THE ROLE OF CELLULAR SENESCENCE IN HEALTH AND DISEASE

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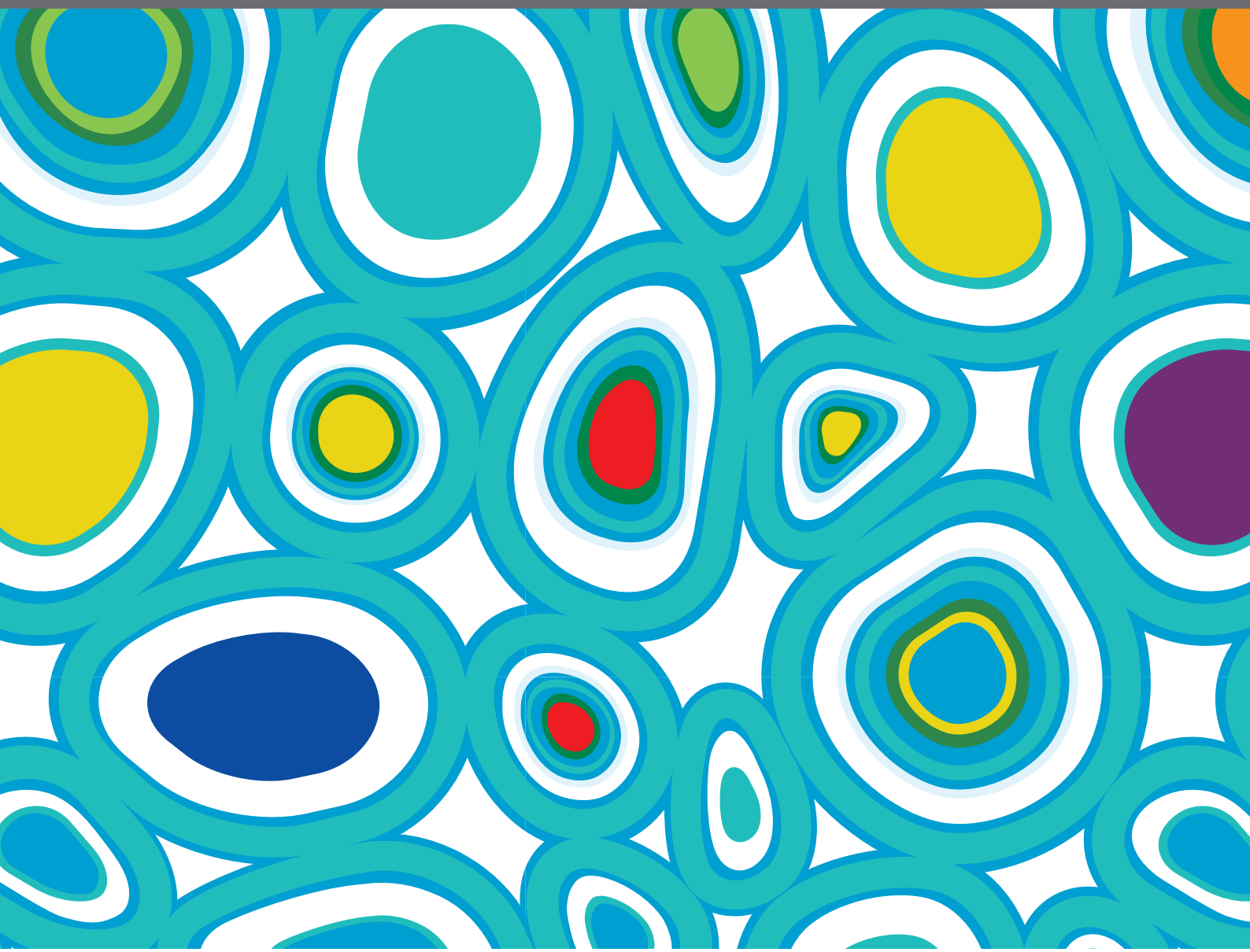

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THE ROLE OF CELLULAR SENESCENCE IN HEALTH AND DISEASE

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EDITORIAL
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Editorial: The Role of Cellular Senescence in Health and Disease

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Keywords: cellular senescence, pathology, development, senotherapy, fibrosis, neurodegeneration, aging

Editorial on the Research Topic

The Role of Cellular Senescence in Health and Disease

Cellular senescence is a stable anti-proliferative state, which has an essential role in cell balance control in diverse physiological and pathological settings (Chan and Narita, 2019; Gorgoulis et al., 2019). Senescence research is a highly dynamic field that has experienced a radical expansion over the last few years with the identification of the role of senescence in a growing list of diseases and physiological processes and the promise for therapeutic interventions based on senescence (Munoz-Espin and Serrano, 2014; Paez-Ribes et al., 2019). The current Research Topic aims to give an overview of the latest advances in this field highlighting the progress in understanding the mechanism of senescence and its link to disease in the nervous system and other organs. The issue includes a wide range of articles, including original research reports, mini-reviews, and reviews that explore diverse angles of this topic, showcasing the current trends in senescence research.

Several contributions in this issue explore the role of senescence in the various components of the nervous system and its involvement in neurodegeneration. Martínez-Cué and Rueda present a comprehensive review that delves into the topic of the contribution of cellular senescence to the pathophysiology of neurodegenerative diseases. The review discusses both molecular mechanisms involved and the evidence that associates cellular senescence with several neurodegenerative disorders including Alzheimer's Disease (AD), Down syndrome, or Parkinson's Disease. Contributing to the discussion of senescence in neurodegeneration, Walton et al. review and discuss the evidence indicating that Aβ oligomers and the process of cellular senescence may be inextricably linked, at least in the early stages of AD. The role of Aβ oligomers as a senescence inducer and the possible role of secondary senescence in AD progression suggest that cellular senescence could possibly be an essential component of the pathological cascade of events within the amyloid cascade hypothesis rather than a separate etiology for AD progression. In their mini-review, Papadopoulos et al. explore another side of neurodegeneration and its relation to cellular senescence, this time with a focus on progressive multiple sclerosis. They review data suggesting involvement of cellular senescence in a wide variety of processes regulating disease progression, such as its role in chronic, non-remitting inflammation, in altered neuronal and glial cell function, in failure of remyelination and in impaired blood-brain barrier integrity. Finally, the possible neuroprotective role of senolytic and senomorphic treatments is discussed. As a mechanistic and methodological complement to these reviews, this special issue also includes an article in the form of a mini-review by Levstek et al., which discusses the topic of telomere attrition in

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neurodegenerative disorders, highlighting the promises and challenges of telomere studies in this context.

Also within the subject of neurobiology, two articles focus on the role of senescence in the auditory system. The article by Magariños et al. deepens our understanding of developmental senescence, highlighting the roles of TGFβ2, and cellular senescence in the regulation of cell fate within the developing inner ear and how this link is highly cell-type dependent. Interestingly, the study shows that TGFβ2 exerts a powerful action in inner ear neurogenesis but, contrary to other embryonic otic cell types, these effects are independent of cellular senescence. The study of cellular senescence in auditory cells is also addressed, this time in the context of aging of mechanotransducer cells, by Lin et al. These authors address the interesting relationship between mitohormesis and cellular senescence by identifying DRP-1 as a possible connector node between the two. They suggest a novel mechanism where DRP-1-induced mitophagy may reduce mitochondrial dysfunction and oxidative stress, thus blunting cellular senescence during aging of otic cells.

Among the pathologies linked to senescence, fibrotic diseases in different organs have attracted a lot of interest. In this issue, Lin and Xu discuss the roles of aging and cellular senescence in idiopathic pulmonary fibrosis, one of the diseases where the pathogenic role of senescence has been more extensively studied. In their review article, the authors focus on the molecular mechanisms by which senescent lung fibroblasts influence this fibrotic disease and the potential for senescence-based interventions in this pathology. Also in this context, Wu et al. review the role of cell cycle deregulation, a hallmark of the senescent phenotype, in renal fibrosis. As in other organs, the link between senescence and fibrosis in the kidney is complex. As highlighted by the authors, injury-associated senescence in tubular epithelial cells may promote fibrosis in a paracrine mechanism mediated by the SASP (Senescence-Associated Secretory Phenotype). However, the specific functional impact of cell cycle deregulation and senescence in renal fibrosis is likely to be more intricate, being influenced by factors like the profibrotic stimulus, renal cell types involved and the distinction between acute and chronic senescence.

Two original reports in this issue provide insights about additional lung diseases, besides fibrosis. In their *in silico* study, Dong et al. identify the role of senescence-associated genes TNFRSF12A and CD38 in chronic obstructive pulmonary disease (COPD), and discuss their contribution to chronic inflammation and accelerated aging that characterize COPD. Through functional pathway enrichment analysis they propose a model where stimulation of TWEAK/TNFRSF12A signaling enhances lung tissue remodeling, while induction of CD38 protein expression contributes to the accelerated aging observed in COPD patients. It is speculated that the balance between TNFRSF12A and CD38 proteins could play a major role in establishing a cycle of unresolvable tissue remodeling in COPD patients' lungs. Pan et al. explore the role of microRNA-221 in the pathogenesis of asthma. They show that inhibition of miR-221 in a murine asthma model reduces airway mucus metaplasia, inflammation, and remodeling. At the same time, miR-221 was found to regulate collagen deposition in the extracellular matrix through the PI3K-AKT pathway, shedding interesting light on miR-221 functions, and highlighting this microRNA as a potential therapeutic target.

A complementary perspective on cellular senescence and age-associated conditions is given in the review article by Li et al. The effects of SIRT6 as an inhibitor of cellular senescence and cell aging, and as an important player in cardiovascular disease through regulation of triglyceride synthesis and cholesterol homeostasis are extensively discussed. Possible clinical applications to target activation of SIRT6 as potential means to delay aging and treat cardiovascular diseases are explored.

Regarding the mechanism of senescence, the article by Shimoni et al. proposes a novel stimulus to add to the list of alterations in cellular homeostasis that have been shown to trigger the senescence response. These include genotoxic damage, oncogene activation, or mitochondrial dysfunction, to name a few. In this article, the authors report that bovine umbilical cord mesenchymal stem cells exposed to mild heat stress acquire features of the senescent phenotype, including cell-cycle arrest and lysosomal Senescence-Associated Beta Galactosidase activity. This phenotype seems to be associated with oxidative stress and mitochondrial damage. It would be interesting to characterize in more detail this response to understand the potential link between abnormal temperature conditions and senescence. Also, the article by Zhou et al. explores the link between cell senescence, intestinal epithelial integrity and gut microbiota. Using a mouse model of loss of function of Bmi-1, an epigenetic repressor of senescence, the authors show that Bmi-1 preserves intestinal epithelial barrier function and microbiota balance by preventing p16Ink4a-mediated senescence, in a process that apparently involves a non-canonical role of the p16Ink4a protein in tight junctions.

One of the major breakthroughs in senescence research in recent years has been the discovery of therapeutical interventions based in senescence. In their review in this issue, Thoppil and Riabowol provide an overview of the existing toolkit of compounds with the ability to selectively kill senescent cells (senolytics) or modify the senescent secretome (senomorphs), as well as senescent cell-specific drug delivery strategies, highlighting their therapeutic potential. The review by Kaur et al. explores a different angle of this subject, discussing alternative strategies to prevent age-induced tissue senescence based in the use of bioactive compounds from plants or food, known as nutraceuticals. Excitingly, some of these synthetic or natural senotherapeutic compounds have already entered clinical trials, opening the prospect to exploit senescence in the clinic.

In summary, we believe that the articles in this issue provide a timely and comprehensive overview of the current research efforts in the senescence field. As editors, we are grateful to the authors for their contributions and we hope that this Research Topic succeeds to convey to the readers the interest and promise of the studies on cellular senescence and its connection with human disease.

AUTHOR CONTRIBUTIONS

All authors contributed to writing and editing the manuscript.

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ORIGINAL RESEARCH
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Inhibition of DRP-1-Dependent Mitophagy Promotes Cochlea Hair Cell Senescence and Exacerbates Age-Related Hearing Loss

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Background: Mitochondrial dysfunction is considered to contribute to the development of age-related hearing loss (AHL). The regulation of mitochondrial function requires mitochondrial quality control, which includes mitophagy and dynamics. Dynamin-related Protein 1 (DRP-1) is believed to play a central role in this regulation. However, the underlying mechanism of DRP-1 in AHL remains unclear. Here, we examined whether the decline of DRP-1-dependent mitophagy contributes to the development of AHL.

Methods: We induced cellular and cochlear senescence using hydrogen peroxide (H_2O_2) and evaluated the level of senescence through senescence-associated β-galactosidase staining. We evaluated mitophagy levels *via* fluorescence imaging and Western Blotting of LC3II and P62. Mitochondrial function was assessed by ATP assay, mtDNA assay, and JC-1.

Results: We found that both the expression of DRP-1 and the mitophagy level decreased in senescent cells and aged mice. DRP-1 overexpression in HEI-OC1 cells initiated mitophagy and preserved mitochondrial function when exposed to H_2O_2, while cells with DRP-1 silencing displayed otherwise. Moreover, inhibition of DRP-1 by Mdivi-1 blocked mitophagy and exacerbated hearing loss in aged C57BL/6 mice.

Conclusion: These results indicated that DRP-1 initiated mitophagy, eliminated mitochondrial dysfunction, and may protect against oxidative stress-induced senescence. These results provide a potential therapeutic target for AHL.

Keywords: presbycusis, oxidative stress, DRP-1, mitophagy, cochlea

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INTRODUCTION

Aging is a physiological phenomenon that occurs in all eukaryotes. Age-related hearing loss (AHL) is one of the most serious health-related social problems (Roth et al., 2011). It is characterized by an age-dependent decline of auditory function that is attributable to the loss and dysfunction of hair cells and spiral ganglion cells in the cochlea of the inner ear (Lin et al., 2013). There is agreement that cumulative mitochondrial dysfunction and Reactive Oxygen Species (ROS) exacerbate hearing loss. Still, the exact molecular mechanism of AHL remains unknown.

A series of cellular activities are regulated in the cellular aging process. Among these, mitochondrial function is considered to be a central event in various diseases in all systems and at all ages. A recent study shows that mitochondrial dysfunction is one of the characteristics of the aging process (López-Otín et al., 2013), and it is thought to induce diseases such as Alzheimer's (Lee et al., 2010) and AHL (Seo et al., 2017).

Cellular activities require mitochondria to provide energy, but meanwhile, adenosine triphosphate (ATP) synthesis oxidative stress is also produced, causing injury to cells and organelles. Excessive oxidative stress and damaged organelles can be eliminated and recycled by an essential process called autophagy (Mizushima et al., 2008). Specific mitochondrial autophagy is called mitophagy and allows dysfunctional mitochondria to go through a process of degradation. Mitophagy, along with mitochondrial biogenesis, maintains mitochondrial function.

Mitochondria constantly undergo fission and fusion to realize mitochondrial quality control. The activation of dynamin-related protein-1 (DRP-1) is required when mitochondria go through fission to create new mitochondria or to separate damaged mitochondria matrices so as to preserve normal mitochondrial function during high levels of cellular stress (Youle and van der Bliek, 2012). The main approach to eliminate injured mitochondria by autophagy is mitophagy. Disturbance of mitophagy may affect normal mitochondrial function and the physiological or pathological cellular condition. DRP-1 is a highly conserved GTPase that plays the core role in mitophagy by regulating the separation of mitochondria (Yoneda et al., 1994). When mitochondrial fission occurs, DRP-1 is recruited from the cytosol to the outer membrane, where it forms a spiral complex around the mitochondria (Ingerman et al., 2005). One situation is symmetrical replicative fission. This is where a healthy mitochondrion divides equally into two daughter mitochondria, which generates new mitochondria by mitochondrial biogenesis or by fusion (Gray et al., 1999). The other situation is where asymmetrical fission is initiated so that damaged mitochondria can be removed through mitophagy (Yang and Yang, 2013). Dysfunctional mitochondria display relatively depolarized membrane potential, which can be targeted for selective autophagy for further elimination.

In our study, we hypothesized that the expression and normal functions of DRP-1 and DRP-1-dependent mitophagy are disrupted in the aged cochlea with AHL. This disruption is considered harmful to the survival and function of cochlear hair cells. To determine whether DRP-1 and mitophagy play a role in aged cochlea with AHL, we examined DRP-1 and mitophagy and their roles in cellular senescence and AHL.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from the Laboratory Animal Center, Sun Yat-sen University (Guangzhou, China). C57BL/6 mice were randomly divided into three groups: one "young control" group (1 month old), one "old control" group (12 months old), one "old Mdivi-1 IP" group (12 months old). Mdivi-1 was administrated intraperitoneally in the amount of 12 mg/kg every 3 days. All experiments were performed according to protocols approved by the Animal Research Center, Sun Yat-sen University.

Auditory Brainstem Response

Auditory brainstem response (ABR) tests were conducted using the Tucker-Davis Technology (TDT) System III (Alachua, FL, USA) as previously described (Pang et al., 2017). In brief, mice were anesthetized, and subdermal needle electrodes were inserted at the vertex (active) and below the left ear (reference) and the right ear (ground). ABR tests were measured at 8, 16, and 32 kHz. The average response to 1,024 stimuli was obtained through reduction of the sound intensity in 5 dB intervals near the threshold, which was defined as the lowest stimulus level where a positive wave was evident.

Tissue Preparation

C57BL/6 mice were decapitated after the ABR tests. The temporal bones were dissected, and the cochleae were obtained and fixed with 4% paraformaldehyde at 4°C overnight and decalcified in 4% sodium ethylenediaminetetraacetic acid for 2 days. For protein preparation, cochlear basilar membranes were dissected from cochlea, snap-frozen in liquid nitrogen, and stored at −80°C.

Cell Culture

Cochlear HEI-OC1 cells (kindly provided by F. Kalinec from the House Ear Institute, Los Angeles, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) and supplemented with 10% fetal bovine serum (Gibco) at 33°C under a 10% CO_2 condition.

Primary Tissue Culture

P2–3 C57BL/6 mice were purchased from Guangdong Medical Laboratory Animal Center (GDMLAC). The temporal bones were dissected, and the cochleae were obtained and then supplemented with 10% fetal bovine serum (Gibco) at 37°C under a 5% CO_2 condition.

Cell Transfection

DRP-1 Silencer (Ruibo, Guangzhou, China), a commercial mixture of siRNA and ASO (Antisense oligonucleotides), was used to knock down the expression of DRP-1 in HEI-OC1 cells. Cell transfections were performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, USA) following the manufacturer's instructions. The HEI-OC1 cells were transfected with lentivirus-mediated green fluorescent protein (GFP)-LC3 to generate GFP-LC3-expressing cells. The lentivirus containing the GFP-LC3 fusion gene was purchased from Hanbio (Shanghai, China). HEI-OC1 cells were seeded into six-well dishes (1×10^5 per well) and infected with the recombinant lentivirus. After culturing in the presence of puromycin for 2 weeks, HEI-OC1 cells with GFP-LC3 were selected.

Western Blot Analysis

Western Blotting was conducted as previously described (Pang et al., 2017). Briefly, proteins were extracted from cells and

cochlea tissues using radioimmunoprecipitation assay lysis buffer (Thermo plus, USA). Protein samples (20 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). After being blocked with 5% nonfat milk, the membranes were incubated with anti-DRP1 (1:1,000, Proteintech, Rosemont, IL, USA), anti-p-DRP1 (1:1,000, Abclonal, Woburn, MA, USA), anti-LC3I/II (1:1,000, CST, USA), anti-P62 (1:1,000, CST, USA), anti-P53 (1:1,000, Abclonal, Woburn, MA, USA), and anti-P21 (1:1,000, Abclonal, Woburn, MA, USA) at 4°C overnight, followed by incubation with secondary antibodies (1:3,000) at room temperature for 1 h. The immunoreactive bands were then detected by enhanced chemiluminescence (Millipore, Burlington, MA, USA). Band intensities were analyzed using ImageJ (NIH, USA). β-actin was applied as loading and internal control.

Cell Viability Assay

Cell viability was detected using CCK-8 kits (DOJINDO, Japan) according to the manufacturer's protocols. Briefly, cells were plated into 96-well plate. At the indicated time after treatments, 10 μl of CCK-8 solution was added to the wells. Cells were then incubated at 37°C for 1 h. Finally, a microplate reader (Labsystems Dragon, Finland) was used to measure the absorbance at 450 nm.

Cell Population Doubling Rate

For the population doubling rate assay, cells were counted, and 1×10^5 cells were plated in every 35 mm dish, pre-treated, replaced with a normal culture medium, and then incubated under permissive conditions. Cells were harvested and counted and re-planted every 72 h. The formula below was used to evaluate the population doubling rate.

$$f(x) = (\log N1 - \log N2)/\log 2$$

(N1 = Number of cells counted, N2 = Number of cells plated)

Senescence-Associated β-Galactosidase Stain

Cellular senescence-associated β-galactosidase staining was conducted using a senescence β-galactosidase staining kit (Beyotime, Shanghai, China) following the manufacturer's instruction. Briefly, cells were plated in 6-well plate and pre-treated. Cells were gently washed with PBS and fixed with fixing solution. After being washed with PBS three times, 1 ml of staining solution was added to each well, which was then sealed with parafilm and incubated at 37°C without CO_2 overnight.

ATP Assay

ATP assay was performed using an ATP Assay Kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Briefly, cells were homogenized with lysis buffer and centrifuged at 12,000 *g* for 5 min at 4°C. An ATP detection reagent was diluted with dilution buffer and added to 96-wells. Then, the samples were added into the wells and mixed with the detection solution. The chemiluminescence intensities of samples and standards were measured with a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA). The levels of ATP were calculated based on the standard curve and normalized to the protein content.

Mitochondrial Fluorescent Probe Staining Analysis

Mitochondrial staining was conducted with the mitochondrial probe MitoTracker Red CMXRos (Yeasen, Shanghai, China) according to the manufacturer's protocols. After being washed with PBS, the cells were counterstained with DAPI for 10 min and imaged with an Olympus BX63 microscope (Olympus, Japan).

Mitochondrial DNA (mtDNA) Content Analysis

Total genomic DNA was extracted from cells using a Universal Genomic DNA Extraction Kit (Takara) according to the manufacturer's protocols. The mtDNA levels were quantified by qPCR on a Roche LightCycler 96 (Roche) using D-loop primers (forward: 5′-GGTTCTTACTTCAGGGCCATCA-3′, reverse: 5′-GATTAGACCCGTTACCATCGAGAT-3′). Nuclear gene beta2-microglobulin (B2M) primers (forward: 5′-ATGGGAAG CCGAACATACTG-3′, reverse: 5′-CAGTCTCAGTGGGGGT GAAT-3′) were used as a nuclear control.

Statistical Analysis

All experiments were independently repeated at least three times. Data were presented as mean ± SD and were analyzed with SPSS and Graphpad Prism 5 software. Student's *t*-test and one-way ANOVA were used for statistical analysis. Values with $p < 0.05$ were considered significant.

RESULTS

Oxidative Stress-Induced Senescence in HEI-OC1 Cells

We first established cellular senescence by inducing oxidative stress. HEI-OC1 cells were briefly exposed to H_2O_2 (1 mM for 1 h), and we then further investigated the cellular molecular change between mitophagy and senescence. Our results revealed that cellular senescence was induced 24 h after H_2O_2 treatment at a rate of 54.4 ± 9.94% HEI-OC1 cells stained with β-gal staining (**Figure 1A**). In the meantime, there was 13.4 ± 2.25% of senescent β-gal-stained cells in the normal control HEI-OC1 cells ($p < 0.0001$, **Figure 1B**). We further assessed cellular senescence with cell viability, population doubling rate, and senescence-associated P53 and P21. Lower cell viability was detected in cells treated with H_2O_2, being 0.63 ± 0.03-fold lower than the control cells ($p = 0.0006$, **Figure 1C**). The population doubling rate was calculated to evaluate the aging pattern. Higher rates indicate a higher speed of cell growth. The population doubling rate dropped to 1.73 ± 0.27 compared to normal cells at 4.21 ± 0.08 ($p = 0.0001$, **Figure 1D**). Cellular senescence-associated P53 and P21 were further assessed by Western Blotting. H_2O_2 treatment of HEI-OC1 cells significantly elevated the expression of P53 and P21 (**Figures 1E–G**). These data demonstrated that H_2O_2 induced cellular senescence in HEI-OC1 cochlear cells.

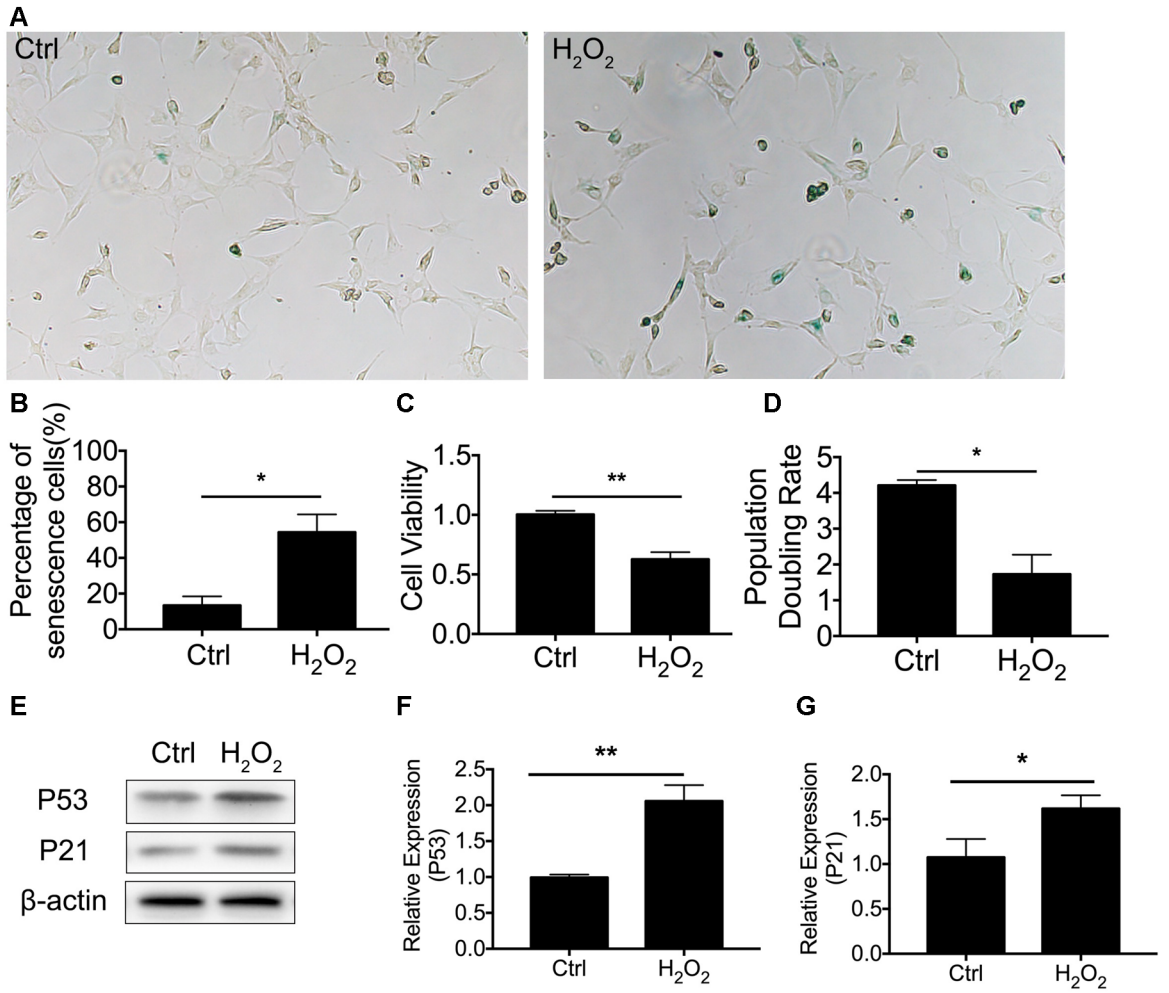

FIGURE 1 | H_2O_2-induced cellular senescence in HEI-OC1 cells. **(A)** β-gal staining of senescent HEI-OC1 cells treated with H_2O_2. **(B)** Percentage of β-gal stained cells. **(C)** Cell viability of 1 mM H_2O_2 treated cells compared with control cells. **(D)** Population doubling rate in HEI-OC1 cells. **(E–G)** Representative Western Blot analysis using antibodies against P53 and P21 to assess cellular senescence. $^*p < 0.05$, $^{**}p < 0.01$.

Oxidative Stress Downregulated the Mitophagy Level and Induced Mitochondrial Dysfunction in Cellular Senescence

To assess whether there was a molecular change between mitophagy and senescence in HEI-OC1 cells, we further examined blockage of the autophagy flux (**Figure 2A**). Western Blotting revealed that 1 mM of H_2O_2 treatment resulted in a decrease of LC3 II of 0.62 ± 0.08-fold relative to control and a 1.77 ± 0.18-fold relative increase of P62 ($p < 0.05$, **Figures 2B,C**). The Western Blotting results revealed the suppression of the autophagy function. To further determine mitophagy, we used transfected HEI-OC1 cells expressing GFP-LC3 and staining with the MitoTracker Red fluorescence probe. The yellow puncta displayed were considered as the merging of mitochondria and autophagosomes (**Figure 2D**). From each group, 20 random cells were counted. The percentage of mitophagosome with H_2O_2 treatment was 10 ± 1.16% compared to the control group, with 16.67 ± 0.88% ($p = 0.0101$, **Figure 2E**). The data indicated that mitophagy was suppressed in H_2O_2-induced senescent HEI-OC1 cells.

