Il6* mRNA expression of the cells in (I). The data are presented as means ± SD from 3 independent experiments. *p <0.05 and **p <0.01 when compared to ctrl or DMSO.

Figure 6. Mitochondria dysfunction precedes lysosomes dysfunction during SIPS development.

NIH3T3 cells were treated with or without H_2O_2 as described in Fig. 1. (A) Intracellular ROS and lysosomal cathepsin B activity within 24 h after H_2O_2 treatment. Intracellular ROS was measured by flow cytometry using DCFH-DA probe. Lysosomal cathepsin B activity was measured by the fluorescence intensity of Magic Red Cathepsin B probe. 0 h means the end time point of H_2O_2 treatment. The data of untreated cells (time point -1 h) was normalized to 1. (B) Intracellular ROS and lysosomal cathepsin B activity at 0, 1, 3, 5 day after H_2O_2 treatment. (C) H_2O_2-treated NIH3T3 cells were cultured in medium with or without 2 μM NAC for 3 days. Cathepsin B activity was visualized by Magic Red Cathepsin B kit. (D) Images of DCFH-DA fluorescence of control or H_2O_2-treated NIH3T3 cells at day 3. 10 μM CCCP was added to the culture medium after H_2O_2 treatment. (E) Images of Magic Red Cathepsin B fluorescence of control or H_2O_2-treated NIH3T3 cells at day 3. 50 μM ambroxol was added to the culture medium after H_2O_2 treatment. (F-H) H_2O_2-treated NIH3T3 cells were incubated with NAC (2 μM), CCCP (10 μM) or ambroxol (50 μM) at different periods of time. Cells were stained with SA-β-gal at day 5. Arrow indicates the proliferating cells in NAC group. Statistics show the percentages of SA-β-gal positive cells in each group. The data are presented as means ± SD from 3 independent experiments. *p <0.05 and **p <0.01 when compared to control or

761 samples not treated with NAC/CCCP/ambroxol.

762

763 **Figure 7. Rapamycin restores mitochondrial and lysosomal function in H_2O_2-treated cells.**

764 NIH3T3 cells were treated with H_2O_2 and incubated with DMSO, rapamycin or bafilomycin A1

765 for 3 or 5 days. (A) Images of JC-1 fluorescence from H_2O_2-treated cells with DMSO,

766 rapamycin or bafilomycin A1 for 3 days. (B) DCFH-DA probed intracellular ROS at day 3. (C)

767 Intracellular ATP levels were measured at day 3. (D) Cathepsin B protein in cells incubated

768 with rapamycin or bafilomycin A1 was examined by Western blots. (E) Intracellular cathepsin

769 B activity at day 3 was visualized by using Magic Red Cathepsin B kit. The data are presented

770 as means ± SD from 3 independent experiments. *$p < 0.05$ and **$p < 0.01$ when compared to

771 DMSO.