Next, we examined whether mitophagy is beneficial to mitochondrial function. We treated the cells with 0.5 μM rapamycin for 8 h to initiate autophagy and mitophagy. To evaluate mitochondrial dysfunction induced by oxidative stress and to determine if mitophagy would rescue mitochondrial function, we evaluated mitochondrial membrane potential by JC-1, ATP content, and mtDNA in HEI-OC1 cells treated with H_2O_2. The mitochondrial membrane potential of each group was evaluated with JC-1 stain by flow cytometry. Cells in the H_2O_2 treatment group displayed a lower percentage of mitochondria with normal potential at a rate of 39.91 ± 0.04% ($p < 0.0001$, **Figures 2F,G,J**). We found that treatment with rapamycin under oxidative stress increased the percentage of mitochondria with normal

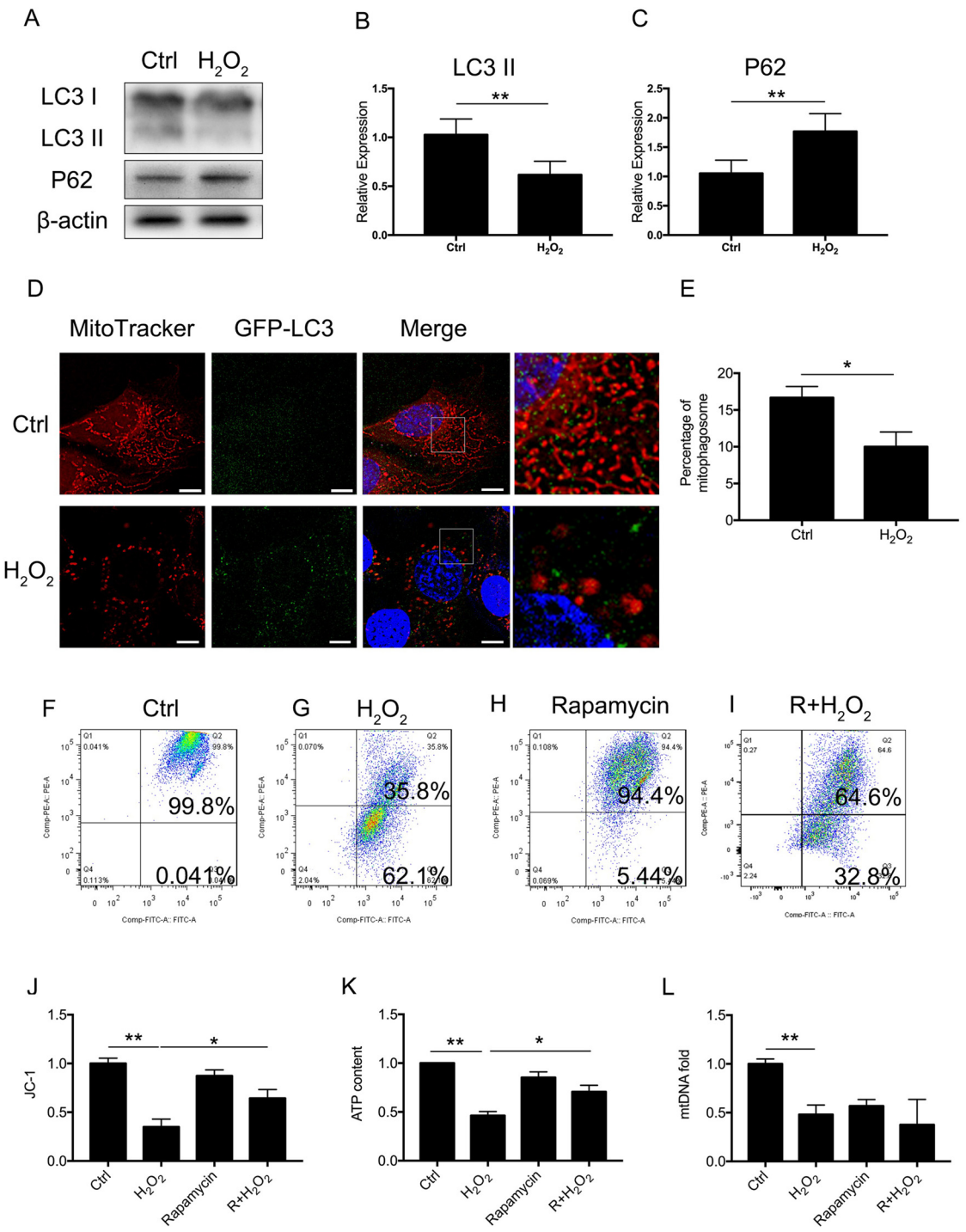

FIGURE 2 | Mitophagy and mitochondrial function of HEI-OC1 cells. **(A)** Representative Western Blot analysis using antibodies against LC3 I/II and P62 to assess autophagy flux. **(B,C)** Relative expression of mitophagy proteins LC3 II and P62 between control cells and H_2O_2-treated cells. **(D)** Immunofluorescence image showing mitophagy of mitochondria and autophagosome in green fluorescent protein (GFP)-LC3-expressing HEI-OC1 cells. **(E)** Percentage of mitophagosome in GFP-LC3-expressing HEI-OC1 cells between control and H_2O_2 treatment. **(F–I)** Cellular mitochondrial membrane potential between control and H_2O_2 treatment according to JC-1 staining and measured by flow cytometry. **(J)** Mitochondrial membrane potential analysis. Rapamycin-initiated autophagy improved mitochondrial membrane potential under H_2O_2 treatment. **(K)** ATP content in cells treated with H_2O_2 or rapamycin. ATP content was largely preserved with rapamycin under H_2O_2 treatment. **(L)** Relative mtDNA fold change, representing relative mitochondria amount. No significant difference between H_2O_2 treatment and rapamycin treatment. $*p < 0.05$, $**p < 0.01$. Scale bar = 10 μm.

potential compared to H_2O_2 treatment alone (p = 0.0152, **Figures 2H,I**). Also, we examined the ATP content under H_2O_2 treatment and also with rapamycin. H_2O_2 decreased the ATP content of HEI-OC1 cells to ∼0.46 ± 0.02-fold that of the control group, while, with pre-treatment with rapamycin, H_2O_2 only resulted in a decline of ATP content to ∼0.71 ± 0.04 fold (p = 0.0058, **Figure 2K**). However, there is no significant difference between H_2O_2 and rapamycin treatment ($p_{(H2O2,rapamycin)}$ = 0.74, $p_{(H2O2,R+H2O2)}$ = 0.28, **Figure 2L**) in the amount of mtDNA. These results suggested that H_2O_2 suppressed autophagy and mitophagy and resulted in mitochondrial dysfunction.

DRP-1 Contributed to Mitophagy and Mitochondrial Function in HEI-OC1 Cells Under Oxidative Stress

DRP-1 plays the core role in mitophagy through regulation of mitochondrial fission. Mitochondrial fission separates injured mitochondria, and this is followed by mitophagy. With a link between declining levels of mitophagy and aging, we hypothesized that the accumulation of damaged mitochondria in senescent cells was due to suppressed mitochondrial fission and subsequent mitophagy. To investigate whether DRP-1 regulates mitophagy, we assessed mitophagy in DRP-1 overexpressing and silencing cells (**Figures 3A,B**). Autophagy flux and cellular senescence were assessed by Western Blotting of LC3II, P62, P53, and P21 (**Figure 3C**). Assessment *via* LC3 II and P62 revealed that autophagy flux was blocked in DRP-1 silencing cells but not in DRP-1 overexpressing cells (p < 0.01, **Figures 3D,E**). We further assessed cellular senescence in DRP-1 overexpressing and silencing cells under H_2O_2 treatment. We evaluated the expression of senescence-associated P53, P21 (**Figures 3F,G**), and β-Gal stain (**Figure 3H**). The elevation of P53 and P21 expression and an increased portion of β-Gal stained cells revealed that silencing DRP-1 suppressed resistance to H_2O_2-induced cellular senescence. We then evaluated mitochondrial function in DRP-1 overexpressing and silencing cells to further understand mitophagy levels. DRP-1 overexpressing or silencing in HEI-OC1 cells resulted in no difference in the percentage of mitochondrial membrane potential compared with control cells. However, when cells faced oxidative stress, DRP-1 overexpression rescued mitochondrial membrane potential, displaying 53.0 ± 0.05% of healthy mitochondria and DRP-1 silencing of 18.8 ± 0.05% (p < 0.05, **Figures 3I,J**). The ATP content in DRP-1 overexpressing cells decreased but remained at 0.71 ± 0.04-fold that of the control group. The mtDNA remained at 0.62 ± 0.04-fold that of the control in DRP-1 overexpressing cells treated with H_2O_2 (p < 0.05, **Figures 3K,L**). In contrast, cells silencing DRP-1 displayed a significant decrease in ATP content to 0.32 ± 0.07-fold that of the control but with mtDNA at 0.90 ± 0.03 that of the control (p < 0.05, **Figures 3H,I**). Together with the blockage of autophagy flux, we may assume that mitophagy in HEI-OC1 cells was also blocked. The data revealed that DRP-1 overexpression initiated mitophagy and that such cells retained most of the mitochondrial function under oxidative treatment, while silencing DRP-1 inhibited mitophagy. Cells retained most of the mitochondria but less ATP content, indicating that mitochondrial function was disturbed in DRP-1 silencing cells under oxidative stress.

Increase of Oxidative Stress Downregulated DRP-1 Expression and Resulted in Mitochondrial Dysfunction in Cochlea

We observed the regulation of DRP-1 in mitochondrial functions and further investigated DRP-1 expression patterns in cochlear explants. Senescence was induced by 0.1 mM H_2O_2 treatment in the cochlear explants. We found a significant decrease of DRP-1 (0.66 ± 0.04-fold of control, p = 0.0017) and s616 phosphorylated DRP-1 (p-DRP1, 0.41 ± 0.08-fold of control, p = 0.0017, **Figures 4A–E**) in the cochlear explants. The mitophagy level of the cochlear explants was determined by the level of autophagy flux and mitochondrial function. Western Blotting showed the LC3 II and P62 protein levels to decrease ($p_{LC3\ II}$ = 0.0164, p_{P62} = 0.0062), suggesting that autophagy flux was also blocked in H_2O_2-treated cochlear explants (p = 0.0188, **Figures 4F,G**). H_2O_2 also resulted in mitochondrial dysfunction in the cochlear explants by down-regulating the ATP content to 0.62 ± 0.08-fold (p = 0.0095) and mtDNA to 0.53 ± 0.09-fold (p = 0.0071) that of control (**Figures 4H,I**). These results indicated that H_2O_2 treatment induced DRP-1 down-regulation and the injury of mitophagy in cochlear explants.

Fission Inhibitor Mdivi-1 Inhibited DRP-1-Mediated Mitophagy and Exacerbated Senescence in Cochlea Under Oxidative Stress

Mitochondrial-division inhibitor Mdivi-1 inhibits mitochondrial fission by deactivation of DRP-1. To determine whether DRP-1 has an effect on cochlear senescence, we assessed the mitophagy function and senescent phenotype in the cochlear explants. We evaluated mitophagy in cochlear explants with pre-treatment with 50 μM of Mdivi-1 for 4 h before H_2O_2 treatment. Mdivi-1 pre-treatment de-activated DRP-1 and significantly down-regulated the mitophagy level under oxidative stress (**Figures 5B,C**). The LC3 II and P62 levels were evaluated to assess autophagy flux and mitophagy (**Figures 5D,E**). Western Blotting of protein extracts of H_2O_2-exposed cochlea pre-treated with Mdivi-1 revealed damaged mitophagy by down-regulation of LC3 II (p = 0.0243) and upregulation of P62 (p = 0.0022, **Figures 5A–E**). H_2O_2-induced cellular senescence was exacerbated by DRP-1 silencing. To further investigate the role of DRP-1 in aged cochlea, we assessed senescence-associated P53, P21 (**Figures 5F,G**), and β-gal stain in the cochlear explants. Mdivi-1 pre-treatment did not solely induce senescence in cochlea but exacerbated the senescent damage induced by H_2O_2 (**Figure 5H**). These results indicated that H_2O_2 inhibited mitophagy and could further induce senescence in cochlea.

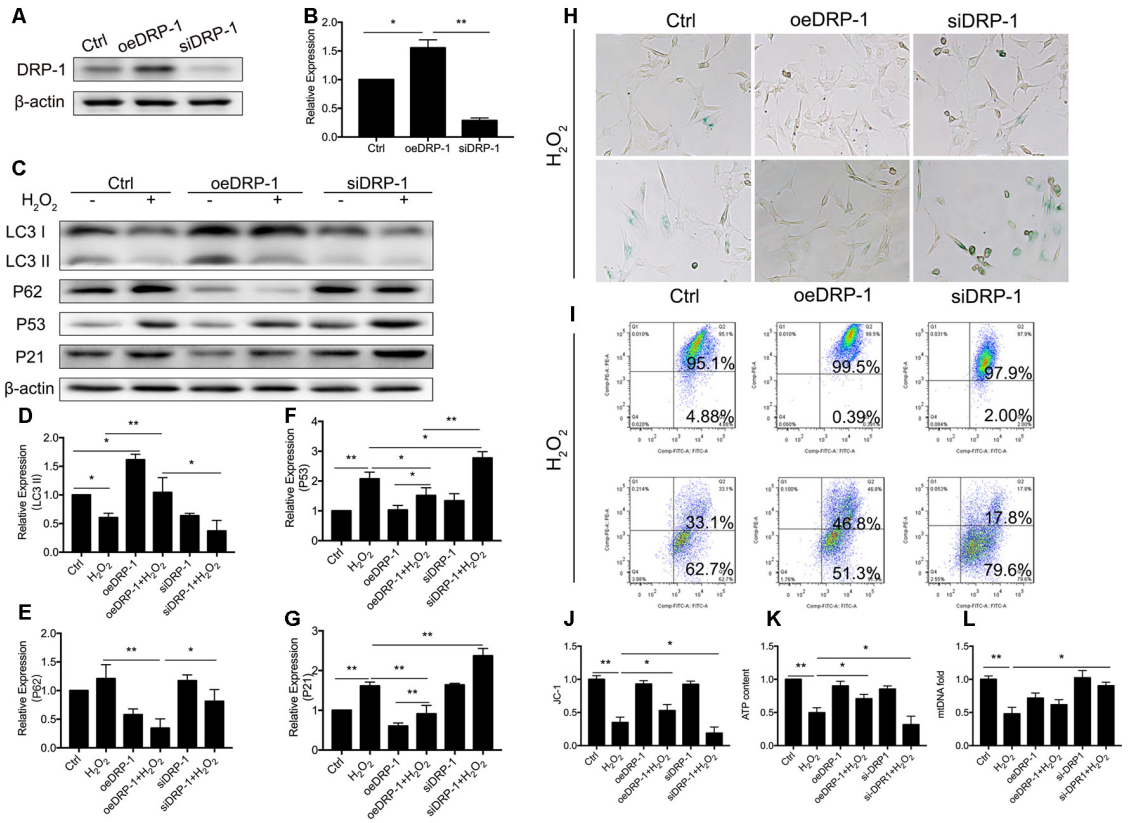

FIGURE 3 | Regulation of Dynamin-related Protein 1 (DRP-1) in cellular senescence and mitochondrial function. **(A)** Representative Western Blot analysis using antibodies against DRP-1 to assess DRP-1 expression. **(B)** DRP-1 expression in overexpressing and silencing HEI-OC1 cells. Relative expression of DRP-1 in overexpressing and silencing HEI-OC1 cells compared to control. **(C)** Representative Western Blot analysis using antibodies against LC3 I/II, P62, P53, and P21 to assess the mitophagy level and cellular senescence in HEI-OC1 cells. **(D,E)** Relative expression of LC3 II and P62. H_2O_2 down-regulated mitophagy in all groups. The relative mitophagy was higher in DRP-1 overexpressing cells compared to the control. The mitophagy level was down-regulated in DRP-1 silencing cells. **(F,G)** Relative expression of P53 and P21. H_2O_2 induced cellular senescence in HEI-OC1 cells, which elevated the expression of P53 and P21. P53 and P21 expression were relatively higher in DRP-1 silencing cells than in DRP-1 overexpressing cells. **(H)** Senescent HEI-OC1 cells with DRP-1 overexpression and silencing, assessed by β-Gal stain. **(I,J)** Mitochondrial membrane potential, evaluated by FCM with JC-1 staining. **(K)** ATP content in cells overexpressing and silencing DRP-1 under H_2O_2 treatment. **(L)** The amount of mitochondria were evaluated by mtDNA measurement. $^*p < 0.05$, $^{**}p < 0.01$.

Mdivi-1 Inhibited Mitophagy and Induced Hearing Loss in Aged Mice

C57BL/6 mice are known for their early-onset hearing loss and are often used as AHL models. To examine the hearing function of C57BL/6 mice, we performed ABR tests on aged and young C57BL/6 mice. We used 1-month-old mice as normal control, where hearing function had fully developed but no hearing loss had occurred. We evaluated the hearing threshold of the normal control group and a Mdivi-1 treatment group of 12 months old, by which time the C57BL/6 mice had developed hearing loss. The Mdivi-1 group was given Mdivi-1 *via* intraperitoneal administration beginning at the 8th month, before the C57BL/6 mice had developed hearing loss. The Mdivi-1 group developed greater threshold shifts at 8, 16, and 32 kHz than control mice and also exhibited greater hair cell loss (**Figures 6A,B**). We evaluated mitophagy using Western Blotting analysis, and the results revealed differences between aged mice and Mdivi-1-treated mice. There was a decrease of LC3 II ($p = 0.013$) and an increase of P62 ($p = 0.0266$, **Figures 6C–E**). Also, we found a decline of expression of DRP-1 and p-DRP-1 in aged cochlea (**Figures 6F–H**). However, there was no significant difference between aged mice and Mdivi-1-treated mice. The results indicated that mitophagy might be an effective way of hair cells survival under age stress and that inhibition of DRP-1 may block mitophagy and induce AHL.

DISCUSSION

Mitochondria form the cellular energy factory that synthesizes and provides ATP. However, dysfunctional mitochondria produce less ATP with more ROS and are more likely to induce apoptosis, necrosis, and senescence. Thus, there is a complicated and delicate mechanism of mitochondrial quality control to maintain mitochondrial function; this consists of mitochondrial fission and fusion, mitochondrial biogenesis, and mitophagy. Mitophagy is a process of selective cellular

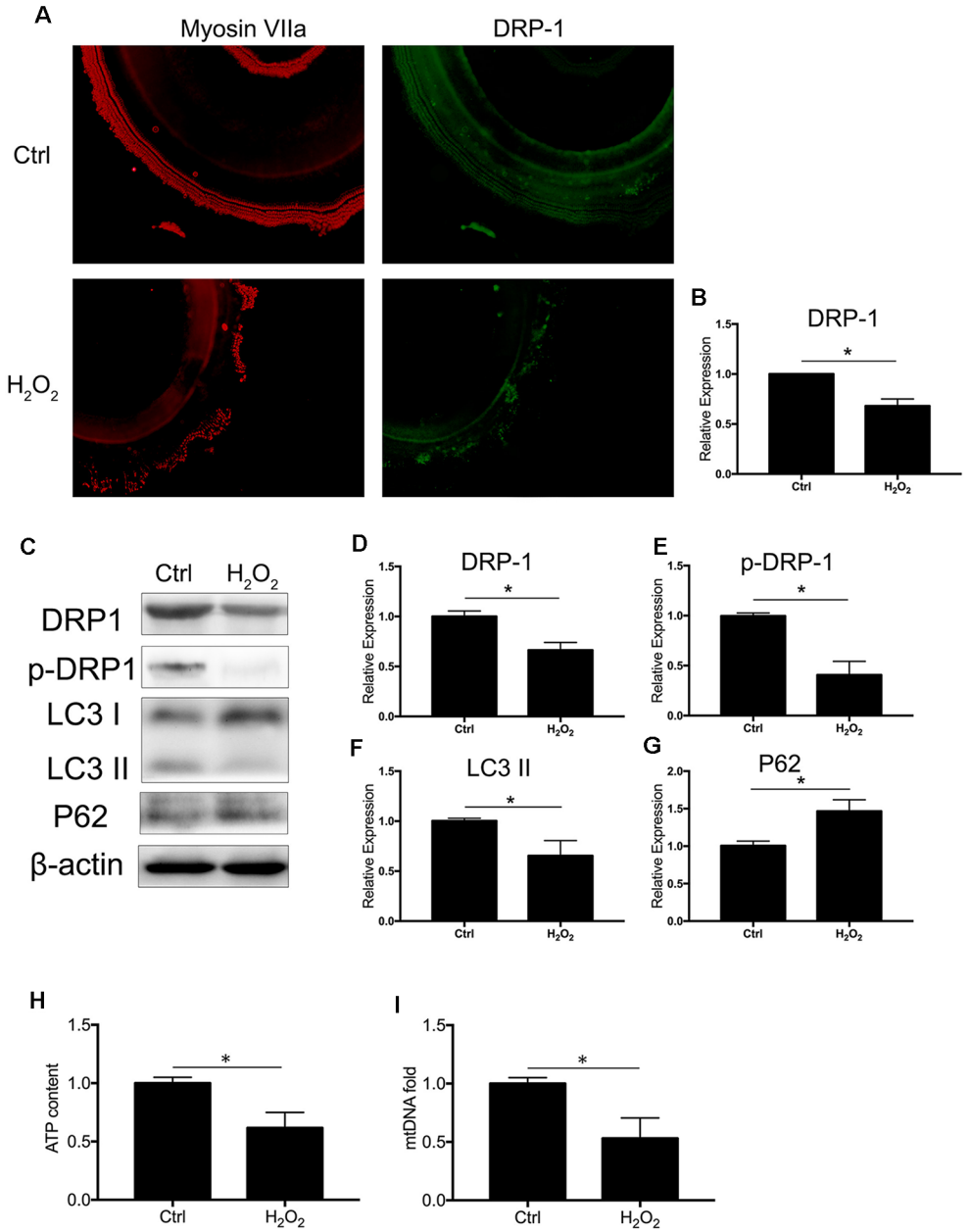

FIGURE 4 | H_2O_2 inhibited DRP-1 and mitophagy in cochlea. **(A)** Representative image of immunofluorescence staining of DRP-1 in cochlear explant. **(B)** Down-regulated expression of DRP-1 in cochlea treated with H_2O_2. **(C)** Representative Western Blot analysis using antibodies against DRP-1, p-DRP-1, LC3 I/II, and P62 to assess the mitophagy level in cochlear explant. **(D,E)** Relative expressions of DRP-1 and p-DRP-1 in H_2O_2-treated cochlea. **(F,G)** Relative expression of LC3 II and P62 in H_2O_2-treated cochlea. **(H,I)** Mitochondrial function evaluated by ATP content and mtDNA. H_2O_2-treated cochlea revealed a low ATP content and mitochondrial amount. $^*p < 0.05$. Scale bar = 300 μm.

defense. Through this process, damaged mitochondrial matrices can be separated and eliminated to maintain mitochondrial function and homeostasis and alleviate cellular injury. The inhibition of mitophagy results in mitochondrial dysfunction and cellular senescence (García-Prat et al., 2016). On the other hand, an increase in mitophagy repairs cellular injury and delays the process of senescence (Dalle Pezze et al., 2014; Manzella et al., 2018).

Mitochondrial fission and fusion depend on a series of GTPases (van der Bliek et al., 2013). DRP-1 is a dynamic-related GTP-binding protein. It is key in controlling mitochondrial fission and is reported to initiate mitophagy (Lemasters, 2005). DRP-1 induces mitophagy to attenuate oxidative damage in other organs like the spinal cord and neurons. Oxidative stress damage also occurs and results in AHL. Here, we investigate whether DRP-1-induced mitophagy plays a role in the process of AHL.

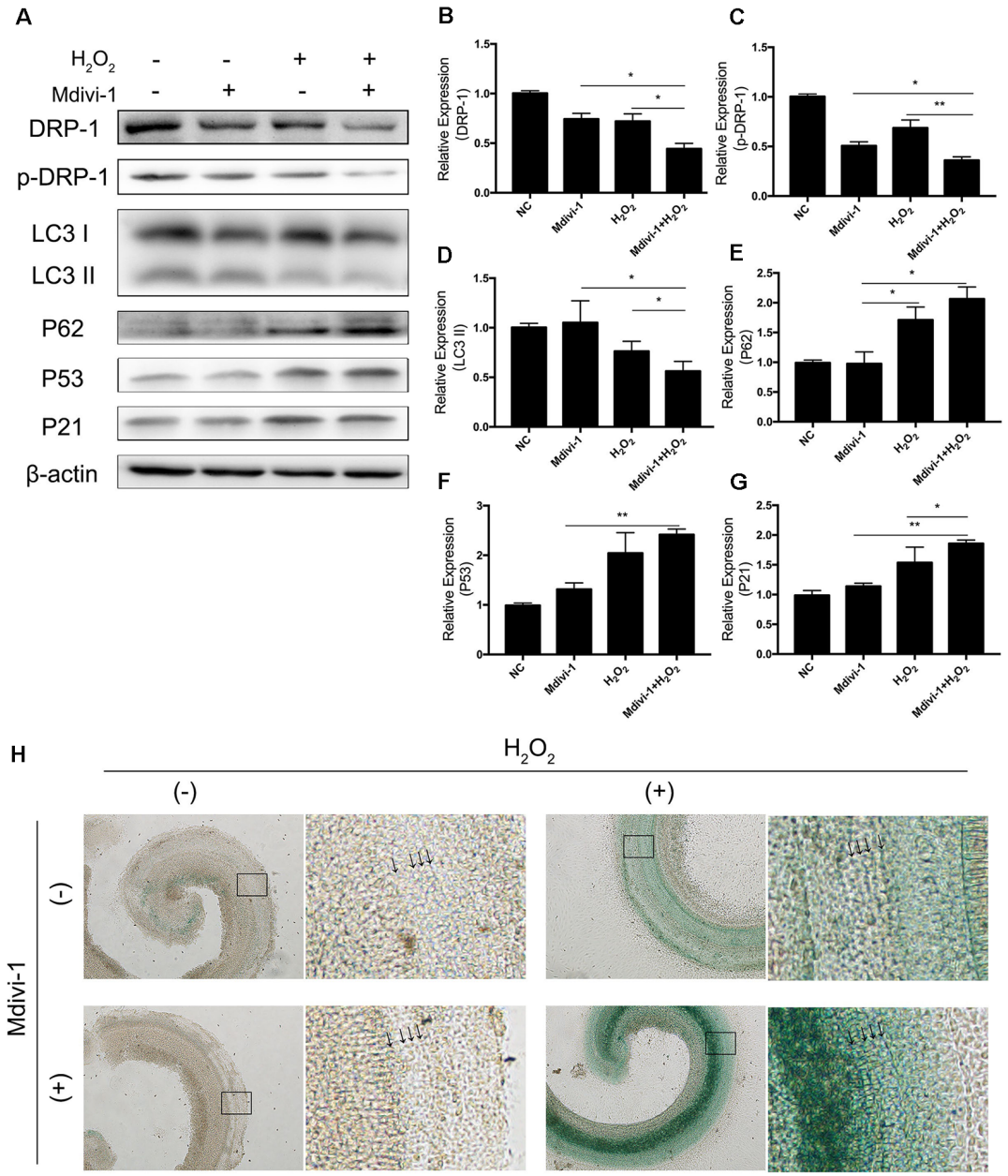

FIGURE 5 | Mdivi-1 exacerbated senescence in H_2O_2-treated cochlea by inhibition of mitophagy. **(A)** Representative Western Blot analysis using antibodies against DRP-1, p-DRP-1, LC3 I/II, P62, P53, and P21 to assess mitophagy level and senescence in cochlear explants treated with H_2O_2 and DRP-1 inhibitor Mdivi-1. **(B,C)** Relative expression of DRP-1 and p-DPR-1. Mdivi-1 inhibited DRP-1 expression and phosphorylation. **(D,E)** Relative expression of LC3 II and P62. Mdivi-1 suppressed the mitophagy level in H_2O_2-treated cochlea. **(F,G)** Relative expression of P53 and P21. Mdivi-1 aggravated H_2O_2-induced senescence in cochlear explants. **(H)** Representative scanned images of the cochlear surface with β-gal staining of cochlea. With Mdivi-1, cochlea had a darker greenish color under H_2O_2 treatment. Black arrows indicate OHC and IHC. $^*p < 0.05$, $^{**}p < 0.01$.

We first established a cellular senescence model of HEI-OC1 cells and studied the expression of DRP-1 and mitophagy levels. We found a decline of DRP-1 expression and mitophagy level along with mitochondrial dysfunction. These results indicated that there was a correlation between DRP-1 and mitochondrial dysfunction in senescent HEI-OC1 cells. We hypothesized that mitochondrial dysfunction was induced by a disturbance in mitophagy. Our previous study revealed that rapamycin can preserve mitochondrial function by initiating autophagy (Pang et al., 2017). We found that in cellular senescence, the disturbed autophagy flux assessed by the LC3 II and P62 proteins confirmed our hypothesis. Cells treated with rapamycin displayed a normal level of ATP but a decreased level of mtDNA, indicating that rapamycin

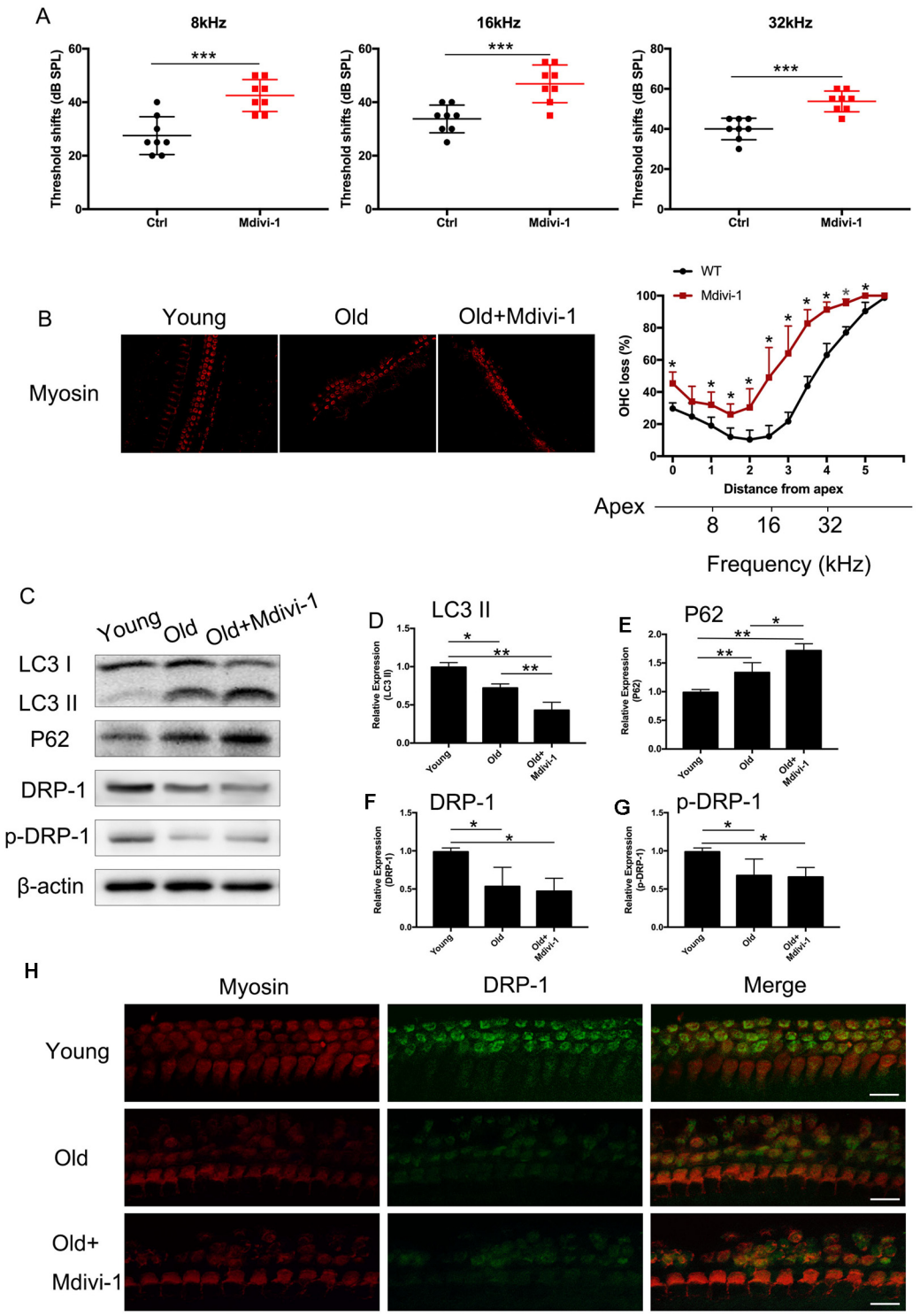

FIGURE 6 | Administration of Mdivi-1 in C57BL/6 mice resulted in greater hearing loss and hair cell loss. **(A)** Auditory brainstem response (ABR) threshold shifts in C57BL/6 mice. One-month-old mice were considered as young control. There was a significant hearing threshold shift in 12-month-old Mdivi-1 IP mice compared to the control mice. Mdivi-1 IP administration resulted in greater shifts compared to in aged mice. **(B)** Hair cell count in the basilar membrane of young, old control, and Mdivi-1 IP mice. **(C–H)** Mitophagy level and DRP-1 expression assessment by LC3 II, P62, DRP-1, and p-DRP-1, comparing the cochlea of young, old control, and Mdivi-1 IP mice. **(H)** Representative image of immunofluorescence staining of DRP-1 in the cochlea of young, old, and old with Mdivi-1 administration mice. Significant down expression of DRP-1 in old-mouse cochlea compared to the young control mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, scale bar = 100 μm.

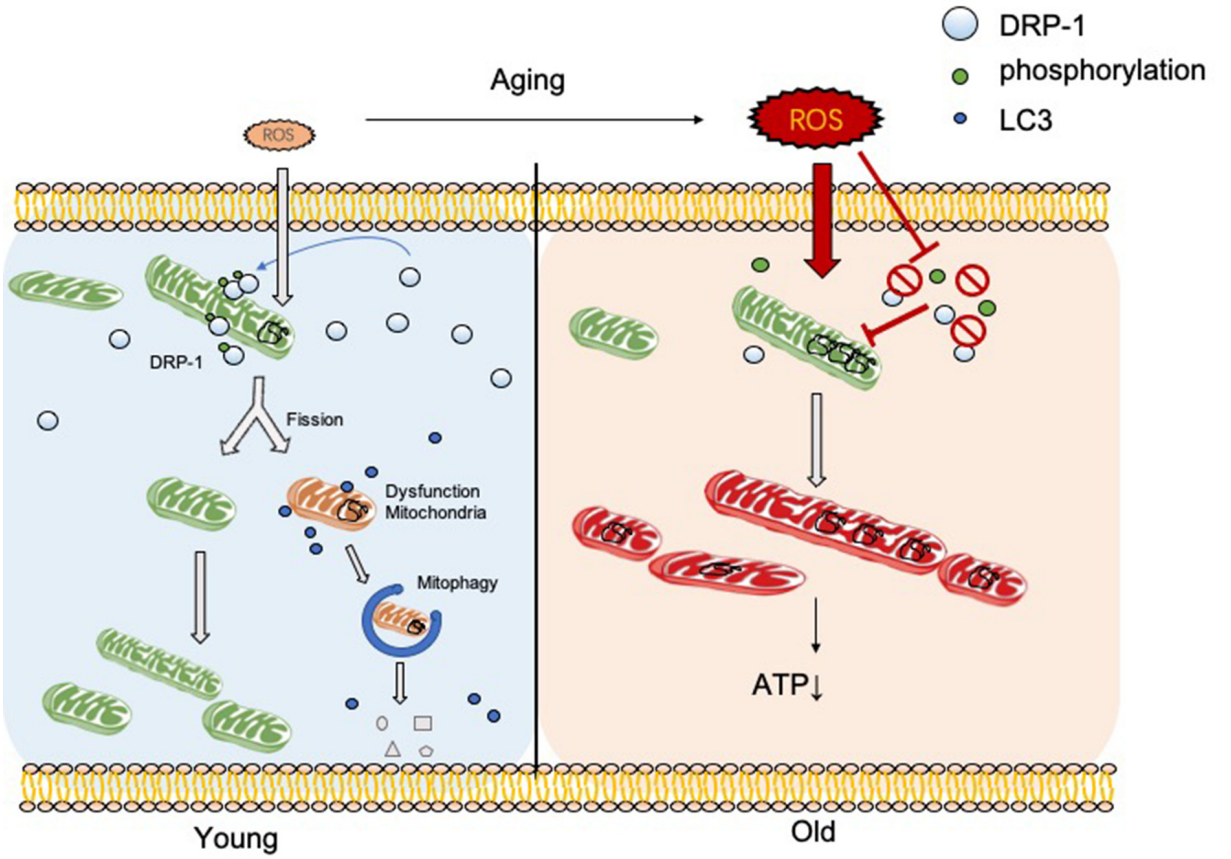

FIGURE 7 | DRP-1 induces separation of damaged mitochondrial matrix before initiation of mitophagy in young cells. With aging, reactive oxygen species (ROS) and oxidative stress increase and inhibit DRP-1 function. Oxidatively damaged mitochondria accumulate and result in a dysfunctional ATP metabolism and thus cellular senescence.

induced autophagy and preserved mitochondrial function. However, with oxidative stress, low ATP content and a lower autophagy level were found in senescent cells. The above results suggested that, in HEI-OC1 cells, oxidative stress suppressed autophagy, which might also suppress mitophagy and induce mitochondrial dysfunction. Other studies have demonstrated that DRP-1-induced mitophagy rescued mitochondrial function. These studies were related to oxidative stress in cardiac aging (Fernández et al., 2018), PD (Martinez et al., 2018), and early-stage AD (Wang et al., 2019). Our research confirmed that the initiation of mitophagy depends on DRP-1 and that DRP-1-induced mitophagy is key in the regulation of oxidative damage in hair cells.

We further investigated DRP-1 and mitochondrial function. We evaluated mitochondrial membrane potential, ATP content, and mtDNA in DRP-1 overexpressing and silencing HEI-OC1 cells. Mitochondrial membrane potential displayed no differences between DRP-1 overexpressing and silencing cells. However, in cells in which senescence had been induced by oxidative stress, we found more injured mitochondria with low membrane potential in DRP-1 silencing cells but relatively less in DRP-1 overexpressing cells. The tendency was the same in ATP content. We defined mitochondrial dysfunction as poor membrane potential and low ATP content. mtDNA, on the other hand, would represent the amount of mitochondria. A low amount of mitochondria, assessed by mtDNA, was found in H_2O_2 treatment and rapamycin treatment. In DRP-1 overexpressing cells, autophagy flux was initiated, while ATP content and membrane potential remained normal. However, mtDNA was mildly decreased in DRP-1 overexpressing cells, suggesting that fewer mitochondria would produce more ATP. In DRP-1 silencing cells treated with H_2O_2, the autophagy flux was disturbed, and mitochondrial dysfunction was displayed. We suggested that failure to eliminate dysfunctional mitochondria was the reason why mtDNA was at a normal level. Also, there were fewer merging fluorescent puncta of mitochondria (red) and lysosome (green). The expression of the autophagy-related proteins LC3 II and P62 decreased. The results may suggest that DRP-1 is the key to regulating mitophagy and mitochondrial function. As cellular senescence developed, the decline of DRP-1 expression resulted in decreased mitophagy levels and an accumulation of dysfunctional mitochondria. We further investigated DRP-1 expression and senescence

in cochlear explants. In senescent cochlear explants, DRP-1 expression and mitophagy levels decreased along with impaired mitochondrial function. These results are similar to those from the cellular experiment. Therefore, we hypothesized that age-related decline in DRP-1 repressed mitophagy and there was an accumulation of impaired mitochondria, which caused cochlear senescence.

In aged C57BL/6 mice as AHL models, we identified the downregulation of DRP-1 expression and mitophagy by evaluating the protein expressions of DRP-1, LC3 II, and P62. DRP-1 and mitophagy were downregulated in aged mice and senescent cochlea. Impaired mitochondria accumulated during aging due to a gradual decline in mitophagy and due to mitochondrial dysfunction. Our previous study revealed that initiating autophagy with rapamycin could notably improve hearing conditions in aged mice compared to AHL mice. In our current study, we applied Mdivi-1, an DRP-1 inhibitor, to C57BL/6 mice. These mice developed even more severe hearing loss than those who went without Mdivi-1. All of the above implied that the inhibition of DRP-1 expression and function resulted in decreased levels of mitophagy and further AHL development. It is possible that there are still unknown mechanisms that alter mitochondrial function (resulting in senescence) that need further study. We propose that DRP-1 could be the center of mitochondrial quality control and could be a key factor in AHL.

In this research, we discovered the relationship between DRP-1 and hair cell senescence (**Figure 7**). We revealed the role of DRP-1 in AHL and examined the effects of inhibition of DRP-1 in hearing. However, there are still questions that remain to be answered in our study. One question regards mitochondrial morphology and mitochondrial function. According to some research, a giant mitochondrial network created through mitochondrial fusion can preserve damaged mitochondria to maintain their function. The dynamic morphology of mitochondria mismatches with mitochondrial function, making this unconvincing. Second, the exact mechanism that controls how oxidative stress suppresses DRP-1 expression and phosphorylation remains unknown. Research has implied that the phosphorylation of DRP-1 may affect the ROS-AMPK pathway (Li et al., 2017), which is one of the critical pathways in oxidative regulation and may be an essential target in the study of senescence and AHL. Third, our study would be more convincing if it were to show whether DRP-1 delays the development of AHL in transgenic mice. Lastly, we revealed that DRP-1 participated in the development of AHL and considered it as a critical part of AHL. We hope to uncover the mechanisms of senescence and AHL by studying DRP-1 and related to DRP-1 that could prevent, delay, or even cure AHL in the future.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

HL and HX: contributed equally to this work. HL: conception and design, designed and performed the experiments, data analysis and interpretation, and manuscript writing. YZ and HX: conception and design, manuscript writing, final approval of manuscript. ZS, JP, LL, HZ, BJ, and WZ: assembly of data and data analysis.

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Senolytics: A Translational Bridge Between Cellular Senescence and Organismal Aging

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Aging is defined as a progressive decrease in physiological function accompanied by a steady increase in mortality. The antagonistic pleiotropy theory proposes that aging is largely due to the natural selection of genes and pathways that increase fitness and decrease mortality early in life but contribute to deleterious effects and pathologies later in life. Cellular senescence is one such mechanism, which results in a permanent cell cycle arrest that has been described as a mechanism to limit cancer cell growth. However, recent studies have also suggested a dark side of senescence in which a build-up of senescent cells with age leads to increased inflammation due to a senescence-associated secretory phenotype (SASP). This phenotype that includes many cytokines promotes tumorigenesis and can exhaust the pool of immune cells in the body. Studies clearing senescent cells from mice using the p16-based transgene INK-ATTAC have shown that senescent cells can impact both organismal aging and lifespan. Here we discuss these advances that have resulted in the development of a whole new class of compounds known as senolytics, some of which are currently undergoing clinical trials in humans for treating a variety of age-related pathologies such as osteoarthritis.

Keywords: aging, senescence, lifespan, senolytics, senomorphics

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AGING

Limited Replicative lifespan was first described by Hayflick and Moorhead (1961), who showed that human diploid fibroblasts have a finite capacity for replication after which they enter a metabolically active but irreversibly arrested proliferative state. Aging has been extensively studied in model organisms such as *Caenorhabditis elegans* and is affected by a variety of factors such as stress, nutrient intake, sex, and gene expression (Moskalev et al., 2015). Several genes have been discovered in these relatively simple model organisms such as DAF-2 (insulin/insulin-like growth factor-1 receptor homolog) which, when mutated results in almost doubling the lifespan of *C. elegans* (Kenyon, 2011). Success in manipulating lifespan in these model organisms ushered in a massive hunt to discover more aging-related genes, mechanisms and therapeutic compounds. At present, The DrugAge database of aging-related drugs lists around 567 distinct chemical perturbagens that can significantly increase lifespan in a subset of non-disease models spanning over 30 species (Barardo et al., 2017). Clinical trials such as The Metformin in Longevity Study (MILES) have been launched recently to assess the anti-aging potential of metformin in delaying age-related ailments

in humans (Crandall, 2015; Piskovatska et al., 2019). One of the essential components for studying aging and age-related diseases in humans are biomarkers that are indicative of chronological age (Calimport et al., 2019). Recently it has been shown that DNA methylation patterns show a strong correlation with chronological age and this "epigenetic clock" is effective in predicting all-cause mortality with age (Horvath, 2013). Analyzing cancer tissues with this epigenetic clock, composed of methylation levels from 353 CpGs, indicated that tissues from cancer patients treated with various therapies appeared to be an average of 36 years older compared to the actual chronological age of the patients, while induced pluripotent stem cells (iPSCs) from the same individuals showed resetting of the clock to an epigenetic age of zero. However, the biological mechanisms behind this epigenetic biomarker remain unknown, especially, due to a lack of correlation with gene expression data.

SENESCENCE

Senescence refers to a state of permanent proliferative arrest characterized by insensitivity to growth factors and mitogens (Kuilman et al., 2010). One of the mechanisms that regulate this insensitivity is dysregulation of normal endocytosis (Wheaton et al., 2001; Rajarajacholan et al., 2013). Senescent cells, were shown to overexpress caveolins, a major component of endocytosis apparatus which prevented their ability to phosphorylate Erk-1/2 phosphorylation post EGF stimulation which was recovered by downregulation via antisense-oligonucleotides. Similar suppression of Erk-1/2 activation was also observed in non-senescent cells post caveolin overexpression (Park, 2002; Park et al., 2002). In cell culture, as observed by Hayflick and Moorhead (1961), a senescence state is achieved upon repeated passaging, and as shown later, it is mainly due to shortening of telomeric DNA found at the end of chromosomes (Harley et al., 1990) that activates an ataxia-telangiectasia mutated (ATM) (Vaziri et al., 1997) and p53-mediated (Atadja et al., 1995) DNA damage response. This was hypothesized first as the end replication problem; as a consequence of semi-conservative DNA replication and later confirmed by Blackburn, Greider, and Szostak (Lundblad and Szostak, 1989; Blackburn, 1991). Senescent cells typically have an enlarged morphology and are most widely detected histochemically by an increased β-galactosidase activity known as senescence-associated β-galactosidase (SA-βGAL) (Itahana et al., 2007), which is correlated to increased autophagy (Young et al., 2009). Other biomarkers of senescence include increased expression of common senescence mediators such as p16, p21, p53, and p47ING1a (Kuilman et al., 2010; Rajarajacholan et al., 2013). However, not all types of senescence result from telomere depletion. For example, up to 50% of mouse embryonic fibroblasts (MEF's) exhibit a senescence phenotype after a mere five passages resulting from oxygen sensitivity due to ROS-induced DNA damage (Parrinello et al., 2003). These interdependent features of cell cycle withdrawal, macromolecular damage, dysregulated metabolism and an altered senescence-associated secretory phenotype (SASP) have been described as hallmarks of the senescence phenotype, although no markers appear to be universal for all types of senescent cells. Therefore, to ensure the accurate identification of senescent cells it has been recommended by the International Cell Senescence Association that multiple markers be used in a three-step senescence identification protocol (Gorgoulis et al., 2019).

MECHANISMS AND STIMULI OF SENESCENCE

A wide variety of stimuli affecting multiple molecular pathways are involved in the induction of a senescence based irreversible arrest in a state resembling G_0 of the cell cycle in mammalian cells (Kuilman et al., 2010; Muñoz-Espín and Serrano, 2014). These pathways are broadly classified into activating either p16 (Alcorta et al., 1996) or p21 (Brown et al., 1997) via the p53 tumor suppresser (Atadja et al., 1995; Vaziri et al., 1997), and these signals converge to produce high levels of active hypophosphorylated Rb (Retinoblastoma) Tumor Suppressor (Chicas et al., 2010; Rajarajacholan et al., 2013). The tumor suppressive nature of cellular senescence is quite evident from the fact that tampering with levels of several of these oncogenes/tumor suppressors can lead to an escape from senescence (Campisi, 1997). Broadly speaking, senescence can be triggered by numerous stresses including DNA-damage, oxidative stress, and oncogene induced stress. DNA-damage induced senescent cells are primarily observed in cancer patients post administration of radiotherapy and chemotherapy (Robles et al., 1999; Gewirtz et al., 2008) and treatment results in accelerated telomere loss (Unryn et al., 2006). Radiotherapy and Chemotherapy not only affect cancer tissue but also induce senescence in surrounding healthy tissue. Several observations have confirmed that exposure to topoisomerase inhibitors such as doxorubicin and etoposide induces senescence in primary diploid human fibroblasts *in vitro* (Robles et al., 1999). Studies have also shown that proteins of the SASP that are released from these surrounding senescent cells can induce an epithelial to mesenchymal transition (EMT) and hence promote malignancy in normally non-aggressive human breast cancer cell lines (Coppé et al., 2008). Another important player in inducing senescence is ING1a from the *IN*hibitor of *G*rowth family of epigenetic regulators. Originally identified in 1996 by subtractive hybridization between cDNAs from normal mammary epithelial cells and transformed breast cancer cells, the *ING* family of genes are evolutionary conserved and ING proteins primarily localize to the nucleus (Garkavtsev et al., 1996). Our lab has shown that the ING1a isoform that is expressed in aging, but not low passage fibroblasts induces senescence when ectopically expressed several-fold faster (24–36 h) compared to other senescence inducing agents such as t-BHP (t-butyl hydroperoxide) and doxorubicin (Rajarajacholan and Riabowol, 2015). We have shown that inhibition of endocytosis is one of the major mechanisms by which ING1a induces senescence (Rajarajacholan et al., 2013; Rajarajacholan and Riabowol, 2015). This occurs via affecting at least three independent members of the Rb pathway including Rb, $p16^{INK4a}$, and

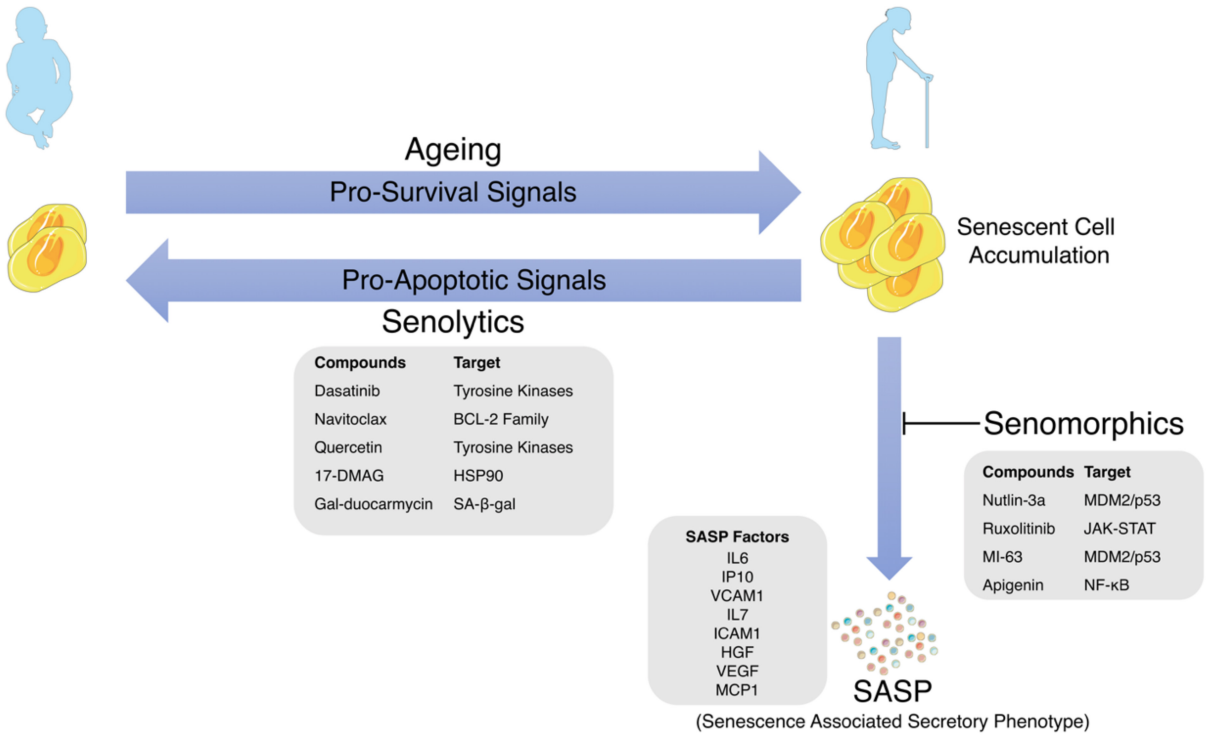

FIGURE 1 | Schematic representation of the link between cellular senescence and aging with examples of SASP Factors, Senolytics and Senomorphics and their targets of action.

$p57^{Kip2}$. ING1a induces expression of Intersectin 2, subsequently altering the stoichiometry of the endocytosis apparatus and blocking signaling by mitogens (Rajarajacholan et al., 2013). This represents a novel mechanism of senescence induction that is independent of telomere attrition and DNA damage signaling.

CELLULAR SENESCENCE AND ORGANISMAL AGING

One of the hallmarks of mammalian aging is the accumulation of irreversibly arrested senescent cells in various tissues (**Figure 1**). In a 2006 primate study, it was observed that senescent cells, as estimated by ATM activation do accumulate and can reach over 15% of the total cell population in aged individuals (Herbig et al., 2006). In contrast to the vast majority of *in vitro* studies, this was one of the first studies showing a clear association between aging and the accumulation of senescent cells *in vivo*. Although this established a strong correlation, efforts were underway to establish causation between the accumulation of senescent cells and aging *in vivo*. In 2011, the Kirkland lab showed that removing $p16^{Ink4a}$ positive senescent cells delayed age-related disorders and increased healthspan in a BubR1 progeroid accelerated model of aging mice (Baker et al., 2011). This was accomplished using a novel transgene composed of a $p16^{Ink4a}$ promoter driving a FKBP–Caspase-8 fusion protein, which is selectively expressed in senescent cells and can be activated by the synthetic drug, AP20187. These transgenic mice showed delayed onset of sarcopenia, cataracts, significantly better exercise test scores and reduction in other age-related pathologies. Later studies confirmed the beneficial effects of senescent cell removal in wild type mice that showed increased median lifespan, delayed tumorigenesis and attenuated age-related multi-organ deterioration (Baker et al., 2016). Removal of senescent cells in mice has also been shown to attenuate markers of age-associated neurodegenerative diseases such as tau hyperphosphorylation and neurofibrillary tangle deposition (Bussian et al., 2018). Since it is likely that even a small percentage of senescent cells can promote substantial deleterious effects, there may be natural mechanisms that clear these senescent cells *in vivo* to maintain overall fitness. One primary response to p53 activation in murine liver carcinoma was found to be the accumulation of senescent cells, which were subsequently shown to be cleared by the innate immune response (Xue et al., 2011). NK cells were also found to eliminate senescent cells *in vivo* by perforin (Prf) mediated granule exocytosis (Sagiv et al., 2013) and $Prf1^{-/-}$ mice showed a 4X accumulation of senescent cells and a 21% decrease in median lifespan, which was alleviated by senescent cell clearance (Ovadya et al., 2018).

Further evidence regarding the connection between cellular senescence and organismal aging comes from accelerated aging syndromes such as HGPS (Hutchinson-Gilford progeria syndrome). A higher proportion of HGPS fibroblasts are SA-β-Gal positive compared to fibroblasts from normal, age-matched controls, and they can be returned to a normal replicative state by exogenous expression of the TERT subunit of telomerase

(Benson et al., 2010). Similarly, expressing progerin, the mutant lamin-A protein known to cause HGPS, in primary cells obtained from healthy individuals result in expression of senescent markers (Cao et al., 2011). These results suggest that progerin-induced telomere dysfunction may result in premature cellular senescence leading to the accelerated aging symptoms observed in HGPS patients.

SENOLYTICS AND SENOMORPHICS

Substantial evidence in the last decade connecting senescent cell accumulation, age-related ailments, and roles in lifespan and healthspan fueled the search for therapeutic compounds that could selectively target senescent cells. A transcriptomic analysis between senescent cells and proliferating cells showed increased expression of pro-survival/anti-apoptotic genes such as Bcl-xL (B-cell lymphoma-extra-large) a member of the Bcl-2 family of proteins that regulates programmed cell death by blocking caspase activation (Zhu et al., 2016). This provided evidence to support the observation that senescent cells accumulate with age by being resistant to a variety of stresses that would normally induce apoptosis (Wang, 1995). Consistent with this idea, siRNAs to reduce Bcl-xL expression selectively reduced survival and viability in senescent cells while not affecting proliferating cells (Zhu et al., 2015). Quercetin and Dasatinib were obtained as hits from a drug screen based on these observations (Hickson et al., 2019). Quercetin: a flavonoid and Dasatinib: an anticancer agent, are known inhibitors of a variety of tyrosine kinases (Huang et al., 1999; Montero et al., 2011). These compounds form one of the first discovered members of the senolytic class of drugs that selectively induce apoptosis in senescent cells. Four years after their initial identification as candidate senolytics, a Dasatinib + Quercetin combination was reported to decrease the senescent cell burden in humans as part of a Phase-1 clinical trial in diabetic kidney disease patients (Hickson et al., 2019). This 2019 study was the first peer-reviewed study to demonstrate the efficacy of senolytics to decrease senescent cell burden in humans. This was carried out after an initial pilot study in early 2019 in 14 idiopathic pulmonary fibrosis (IPF) patients was completed to evaluate the feasibility of implementing a senolytic treatment (Justice et al., 2019). What now remains to be determined is whether future clinical trials will demonstrate any positive medical outcomes resulting from decreased senescent cell burden in diabetes and other age-associated ailments.

Another senolytic strategy is the inhibition of the interaction between Mouse Double Minute 2 (MDM2) and p53 that usually results in the ubiquitination and proteasomal degradation of p53 (Nag et al., 2013; Wade et al., 2013). The MDM2 protein acts as an E3 ubiquitin ligase and facilitates p53 degradation. *MDM2* contains two promoters: P1 and P2. P1 is constitutively active at low levels while the P2 promoter has p53 binding sites and acts as a negative regulator of p53 (Hollerer et al., 2019). The MDM2 antagonists Nutlin-3a and MI-63 have been shown to increase p53 levels and attenuate the secretory phenotype of senescent cells (SASP) (Wiley et al., 2018). These represent a new class of compounds known as "Senomorphics," which attenuate the expression of cytokines such as IL-6 (Interleukin-6) that make up the SASP. UBX0101, a compound from the Unity Biotechnology pipeline is another promising senolytic that inhibits the MDM2/p53 interaction and is currently undergoing Phase 1 clinical trial against osteoarthritis. UBX0101 administration was reported to decrease classical osteoarthritis-related phenotypes such as cartilage erosion and joint pain in mice by p53-mediated clearing of senescent cells. This study suggested that senescent cells might promote osteoarthritis (Jeon et al., 2017), another medically relevant age-related pathology.

Heat Shock Protein 90 (HSP90) might represent another target for senolysis. This protein is one member of a large family of chaperones that help other proteins to fold and refold after cell stress and is indirectly involved in a wide variety of cellular processes such as DNA repair, heat stress response and neurodegenerative pathologies (Schopf et al., 2017). While HSP90 levels remain relatively similar in senescent and non-senescent cells, AKT (Protein Kinase B), which is a downstream effector of HSP90 is highly expressed in senescent cells. Inhibiting HSP90 destabilizes AKT and results in increased apoptosis (Karkoulis et al., 2013). HSP90 inhibitors such as 17-DMAG were discovered through a screen for senolytic compounds based on SA-βGAL positive cell count as an endpoint. 17-DMAG was further shown to be senolytic *in vivo* by decreasing p16 positive cells and extending healthspan in Ercc1$^{-/\Delta}$ mice (Fuhrmann-Stroissnigg et al., 2017).

THE NEXT GENERATION OF SENOLYTICS

Since senescence is a complex phenotype involving multiple pathways and proteins, it is unlikely that a single senolytic compound targeting a single protein will be able to eliminate all types of senescent cells. For example, Quercetin is more selective against senescent human endothelial cells, while Dasatinib is far more effective against senescent human primary preadipocytes (Hickson et al., 2019). Similarly, while Navitoclax, a senolytic agent targeting the Bcl-2 family of proteins is selective against senescent human umbilical vein epithelial cells (HUVECs) and IMR90 human lung fibroblasts, it is not particularly effective against primary human preadipocytes (Zhu et al., 2016). Senolytic compounds that target senescent cells more broadly while maintaining low cytotoxicity by increased selectivity solely for cells that are truly senescent are therefore of great interest! Elevated activity of the lysosomal β-galactosidase, which is a nearly universal characteristic of senescent cells (Lee et al., 2006) and has been exploited as a senescence biomarker can also be utilized for drug selectivity against a wide range of senescent cells. One example is a drug delivery system utilizing encapsulation of cytotoxic drugs with galacto-oligosaccharides (Muñoz-Espín et al., 2018). These capsules that can be loaded with a variety of cytotoxic compounds, take advantage of the high senescent cell lysosomal β-galactosidase activity to preferentially release their cargo within senescent cells. A very recent study also used a similar strategy involving galactose-modified prodrug. Here, preferential processing of galactose-modified duocarmycin

(GMD), a cytotoxic conjugate, was used to eliminate a wide variety of senescent cell types (Guerrero et al., 2019). Targeting cell surface proteins enriched in senescent cells such as DPP4 (dipeptidyl peptidase 4) can also be used to enhance selectivity (Kim et al., 2017). Another cell surface protein, the CD9 receptor has also been used to target senescent cells and their secretory phenotype with anti-CD9 monoclonal antibody encapsulated, lactose-wrapped nanoparticles, loaded with rapamycin as the payload (Thapa et al., 2017). These nanoparticles have dual selectivity toward senescent cells based on both increased CD9 receptors on the cell surface and increased lysosomal β-galactosidase expression.

CONCLUSION

The role of cellular senescence in organismal aging has been well established in the past two decades in a variety of organisms (Fick et al., 2012; Heidinger et al., 2012; Muñoz-Lorente et al., 2019). From initial thoughts indicating that senescence served primarily as an anti-cancer mechanism, we note the emerging view of senescence also acting as a driving force behind a wide variety of age-related pathologies (Fernandes et al., 2016). More recent studies in the last decade, which resulted in the development of transgenes and chemical perturbagens, have finally given us the tools to selectively manipulate the quantity of senescent cells *in vivo*. Thorough characterization of their deleterious secretory phenotype, that varies in different cell types, continues to improve our understanding of how they contribute to the wide variety of age-related diseases observed in our populations. Finally, as the first Senolytics enter clinical trials, we are on the cusp of establishing a translational bridge between cellular senescence and organismal aging (Muñoz-Espín and Serrano, 2014; Calimport et al., 2019; Gorgoulis et al., 2019) that may help ameliorate the burden of many age-related pathologies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Cellular Senescence in Neurodegenerative Diseases

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Cellular senescence is a homeostatic biological process characterized by a permanent state of cell cycle arrest that can contribute to the decline of the regenerative potential and function of tissues. The increased presence of senescent cells in different neurodegenerative diseases suggests the contribution of senescence in the pathophysiology of these disorders. Although several factors can induce senescence, DNA damage, oxidative stress, neuroinflammation, and altered proteostasis have been shown to play a role in its onset. Oxidative stress contributes to accelerated aging and cognitive dysfunction stages affecting neurogenesis, neuronal differentiation, connectivity, and survival. During later life stages, it is implicated in the progression of cognitive decline, synapse loss, and neuronal degeneration. Also, neuroinflammation exacerbates oxidative stress, synaptic dysfunction, and neuronal death through the harmful effects of pro-inflammatory cytokines on cell proliferation and maturation. Both oxidative stress and neuroinflammation can induce DNA damage and alterations in DNA repair that, in turn, can exacerbate them. Another important feature associated with senescence is altered proteostasis. Because of the disruption in the function and balance of the proteome, senescence can modify the proper synthesis, folding, quality control, and degradation rate of proteins producing, in some diseases, misfolded proteins or aggregation of abnormal proteins. There is an extensive body of literature that associates cellular senescence with several neurodegenerative disorders including Alzheimer's disease (AD), Down syndrome (DS), and Parkinson's disease (PD). This review summarizes the evidence of the shared neuropathological events in these neurodegenerative diseases and the implication of cellular senescence in their onset or aggravation. Understanding the role that cellular senescence plays in them could help to develop new therapeutic strategies.

Keywords: senescence, Alzheimer's disease, Down syndrome, Parkinsion's disease, neurodegenaration

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INTRODUCTION

Cellular senescence, a homeostatic process that reduces proliferation and helps to prevent the propagation of damaged cells (Vicencio et al., 2008; Coppé et al., 2010; Faragher et al., 2017; Yanagi et al., 2017), has been proposed to be a type of cell differentiation (Stein et al., 1991). Among its most relevant biological functions are the counteraction of uncontrolled cell proliferation, which avoids the formation of tumors, and the facilitation of the elimination of cells that are damaged or that are no longer necessary (Kültz, 2005). Cellular senescence has an essential physiological role during development (Muñoz-Espín and Serrano, 2014; Barbouti et al., 2019).

According to its duration and characteristics, senescence has been categorized into acute or chronic senescence (van Deursen, 2014). Acute senescence is implicated in normal biological processes during embryonic development or tissue repair. While, chronic senescence, induced by prolonged exposure to stress (Salama et al., 2014; van Deursen, 2014; Childs et al., 2015; Velarde and Menon, 2016; Watanabe et al., 2017; Ogrodnik et al., 2019), produces cellular and tissue alterations. Although both types of senescent cells display similar characteristics *in vitro* and *in vivo*, they are triggered by different stimuli and have differential consequences on the tissue.

Chronic cellular senescence contributes to functional alterations associated with aging and neurodegenerative diseases (Howcroft et al., 2013); the number of cells with this phenotype increases with healthy aging (Herbig et al., 2004; Lawless et al., 2010).

Different categories of chronic senescence have been identified, including replicative senescence, stress-induced premature senescence (SIPS), and mitochondrial dysfunction-associated senescence, among others (Kuilman et al., 2010).

Replicative senescence is the process in which human fibroblasts cultured *in vitro* end up displaying the hallmarks of senescence (a rise in protein p21 levels cell cycle arrest, SA-β-Gal activation, and morphological alterations; Romanov et al., 2012; Sanders et al., 2013). Replicative senescence is currently considered a model of aging (Chen et al., 2007).

Different types of cells of the central nervous system (CNS) can become senescent, including astrocytes (Pertusa et al., 2007; Mansour et al., 2008; Salminen et al., 2011), microglia (Evans et al., 2003; Flanary and Streit, 2004; Flanary et al., 2007; Bitto et al., 2010), oligodendrocytes (Al-Mashhadi et al., 2015), neurons (Sedelnikova et al., 2004; Jurk et al., 2012), and Neural Stem Cells (NSCs; Ferrón et al., 2004; He et al., 2013; Li et al., 2016). Senescence of these cell types have been implicated in the etiopathology of several neurodegenerative diseases (Streit et al., 2004, 2009; Conde and Streit, 2006; Baker et al., 2011; Bhat et al., 2012; He et al., 2013; Nasrabady et al., 2018; Ohashi et al., 2018), including Alzheimer's disease (AD), Parkinson Disease (PD), frontotemporal dementia, amyotrophic lateral sclerosis, and multiple sclerosis (Yurov et al., 2014; Biron-Shental et al., 2015). This review summarizes the main findings on the role of cellular senescence in the neurodegenerative diseases AD, Down syndrome (DS), and PD.

Cellular Senescence: Phenotype, Triggering Mechanisms and Implication in Neurodegenerative Diseases

Senescent cells are characterized by:

(i) Permanent cell cycle arrest, due to the blockade to the entrance to the S phase of the cycle (Stein et al., 1999; Krenning et al., 2014).

(ii) Senescence-associated secretory phenotype (SASP), which consists of the synthesis and release of proinflammatory chemokines, cytokines, growth factors and metalloproteinases (Coppé et al., 2010; Acosta et al., 2013; Chen et al., 2015) responsible of neuroinflammatory processes (Coppé et al., 2010; Freund et al., 2010; Özcan et al., 2016).

During aging and in neurodegenerative diseases, microglial cells display altered morphology characteristic of senescence (Flanary and Streit, 2004; Streit et al., 2004; Flanary et al., 2007). However, it has to be taken into account that increased release of pro-inflammatory cytokines due to microglia or astroglia activation is not necessarily related to cellular senescence. During healthy aging, the brain suffers mild chronic inflammation (Yankner et al., 2008). There is evidence that this state is caused by the dysregulation of microglial activation (Sheng et al., 1998; Frank et al., 2006; von Bernhardi, 2007; Mosher and Wyss-Coray, 2014; von Bernhardi et al., 2015a), which enhances the release of numerous proinflammatory cytokines, including IL1α, IL-2, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-22, IL-23, IL6, IFN-γ and TNFα (Sheng et al., 1998; Njie et al., 2012; von Bernhardi et al., 2015b; Minciullo et al., 2016; Ventura et al., 2017; Rea et al., 2018). Inflammation induces a higher release of proinflammatory cytokines in old than in young cells (Combrinck et al., 2002; Cunningham et al., 2005; Sierra et al., 2007; Henry et al., 2009).

In neurodegenerative diseases, neuronal damage also dysregulates microglia activation and induces an increase in the release of pro-inflammatory mediators (von Bernhardi, 2007; López-Otín et al., 2013).

(iii) Altered mitochondrial function and morphology, which is one of the most important triggers of senescence, mainly through the induction of oxidative stress, which will alter cellular signaling and SASP (Takahashi et al., 2005; Passos et al., 2007, 2010; Correia-Melo and Passos, 2015).

High levels of oxidative stress have been demonstrated to induce cellular senescence during aging (De Haan et al., 1996), promoting neuronal DNA damage (Chow and Herrup, 2015), a deregulated DDR (Sedelnikova et al., 2004), an alteration in the entry and progression of the cell cycle and changes in cell morphology (Monti et al., 1992), premature replicative senescence and an accelerated rate of telomere attrition per cellular replication (von Zglinicki et al., 2000; Serra et al., 2003).

Mitochondria is a major site of production as well as a target of reactive oxygen species (ROS), and its endogenous antioxidant pathways are essential to maintain physiological redox signaling (Brand, 2014). Furthermore, mitochondrial ATP production is necessary for neural activity. But aging deteriorates mitochondrial integrity and function leading to reduced ATP production, to enhanced ROS formation, mutations in mitochondrial DNA (Richter et al., 1988; Shigenaga et al., 1994), facilitating neurodegeneration (Dröge and Schipper, 2007).

Finally, mitochondrial ROS can also induce telomere attrition, and dysfunction (Passos et al., 2007) and mitochondrial dysfunction can also facilitate senescence by inducing alterations in the cell metabolism (Ziegler et al., 2015; Liguori et al., 2018).

(iv) Changes in cellular metabolism, partially due to the altered mitochondrial function, producing an increase in lysosomal senescence-associated-β-galactosidase (Lee et al., 2006; Weichhart, 2018), accumulation of lipofuscin in the cytoplasm (Georgakopoulou et al., 2013; Höhn and Grune, 2013), and a reduction in fatty acid synthesis (Wu et al., 2017). Due to the high energy demand, and increased oxidative stress the cells can

accumulate high levels of DNA damage. This damage increases during normal aging, when the DNA repair capacity is reduced (Maynard et al., 2015).

(v) DNA damage that alters chromatin structure and activates the DNA damage response (DDR) (Nakamura et al., 2008; Rodier et al., 2011).

Telomere Attrition and Telomere Dysfunction

During replicative senescence, telomeres, DNA–proteins that protect chromosome ends from degradation, and inappropriate recombinations or fusions shorten with every cell division (Blackburn, 1991; Zakian, 1995; Chan and Blackburn, 2004; Hockemeyer et al., 2005). Telomere shortening reduces the protection of the ends of the chromosomes and leaves them exposed to DNA damage that can be similar to DNA double-strand break (d'Adda di Fagagna et al., 2003; Takai et al., 2003; Herbig et al., 2004; Rodier et al., 2009, 2011) that triggers the DDR. During senescence, the increase in DDR leaves these cells unable to perform DNA repair (Galbiati et al., 2017). Under these circumstances, the cell stops dividing (Harley et al., 1990; Harley, 1991; de Lange, 2002; d'Adda di Fagagna et al., 2003) leading to the cell cycle arrest characteristic of senescence.

Telomere shortening has been proposed to be a major mechanism in aging, and age-related pathology and a marker of cellular senescence (Greider and Blackburn, 1987; Olovnikov, 1996; Bernatdotte et al., 2016). The role of telomere attrition in aging and its correlation with senescence has been demonstrated in studies in primates, mice and humans (Blasco et al., 1997; Lee et al., 1998; Herbig et al., 2006; Hewitt et al., 2012; Kong et al., 2013; López-Otín et al., 2013; Birch et al., 2015).

Besides cellular replication, several stimuli can affect telomeres. There is evidence that mitochondrial dysfunction induces telomere damage, and that telomere damage can produce mitochondrial alterations (Zheng et al., 2019). Besides, oxidative stress accelerates telomere damage producing senescence (von Zglinicki et al., 2000; von Zglinicki, 2002; Saretzki et al., 2003; Serra et al., 2003), and the intracellular oxidative stress of a cell correlates with telomere attrition and with its replicative potential (Richter and von Zglinicki, 2007).

Although, as mentioned above, many studies have shown a relation between telomere attrition, senescence and aging (Harley et al., 1990; Allsopp et al., 1992; Hao et al., 2005; Heidinger et al., 2012; Kaul et al., 2012; Reichert et al., 2013), telomere length does not always correlate with senescence (Karlseder et al., 2002; Stewart et al., 2003).

Several reports failed to associate telomere length and mortality risk in elder humans (Bischoff et al., 2006; Li et al., 2015). Also, some studies have demonstrated that telomere dysfunction is not dependent on its length. In mice, and baboons longer telomeres with DNA-damage have been associated with aging (Fumagalli et al., 2012; Hewitt et al., 2012; Jurk et al., 2014).

Finally, as previously mentioned, telomere dysfunction is not only related to senescence in healthy aging but it is also found in patients with dementia (Kota et al., 2015), AD (Cai et al., 2013), and PD (Maeda et al., 2012).

Non-telomeric DNA Damage

Genomic Instability and DNA Damage and Repair

The continuous activation of DDR produces senescence in neurons (Fielder et al., 2017). DNA damage induces senescence, enhanced oxidative stress and an associated increase in β-galactosidase activity in neurons of old mice (Jurk et al., 2012). Because the efficiency of DNA repair decreases with age, and more complex, and less efficient DNA repair mechanisms are used, the increased DNA damage can lead to neurodegeneration (Vaidya et al., 2014).

(vi) Epigenetic modifications. Epigenomic changes in senescent cells include an imbalance in repressive and active histone marks, heterochromatic alterations including formation of senescence-associated heterochromatic foci (SAHF), histone variants, altered nucleosomal composition, alterations in DNA methylation pattern, alterations in nuclear lamina-chromatin interactions and on 3D genome organization that contributes to cellular dysfunction (Yang and Sen, 2018; Wagner, 2019). Micro-RNAs, also participate in the regulation of cellular senescence (Komseli et al., 2018); resistance to apoptotic death, controlled by the p53 and p21 stress response pathway (Tang et al., 2006).

Epigenetic modification such as histone modifications and specific DNA methylation changes, including alterations in the activity of methylation enzymes (Vanyushin et al., 1973), occur during aging and are associated with the neuropathology and the progression of various neurodegenerative diseases (Berson et al., 2018; Prasad and Jho, 2019; Wagner, 2019). During aging, these changes in DNA methylation and histone modifications alter chromatin architecture (Tsurumi and Li, 2012). Aged tissues also display alterations in the expression of some micro RNAs (Cencioni et al., 2013).

(vii) Morphological changes. Senescent cells display changes in the organization of nuclear lamina that modify nuclear morphology and gene expression (Freund et al., 2012). Due to cytoskeletal rearrangements, senescent cells display an increased size, a flat and irregular shape and changes in cell membrane composition (Ohno-Iwashita et al., 2010; Druelle et al., 2016; Cormenier et al., 2018).

(viii) Altered proteostasis. Senescence cells display increased unfolded protein response (UPR) associated with endoplasmic reticulum (ER) stress, which participates in the increase in ER size and the changes in the shape and size of these cells (Ohno-Iwashita et al., 2010; Cormenier et al., 2018).

Proteostasis refers to the balance and correct function of the proteome and requires proper synthesis, folding, quality control, and degradation rate of proteins. In eukaryotic cells, it depends on the accurate regulation of the proteasome, on the lysosomal system and autophagy, an intracellular degradation system of damaged proteins (López-Otín et al., 2013). Senescent cells lose protein homeostasis due to nucleolar dysfunction, autophagy and lysosomal anomalies and alterations in UPR indicative of ER stress. Correct proteostasis reduces the secretion of inflammatory cytokines (Tanaka and Matsuda, 2014), while its alteration (including protein misfolding aggregation and deposition) are a hallmark of many neurodegenerative diseases (see below for PD and AD).

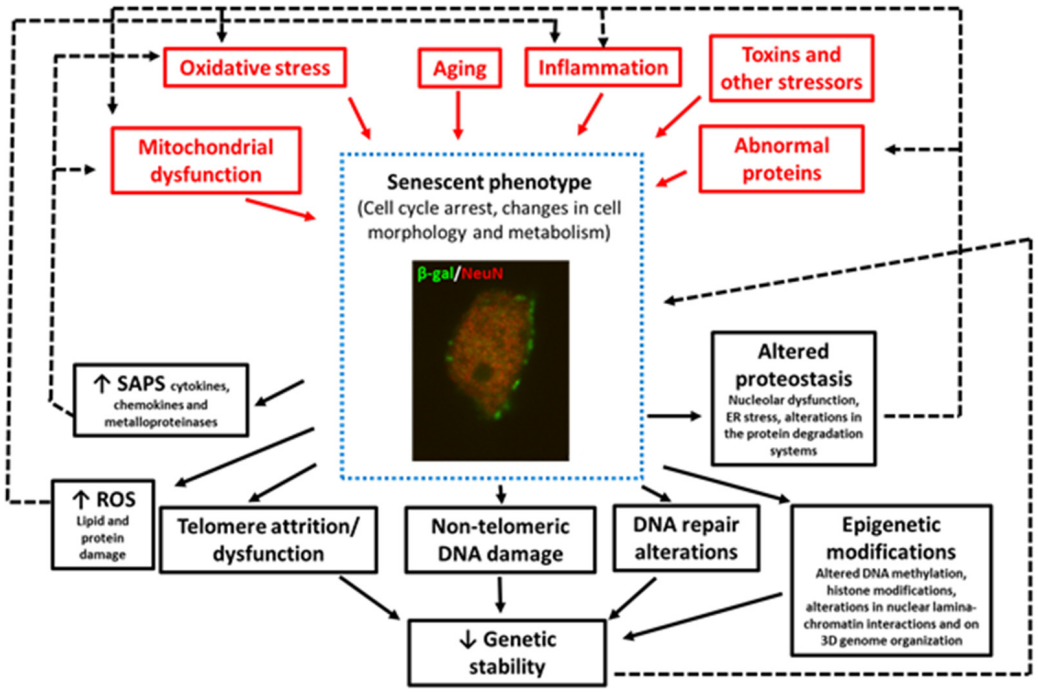

FIGURE 1 | Image of a granular neuron (NeuN+) of the hippocampus of an aged wildtype mouse showing senescent phenotypes SA-β-Gal expression, and changes in cell morphology. This figure summarizes the triggering stimuli (in red) and the consequences (in black) of the increase in the number of cells with senescent phenotypes in neurodegenerative diseases. The black dotted lines represent the positive feedback mechanism that aggravates aging and neurodegeneration.

In summary, some of the factors that trigger senescence in pathological conditions are oxidative SIPS (Hernandez-Segura et al., 2018), mitochondrial dysfunction (Wiley et al., 2017), DNA-damage (Dörr et al., 2013), telomere attrition or dysfunction (Hayflick and Moorhead, 1961), aberrant gene activation (Gorgoulis and Halazonetis, 2010), epigenetic modifications (Yang and Sen, 2018; Wagner, 2019), and impaired autophagy (Kang et al., 2011), although other stressors can also induce senescence (**Figure 1**).

Finally, as previously mentioned, there is evidence that cellular senescence contributes to the pathogenesis of different neurodegenerative diseases through various mechanisms (**Figure 1**) including:

(i) The promotion of chronic inflammation: (Coppé et al., 2010). Thus, neuroinflammation is not only a trigger but also a result of senescence that can perpetuate the damage to the cell or neighboring cells (Nelson et al., 2012; Acosta et al., 2013; Ribezzo et al., 2016).

(ii) The promotion of oxidative stress and mitochondrial dysfunction. There is a positive feedback mechanism between senescence, oxidative stress, and mitochondrial dysfunction (Pole et al., 2016; **Figure 1**).

(iii) The reduction of the regenerative capacities of the nervous system: adult neurogenesis has been demonstrated in rodents (Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011) and humans (Cipriani et al., 2018). Importantly, it has been recently demonstrated that neurogenesis is reduced in aged individuals and AD patients (Moreno-Jiménez et al., 2019). Due to the cell cycle arrest associated with cellular senescence, the reduced regenerative capacities of the brain (i.e., neurogenesis), would facilitate neurodegeneration.

(iv) Loss of function: finally, cell-cycle arrest and other alterations associated with cellular senescence alter the function of neurons and different CNS cell types (Purcell et al., 2014).

Aging increases the number of senescent cells (Rodier and Campisi, 2011) and the loss of neurons (De Stefano et al., 2016), compromising brain function and triggering or aggravating a neurodegenerative disease. Because senescent cells cannot maintain tissue function or repair its damage, if this state is maintained chronically, cellular aging and degenerative diseases would be aggravated.

In this review, we summarize the data that demonstrate the role of cellular senescence in the neurodegenerative diseases AD, DS, and PD (**Table 1**). Despite the differences in pathology among these three diseases, they are characterized by senescence and progressive loss of neurons leading to functional alterations (Nussbaum and Ellis, 2003).

CELLULAR SENESCENCE IN ALZHEIMER'S DISEASE

AD is a chronic neurodegenerative disease that accounts for 60–70% of cases of dementia. The main pathological hallmarks of AD are dementia and cognitive impairment, amyloid plaques, neurofibrillary tangles (NFTs) of hyperphosphorylated tau proteins, and loss of neurons and

TABLE 1 | Summary of senescent phenotypes in normal aging, Alzheimer's disease (AD), Down syndrome (DS), Parkinson Disease (PD).

Senescence phenotypes	Normal aging	AD	DS	PD
Cell cycle arrest	Cell cycle arrest (alterations in cell and tissue functions).	Cell cycle prolongation and re-entry.	Cell cycle arrest and elongation.	Cell cycle arrest (alterations in the expression of several genes implicated in the cell cycle).
SASP	Brain mild chronic inflammation, changes in microglia morphology, altered microglia activation, enhanced release of proinflammatory mediators.	Microglia overactivation, enhanced release of proinflammatory cytokines and other SASP that aggravate amyloid and tau pathology.	Microglia overactivation, proinflammatory mediators' over-expression, increased release of proinflammatory cytokines from early late stages, as the person ages this is enhanced and aggravates AD neuropathology.	Activation of microglia and pro-inflammatory mediators release that has a role in the dopaminergic loss.
Oxidative stress and mitochondrial dysfunction	Increased ROS and mtROS. Altered mitochondrial integrity and function that compromises cell metabolism (reduces ATP) and induces damage DNA.	Increased ROS and altered mitochondrial structure and function that produces cellular changes associated with senescence.	Increased ROS and mtROS from embryonic stages. Altered mitochondrial structure and function that compromises cell metabolism (reduces ATP production). As the person ages, oxidative stress is enhanced and aggravates AD neuropathology. Oxidative stress-induced damage of DNA, lipids, and proteins.	Increased ROS and mtROS. Altered mitochondrial structure and function. Mutation of genes associated with pathways of mitochondrial dysfunction.
Telomeric DNA damage	Telomere attrition (replicative senescence) and damage.	Telomeric DNA damage. Regarding telomere shortening: controversial results.	Shorter telomeres correlate with the degree of dementia in DS individuals with AD.	Contradictory results regarding telomere length.
Non-telomeric DNA damage and DNA repair mechanism	Increased DNA damage accumulation and alteration in DDR.	Increased DNA damage and alterations in DDR.	Oxidative stress and other stressors enhance DNA damage accumulation. Overexpression of the USP16 gene and the reduced DNA POLb alter DNA repair mechanisms and chromatin remodeling. Genomic instability.	Genetic instability due to gene mutations, altered gene expression or regulation leading to cell cycle alterations. Impaired DNA repair mechanisms that increase the duration of cell cycle.
Epigenetic modifications	Alterations in histones, DNA methylation pattern, chromatin architecture, and micro RNAs expression.	Aberrant phosphorylation of histones, changes in DNA methylation of AD critical genes. Mislocated chromatin organizing proteins and epigenetic regulators.	Histone modifications, DNA hypermethylation, alteration in small non-coding RNAs implicated in premature aging and cognitive defects.	Changes in DNA methylation, posttranscriptional modification of histones.
Morphological changes	Increased size, flat and irregular shape, changes in membrane composition.	Increased size, flat and irregular shape, changes in membrane composition.	Increased size, flat and irregular shape, changes in membrane composition.	Increased size, flat and irregular shape, changes in membrane composition.

(Continued)

TABLE 1 | (Continued)

Senescence phenotypes	Normal aging	AD	DS	PD
Altered proteostasis	Nucleolar dysfunction, altered transcription, endoplasmic reticulum stress, proteasome and autophagy anomalies.	Loss of protein homeostasis. Nucleolar dysfunction. Accumulation of abnormal proteins (amyloid peptides and hyperphosphorylated tau).	Increased gene copy number alters proteostasis. Alterations in the ribosome biogenesis machinery (increases number of nuclear organizer regions resulting in excessive rRNA and ribosomal protein synthesis leading to waste energy. Alterations in the protein degradative systems (autophagy and proteasome). Altered proteostasis leads to increases in amyloid aggregates and tau hyperphosphorylation.	Accumulation of misfolded proteins. Accumulation of neurotoxic α-synuclein associated with Lewy bodies. Autophagic/lysosomal dysfunction.
Others	Accumulation of lipofuscin		Early aging, and development of senescence phenotype in the brain and other tissues.	

synapses (Selkoe and Hardy, 2016). However, there is increasing evidence that some pathological events that appear years earlier than the former have a prominent role in the development of amyloid plaques and NFTs. These events include increased oxidative stress, neuroinflammation, and cellular senescence due to DNA damage and altered proteostasis. Numerous reports have demonstrated their role in the increase in Aβ burden, tau hyperphosphorylation, neuronal death and an accelerated cognitive decline (Hardy and Higgins, 1992; Gitter et al., 1995; Chong, 1997; Weldon et al., 1998; Eikelenboom et al., 2006; Hardy, 2006; Sipos et al., 2007; Wilcock, 2012).

Cellular senescence has been demonstrated to play an important role in the onset and aggravation of AD (Bhat et al., 2012; Boccardi et al., 2015). Increased senescence is found in different cell types of AD brains, including astrocytes, microglia, and neurons as demonstrated by their enhanced SA-β-gal expression (He et al., 2013), p53 expression, a mediator of cellular senescence (Arendt et al., 1996; McShea et al., 1997; Luth et al., 2000; Yates et al., 2015), an increase in the release of SASP components (Erusalimsky, 2009), DNA damage (Myung et al., 2008), telomere attrition or damage (Flanary and Streit, 2004), and senescence-like morphological changes (Streit et al., 2004).

Increased levels of senescent cells with higher SA-β-gal and p53 levels are also found and in plasma samples from AD patients and mouse models of AD (de la Monte et al., 1997; Tiribuzi et al., 2011; Magini et al., 2015; Caldeira et al., 2017).

Several studies have demonstrated an association between senescence and AD neurodegeneration. In cultured neurons, the expression of several senescent-associated genes was upregulated after the exposure to Aβ (Wei et al., 2016). These results were also confirmed *in vivo*, using the 5× FAD mouse model of AD that displays progressive Aβ deposition. After 7 months of age, this model shows the upregulation of some essential senescent—related genes in the hippocampus (Wei et al., 2016). Besides, a murine model that overexpresses human tau and develop NFT deposition also shows increased expression of several senescent—associated genes in the hippocampus and cortex (Bussian et al., 2018).

Besides, the administration of Aβ oligomers to oligodendrocyte progenitor cells can also induce senescence (Zhang et al., 2019) and in murine neural stem cells, Aβ42 peptides increase the number of SA-β-Gal positive cells (He et al., 2013). The removal of senescent cells in mice reduces Aβ accumulation and enhances their cognitive abilities (Zhang et al., 2019). Besides, hyperphosphorylation of tau can induce senescence in glial cells (Musi et al., 2018).

The fact that many of the early alterations found in AD (i.e., neuroinflammation, oxidative stress, DNA damage and changes in DNA repair and altered proteostasis) trigger or are associated with cellular senescence, has led to the suggestion that this process has a crucial role in the etiopathology of AD.

Neuroinflammation

The brains of AD patients and of mouse models of AD have higher levels of inflammation due to microglia activation that produces pro-inflammatory cytokines and other SASP mediators (Streit et al., 2004; Hickman et al., 2008; Rawji et al., 2016;

Olivieri et al., 2018). Neuroinflammation in the brain of AD patients is influenced by many SASPs mediators (Bauer et al., 1991; Huell et al., 1995; Kiecolt-Glaser et al., 2003) that have a prominent role in the onset of senescence (Flanary and Streit, 2004; Flanary et al., 2007). IL-6, a proinflammatory cytokine, is upregulated in the aged brain and AD (Bauer et al., 1991; Huell et al., 1995; Kiecolt-Glaser et al., 2003) and its overexpression has been shown to induce neurodegeneration (Campbell et al., 1993). The brains and lymphocytes of AD patients and AD mouse models also present increased activity of the SASP regulator p38MAPK (Sun et al., 2003). This enhanced p38MAPK activity upregulates the levels of the pro-inflammatory cytokines IL-6, IL-1, TGF-β and TNF-α levels in AD brains (Bauer et al., 1991; Huell et al., 1995; Freund et al., 2012; Lai et al., 2017; Rea et al., 2018), CSF and serum (Wood et al., 1993; Cacabelos et al., 1994; Blum-Degen et al., 1995; Luterman et al., 2000; Swardfager et al., 2010; Tarkowski et al., 2003; Gezen-Ak et al., 2013; Dursun et al., 2015). AD brains also present increased levels of other SASPs mediators: the metalloproteinases MMP-1, MMP-3, and MMP-10 (Leake et al., 2000; Yoshiyama et al., 2000; Bjerke et al., 2011; Horstmann et al., 2018). The fact that SASP meditators have a prominent role in the onset of senescence (Flanary and Streit, 2004; Flanary et al., 2007), provide support for the relationship between neuroinflammation and senescence in AD neuropathology.

Neuroinflammation aggravates AD (Guerreiro et al., 2013) mainly because it increases APP and Aβ expression in this disease. Pro-inflammatory cytokines induce the formation of Aβ oligomers, the phosphorylation of tau, and ROS production (Sastre et al., 2003; Blurton-Jones and Laferla, 2006; Steele et al., 2007). In turn, Aβ peptides and APP activate glial cells (Dickson et al., 1993; Barger and Harmon, 1997), produce an enhanced release of pro-inflammatory mediators such as IL-1 and IFNγ in the brains of AD patients (Ho et al., 2005; Meager, 2004, 2005). Under these circumstances, in AD, microglia internalize less Aβ (Floden and Combs, 2011; Njie et al., 2012) and are less able to process it (Nixon et al., 2001; Hickman et al., 2008; Mawuenyega et al., 2010). The higher release of pro-inflammatory cytokines reduces the ability of the cells to remove Aβ and facilitates its accumulation. Thus, there is a positive feedback between cytokine release, Aβ, and APP expression, phosphorylated tau and neurodegeneration (Wilcock and Griffin, 2013).

Oxidative Stress and Mitochondrial Dysfunction

Oxidative stress is another crucial mechanism that contributes to accelerating aging and cognitive dysfunction in AD and during aging (López-Otín et al., 2013). AD patients and mouse models of AD, present increased oxidative stress and mitochondrial dysfunction, similar to that observed in senescence. These events are present in the early stages of AD and precede the major pathologic hallmarks, such as senile plaques and NFTs (Yates et al., 2015; Ott et al., 2018).

In AD mitochondrial function and structure are impaired (Cadonic et al., 2016; Tai et al., 2017), which has a critical role in the progression of SIPS (Gao et al., 2017). Besides, the enhanced production of ROS decreases ATP synthesis. Because DNA repair is a mechanism that requires a high amount of energy, and DNA is heavily damaged in cells exposed to oxidative stress (Monti et al., 1992), these cells would need a high amount of energy to perform DNA repair. Thus, mitochondrial dysfunction reduces the ability of the cell to repair these alterations.

As mentioned in the "Introduction" section, during aging, high levels of oxidative stress induce in cells a state of senescence that displays its main pathological characteristics, including DNA damage, altered DDR, alterations in the cell cycle and cell morphology and telomere damage (Monti et al., 1992; De Haan et al., 1996; von Zglinicki et al., 2000; Serra et al., 2003; Chow and Herrup, 2015; Kawanishi and Oikawa, 2004; Jennings et al., 2000; Liu et al., 2003). Thus, a link between oxidative stress and cellular senescence has been postulated in AD.

Evidence for the relation between oxidative stress and cellular senescence in AD comes from studies in mouse models of AD in which the animals were subjected to chronic oxidative stress (SIPS) and found cellular changes identical to those found in other types of senescence: i.e., an increase in SA-β-gal expression, cell cycle arrest and alterations in cellular morphology (Toussaint et al., 2000; Ma et al., 2014). Besides, fibroblasts from AD patients produced more ROS, a slowing in the growth rate, an increase in the expression of p53 and p21 and senescence-like phenotype (Toussaint et al., 2000; Naderi et al., 2006).

DNA Damage and Repair

As mentioned above, DNA damage and alterations in DNA repair are two of the main characteristics of cellular senescence (Sedelnikova et al., 2004) and are associated with aging (Brosh and Bohr, 2007). In AD, enhanced DNA damage and reduced DDR has also been demonstrated, and these alterations seem to accelerate the progression of the disease (Lovell et al., 1999).

Telomere attrition and damage have been proposed to be a potential contributor in the pathogenesis of several neurological disorders including AD (Eitan et al., 2014; Boccardi et al., 2015; Forero et al., 2016). However, there are contradictory reports on the role of telomeres alterations on AD neuropathology.

Some studies have shown that telomere shortening or alteration might be implicated in AD pathology (Aβ burden, hyperphosphorylation of tau and dementia) (Grodstein et al., 2008; Jenkins et al., 2008; Guan et al., 2012).

In monocytes of AD patients, shorter telomeres were found (Hochstrasser et al., 2012). Shorter telomere length was also found in patients with AD than in those with mild cognitive impairment than in healthy controls (Scarabino et al., 2017). Moreover, a meta-analysis of 13 studies concluded that there is evidence that AD patients are more likely to present telomere attrition (Forero et al., 2016).

However, other studies did not find changes in the length of the telomeres of patients with AD (Hinterberger et al., 2017). The telomere length of tissue obtained from the cerebellum of AD patients did not differ from age-matched controls (Lukens et al., 2009). In studies that evaluated telomere length in patients with different types of dementia (Zekry et al., 2010) or with dementia of AD types, no differences were found between affected patients and controls (Takata et al., 2012; Hinterberger et al., 2017). Also, in a mouse model of AD,

telomere shortening reduces Aβ burden and improves cognition (Rolyan et al., 2011), although the animals still showed enhanced DNA damage and neurodegeneration.

Therefore, more studies are necessary to clarify the role of telomere attrition in AD neuropathology.

Additionally, an association between reduced lifespan, elevated levels of DNA damage, and a prolongation of the cell cycle duration have been demonstrated (Weirich-Schwaiger et al., 1994). In AD there is also evidence that there are significant alterations of cell cycle re-entry of post-mitotic neurons (Vincent et al., 1996; Nagy, 2005; Herrup and Yang, 2007).

During senescence, the accumulated DNA errors alter cellular functions such as transcription and DNA repair (Hackett et al., 1981; Cleaver et al., 1983; Mayne, 1984; Friedberg, 1985; Klocker et al., 1985). These alterations are more evident during aging (Staiano-Coico et al., 1983; Nette et al., 1984; Dutkowski et al., 1985; Mayer et al., 1989; Roth et al., 1989). Moreover, in AD, an enhanced DDR is found in the hippocampus and lymphocytes, as demonstrated by the presence of elevated levels of the phosphorylated histone γH2AX (H2A histone family member X) (Silva et al., 2014; Siddiqui et al., 2018).

In neurodegenerative diseases, epigenetic modifications, such as alteration in DNA methylation occur early in the disease process, affect to particular genes and correlate with misfolded proteins in specific brain regions (Armstrong et al., 2019; Prasad and Jho, 2019).

AD, PD, and DS among other neurodegenerative diseases have common aberrant DNA methylation profile that mainly affects the expression of critical genes involved in various signaling pathways that are implicated in several pathological hallmarks such as presence of Aβ plaques, NFT or α-synuclein inclusions that are present in some of them (Sanchez-Mut et al., 2016; Armstrong et al., 2019; Prasad and Jho, 2019).

Among the epigenetic alterations found in AD, DNA methylation and histone modifications show evident deregulation. It has been demonstrated that in AD brains changes in DNA methylation affect to a few common gene loci which play an essential role in the formation of Aβ plaques (Lord and Cruchaga, 2014; Watson et al., 2016; Qazi et al., 2018; Armstrong et al., 2019; Esposito and Sherr, 2019; Prasad and Jho, 2019). Among them, of particular interest are the differences in methylation in different regions of the *APP* gene promoters in the brains of humans with AD and these changes have been associated with neuropathological markers of this disorder (Bradley-Whitman and Lovell, 2013). Besides, in AD brains histone modifications have been linked to the reduction in transcription of genes that are implicated in neuronal physiology and increased the transcription of genes that are normally silenced (Berson et al., 2018). The phosphorylated histone H3 presents an altered localization of the cytoplasm, while the levels of the acetylated histone H4 are decreased in AD patients (Kwon et al., 2016).

Also, chromatin organizing proteins and epigenetic regulators are mislocated in AD, affecting chromatin architecture (Winick-Ng and Rylett, 2018). Finally, in AD, other epigenetic modifications such as alterations in chromatin remodelers, phosphorylation of histones have been found (Esposito and Sherr, 2019), and might be associated with senescence.

Proteostasis

Altered proteostasis is a phenotypic hallmark of senescent cells. The integrity of the nucleolar ribosome biogenesis machinery and the ER, as well as the correct function of the degradation machinery (ubiquitin-proteasome and lysosomal pathways), are essential for maintaining proteostasis (Labbadia and Morimoto, 2015). The perturbation of proteostasis leads to misfolded protein accumulation and proteotoxic stress (Hetz and Mollereau, 2014; Hipp et al., 2014), which also contributes to neurodegeneration. Senescent cells lose protein homeostasis due to nucleolar dysfunction, autophagy and lysosomal anomalies and alterations in UPR indicative of ER stress. The majority of neurodegenerative disorders, present impairments of degradative compartments such as lysosomes and autophagosomes.

Protein dysfunction has been associated with numerous human neurodegenerative diseases such as AD, PD, amyotrophic lateral sclerosis, spinocerebellar ataxia, and Huntington's disease (Yurov et al., 2014). In these diseases, high levels of different toxic protein aggregates due to a loss of protein homeostasis have been found. In particular, in AD, the formation of Aβ plaques (Ow and Dunstan, 2014), which is directly associated with the expression of SASP-associated factors (Bhat et al., 2012), and of NFT, composed by hyperphosphorylated tau protein, increase with the acquisition of a senescent phenotype and at the same time both induce senescence (Zare-Shahabadi et al., 2015; Chung et al., 2018; Mendelsohn and Larrick, 2018).

These alterations have been demonstrated to contribute to the etiology and progression of AD (Ihara et al., 2012; Nixon, 2013; Hetz and Mollereau, 2014). In this disorder, the most common form of protein aggregation, in Aβ plaques (Ow and Dunstan, 2014), which is directly associated with the expression of SASP-associated factors (Bhat et al., 2012), is one of the primary triggers and consequences of cellular senescence. The other abnormal protein found in AD brains is hyperphosphorylated tau in the form of NFTs, which have been reported to induce senescence (Mendelsohn and Larrick, 2018).

CELLULAR SENESCENCE IN DOWN SYNDROME

DS, or trisomy of chromosome 21, is the primary cause of cognitive disability of genetic origin (Shin et al., 2009). These cognitive alterations are due to defects in growth and differentiation of the CNS that appear during early prenatal stages (Haydar and Reeves, 2012; Lott, 2012). DS is characterized by premature aging and early appearance (around the fourth decade of life) of neuropathology identical to the one found in sporadic AD, including amyloid plaques, NFTs, neurodegeneration, and synapse loss (Teipel and Hampel, 2006; Sabbagh et al., 2011; Cenini et al., 2012; Lott, 2012; Wilcock and Griffin, 2013; Casanova et al., 1985; McGeer et al., 1985). Both, premature aging and AD pathology are thought to be the leading

causes of the earlier mortality of this population (Schupf and Sergievsky, 2002; Zigman and Lott, 2007; Prasher et al., 2008; Esbensen, 2010).

Neuroinflammation

The DS brain also present neuroinflammatory changes typical of AD, such as microglial activation and increased release of pro-inflammatory cytokines (Griffin et al., 1989; Park et al., 2005; Griffin, 2006; Wilcock and Griffin, 2013). There is compelling evidence that, in DS, the increased release of cytokines due to microglia activation, enhances the production of ROS, aggravates synaptic dysfunction, and neurodegeneration, and reduces neurogenesis (Town et al., 2005; Fuster-Matanzo et al., 2013; Llorens-Martín et al., 2014; Lyman et al., 2014; Rosi et al., 2012). The aggravation produced by neuroinflammation on neurodegeneration and the reduced neurogenesis (see Rueda et al., 2012), render DS individuals more susceptible to neuropathological events that can accelerate the onset of dementia (Teipel and Hampel, 2006).

Enhanced microglial activation also produces an increased expression of proinflammatory cytokines in different areas of the brain of a mouse model of DS (Hunter et al., 2004; Lockrow et al., 2011; Roberson et al., 2012; Rueda et al., 2018). Reducing neuroinflammation by administering an antibody against the pro-inflammatory cytokine IL17A to these mice, reduces cellular senescence in the hippocampus and cortex of these animals, improves their cognitive abilities and reduces some of the signs of neurodegeneration (Rueda et al., 2018). These results suggest a direct link between cellular senescence, neuroinflammation and neurodegeneration in DS.

Oxidative Stress and Mitochondrial Dysfunction

Another crucial mechanism that contributes to accelerating aging and cognitive dysfunction in DS is oxidative stress. In this syndrome, it is present from embryonic stages producing a reduction in neuronal proliferation, differentiation, connectivity, and survival (Monti et al., 1992; Busciglio and Yankner, 1995; Perluigi et al., 2011). As the person with DS ages, the levels of oxidative stress increases, contributing to the progression of cognitive and neuronal degeneration (Busciglio et al., 2007; Lockrow et al., 2009; Shichiri et al., 2011; Perluigi and Butterfield, 2012; Rueda et al., 2012; Parisotto et al., 2016). As previously mentioned, oxidative stress is an essential factor that causes cellular senescence (Monti et al., 1992; He et al., 2013; Rodríguez-Sureda et al., 2015).

Furthermore, mitochondrial dysfunction is also present in DS cells during embryonic life (Perluigi et al., 2011), leading to profound alterations in energy metabolism due to the reduced synthesis of ATP synthesis (Valenti et al., 2010). ATP is also reduced *in vitro* in DS fibroblasts, which could contribute to premature cell aging (Rodríguez-Sureda et al., 2015).

In DS, early induction of senescence by aberrant intracellular oxidant activity and antioxidant defense has been demonstrated in fibroblasts, skin tissue, lymphocytes (De Haan et al., 1996; Kalanj-Bognar et al., 2002; Cristofalo et al., 2004; Kimura et al., 2005; Biron-Shental et al., 2015; Rodríguez-Sureda et al., 2015) and in the amniotic fluid of women carrying DS fetuses (Perluigi et al., 2011; Amiel et al., 2013). Thus, senescence is present in DS from prenatal stages and can be responsible for different altered phenotypes. But, enhanced senescence is a prominent phenomenon during all the life stages of a DS individual and is aggravated with aging and the appearance of AD neuropathology.

In agreement with these results, it has been demonstrated that the hippocampus of a mouse model of DS shows higher amounts of oxidative damage (protein and lipid oxidative damage), Aβ expression and tau phosphorylation, as well as increased density of senescent cells in different hippocampal regions (i.e., DG, CA1, CA3, and hilus; Corrales et al., 2013; Parisotto et al., 2016; García-Cerro et al., 2017; Rueda et al., 2018). Other authors have also found the early presence of SA-β-gal activity in both cultured fibroblasts and skin tissue from DS mice (Contestabile et al., 2009b). Finally, chronic administration of melatonin, a potent antioxidant, to this mouse model of DS reduces hippocampal senescence and oxidative damage, improves the cognitive abilities of these mice, enhances neurogenesis and reduces their neurodegenerative phenotype (Corrales et al., 2013, 2014; Parisotto et al., 2016). These results provide support for the association between oxidative stress and senescence in DS.

DNA Damage and Repair

Hematopoietic stem cells and satellite cells of skeletal myofibers in the Ts65Dn mouse model of DS accumulate DNA damage and prematurely develop a senescent phenotype (Adorno et al., 2013; Wang et al., 2016; Pawlikowski et al., 2018). Furthermore, in this murine model, the ubiquitin-specific-peptidase 16 (*Usp16*) gene is triplicated, leading to the overexpression of the Usp16 enzyme that controls the ubiquitination state of the histone 2A (Ub-H2A) and contributes to regulate the DDR chromatin remodeling and cell cycle progression (Joo et al., 2007). Thus, in these mice, both the overexpression of the *Usp16* gene, which may affect the DNA damage/repair signaling pathways and the high levels of brain oxidative stress and neuroinflammation may produce and excessive accumulation of DNA damage leading to genomic instability and neurodegeneration or premature cellular senescence.

In DS, there is also evidence of prenatal accumulation of DNA damage and of the reduction of the ability to repair DNA (Agarwal et al., 1970; Raji et al., 1998; Pogribna et al., 2001; Cabelof et al., 2009; Nižetić and Groet, 2012; Di Domenico et al., 2015). Besides, in response to DNA damage, DNA polymerase b (POLb), whose function is to participate in base excision repair (BER) is induced; however, in DS fibroblasts its expression is reduced, thereby reducing the ability to repair DNA damage. This reduction is sufficient to induce senescence (Ahmed et al., 2018; Cabelof et al., 2002, 2003; Cabelof, 2007).

In DS, both alterations in the DNA damage/repair signaling pathways, and the high levels of brain oxidative stress may produce and excessive accumulation of DNA damage leading to genomic instability and neurodegeneration or premature cellular senescence. Hence, this mechanism might play an essential role in the onset and aggravation of the AD-associated cognitive decline.

DNA repair mechanisms eliminate damages and elongate cell cycle duration to give the repair enzymes time to work. However, alterations in DNA repair mechanisms producing accumulated DNA damage can delay the cell cycle until mitotic activity ceases, leading to premature senescence and sometimes cell death. In DS, enhanced DNA damage and impairment in DNA repair impairs mitosis and delays the cell cycle leading to senescence. There is evidence of delayed cell cycle in DS individuals and mouse models of this syndrome (Contestabile et al., 2007, 2009a).

As mentioned above, telomere attrition is one of the best-characterized senescence-triggering mechanisms. Telomeres are shorter in DS amniocytes, and placentas of fetuses with DS present an increased percentage of trophoblasts with senescence phenotypes (Sukenik-Halevy et al., 2011; Amiel et al., 2013; Biron-Shental et al., 2015). For this reason, it has been suggested that it could be an early biomarker of premature senescence (Vaziri et al., 1993). Also, a significantly higher loss of telomere sequences was observed *in vitro* and *in vivo* in lymphocytes from DS individuals (0–45 years old) (Vaziri et al., 1993; de Arruda Cardoso Smith et al., 2004). Jenkins et al. (2008) also demonstrated that shorter telomere length correlates with the degree of dementia and AD-associated neurodegeneration in DS individuals. These results suggest that senescence can play an essential role in the aging of the immune system in DS (Vaziri et al., 1993), leading to the altered microglia function and neuroinflammation. Thus, in DS, this type of DNA damage can induce senescence from prenatal stages through the entire lifespan of the individual.

Epigenetic Modifications

Epigenetic alterations such as DNA and histone modifications are also found in patients with DS. Among them, DNA hypermethylation, histone modifications, histone core variants and changes in small non-coding RNAs are implicated in several phenotypic characteristics of DS such as memory impairment, premature aging and neurodevelopmental defects (Mentis, 2016).

Although some of these alterations are associated with premature aging in DS, the exact implication of epigenetic modifications in cellular senescence found in this syndrome needs to be elucidated.

Proteostasis

Abnormalities in chromosome number disrupt proteostasis (Oromendia et al., 2012; Oromendia and Amon, 2014; Stingele et al., 2012). Gene copy number changes due to aneuploidy can affect the number of proteins expressed, their functions and the protein quality-control and repair machinery of the cell (Sheltzer et al., 2011; Oromendia et al., 2012; Thorburn et al., 2013; Yurov et al., 2014).

Thus, in DS, the extra chromosome can alter proteostasis (Lockstone et al., 2007). Trisomy may also affect the 3D nuclear architecture of the genome resulting in changes in gene interactions and gene expression that may impact proteostasis regulation (Vermunt et al., 2019).

Regarding protein synthesis, only a few studies have analyzed the ribosome biogenesis machinery in DS. Demirtas (2009), using a classic silver staining procedure to study the arrangement of the nucleolar organizer regions (NORs), found that cells from DS infants presented an increased number of AgNOR dots. Because ribosome biogenesis is a highly energy-consuming process, these authors suggest that this response to trisomy 21 might result in unnecessary rRNA and ribosomal protein synthesis, which leads to a waste in energy. Also, reduced expression of specific transcription, splicing, and translation factors has been found in cortices of fetuses with DS (Freidl et al., 2001), suggesting a deranged protein synthesis in them.

Moreover, in DS, both intracellular degradative systems, UPS and autophagy, seem to be affected. Chronic exposure to oxidative stress, as occurs in the DS brain, also causes protein oxidation of members of the proteostasis network, resulting in accumulation of unfolded/damaged protein aggregates and dysfunction of intracellular degradative system, such as autophagy and the ubiquitin-proteasome systems (UPS), contributing to neurodegeneration (Di Domenico et al., 2013; Hetz and Mollereau, 2014). In the hippocampus of Ts65Dn mice altered autophagy, due to reduction of autophagosome formation, has been found (Tramutola et al., 2016). In DS human fibroblasts and in the brain of two segmental trisomy models of DS, the Dp16 and Dp17 mice, increased ubiquitination, and disrupted proteasome activity has been reported (Aivazidis et al., 2017). Finally, in the cerebellum of the Ts65Dn mouse, a dysfunction of the UPS, due to reduced proteasome activity and a parallel increase of ubiquitinated proteins, has been implicated in the degeneration of Purkinje cells (Necchi et al., 2011). Consequently, ubiquitinated or polyubiquitinated proteins are not degraded at a normal rate and are stored in the nuclei of cerebellar neurons of this model (Necchi et al., 2011).

Besides, disruption in the proteostasis network could contribute to the accumulation of protein aggregates, such as amyloid deposits and NFTs (Di Domenico et al., 2013). Alterations of the proteostasis network are present in individuals with DS years before the age-related cognitive decline, and AD-associated dementia is detected.

In summary, DS is characterized by early senescence that continues throughout the lifespan of the individual and is very likely aggravated by the appearance of AD neuropathology.

CELLULAR SENESCENCE IN PARKINSON DISEASE

PD is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra, the presence of cytoplasmic protein aggregates, known as Lewy bodies, that contain a variety of proteins, including ubiquitin and α-synuclein (Nussbaum and Ellis, 2003; Thomas and Beal, 2011). These pathological features result in an impairment of motor control, including difficulty to initiate movements, loss of balance, rigidity, and tremor, and cognitive deterioration (Poewe et al., 2017). The most important risk factors for the development

of PD are aging, a genetic predisposition and exposure to toxins (Chinta et al., 2013).

Similarly to AD and DS, PD neuropathology is aggravated by neuroinflammation mitochondrial dysfunction, α-synuclein accumulation, oxidative stress, and cellular senescence (Poewe et al., 2017).

There is widespread evidence that cellular senescence plays an essential role in the pathogenesis of PD. Patients with PD present enhanced levels of SA-β-gal in their CSF (van Dijk et al., 2013) and in their brain tissue (Chinta et al., 2018). Higher numbers of senescent astrocytes are also present in the substantia nigra of patients with PD (Chinta et al., 2018). Besides, paraquat, a herbicide that has been proposed to be implicated in the appearance of some types of PD, induces senescence in human astrocytes (Chinta et al., 2018).

Neuroinflammation

Similarly to AD and DS, inflammation is prominent in PD (Chinta et al., 2018). Activation of microglia is thought to be one of the main determinants of dopaminergic loss in the substantia nigra (McGeer et al., 1988), hippocampus, cingulate and temporal cortex (Imamura et al., 2003). α-synuclein aggregation activates microglial cells in PD (Zhang et al., 2005). The result of microglia activation is in the increased levels of the pro-inflammatory mediators IL-1, IL-6, TNFγ and TNF-α in the CSF, serum, and dopaminergic regions of the striatum from patients with PD (Mogi et al., 1994a,b; Blum-Degen et al., 1995; McCoy et al., 2006; Mount et al., 2007; Brodacki et al., 2008; Scalzo et al., 2010; Lindqvist et al., 2012; Dursun et al., 2015).

The high presence of senescent cells and aged astrocytes in PDs brains suggests that senescence-induced neuroinflammation might be an important mechanism for PD neurodegeneration and a potential therapeutic target for this disease.

Oxidative Stress and Mitochondrial Dysfunction

Oxidative stress plays an essential role in the etiology and progression of PD (Trist et al., 2019). Even in the early stages of the disease, before a significant loss of dopaminergic neurons, PD patients present elevated oxidative stress (Ferrer et al., 2011). Thus, oxidative stress could be partially responsible for neurodegeneration, in addition to been enhanced as a result of the neuronal loss. As demonstrated in AD and DS, it seems that positive feedback is produced between both insults that probably aggravates the disease.

Mitochondrial dysfunction also plays a vital role in PD etiopathology. In familial PD, different mutations in the genes associated with pathways of mitochondrial dysfunction have been demonstrated, and some of these pathways are also altered in sporadic PD (Park et al., 2018). The production of ROS during ATP synthesis is higher in dopaminergic neurons of the substantia nigra. Due to their size and complexity, they need more elevated amounts of ATP to maintain resting membrane potential, propagate action potentials, and enable synaptic transmission (Pissadaki and Bolam, 2013).

These alterations in mitochondrial function and oxidative stress are likely to be a result of senescence.

DNA Damage and Repair

DNA damage and repair have been proposed as important actors altering the function of the dopaminergic system in PD (Sepe et al., 2016). Telomere shortening plays a causal role in cellular aging (Hastie et al., 1990), and it has been proposed as a potential contributor in the pathogenesis of PD (Anitha et al., 2019).

The studies of telomere length in PD patients had provided contradictory results. Some of them provide support for shorter telomere length in different cell types or tissues (Tomac and Hoffer, 2001; Guan et al., 2008; Koliada et al., 2014) while others do not find evidence of this alteration in PD (Guan et al., 2008; Wang et al., 2008; Eerola et al., 2010; Watfa et al., 2011; Maeda et al., 2012; Degerman et al., 2014; Eitan et al., 2014; Forero et al., 2016). A meta-analysis of all these studies concluded that PD patients do not present changes in telomere length (Forero et al., 2016). Thus, new studies are necessary to clarify the putative role of replicative senescence and telomere shortening in different brain regions and cell types in PD.

However, it has been demonstrated that PD is characterized by genetic instability due to gene mutations, altered gene expression, or regulation. The expression of some genes implicated in the cell cycle, such as *p16INK4a* (Chinta et al., 2018) and *pRb* (Ihara et al., 2012), are upregulated in PD brains. This enhanced genetic expression is likely to produce alterations in the cell cycle in this disorder.

Furthermore, alterations in epigenetic modulation seem to play an important role in the etiopathology of PD. The brains of these patients present changes in DNA methylation, post-translational modifications of histones and of microRNAs that regulate pathways involved in the pathophysiology of PD (Armstrong et al., 2019; Prasad and Jho, 2019; Renani et al., 2019).

However, the role of the alterations of DNA methylation in the pathological hallmarks of this disease, including cellular senescence, is still unclear.

Proteostasis

Another characteristic that PD shares with other neurodegenerative diseases is altered proteostasis. In PD, there is an accumulation of misfolded proteins in the brain (Martinez et al., 2019). All patients with PD present insoluble α-synuclein fibrils, which damage neurons, and are associated with intracellular Lewy bodies (Melki, 2015). Alterations in the proteostasis network seem to play a prominent role in the protein aggregation found in PD, mainly due to changes in the function of the ER, the organelle involved in protein folding and quality control. ER stress produces the UPR to try to restore proteostasis.

In this disease, the increase in senescent cells is thought to be directly associated with the enhanced aggregation of α-synuclein (Chinta et al., 2018).

It has been proposed that mitochondrial dysfunction and oxidative stress could be partially responsible for this α- synuclein accumulation (Dias et al., 2013; Rocha et al., 2018).

Finally, the autophagic/lysosomal dysfunction also plays an essential role in the pathogenesis of the disease, since different genetic mutations that produce defects in these

pathways are found in PD patients (Rivero-Ríos et al., 2016; Pitcairn et al., 2018).

In summary, cellular senescence is also a pathological event of crucial importance in PD, and shares with other neurodegenerative diseases many of the factors that trigger it and aggravate the neuropathology (i.e., oxidative stress, mitochondrial dysfunction, altered proteostasis, among others).

CONCLUDING REMARKS

Senescent cells accumulate with aging and in neurodegenerative pathologies, and there is extensive evidence that they might be implicated in the etiopathology of these diseases. Here, we reviewed the data that demonstrate that cellular senescence is a common pathological feature of PD, AD, and DS. These three diseases also share some of the mechanisms that promote senescence and that allow this permanent state of cell arrest to induce or aggravate neurodegeneration. Furthermore, extensive senescence has also been found in other neurodegenerative diseases such as Huntington Disease, Multiple Sclerosis, and Rett syndrome. Thus, senescence seems to be a common mechanism for neurodegeneration that needs to be further investigated.

At present, there are no efficient neuroprotective treatments that can prevent or delay the progression of the disease for AD in individuals with or without DS or for PD. Thus, new neuroprotective therapeutic approaches are needed.

Because of the central role of cellular senescence in the etiopathology of different neurodegenerative diseases, therapies that reduce senescence could be a promising approach to prevent the loss of cells and the alteration of their function. These therapies have been more extensively investigated in the case of AD (He et al., 2013; Kirkland and Tchkonia, 2017).

Several therapeutic strategies, known as senotherapies, to selective eliminate tissue-specific senescent cells, have been developed (Childs et al., 2017; Kritsilis et al., 2018; Kim and Kim, 2019). However, almost none have been tested in models of different neurodegenerative diseases, except AD. Clearance of senescent cells extended lifespan in normal mice (Baker et al., 2016) and in a progeroid mouse model (Baker et al., 2011). Finally, a recent work has demonstrated that pharmacological elimination of senescent astrocytes and microglia cells using a senolytic compound (ABT263) attenuates tau phosphorylation, modulates tau aggregation, prevents the upregulation of senescence-associated genes in the cortex and hippocampus and improves short-term memory in a transgenic mouse model that overexpresses human tau (Kirkland and Tchkonia, 2017; Bussian et al., 2018). Thus, senotherapeutic agents may serve as powerful tools to prevent neurodegenerative diseases.

Importantly, in many of these disorders, senescence is an early event that appears years before other signs of neurodegeneration and in the case of DS, even at embryonic stages, providing a broad therapeutic window to prevent or delay some of the pathological processes associated.

Finally, many of the mechanisms implicated in the onset of senescence and that also appear years earlier than neurodegeneration (i.e., oxidative stress, mitochondrial damage, neuroinflammation, DNA damage, altered proteostasis), are also a result of senescence, therefore aggravating the disease (**Figure 1**). Thus, therapies targeting these earlier events could exert a double benefit preventing the onset of the neurodegenerative disease or delaying its progress.

AUTHOR CONTRIBUTIONS

CM-C and NR wrote and edited the manuscript.

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An Appraisal on the Value of Using Nutraceutical Based Senolytics and Senostatics in Aging

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The average human life expectancy has increased globally, and continues to rise, owing to the substantive progress made in healthcare, medicine, sanitation, housing and education. This ultimately enriches society with a greater proportion of elderly people. Sustaining a healthy aged population is key to diminish the societal and economic impact of age-related infirmities. This is especially challenging because tissue function, and thus wellbeing, naturally progressively decline as humans age. With age increasing the risk of developing diseases, one of the therapeutic options is to interfere with the molecular and cellular pathways involved in age-related tissue dysfunction, which is in part caused by the accumulation of senescent cells. One strategy to prevent this could be using drugs that selectively kill these cells (senolytics). In parallel, some compounds have been identified that prevent or slow down the progression of senescence or some of its features (senostatics). Senolytic and senostatic therapies have been shown to be efficient *in vivo*, but they also have unwanted dose-dependent side effects, including toxicity. Important advances might be made using bioactive compounds from plants and foods (nutraceuticals) if, as is proposed, they offer similar effectiveness with fewer side effects. The focus of this review is on the use of nutraceuticals in interfering with cellular senescence.

Keywords: senescence, senolytics, senostatics, nutraceuticals, aging

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INTRODUCTION

Aging is the time-related deterioration of physiological functions necessary for survival and fertility (Gilbert, 2000). On a cellular level, aging is accompanied by the accumulation of cells that adopt a specific phenotype, known as senescence, a process which is conserved across species. Senescence is mainly characterized by an irreversible cell cycle arrest and the secretion of a group of factors, known as the senescence-associated secretory phenotype (SASP), whilst maintaining metabolic activity (Wiley and Campisi, 2016). Over time, senescent cells accumulate in all tissues and organs, contributing to their functional deterioration (van Deursen, 2014). The permanent cessation of cell proliferation is seen as a way to avoid neoplastic outgrowth, which means that in this context, senescence can be considered a tumor supressor mechanism (Dimri, 2005). For growth arrest to take place, senescent cells increase the expression of cyclin dependent kinase inhibitors (CDKIs) expression such as p21, p16 or p27 (Alcorta et al., 1996; Hernández-Segura et al., 2018).

The SASP, which is capable of promoting tissue repair and regeneration, creates a microenvironment that can drive neighboring cells into proliferation or even senescence, thus contributing to the disruption of tissue homeostasis (Rodier and Campisi, 2011). Therefore, with beneficial attributes early in life and detrimental consequences late in life, the senescence phenomenon is as an example of so-called antagonistic pleiotropy (Rodier and Campisi, 2011; Giaimo and d'Adda di Fagagna, 2012).

Due to general lack of specificity, multiple markers have to be considered and validated in conjunction for the identification of senescent cells. These markers include, but are not limited to, senescence associated beta-galactosidase staining (SA-β-Gal), cell cycle arrest, morphological and nuclear changes, and expression of certain proteins (Hooten and Evans, 2017). It is important to differentiate senescent cells from quiescent and terminally differentiated cells such as neurons, macrophages and muscle cells (Rodier and Campisi, 2011). Quiescent cells retain the ability to re-enter the growth cycle when stimulated with growth signals and are key to tissue regeneration by maintaining a constant state of balanced proliferation (Yao, 2014).

CAUSES OF CELLULAR SENESCENCE

Replicative Senescence

Hayflick and Moorhead first observed senescence in somatic cells serially passaged in culture (Hayflick and Moorhead, 1961). Normal cells undergo multiple mitotic cell divisions, eventually reaching a limit at which their proliferative capabilities are lost. This is now referred to as replicative senescence. Human fibroblasts, the cell type originally described, had a limit of around 50 passages. Since then, other forms of senescence have been discovered and applied in models of *in vitro* and *in vivo* research.

Most types of senescence are largely triggered through the DNA damage response pathways (DDR) (Fumagalli et al., 2014). The DDR consists of upstream components such as ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3 related) kinases, which activate cell cycle checkpoint proteins CHK1 and CHK2. Eventually, cyclin dependent kinases (CDKs) are inhibited to arrest the cell cycle (Jackson and Bartek, 2009). In senescent cells, the main proteins involved in proliferative arrest are CDKIs p21 and p16. These are found to be overexpressed and are often used as markers for cell cycle arrest and senescence.

In replicative senescence, activation of the DDR is triggered through the shortening of telomeres. The change in DNA content following loss of telomeric length is recognized as damage, resulting in the initiation of the DDR response (Serrano and Muñoz-Espín, 2014). Telomeres are complexes composed of proteins and nucleotides of TTAGGG repeats at the ends of eukaryotic chromosomes that are considered protective structures (Bernadotte et al., 2016). When a cell divides, chromosomes are replicated and telomeres shorten in length with each cell division. An increase in the presence of telomere associated foci is seen in connection to aging (Hewitt et al., 2012). Loss of telomeric length has been suggested to be the biomarker of choice in replicative senescence (Bernadotte et al., 2016). Of note, lifestyle choices such as smoking, diet, stress and exercise can have an effect on telomere attrition (Shammas, 2011). Telomere attrition occurs in cells in which the expression of telomerase is repressed, as is the case in adult human somatic cells (Calado and Dumitriu, 2013). By contrast, mice regulate telomere length and telomerase activity differently from humans and are therefore inherently limited in studies of replicative aging (Zhang et al., 2016).

Stress-Induced Premature Senescence (SIPS)

DNA single and double strand breaks (induced by exposure to radiation, overexpression of oncogenes, oxidative stress, etc.) result in the activation of the DDR and can eventually lead to a stress-induced premature senescence (SIPS), which is independent of telomere length (Boothman and Suzuki, 2008). *In vitro* SIPS models have been established using oxidative stress, ionizing radiation or DNA damage causing agents such as bleomycin (González-Hunt et al., 2018).

Overexpression of the *ras* oncogene in human and rodent cells was found to elicit a phenotype similar to cellular senescence, supporting the fact that senescence is a tumor-suppressor mechanism. Since then, other oncogenes have also been found to induce senescence including; Raf, c-Myc, Akt and E2F3 (Serrano et al., 1997; Astle et al., 2011; Qian and Chen, 2012; Ko et al., 2018).

In vivo models of SIPS include accelerated aging of mice as a result of gene defects. For example, mutations in the *LMNA* gene of mice can result in the onset of Hutchinson-Gilford progeria syndrome, deficiencies in *nfkb1* can lead to early aging phenotype in middle-aged mice and WRN protein deficient mice develop Werners syndrome, a disease of premature aging (Kõks et al., 2016). Recent models include the syngeneic transplantation of senescent cells in mice, leading to signs of physical dysfunction (Xu et al., 2018). These models have become the basis for senescence research, specifically for the testing of potential anti-aging drugs.

CHARACTERISTICS OF CELLULAR SENESCENCE

As outlined below and illustrated in **Figure 1**, senescent cells display changes in morphology, increases in certain cell cycle related proteins, nuclear changes and the presence of SASP.

Morphology

Senescent cells in culture have a characteristic morphology that makes them distinguishable from proliferating cells (Dimri, 1995). Once in senescence, cells become flatter and the nucleus becomes enlarged. The cause for these morphological changes is not fully understood but the increase in cell size may be associated with the process of cell cycle arrest, where the imbalance of DNA: cytoplasm ratio in itself may contribute to aging (Neurohr et al., 2019).

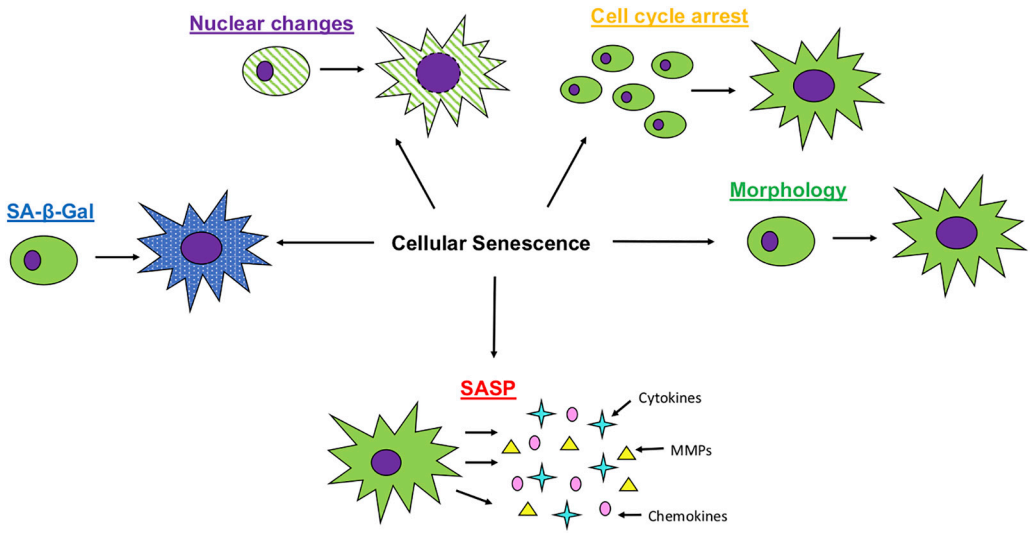

FIGURE 1 | Characteristics of Cellular Senescence. Senescent cells will undergo multiple changes, with morphology becoming larger and flatter, an increased expression of SA- β -Gal activity, loss of nuclear membrane integrity, cease in cell proliferation and the production of SASP, including an increased expression of matrix metalloproteases (MMPs), cytokines and chemokines.

SA-β-Gal Activity

The cytoplasmic content of senescent cells also changes, with an increase in lysosomal SA- β-Gal activity. This enzyme is thought to exist in cell lysosomes, which are known to increase in size as cells age. Therefore, a high SA- β-Gal activity would be expected in senescent cells. However, this is not considered to be exclusive, as it can also be found in other cell types of non-senescent nature and in some cases may not be established in senescence (Dimri, 1995; Lee et al., 2006; Huang and Rivera-Pérez, 2014).

Cell Cycle Arrest

The CDK/Cyclin complexes are necessary to progress through the cell cycle. In senescent cells, this interaction between cyclins and CDKs is blocked by CDKIs (Blagosklonny, 2011). The *INK4A* gene encodes the p16 protein, which inhibits the Cyclin D/CDK4/6 complex to stop the progression of G1 phase to S phase (Jingwen et al., 2017). It is often used as a marker for senescent cells due to its increased levels of expression. The *CDKN1A* gene encodes p21, which inhibits the Cyclin E/CDK2 and Cyclin B/CDK1 complexes (Jingwen et al., 2017). The inhibitor is commonly used a marker as well, but is less specific, since it can be upregulated in other situations (Hernández-Segura et al., 2018).

Nuclear Changes

In addition to the increase in nuclear size, senescent cells have changes to the nuclear lamina, which impact on nuclear stability (Hernández-Segura et al., 2018). The nuclear lamina is made up of lamin proteins, which are type V intermediate filaments, and is disassembled and reassembled with every mitotic division (Freund et al., 2012). Lamin B1 is expressed differentially between pre-senescent and senescent cells: in various primary human fibroblast cell lines, a decrease of protein and mRNA expression of Lamin B1 can be observed, suggesting its use as a senescent cell biomarker (Freund et al., 2012).

Changes in chromatin structure are also exhibited in senescent cells. Heterochromatin becomes more prominent, with areas of nucleus having denser DAPI (diamidinophenylindole) stained foci that have been called senescence associated heterochromatin foci (SAHFs) and may be involved in gene silencing of proliferation genes (Aird and Zhang, 2012). These SAHFs can also be distinguished with specific epigenetic markers, such as H3K9me3, H3K27me3, and macroH2A, a facultative heterochromatin histone variant (Parry and Narita, 2016). DNA methylation patterns change during the process of aging as well, with hypomethylation in heterochromatic regions and focal hypermethylation in gene-associated cytosine-guanine islands (CGIs) (Ciccarone et al., 2018). Histone modifications can cause repression of certain genes, silencing those involved in cell cycle progression.

SASP

The SASP consists of a collection of molecules secreted by senescent cells that shape the microenvironment. These molecules include proteases and growth factors, chemokines and cytokines such as IL-6, IL-8 and IL-1β (Borodkina et al., 2018). Interleukins take part in activating signaling pathways such as Jak/STAT, PI3K, and MAPK (Garbers et al., 2013). Proteases, such as matrix metalloproteases (MMPs) and serine proteases, are involved in the development and regeneration of the extracellular matrix. The inhibitors of these proteins and signaling molecules are also secreted by senescent cells to regulate their functions (Borodkina et al., 2018), so the net effect is dependent on this balance. Although the SASP works to promote tissue repair and regeneration and encourages immune surveillance, it

can also be detrimental to health. For example, through paracrine induction of epithelial to mesenchymal transition and attraction of immunosuppressive immune cells, it can contribute to tumorigenesis and metastasis in cancer (González-Meljem et al., 2018).

CHEMICAL APPROACHES TO REGULATE SENESCENT CELL ACCUMULATION AND ACTIVITY

The characterization of compounds that could counteract the process of senescence has recently become a priority, given its immense therapeutic potential. These anti-senescence compounds are divided into two categories: senolytics and senostatics. Senolytic drugs are agents that selectively induce apoptosis of senescent cells. They were first discovered by Zhu et al. (2015), in whose work the combination of quercetin and dasatinib showed potential apoptotic activity in eliminating senescent cells (Zhu et al., 2015). On the other hand, senostatics are drugs capable of interfering with the progression of cells entering senescence or modulate their activity by reducing SASP generation (Short et al., 2019).

Senolytics can work by overriding anti-apoptotic pathways in senescent cells (Short et al., 2019). One of the most well studied senolytics is the combination of dasatinib and quercetin. Dasatinib is an established anti-cancer drug that, when administered in mice alongside quercetin, selectively eliminates senescent cells, leading to improved physical function and increased survival *in vivo* (Xu et al., 2018). This combination is now being tested as part of clinical trials to investigate its potential in patients with idiopathic pulmonary fibrosis, with preliminary results already showing improvements in cardiopulmonary function (Justice et al., 2019). Many compounds are under investigation for their potential senolytic properties. Other synthetic senolytic drugs include BCL inhibitor ABT-737, Panobinostat, HSP90 inhibitors or cardiac glycosides (Yosef et al., 2016; Fuhrmann-Stroissnigg et al., 2017; Samaraweera et al., 2017; Xu et al., 2018; Guerrero et al., 2019; Triana-Martínez et al., 2019).

Senostatics can work by inhibiting paracrine signaling or by counteracting the effects of the SASP in senescence (Short et al., 2019). They can also prevent the emergence of senescent cells by blocking fundamental steps of the effector mechanisms of the phenotype, such as activation of the p53 pathway (Althubiti et al., 2016). Examples of senostatic that have proven to be effective therapies in mouse models include rapamycin and metformin (Short et al., 2019). Rapamycin is a naturally derived antibiotic with additional anti-fungal and immunosuppressant properties. The macrolide compound is an mTOR inhibitor and it delays the progression of senescence and improves health in animal models (Blenis et al., 2014; Wang et al., 2017). Metformin, originally derived from *Galega officinalis* also known as Goat's Rue (Bailey, 2017), is a well-established anti-diabetic drug that, has previously been shown to inhibit the SASP by interfering with the NFκB pathway (Moiseeva et al., 2013).

NUTRACEUTICAL-BASED SENOLYTICS AND SENOSTATICS

Synthetic compounds with senolytic or senostatic properties can be effective, however, they are not specific, and systemic side effects can be severe and deleterious to healthy cells (Malavolta et al., 2018). Hence, a movement toward the research of natural based compounds (nutraceuticals) with potential anti-senescence properties has begun. Nutraceuticals are bioactive compounds derived from food, including plant material, with physiological benefits in the prevention or treatment of disease (Rafieian-Kopaei et al., 2014). For instance, polyphenols, found in high abundance in plants, are bio-active compounds with anti-oxidant and anti-inflammatory properties making them potential senostatics by negating the pro-oxidant and pro-inflammatory signaling of senescent cells (Gurău et al., 2018). The aim remains to find potential anti-aging therapies that are effective but exhibit minimal side effects, and some natural plant-based compounds could fit this criterion. Below, we will discuss studied examples of nutraceuticals that could act as senolytics or senostatics as illustrated in **Figure 2**.

Olive Phenols

The olive oil plant contains high amounts of phenolic compounds, which exhibit potential beneficial properties regarding cardiac health, cancer protection and antimicrobial effects (Tuck and Hayball, 2002; Marković et al., 2019). *In vitro,* two olive phenols called hydroxytyrosol (HT) and oleuropein aglycone (OLE) have shown to counteract senescence via significant reductions in SA-β-Gal staining, p16 levels and SASP levels in pre-senescent human lung fibroblasts (MRC5) and neonatal human dermal fibroblasts (Menicacci et al., 2017). HT has also been investigated in a UVA-induced senescence model in human dermal fibroblasts (HDFs) and significant reductions in SA-β-Gal positivity and mRNA expression levels for SASP related genes were observed after treatment (Jeon and Choi, 2018).

OLE reduces oxidative stress and inhibits mTOR, which is a key modulator in aging (Johnson et al., 2013; Sun et al., 2017). However, there are no data yet on the potential effects of OLE on organismal ageing. During the process of aging, the accumulation of misfolded and damaged proteins takes place, and these are degraded and removed through proteasome activity (Sáez and Vílchez, 2014). OLE has shown to improve proteasome activity, thereby delaying senescence in human fibroblasts. Furthermore, continuous OLE treatment of early passage human embryonic fibroblasts was shown to reduce ROS levels, curb the progression of the senescence phenotype by abating the changes in morphology seen in senescence, and lower cell mortality (Katsiki et al., 2007; Giovannelli, 2012; Menicacci et al., 2017).

Green Tea Catechins

Catechin is a tannin found in green tea that has high antioxidant properties. As a polyphenol, it exists in multiple forms, including Epigallocatechin gallate (EGCG), which is found abundantly in tea leaves (Unno, 2016). Research investigating the effects of EGCG against replicative senescence in primary cells (rat vascular smooth muscle cells, HDF (human dermal fibroblasts)

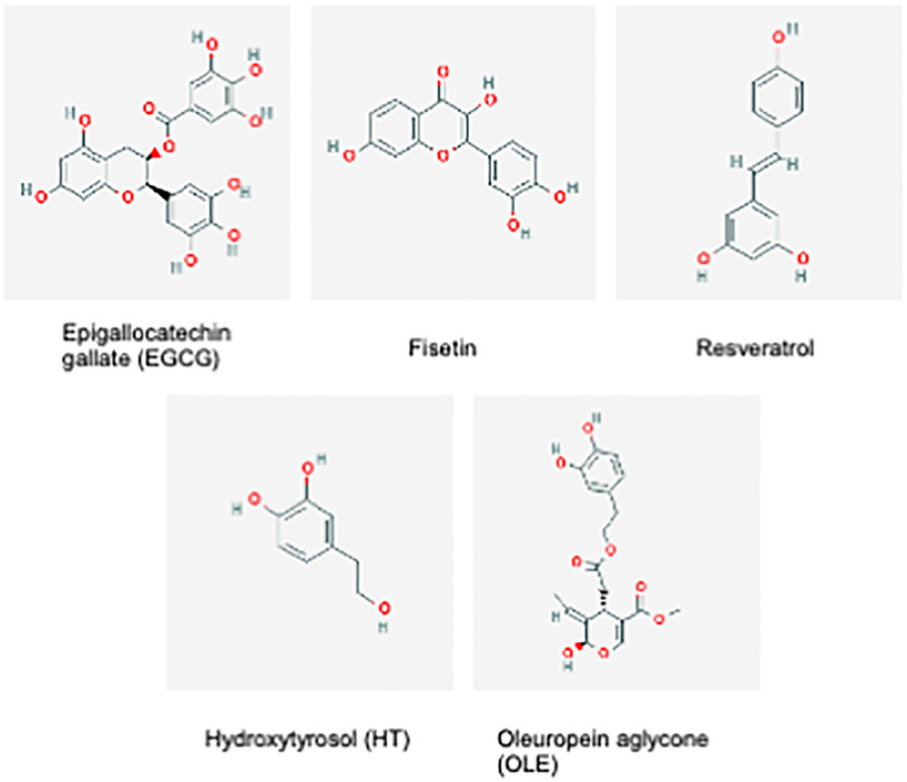

FIGURE 2 | Chemical structures of Epigallocatchin gallate (EGCG), Fisetin, Resveratrol, Hydroxytyrosol (HT) and Oleuropein aglycone (OLE). These compounds consist of either phenolic of polyphenolic alcohol groups (National Center for Biotechnology Information, 2019a,b,c,d,e).

and human articular chondrocytes), has shown the potential senostatics effects of the nutraceutical (Han et al., 2012). SA-β-Gal staining of HDF cells treated with EGCG at early and late passages showed fewer positive cells in treated than controls (Han et al., 2012). Moreover, p53 was shown to be significant reduced in EGCG treated cells (Han et al., 2012). However, cell cycle analysis of HDFs with and without EGCG treatment showed that treated cells had a similar percentage of cell in S phase as control cells.

EGCG was found to suppress premature senescence in preadipocytes, with treated cells showing significant downregulation of ROS, SASP and p53 mediated cell cycle arrest, in addition to the suppression of the anti-apoptotic protein BCL-2 and an increase in cell death (Kumar et al., 2018). This suggests that EGCG could have senolytic properties.

The effect of green tea catechins in aging was studied using a senescence accelerated mouse model ($SAMP_{10}$), which exhibits a short life span, cerebral atrophy and cognitive dysfunction (Unno et al., 2006). Between the ages of 1 and 15 months, mice were fed 35 mg/kg/day of green tea catechins. Analysis of blood and brain tissue found a decrease in oxidative DNA damage in the catechin-fed mice along with higher memory retention (Unno et al., 2006). A study investigating the effects of EGCG in healthy rats showed a median increase of lifespan by 8–12 weeks compared to controls. There was a significant decrease in liver and kidney IL-6 and ROS levels of the EGCG treated group, both of which are known to be upregulated with increasing age (Niu et al., 2013). This shows the potential beneficial systemic effects of EGCG in various organs.

However, an Intervention Testing Program was carried out, in which green tea extract was administered to a genetically heterogenous mouse model from a young age. Results showed no significant changes to lifespan (Strong et al., 2012). Although, the limited research of EGCG in connection to aging does not allow for definite conclusions, it has been widely used as a cosmetic for its potential skin protective effects its widely researched as a cosmetic additive having shown skin protective effects (Kim et al., 2018) and is available commercially in the form of green tea extract tablets.

Fisetin

Fisetin is bioactive flavonol molecule found in fruits and vegetables such as cucumber, apple, grape and onions, with the highest concentration being found in strawberries (Khan et al., 2013). It has established antioxidant, apoptotic and anti-proliferative qualities (Khan et al., 2013). Application of 5 μM Fisetin for 48 h led to a significant reduction in SA-β-Gal positivity in oxidative-stress induced senescent murine embryonic fibroblasts (Yousefzadeh et al., 2018b). Progeroid $Ercc1^{-/\Delta}$ and aged wild type mice receiving a diet supplemented with fisetin for two 14-day periods over 14 weeks at a concentration of 60 mg/kg/day had significantly lowered p16 expression in adipose tissue, in addition to decreased SASP.

Naturally aged C57BL/6 mice treated orally at 22–24 months with 100 mg/kg fisetin for 5 days showed a reduction in senescent cells in white adipose tissue (Yousefzadeh et al., 2018b). This result was mimicked in human adipose tissue explants that had been treated with fisetin and cultured *ex vivo*, showing reduction in SASP markers, IL-6, IL-8, MCP-1, and SA-β-Gal activity (Yousefzadeh et al., 2018b). Additionally, fisetin treatment at 85 weeks of age significantly prolonged life-span of these mice by an additional 3 months.

This research has given rise to the Alleviation by Fisetin of frailty, Inflammation and related measures in Older Adults (AFFIRM-LITE) clinical trial. Currently in the recruiting phase, the trial hopes to recruit 40 participants between the ages of 70–90 to take an oral 2-day dose of a placebo or fisetin at 20 mg/kg/day with analysis focusing on markers of frailty, inflammation, insulin resistance and bone metabolism (Clinicaltrials.gov, 2019a).

Resveratrol

Resveratrol is a stilbene polyphenol commonly found in pigmented fruits and vegetables, such as grapes and berries (Risuleo, 2016; Salehi et al., 2018). Biological activities of resveratrol include being antitumor, phytoestrogenic, antioxidant and antiviral (Risuleo, 2016). In senescence, resveratrol has been investigated for its effects on WI-38 human fibroblasts and HT-1080 cells with inducible ectopic p21. With the application of resveratrol at 50 μM, the senescent morphology of both SIPS model-based cell types was prevented, suggesting a senostatic effect. Moreover, at these concentrations, there was a 2-fold increase in cell number, showing that resveratrol helps overcome the cell cycle arrest. Of note, the polyphenol proved to be cytotoxic only at concentrations over 200 μM (Demidenko and Blagosklonny, 2009).

Resveratrol treatment in endothelial progenitor cells (EPCs) also showed prevention of replicative senescence, with reduced SA-β-Gal positive staining in comparison to controls (Xia et al., 2009). Treatment also demonstrated an increase in proliferative and migrating capabilities of EPCs, alongside a dose-dependent increase in telomerase activity, further highlighting the potential anti-senescence effects of resveratrol. This increase in telomerase activity was attributed to the activation of the PI3K-AKT pathway, which was shown to be phosphorylated in a resveratrol dose-dependent manner (Xia et al., 2009).

Moreover, when a large-scale *in vivo* study of resveratrol in genetically heterogenous (outbred) mice was conducted in parallel with rapamycin treatment, analysis of activity showed that there was no significant difference between control and resveratrol-treated mice. However, rapamycin-treated mice showed a 10% and 18% increase in median survival in males and females, respectively (Miller et al., 2010). The study highlighted the minimal effect resveratrol has on overall survival *in vivo*, even if *in vitro* studies suggested potential anti-senescence properties. In line with this, *in vivo* research from an Interventions Testing Program, which investigated the effect of administering resveratrol to a genetically heterogenous mouse model from a young age, showed no significant positive or negative effects in lifespan (Strong et al., 2012).

Nevertheless, a clinical trial involving the administration of resveratrol in older patients is underway, with participants taking a placebo, 1000 mg or 1500 mg per day in capsule form. The study will look at mitochondrial and physical function, focusing on the effects in muscle. Measures of output include levels of mitochondrial enzymes, walking speed, blood glucose, blood pressure, AMPK protein levels and resistance to muscle fatigue (REVIVE – Clinicaltrials.gov, 2019b). Despite studies and research still being conducted on resveratrol, it is also widely available to the public in tablet form, and is found in many anti-aging based cosmetics.

CONCLUSION: A LONG ROAD AHEAD

With life expectancy on the increase and a greater geriatric population with chronic illnesses, management of the elderly should be focused on prevention of tissue dysfunction and maintaining a better quality of life for longer times, what is known as increasing health span. In the last decade, the field of senescence has seen the emergence of senolytic therapies, which are being investigated intensively through *in vivo* research and, recently, a move toward clinical trials. Currently, a universal standard for senescence research isn't recognized. Different groups were able to create models *in vitro* using multiple inducers to cause DNA damage. All research should aim to test nutraceuticals first on an *in vitro* model preferably in human derived cells which have entered replicative senescence through multiple passaging. However, this kind of cell preparation would take a long time to reach senescence and only produce a finite number of cells for testing. Alternatively, using an *in vitro* mouse replicative senescence model is more feasible, as cells they stop proliferating after 5–6 passages (Khan and Gasser, 2016). Senolytics or senostatics could next be applied *in vivo* using aged wild-type mice. Despite this, multiple mouse models exist to test senolytic and senostatic application such as progeroid mice, which mimic the human syndrome known as progeria. Mice naturally reach full term within 6 months and can arise from $BubR1^{H/H}$, $Zmpste24^{-/-}$, $Sod1^{-/-}$ or $Ercc^{-/}\Delta$ strains (Yousefzadeh et al., 2018a).

Senolytics could be used as a preventative in the elderly, as a supplement to clear senescent cells to thus improve or maintain tissue and organ health. They are also being looked at as an adjuvant cancer therapy, with the aim of clearing treatment-induced senescent cells and thus reducing the probability of relapse (Tabasso et al., 2019). They are also being investigated in telomere biology. Telomerase is capable of reversing telomere erosion and has been targeted in peripheral blood mononuclear cells by plant derived telomerase activators (Tsoukalas et al., 2019). TA-65, a derivative of *Astragalus membranaceus*, has been shown to elongate telomerase in a telomere deficient mouse model (de Jesus et al., 2011).

Recent studies argue for a pathogenic role of senescent cells, which would contribute to a range of aging related diseases, such as osteoarthritis (Jeon et al., 2018), cardiovascular disease (Childs et al., 2018) and cataract (Fu et al., 2016). Senescent cells are found in aging related cognitive decline but

also in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Baker and Petersen, 2018) which are typically identified clinically and further characterized through imaging or at autopsy. Therefore, the possible application of senolytics in a wide range of clinical scenarios is becoming an attractive concept (Paez-Ribes et al., 2019).

However, for senolytics to be widely used in aged but otherwise healthy populations to prevent tissue dysfunction, unwanted side effects have to be kept to the minimum. The use of nutraceutical based senolytics could result in fewer complications, while retaining anti-senescent effects. Despite promising *in vitro* reports, the data on the *in vivo* efficacy of nutraceutical senolytics is still sparse and, in some cases, contradictory. Thus, more research is still needed to determine whether they could be an attractive alternative to the most used chemical senolytics, such as dasatinib + quercetin, which have shown promising results in preliminary short-term clinical trials (Hickson et al., 2019).

It also has to be taken in consideration that this therapeutic field is new, and the use of nutraceuticals as senolytics may also come with its drawbacks. The potential toxicity of the compounds and their adverse interactions with existing therapies for other health issues, needs to be carefully investigated. Because of this, it is worrying that some of these compounds have reached the wider public without proper validation or complete safety studies. Caution should be exerted when dealing with them. The adoption of nutraceutical senolytics as harmless complements may need to be discouraged until more information is available.

AUTHOR CONTRIBUTIONS

AK researched and composed the manuscript. CS discussed the data. CS and SM edited the manuscript.

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REVIEW
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Senescence as an Amyloid Cascade: The Amyloid Senescence Hypothesis

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Due to their postmitotic status, the potential for neurons to undergo senescence has historically received little attention. This lack of attention has extended to some non-postmitotic cells as well. Recently, the study of senescence within the central nervous system (CNS) has begun to emerge as a new etiological framework for neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). The presence of senescent cells is known to be deleterious to non-senescent neighboring cells via development of a senescence-associated secretory phenotype (SASP) which includes the release of inflammatory, oxidative, mitogenic, and matrix-degrading factors. Senescence and the SASP have recently been hailed as an alternative to the amyloid cascade hypothesis and the selective killing of senescence cells by senolytic drugs as a substitute for amyloid beta (Aß) targeting antibodies. Here we call for caution in rejecting the amyloid cascade hypothesis and to the dismissal of Aß antibody intervention at least in early disease stages, as Aß oligomers (AßO), and cellular senescence may be inextricably linked. We will review literature that portrays AßO as a stressor capable of inducing senescence. We will discuss research on the potential role of secondary senescence, a process by which senescent cells induce senescence in neighboring cells, in disease progression. Once this seed of senescent cells is present, the elimination of senescence-inducing stressors like Aß would likely be ineffective in abrogating the spread of senescence. This has potential implications for when and why AßO clearance may or may not be effective as a therapeutic for AD. The selective killing of senescent cells by the immune system via immune surveillance naturally curtails the SASP and secondary senescence outside the CNS. Immune privilege restricts the access of peripheral immune cells to the brain parenchyma, making the brain a safe harbor for the spread of senescence and the SASP. However, an increasingly leaky blood brain barrier (BBB) compromises immune privilege in aging AD patients, potentially enabling immune infiltration that could have detrimental consequences in later AD stages. Rather than an alternative etiology, senescence itself may constitute an essential component of the cascade in the amyloid cascade hypothesis.

Keywords: Alzheimer's disease, Neurodegeneration, senescence, senescence associated secretory phenotype, cell cycle, reactive oxygen species

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INTRODUCTION

Alzheimer's disease (AD) is an as of yet incurable neurodegenerative disorder (Selkoe and Hardy, 2016). Its cardinal features are senile plaques formed by nonvascular extracellular deposits of amyloid fibrillary amyloid beta (Aß) and intra-neuronal neurofibrillary tangles (NFT) consisting of aggregates of hyperphosphorylated tau protein (Hyman et al., 2012; Deture and Dickson, 2019). The amyloid cascade hypothesis posits Aβ is the cause of AD, triggering the formation of NFT, neuronal cell loss, vascular damage, and dementia (Selkoe and Hardy, 2016). In the modernized version of the amyloid cascade hypothesis, rather than senile plaques it is now thought that soluble Aβ oligomers (AßO) are the major driver of AD. Aβ peptides result from enzymatic cleavage of the disease-associated amyloidogenic processing of APP (O'Brien and Wong, 2011). Aβ peptides of different lengths can seed formation of AßO, protofibrils, fibrils, and senile plaques (Larson and Lesné, 2012; Karran and De Strooper, 2016; Selkoe and Hardy, 2016; Deture and Dickson, 2019; Panza et al., 2019). The 40 (Aβ40) and 42 (Aβ42) amino acid length peptides are the most intensely studied, with Aβ42 being the most amyloidogenic.

Beyond the fact that extracellular Aβ deposits are a pathological diagnostic hallmark (Hyman et al., 2012), genetic data supporting the amyloid cascade hypothesis is rather powerful (Selkoe and Hardy, 2016). Clinical trial "failures" of Aβ-depleting antibodies are also powerful argument against the hypothesis (Karran and De Strooper, 2016; Panza et al., 2019). There are several antibodies designed to target Aβ, which in turn elicit their putative therapeutic effects by targeting different species of Aβ (Panza et al., 2019). Clearance of amyloid plaques by AN-1792 did not prevent disease progression (Holmes et al., 2008). As noted, neurotoxicity is thought to depend on AβO (Selkoe and Hardy, 2016). Adecanumab targets the neurotoxic effects associated with oligomers as well as prompting the dissolution of Aβ plaques (Sevigny et al., 2016; Panza et al., 2019). Despite this, by the first quarter of 2019 the Aducanumab ENGAGE (NTC02484547) and EMERGE (NTC02477800) trials were halted as a result of futility analysis (Schneider, 2020).

In the wake of this mounting discouraging clinical evidence, encouraging reports simultaneously begun to emerge supporting a new class of drugs known as senolytics as a novel therapeutic avenue for AD (Bussian et al., 2018; Musi et al., 2018; Zhang et al., 2019). Senolytics elicit the selective killing of senescent cells (Kirkland et al., 2017) and there was already proof of concept for their potential use in Parkinson's disease (PD; Chinta et al., 2018). Most senescent cells develop a senescence associated secretory phenotype (SASP) involving the secretion of cytokines, chemokines, mitogenic factors, and proteases that can damage the surrounding microenvironment (Acosta et al., 2008; Coppé et al., 2008, 2010a,b; Kuilman et al., 2008; Neves et al., 2015). Senolytics eliminate senescent cells and therefore the SASP (Kirkland et al., 2017). Within the context of AD, proof of concept for senolytic therapies was first provided in mice expressing wild type or mutated human tau isoforms but lacking Aβ pathology (Bussian et al., 2018; Musi et al., 2018). A role for Aβ in the development of cellular senescence was rejected (Musi et al., 2018). Paradoxically a senolytic intervention was later proven successful in transgenic mice presenting Aβ without tau pathology (Zhang et al., 2019), underscoring that each of the proposed mechanisms of action of senolytic intervention are radically different.

Unexpectedly, by the last quarter of 2019 a subgroup of patients receiving high-dose Aducanumab treatments were claimed to have met its target outcomes in prodromal and early AD patients. Although not free of skepticism (Schneider, 2020), this has prompted the re-launch of a clinical trial to assess high dose Adecanumab to begin in March 2020 (NCT04241068). It is possible that Aβ may trigger a pathological cascade of events that may evolve on its own independent of the continued presence of Aβ and therefore its clearance after the cascade has already been set in motion may be too late (Selkoe and Hardy, 2016). Implementing early intervention in AD is particularly complex considering that the first signs of Aβ pathology can precede clinical AD by 15 to 25 years (Bateman et al., 2012; Villemagne et al., 2013; Vermunt et al., 2019). Both proponents and detractors of the amyloid cascade hypothesis seem to be in agreement on one thing; Aβ clearance is not likely effective in mild-to-moderate clinical AD. Antibodies such as Aducanumab may therefore only be effective at early stages, prior to the activation of the cascade. We arguably need to shift to therapies suppressing the cascade and yet we do not know what the cascade is. As we will review, the cascade may be senescence itself.

CELLULAR SENESCENCE

Senescence is traditionally regarded as an oncosuppressive mechanism that imposes an irreversible cell cycle withdrawal (Gorgoulis et al., 2019). The classical senescence-inducing stressor is DNA damage signaling associated to telomere attrition, better known as replicative senescence (RS; Hayflick and Moorhead, 1961; Gorgoulis et al., 2019). RS marks the end of the replicative lifespan of the cell, but cells can undergo senescence before reaching it. This is often termed stress-induced premature senescence (SIPS). Multiple stressors can result in SIPS including reactive oxygen species (ROS), oncogenes, and ionizing radiation (IR; Gorgoulis et al., 2019). Although there are exceptions, most stressors result in SIPS by causing persistent DNA damage either directly or indirectly (Alimonti et al., 2010; Freund et al., 2011; Ziegler et al., 2015; Wiley et al., 2016; Gorgoulis et al., 2019). ROS and IR can directly elicit DNA damage, while oncogenes often–albeit not invariantly-do so indirectly by aberrantly activating the DNA replication machinery (Halazonetis et al., 2008; Lecot et al., 2016; Gorgoulis et al., 2019). SIPS is often associated with cell cycle dysregulation but nevertheless cells that are not mitotically active can undergo senescence (Toledo et al., 2008; Alimonti et al., 2010).

Evidence for SIPS in neurons and non-neuronal cell types has been provided in *in vitro* and *in vivo* models of AD (Bhat et al., 2012; He et al., 2013; Zhang et al., 2019), and senescence markers have been described in neurons of AD patients (Arendt et al., 1996, 1998; McShea et al., 1997; Lüth et al., 2000). As

we will argue, there is reasonable evidence that AβO is a SIPS-inducing stressor. Senescence is a largely irreversible phenotype (Gorgoulis et al., 2019). It follows that the clearance of AβO should prevent the onset of cellular senescence but not revert it once it is established. If senescence is the actual cascade of the amyloid cascade hypothesis it may be largely irrevocable, potentially explaining the failure of some Aβ-targeting antibodies in clinical trials.

SENESCENCE MARKERS

Although it is not always the case, when it comes to neurons it is common to see use of the term "senescent-like phenotype" (Walton and Andersen, 2019). Senescent-like is a conservative denomination that reflects potentially insurmountable challenges in the study of senescence in neurons.

There are no universal markers of senescence and therefore use of a single senescent marker is not a reliable mean of proving senescence in any cell type (Hernandez-Segura et al., 2017, 2018; Gorgoulis et al., 2019). For example, a widely used senescence marker in non-neuronal cells is senescence-associated-beta-galactosidase (SA-ß-Gal; Debacq-Chainiaux et al., 2009). However, SA-ß-Gal has been shown to be up-regulated in neurons that lack other senescence markers (Piechota et al., 2016; Musi et al., 2018; Walton and Andersen, 2019). SA-ß-Gal is lysosomal and reflects the increased lysosomal mass in senescent cells but is not necessary nor causes senescence (Kurz et al., 2000; Lee et al., 2006; Hernandez-Segura et al., 2018; Gorgoulis et al., 2019). SA-ß-Gal in neurons has indeed been argued to simply reflect senescence-unrelated lysosome biogenesis (Piechota et al., 2016; Musi et al., 2018; Walton and Andersen, 2019). In order to prove neuronal senescence, multiple markers of senescence should be used which may include $p16^{INK4A}$, $p21^{CIP1}$, Lamin B1, HMGB1, and amongst others (Hernandez-Segura et al., 2018; Gorgoulis et al., 2019). The phenotype should also be relatively stable, as cellular senescence is considered an irreversible phenotype. With the aforementioned in mind, we propose that: 1. Multiple senescence markers need to be used to assess senescence in neurons; 2. The mechanism of action of any identified senescence-inducing stressor should be consistent with that in mitotically-competent cells; and 3. The phenotype should still persist after the senescence-inducing stressor has been removed. If successfully demonstrated, this would provide a convincing characterization of neuronal senescence.

Arguably the gold standard for identifying cellular senescence is demonstrating an irreversible block on cellular proliferation. Normally differentiated neurons never proliferate under physiological conditions (Frade and Ovejero-Benito, 2015). When non-physiological means are used to force neuronal cell division, the rate of success is under 5% and thus far entails detection of only a single cell division (Walton et al., 2019). Because neurons in general do not proliferate, it is not possible to prove that an irreversible block on proliferation is caused by senescence rather than their native postmitotic state. The best we can hope for is to characterize a senescent-like phenotype that is consistent with senescence. Nevertheless, given that this review concerns several cell types, to avoid cumbersome phrasing we will hereafter use the term "senescence" for neurons, and glial cells alike.

NEURONAL CELL CYCLE ENTRY IN AD

Aberrant cell cycle entry in neurons of AD patients is well established (Frade and Ovejero-Benito, 2015). It is important to distinguish this from adult neurogenesis. Adult neurogenesis entails the physiological proliferation of neuronal precursor cells (NPCs), which latter differentiate into two specific neuronal types in very restricted niches (Gross, 2000; Kempermann et al., 2018). In contrast, aberrant activation of the cell cycle in AD patients takes place within neurons and is strictly pathological (Frade and Ovejero-Benito, 2015). Cell cycle reactivation in neurons of AD patients does not result in cell division (Frade and Ovejero-Benito, 2015). Neurons in the brains of AD patients have been shown to survive for extended periods of time after cell cycle entry (Arendt et al., 2010). This is consistent with cells having undergone cellular senescence after an abortive cell cycle (Kastan and Bartek, 2004; Halazonetis et al., 2008; Vitale et al., 2011; Johmura et al., 2014). In support of this, up-regulation of the senescence marker $p16^{INK4A}$ was reported within pyramidal neurons of AD patients in several older historic studies, suggesting the potential involvement of senescence in AD as early as two decades ago (Arendt et al., 1996, 1998; McShea et al., 1997; Lüth et al., 2000).

Given that potential evidence for senescence in AD patients has existed for years, it is somewhat curious that research of senescence in neurobiology is only now blooming (Walton and Andersen, 2019). The historic paucity of studies of senescence in AD may be explained by a misunderstanding of mitotic cell biology. For many years, forcing primary neurons in culture to enter the cell cycle resulted in cell death (Frade and Ovejero-Benito, 2015). In one way or another, many thought that cell death was a consequence of the postmitotic status of neurons, likely influenced by the erroneous presumption that mitotically-competent cells never undergo cell death upon cell cycle entry. Oncogenes can and do force cell cycle entry in mitotically-competent cells, where cell death is an indispensable response to prevent carcinogenic cell division (Kastan and Bartek, 2004; Brito and Rieder, 2006; Halazonetis et al., 2008; Vitale et al., 2011; Johmura et al., 2014). The first *in vitro* models of an abortive cell cycle with viable exit were achieved by inactivating the same machinery that causes oncosuppressive cell death and senescence in mitotically-competent cells (Barrio-Alonso et al., 2018; Walton et al., 2019). Hence, cell death after forced cell cycle suggests that neurons possess at least one of two major oncosuppressive mechanisms present in virtually all mitotically-competent cells. The other described mechanism for oncogenic suppression is cellular senescence (Kastan and Bartek, 2004; Halazonetis et al., 2008; Vitale et al., 2011; Childs et al., 2014; Johmura et al., 2014; Lecot et al., 2016; Gorgoulis et al., 2019).

As noted, senolytics have been demonstrated to have a therapeutic effect in a tau transgenic mice models

(Bussian et al., 2018; Musi et al., 2018). Whether it is the killing of senescent neurons or senescent glia remains unclear (Walton and Andersen, 2019). In the case in which it was argued that senescent neurons were killed, it was speculated that senescence was caused by NFT mediated cell cycle entry (Musi et al., 2018). Indeed, there are some reports of tau-induced cell cycle entry in neurons (Andorfer et al., 2005; Bonda et al., 2009; Jaworski et al., 2009; Seward et al., 2013a; Hradek et al., 2014), albeit entry mediated by NFT has been contested (Jaworski et al., 2009), and at least in some cases it involves Aβ (Seward et al., 2013a; Hradek et al., 2014). When compared to tau-related models, cell cycle deregulation has been vigorously researched in models of Aβ pathology (Copani et al., 1999, 2006; Giovanni et al., 1999, 2000; Park et al., 2000; Wu et al., 2000; Kruman et al., 2004; Sortino et al., 2004; Yang et al., 2006; Caraci et al., 2008; Majd et al., 2008; Varvel et al., 2008, 2009; Bhaskar et al., 2009; Lopes et al., 2009, 2010; Li et al., 2011; Modi et al., 2012, 2015; Seward et al., 2013b; Hradek et al., 2014; Merlo et al., 2015; Caraci et al., 2016; Leggio et al., 2016; **Table 1**). Virtually all reports report cell cycle related cell death, which is of relevance for senescence. As noted above, aberrant cell cycle activation should result in cell death or senescence. For example, the E2F1 transcription factor, a major driver of G1/S transition, can also result in cell death or senescence if aberrantly activated (Johnson and DeGregori, 2012). Fittingly, Aβ-mediated cell death is at least in part mediated by E2F1 (Giovanni et al., 1999, 2000). It may be argued that if aberrant cell cycle entry is the means by which neurons senesce in AD, Aβ is the most likely cause.

Aβ, ROS, AND SENESCENCE

Amyloid beta pathology models have been used to study senescence despite the lack of studies in neuronal cell cycle dysregulation. Aβ peptide administration is shown to induce senescence in astrocytes *in vitro* and the number of astrocytes with a senescent phenotype are increased in AD patients (Bhat et al., 2012). Increased expression of p16 is reported in neurons of an AD mouse model and AβO reportedly increase p16 levels *in vitro* (Wei et al., 2016). Mechanisms involving ROS are known to induce senescence in mitotically competent cells (Gorgoulis et al., 2019). AβO exposure induces senescence via ROS in NPCs from wild type (WT) and AD mouse models (He et al., 2013). DNA damage can result in persistent DDR and p21-mediated mitochondrial dysfunction leading to increased ROS production (Passos et al., 2010). ROS regenerates DNA damage, locking cells into senescence. A similar senescent-like phenotype has been described in the neurons of aging mice (Jurk et al., 2012). Thus, increases in ROS downstream of AβO is a plausible mechanism for senescence induction.

There is extensive research linking Aβ to ROS in neurons. Extracellular actions of AβO elicits neurotoxic effects via ROS production and Ca^{2+} dysregulation by binding to N-Methyl-D-Aspartate receptor (NMDAr) on excitatory synapses (De Felice et al., 2007; Lacor et al., 2007; Shelat et al., 2008; Gunn et al., 2016; Smith and Strittmatter, 2017). In fit, toxic, or physiological ROS levels are produced downstream of NMDAr excitotoxic or normal activation, respectively (Dugan et al., 1995; Reynolds and Hastings, 1995; Ward et al., 2000; Brennan et al., 2009). AβO exposure *in vitro* induces ROS and JNK pathway activation (Kadowaki et al., 2005), up-regulation of p38, and Ca^{2+} influx (Drews et al., 2016). Intracellularly-acting AβO has been associated with oxidative stress and ROS production via mitochondrial dysfunction, Ca^{2+} perturbation, and trace element interactions. AβO impacts mitochondrial dysfunction via inhibition of nuclear protein import (Sirk et al., 2007; Cenini et al., 2016), abnormal fission and fusion dynamics (Barsoum et al., 2006; Zhang et al., 2008; Hung et al., 2018), ATP synthase activity impairment (Schmidt et al., 2008; Cha et al., 2015; Beck et al., 2016), and an up-regulation of mitochondrial production of ROS (Rhein et al., 2009; Mao and Reddy, 2011; Mossmann et al., 2014; Kaminsky et al., 2015; Hung et al., 2018; Fang et al., 2019). AβO accumulation within the mitochondria directly interacts with ABAD and cyclophilin D, promoting ROS leakage, membrane potential change, and Ca^{2+} dysregulation (Lustbader et al., 2004; Hemmerová et al., 2019; Morsy and Trippier, 2019). Perturbation of mitochondrial or endoplasmic reticulum (ER)-mediated Ca^{2+} homeostasis may underlie intracellular-mediated Aβ excitotoxicity. Intracellular AβO modulates resting cytosolic free Ca^{2+} levels (Sanz-Blasco et al., 2008; Demuro and Parker, 2013; Müller et al., 2018; Jadiya et al., 2019), remodels intra-organellar Ca^{2+} by disruption of mitochondria-associated ER membranes (MAMs; Müller et al., 2018), and alters Ca^{2+} release from internal stores (Sanz-Blasco et al., 2008; Müller et al., 2018; Calvo-Rodriguez et al., 2019; Jadiya et al., 2019), which can lead to ROS formation and further pathological oligomerization of Aβ (Kadowaki et al., 2005; Meli et al., 2014; Kaminsky et al., 2015; Boyman et al., 2020). Further, production of ROS can be mediated by Aβ interaction with transition metals, specifically copper or iron, to produce hydrogen peroxide, and superoxide via Fenton reaction (Jomova et al., 2010; Cheignon et al., 2018; Masaldan et al., 2018; Butterfield and Halliwell, 2019; Gomes et al., 2019). AβO-dependent increase in ROS and activation of the cell cycle machinery are alternative ways in which AβO can induce potentially senescence in several cell types (**Figure 1**).

THE DELETERIOUS EFFECTS OF SENESCENCE

Within a senescence-based hypothesis of AD, the consensus is that the SASP is the culprit for subsequent observed disease phenotypes (Bussian et al., 2018; Musi et al., 2018; Walton and Andersen, 2019; Zhang et al., 2019). Although the SASP is a very heterogeneous phenotype (Basisty et al., 2020), secreted components often include interleukin IL-6, chemokine IL-8, or TGFβ (Coppé et al., 2010a; Neves et al., 2015; Gorgoulis et al., 2019). Prominent SASP regulators include p38MAPK, NF-kB C/EBPβ, GATA4, and mammalian target of rapamycin (mTOR). Senescent markers are up-regulated in the astrocytes of AD patients and Aβ has been reported to elicit senescence in astrocytes *in vitro* via ROS accompanied by p38, IL-6, and IL-8 up-regulation (Bhat et al., 2012). Further, oligodendrocyte

TABLE 1 | Literature describing cell cycle entry downstream of Aβ treatment or in mouse models of AD.

					Max Percent Putative Cell Cycle Entry Reported			
References	**Model**	**Maturation**	**Method**	**Neuron marker**	**Aβ25-35**	**Aβ1-40**	**Aβ1-42**	**Control**
Copani et al., 1999		8–12 DIV	DNA content	Ref Cult	2–10%	2–10%	2–10%	0.1–2%
Wu et al., 2000	Culture	5–8 DIV	BrdU	MAP2	2–10%			2–10%
Copani et al., 2002	Culture	8–9 DIV	DNA content	Ref Cult	2–10%			0.1–2%
Sortino et al., 2004	Culture	NS	DNA content	Ref Cult	10–20%			0.1–2%
Kruman et al., 2004	Culture	4–8 DIV	DNA Content	MAP2			10–20%	2–10%
Copani et al., 2006	Culture	8–12 DIV	DNA Content	Ref Cult	2–10%			0.1–2%
Majd et al., 2008	Culture	Adult mice	Cyclin D1 IHC	NS			50–75%	0.1–2%
Majd et al., 2008	Culture	Adult mice	Cyclin B1 IHC	NS			20–50%	0%
Caraci et al., 2008	Culture	8–11 DIV	DNA Content	Ref Cult	2–10%			0.1–2%
Varvel et al., 2008	Culture	7–8 DIV	BrdU	MAP2			20–50%	2–10%
Varvel et al., 2008	R1.40	6–12 M	Cyclin A		R1.40	20–50%		0–10%
Varvel et al., 2008	R1.40	6–12 M	Cyclin D		R1.40	50–75%		0–10%
Varvel et al., 2008	R1.40	6–12 M	Polyploid		R1.40	10–50%		0–10%
Bhaskar et al., 2009	Culture	21–22 DIV	BrdU	MAP2			20–50%	2–10%
Bhaskar et al., 2009	Culture	21–22 DIV	BrdU	MAP2	R1.40	20–50%		10–20%
Varvel et al., 2009	R1.40	6 M	Cyclin D	NeuN	R1.40	20–50%		
Varvel et al., 2009	R1.40	6 M	Cyclin A	NeuN	R1.40	20–50%		
Varvel et al., 2009	R1.40	6 M	Polyploid	NeuN	R1.40	10–20%		
Lopes et al., 2010	WT ICV	8 W	Cdk4 IHC HP	NS		10–20%		2–10%
Lopes et al., 2010	WT ICV	8W	Cyclin D1 IHC HP	NS		2–10%		2–10%
Li et al., 2011	R1.40	6–12 M	PCNA		R1.40 (HP)	15–30%		0–5%
Li et al., 2011	Tg2576	9–11 M	PCNA		Tg2576 (HP)	0–5%		0–5%
Li et al., 2011	5XFAD	6 M	PCNA		5XFAD (HP)	0–5%		0–5%
Li et al., 2011	APP8.9	14 M	PCNA		APP8.9 (HP)	0–5%		0–5%
Li et al., 2011	APP/PS1	6–7 M	PCNA		APP/PS1 (HP)	0–5%		0–5%
Modi et al., 2012	Culture	5 DIV	BrdU	NS			20–50%	2–10%
Hradek et al., 2014	3xTgAD	5–16 M	ppRb807	NS	3xTgAD	0.1–10%		
Hradek et al., 2014	3xTgAD	18–20 M	ppRb807	NS	3xTgAD	20–50%		
Hradek et al., 2014	3xTgAD	5–16 M	ppRb807	NS	3xTgAD	0.1–10%		
Hradek et al., 2014	3xTgAD	18–20 M	ppRb807	NS	3xTgAD	20–50%		
Merlo et al., 2015	Culture	NS	DNA Content	Ref Cult		10–20%		2–10%
Merlo et al., 2015	Culture	NS	Cyclin A2 ICC	NS		2–10%		2–10%
Modi et al., 2015	Culture	NS	BrdU	NS			10–20%	0.1–2%
Leggio et al., 2016	WT ICV	7 weeks	Cyclin A2 IHC Cx	NeuN			20–50%	2–10%
Leggio et al., 2016	WT ICV	7 weeks	Cyclin A2 IHC Hp	NeuN			20–50%	10–20%

We have not included research that assess cell cycle entry by bulk analysis (e.g., Western Blot, qPCR) as neuronal cultures invariantly have astrocyte contamination and brain lysates also contain glial cells. Consequently, distinguishing cell cycle activity in neurons from that of other cells is not possible. The models we have included are neuronal cell cultures (Culture), transgenic AD mouse models (R1.40, Tg2576, 5XFAD, APP8.9, APP/PS1, and 3xTgAD), and acute AD mouse models based on intracerebroventricular microinjection of Aβ peptides (ICV). "Method" refers to the manner in which cell cycle entry was assessed. Certain cell cycle markers are present under basal conditions and thus the data should be interpreted with caution. "Neuron marker" refers to the manner in which the authors have identified neurons to separate them from contaminating glial cells. "Ref Cult" is Reference Culture and alludes to cultures in which neuronal markers have been used to estimate glial cell contamination. Putative percent cell cycle entry is approximate. Aβ isoforms cannot be identified in transgenic models and therefore is not specified. Days in vitro (DIV), immunohistochemistry (IHC), hippocampus (Hp), cortex (Cx), and immunocytochemistry (ICC).

precursor cells (OPC) in an AD mouse model show a pro-inflammatory phenotype along with increases p16 and p21 expression near Aβ plaques and Aβ can induce senescence in cultured OPCs (Zhang et al., 2019). IL-6 and TGFβ mRNA are upregulated in AD patients (Luterman et al., 2000; Gruol, 2015), but inflammatory response from resident immune cells is also prominent in AD and cannot be ruled out (Heneka et al., 2015). However, active p38 overlaps and immunoprecipitates with NFT from neurons of AD patients, but not from healthy controls (Zhu et al., 2000). Aβ activation of p38 has been placed upstream of pathological tau phosphorylation in neuron cultures (Origlia et al., 2008; Munoz and Ammit, 2010). Aβ activates NF-kB in primary neurons and is found in astrocytes and neurons in proximity to senile or diffuse plaques as well as NFT-positive neurons in AD patients, but not in healthy controls (Terai et al., 1996; Kaltschmidt et al., 1997; Ferrer et al., 1998; Snow and Albensi, 2016). A "senescent-like" phenotype described in neurons of aging mice involves p38, ROS and intraneuronal IL-6

suggesting neurons may develop a "SASP-like" phenotype during normal aging (Jurk et al., 2012), which, in light of aforementioned evidence, could be exacerbated in AD.

It is plausible that AβO can result in senescence, with subsequent increases in p38 and NF-kB activity reflecting the development of the SASP. Given the association of active p38 and NF-kB with NFTs (Munoz and Ammit, 2010; Gruol, 2015), tau pathology may be part of the SASP and therefore a feature of senescence whether the latter is caused by Aβ or not (**Figure 1**). Hence, the cascade of the amyloid cascade hypothesis may be irreversible if it is indeed cellular senescence and deleterious if it also involves the SASP.

SECONDARY SENESCENCE

Senescent cells can induce senescence in neighboring non-senescent cells via a process known as paracrine senescence or secondary senescence (hereafter secondary senescence; Gorgoulis et al., 2019). Secondary senescence is dependent on SASP factors and has been shown to be transmitted by either diffusible factors, gap-junctions, or both (Hubackova et al., 2012; Nelson et al., 2012, 2018; Acosta et al., 2013; Jurk et al., 2014; da Silva et al., 2019). It may be expected that a population of senescent astrocytes, microglia, OPCs, and or NPCs radiate away from Aβ foci by secondary senescence. Consequently, these senescent cells would be expected to be found near regions of Aβ burden but not necessarily elsewhere, which does not appear to be consistent with what is known about AD progression. Senile plaques and NFT in AD largely follow a stereotypical pattern of neuroanatomical distribution (Hyman et al., 2012; Deture and Dickson, 2019). This has led to the Thal staging of amyloid phases (Thal et al., 2002) and the Braak staging based on hyperphosphorylated tau and NFTs (Braak and Braak, 1991; Braak et al., 2006). In Braak stages I and II, NFTs first develop in the trans-entorhinal cortex and layers two and four of the entorhinal cortex. In stages III and IV, NFT burden is also present in the hippocampus. In the last phase, and stages V and VI, tau pathology is spread to the neocortex. Paradoxically, the neocortex is the first site of Aβ deposits in Thal phase 1 (Thal et al., 2002). In phase 2/3 there is a spread to allocortical brain regions, including the hippocampus and entorhinal cortex. Stage 3, 4, and 5 entail a spread into subcortical and cerebellar regions. Albeit not impossible, this progression is hard to reconcile with the spread of AD neuropathology being driven by senescent astrocyte, microglia, OPCs, and or NPCs. In contrast, neurons emit long-range axonal projections that can span the entire brain. If cortical neurons senescence and develop the SASP in response to AβO, then SASP and secondary senescence can reach distant regions that are free of Aβ. Importantly, the trans-entorhinal region converges widespread afferent projections from the neocortex (Vismer et al., 2015). At least in theory, a sparse population of neocortical senescent neurons spawned by diffuse AβO can converge their axonal projections into trans-entorhinal and entorhinal cortices, potentially allowing spread of the SASP and secondary senescence from the entire cortex. While cellular senescence and the SASP may, respectively, render the amyloid cascade irreversible and deleterious, secondary senescence can explain why the cascade results in the striking topographical spread of AD neuropathology.

SENESCENCE IMMUNE SURVEILLANCE AND IMMUNE PRIVILEGE

Outside the central nervous system (CNS) senescent cells are normally cleared by the innate immune system (Lujambio, 2016).

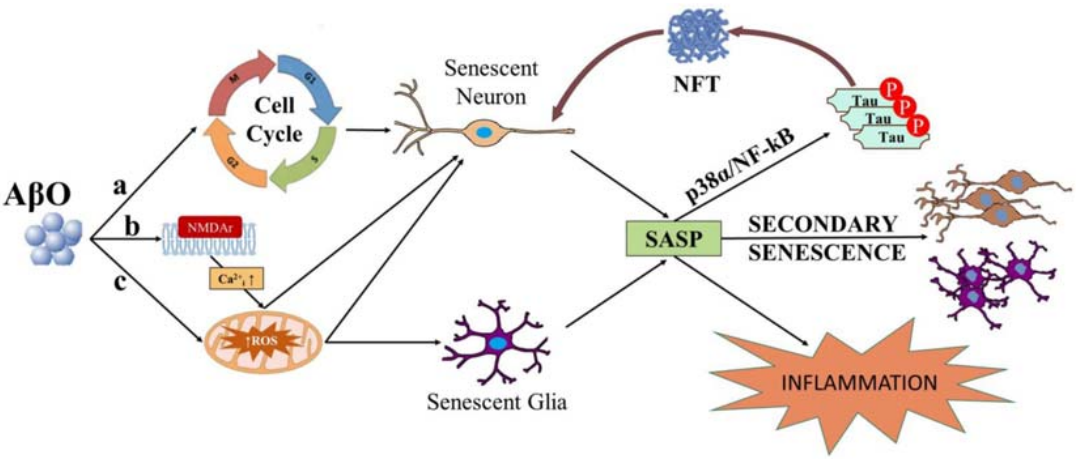

FIGURE 1 | Diagram depicting possible ways in which AβO can induce senescence in neurons and or glial cells. AβO can induce unscheduled cell cycle entry in neurons leading to senescence (a). AβO can also induce ROS, either downstream of aberrant activation of post-synaptic receptors (e.g., NMDAr) in neurons (b) or independent of receptor activation in neurons and or glial cells (c). Senescent neurons and or glial cells may develop the SASP. SASP factors released from senescent neurons and or glial cells can result in inflammation and secondary senescence, spreading the disease. Major SASP regulators p38 and NF-kB may also elicit tau hyperphosphorylation, potentially linking the SASP to NFTs in neurons.

Senescent cells have been shown to be cleared by natural killer cells (NKs; Iannello et al., 2013; Eggert et al., 2016; Sagiv et al., 2016; Antonangeli et al., 2019; Muñoz et al., 2019; Pereira et al., 2019) and macrophages (Xue et al., 2007; Krizhanovsky et al., 2008; Kang et al., 2011; Muñoz-Espín et al., 2013). The way in which NKs kill senescent cells in the periphery is well-understood (Antonangeli et al., 2019). The activation of NKs depends on a complex interplay between their activator and inhibitory receptors. Specifically, human senescent cells up-regulate MICA, and ULBP2, ligands for the stimulatory receptor NKG2D (Antonangeli et al., 2019). Given the CNS is under immune privilege, T-cells, NKs, and peripheral macrophages normally have limited access to the meninges and choroid plexus and far-limited access to the CNS parenchyma (Galea et al., 2007; Korin et al., 2017; Benakis et al., 2018). Thus, regardless of which CNS cell types undergo senescence, the clearance of senescent cells is likely limited in healthy non-aged individuals. Senescence cells, the SASP and secondary senescence, may therefore continue in the brain for years to decades.

A feature of aging that is exacerbated in AD is the progressive dysfunction of the blood brain barrier (BBB) resulting in immune cell infiltration (Gorlé et al., 2016; Sweeney et al., 2018; Nation et al., 2019). In AD, the question arises as to whether loss of immune privilege with advancing age eventually enables immune cells to kill senescent that have accumulated for years in the CNS. Infiltrating monocytes and their derived macrophages have been studied in AD (Herz et al., 2017), albeit their role is unlikely linked to senescent cell killing. In as far as microglia are considered CNS resident macrophages, a case for microglia-mediated killing of senescent cells could potentially be made. This would require infiltrating $CD4^+$ T-cells, as peripheral macrophages appear to depend on these cells to kill senescent cells outside the CNS (Kang et al., 2011). To the best of our knowledge, there is no evidence that microglia selectively kill senescent cells. Studies have focused on infiltrating $CD8^+$ T-cells in AD (Lindestam Arlehamn et al., 2019), some with surprising results (Gate et al., 2020), yet these are cells from the adaptive immune system that do not seem to play a relevant role in senescence immune surveillance (Antonangeli et al., 2019). With regards to NK cells, studies in AD patients and AD mouse models have assessed peripheral but not infiltrating NKs (Solana et al., 2018). Unfortunately, from these studies it is hard to infer what may be happening within the brain parenchyma.

There is abundant evidence that NK cells can infiltrate and kill brain cells under other pathological conditions such as ischemia and NK are known to kill stressed neurons in co-culture (Backström et al., 2003, 2007; Poli et al., 2013; Gan et al., 2014; Zhang et al., 2014; Li et al., 2017; Wang et al., 2018). Interestingly, under non-stressed conditions primary hippocampal neurons have been reported to be protected against NK cell killing by the lack of expression of NKG2D ligands (Backström et al., 2003, 2007). These same stress ligands are up-regulated in senescent cells and target them for killing by NKs (Antonangeli et al., 2019). Whether NKs can infiltrate the brain at latter stages of the disease and selectively eliminate senescent neurons, astrocytes or other CNS cell types will require further studies, although existing data fits with this possibility.

CURRENT CONFLICTS AND FUTURE DIRECTIONS

Evidence that senolytic intervention may be an effective treatment for AD was first reported in tau transgenic mice models of frontotemporal dementia (FTD; Bussian et al., 2018; Musi et al., 2018), which do not present amyloid plaques but are arguably an AD-like tau pathology model. Shortly thereafter, senolytics were proven therapeutic in APP/PSEN1 AD transgenic models which present plaques but not tau pathology (Zhang et al., 2019). The beneficial effects of senolytic intervention are attributed to either the selective killing of senescent astrocytes and microglia–not neurons-(Bussian et al., 2018) or the selective killing of neurons–not astrocytes-(Musi et al., 2018) in these tau models and to the selective killing of senescent OPCs – not astrocytes nor microglia-in the APP/PSEN1 AD model (Zhang et al., 2019). The use of AD and FTD models may explain why senescent OPCs appear to be the culprit in the AD mouse model but not in the FTD models. The selective killing of neurons versus glial cells within the FTD models is harder to reconcile, albeit different transgenic tau models were used (Bussian et al., 2018; Musi et al., 2018). The future of senolytics requires resolving the cell type that is killed, but more importantly whether they kill neurons or not (Walton and Andersen, 2019). A prime objective in future studies should include a thorough brain-wide assessment of neuronal cell death after senolytic intervention.

Cdkn2a up-regulation as measured by qPCR was reported to be absent in 15 month old 3xTg-AD mice by Musi et al. (2018). Cdkn2a encodes not only the widely used senescence marker $p16^{INK4A}$ but also $p19^{ARF}$ ($p14^{ARF}$ in humans), the latter of which can have opposing functions (Baker et al., 2008). Zhang et al. (2019) report an increase in Cdkn2a in Aβ-producing APP/PS1 transgenic mice crossed with the INK-ATTAC mice. The INK-ATTAC transgene expresses a fluorescent reporter from the p16 promoter which, contrary to qPCR, and allows identification of specific cell types transcribing p16. The conflicting results between the 3xTg-AD (Musi et al., 2018) and the APP/PS1 (Zhang et al., 2019) mice can be resolved by considering reported temporal expression of intraneuronal versus extraneuronal Aβ species in the 3xTg-AD mice. Intraneuronal AβO is present at 6 months, followed by a dip in expression that is only fully restored at 20 months (Oddo et al., 2006). While intraneuronal Aβ peptides are present at 6 months of age, extracellular Aβ is not readily evident until 18 months of age (Oddo et al., 2008). Hence, at 15 month of age, neither extracellular nor intracellular AβO pathology is fully developed in the 3xTg-AD. Attention should be paid to the presence of monomeric, oligomeric, and fibrillary Aβ as well as whether it is intracellular and or extracellular. Particularly considering

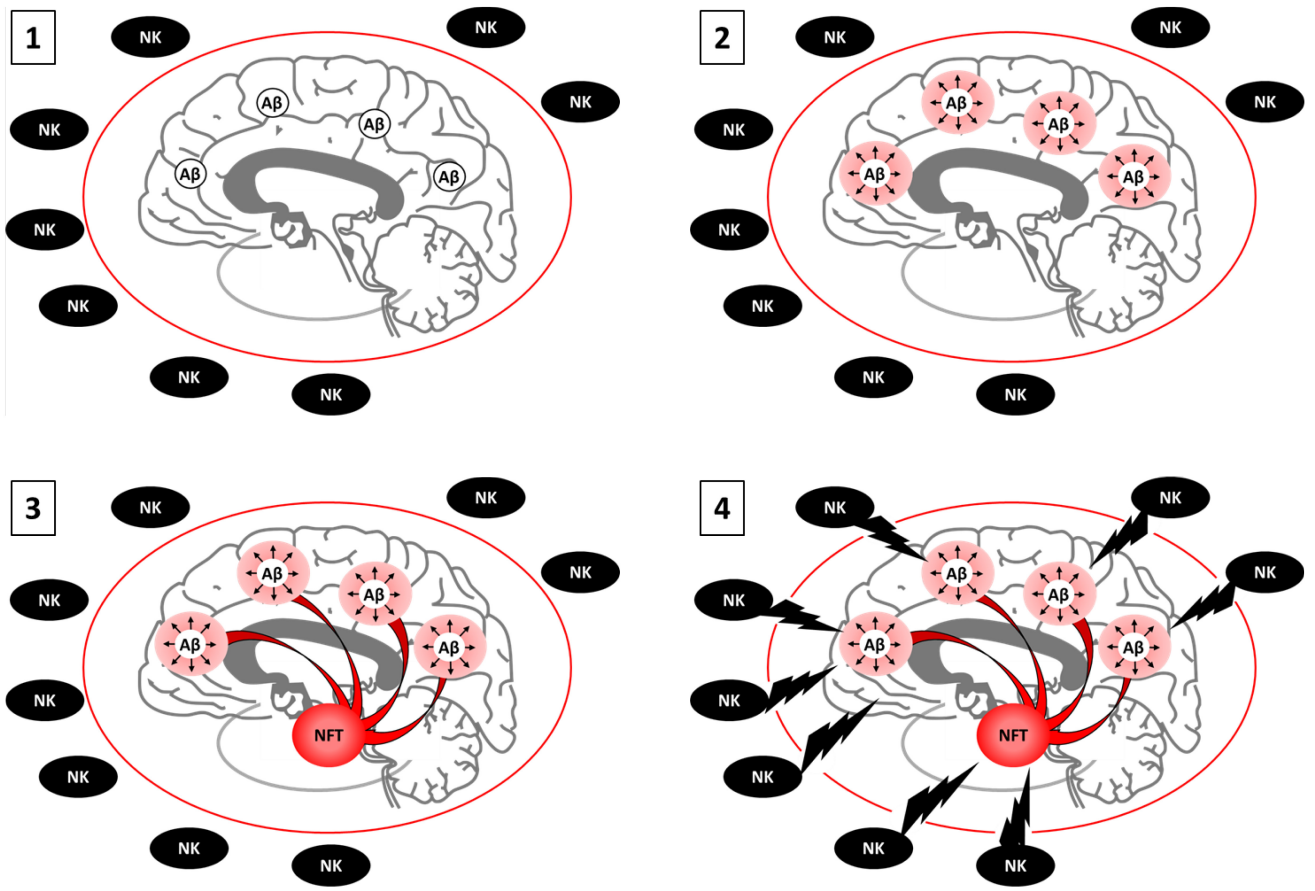

FIGURE 2 | Proposed model of the amyloid-senescence hypothesis. **1.** AβO burden appears 15 to 25 years prior to clinical onset of AD. At this point, antibody-mediated clearance is effective. **2.** AβO causes foci of senescent neurons, astrocytes, microglia, OPCs, and or NPCs by inducing aberrant cell cycle entry and or persistent ROS and DNA damage. Senescence cells and the SASP cause oxidative stress, inflammation, and initial stages of cognitive impairment. At early stages, the BBB is healthy. NKs cannot access the brain parenchyma to clear senescent cells, which remain viable. Antibody mediated clearance of AβO is no longer effective because senescence is irreversible. Senolytics can stop disease progression **3.** Foci of senescent neurons from across the cortex project into the transentorhinal cortex, where deleterious SASP and secondary senescence builds for years to decades. The SASP is accompanied by tau hyperphosphorylation and NFT. Cognitive impairment is accentuated. If too many senescent neurons are present, senolytics may prove fatal as neurons cannot regenerate. Senostatics, which target the SASP without killing cells, are an alternative. **4.** Age-associated disruption of the BBB enables NK cell infiltration and the killing of a large pool of senescent neurons, astrocyte, microglia, OPCs, and or NPCs, increasing inflammation and marking the onset of clinical AD. Immune suppression may be the only viable alternative at this stage. Boosting acetylcholine levels can help suppress the NK cell response. Individuals with senile plaques and NFT with a healthy BBB may remain relatively spared.

that extracellular amyloid plaques are a diagnostic hallmark of AD (Hyman et al., 2012; Deture and Dickson, 2019), some AβO act exclusively extracellularly (Larson and Lesné, 2012), extracellular AβO preparations can interact with receptors and the plasma membrane itself (Chen et al., 2017), and kill primary neurons (Yankner et al., 1990), alter synaptic functions (Verdier et al., 2004), induce tau-hyperphosphorylation (De Felice et al., 2008), and the vast majority of the *in vitro* experiments referenced above regarding ROS and the cell cycle are based on extracellular Aβ administration. Future work is needed to assess senolytic interventions in transgenic mice with combined Aβ and tau pathology at stages in which both extracellular and intracellular Aβ pathologies are fully developed.

CONCLUSION

Failure of Aβ antibody-mediated clearance in clinical trials challenging the amyloid cascade hypothesis occurred around the same time that senolytic interventions began emerging as a therapeutic alternative for the treatment of AD. Senescence and the amyloid cascade hypothesis have generally been presented as separate etiological phenomena during the progression of AD. This unfortunately has led to a disregard of the abundant literature that directly and indirectly supports the ability of AβO to induce cellular senescence. Aβ pathology models have been shown to induce senescence in astrocytes (Bhat et al., 2012), OPCs (Zhang et al., 2019), and NPCs (He et al., 2013). As discussed above, AβO has also been shown to induce

aberrant cell cycle entry and ROS in neurons, placing it upstream of stressors that are known to induce senescence in mitotically-competent CNS cell types (Gorgoulis et al., 2019). Rather than senescence being an alternative etiology to the amyloid cascade hypothesis, we describe aspects of senescence that potentially allow substitution of the term "senescence" for "cascade" which we propose as a novel amyloid-senescence hypothesis (**Figure 2**). Future studies will be required to determine whether senescence provides the "cascade" in the amyloid cascade hypothesis. However, based on the current literature, it is likely too early to reject an amyloid-senescence hypothesis out of hand.

AUTHOR CONTRIBUTIONS

CW, DB, and WN wrote the manuscript. JA edited the manuscript.

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TNFRSF12A and CD38 Contribute to a Vicious Circle for Chronic Obstructive Pulmonary Disease by Engaging Senescence Pathways

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Pathogenesis of chronic obstructive pulmonary disease (COPD) is dependent on chronic inflammation and is hypothesized to represent organ-specific senescence phenotype. Identification of senescence-associated gene drivers for the development of COPD is warranted. By employing automated pipeline, we have compiled lists of the genes implicated in COPD (N = 918) and of the genes changing their activity along with cell senescence (N = 262), with a significant ($p < 7.06e^{-60}$) overlap between these datasets (N = 89). A mega-analysis and a partial mega-analysis were conducted for gene sets linked to senescence but not yet to COPD, in nine independent mRNA expression datasets comprised of tissue samples of COPD cases (N = 171) and controls (N = 256). Mega-analysis of expression has identified *CD38* and *TNFRSF12A* ($p < 2.12e^{-8}$) as genes not yet explored in a context of senescence–COPD connection. Functional pathway enrichment analysis allowed to generate a model, which explains accelerated aging phenotypes previously observed in COPD patients. Presented results call for investigation of the role of TNFRSF12A/CD38 balance in establishing a vicious cycle of unresolvable tissue remodeling in COPD lungs.

Keywords: senescence, lung, chronic inflammation, aging, tissue remodeling, network analysis

INTRODUCTION

Aging is a fundamental biological process accompanied by changes in the structure and functions of vital organs, including the lungs. Chronic obstructive pulmonary disease (COPD) is associated with persistent airway inflammation and manifests as a steady decline in the lung function, increase in gas trapping, enlargement of the distal air spaces, and the loss of elastic recoil (Vestbo, 2014). According to the 2007–2012 NHANES survey, approximately 5.2% of US adults aged 40–79 were diagnosed with COPD (Tilert et al., 2018).

Current understanding of the pathophysiology of COPD emphasizes pivotal role of chronic inflammation, which is evident from an increase in neutrophils, macrophages, and $CD8^+$ T-cell counts throughout the airways, and related release of inflammatory mediators including IL−8, TNF−α, leukotrienes, and reactive oxygen species (ROS) (Barnes, 2014). It is generally accepted that both cellular and molecular pro-inflammatory players act in concert to form an inflammation

promoting feedback loop. Some experts, however, suggest that the confinement of the process of inflammation in the airway wall, where the COPD-related remodeling occurs, is debatable (Eapen et al., 2017). Moreover, epidemiological studies point toward a strong systemic component of COPD, with is often accompanied by muscle wasting, cachexia, and cardiovascular involvement (Rubinsztajn et al., 2019). This is one of the reasons why COPD is now considered as an example of organ-specific accelerated aging, with increase in oxidative stress and alteration in the extracellular matrix leading to the development of senescent cell phenotypes (Mercado et al., 2015). Previous gene expression studies produced a list of hundreds of genes, which are both linked to COPD and aging promoting (Brandsma et al., 2017; de Vries et al., 2018), paving a solid foundation to explore the association between COPD and aging at a genetic level. Here, we hypothesize that genes with activity or expression levels increased in aged individuals may also play roles for the etiology of COPD.

To test this hypothesis, we conducted large-scale literature-based disease–gene relationship analysis to compile the lists of genes implicated in COPD and, separately, in senescence, and subtracted the genes previously implicated in both of these phenotypes. For the genes previously identified as senescence-related but not yet highlighted by any COPD studies, a mega-analysis and a partial mega-analysis were completed to evaluate their expression patterns in multiple independently collected COPD-related transcriptomic datasets. Functional pathway enrichment analysis allowed us to generate a model that centers on two novel COPD genes, *CD38* and *TNFRSF12A*, and explains the accelerated aging phenotypes previously observed in COPD patients.

MATERIALS AND METHODS

The workflow was organized as follows. First, a large-scale literature mining effort for COPD-related and aging-promoting related gene sets were undertaken in the Pathway Studio environment; these gene sets were compared to identify common genes and age-promoting specific genes. Then, for each gene from the list implicated in aging alone, a mega-analysis, followed by a partial mega-analysis were conducted using nine publicly available COPD expression datasets retrieved from Gene Expression Omnibus (GEO)[1]. For these genes that showed a significant change in expression across analyzed datasets, a Gene Set Enrichment Analysis (GSEA) and literature-based functional pathway analysis were conducted. In addition, possible influences of sample size, population region, and study date on the gene expression levels in COPD were investigated by a multiple linear regression (MLR) model.

Extraction of Relation Data From Literature

Relation data for genes previously associated with either senescence or COPD were extracted from existing literature in the Pathway Studio environment[2] and arranged in the database Aging_COPD, hosted at http://database.gousinfo.com. The downloadable form in Excel is available at http://gousinfo.com/database/Data_Genetic/Aging_COPD.xlsx.
Beside the list of analyzed genes (Aging_COPD→Aging_alone genes, Aging_COPD→COPD_alone genes, and Aging_COPD→Common genes), supporting references for each disease-gene relation were retrieved (Aging_COPD→Ref for Aging_alone genes, Aging_COPD→Ref for COPD_alone genes, and Aging_COPD→Ref for Common genes) to include both titles of respective papers and particular sentences describing identified relationships. The database allows automated mining for supportive statements underlining the association of each candidate gene with senescence and/or with COPD.

Selection of Gene Expression Datasets

To compile the list of gene expression datasets, publicly available, the GEO database was searched using the keyword "chronic obstructive pulmonary disease," which has returned 171 entries. Datasets were extracted with no selection bias and covered the entire GEO contents. The following standards were applied for the further filtering: (1) The organism is *Homo sapiens*; (2) The data type is RNA expression; (3) The sample size is no less than 10; and (4) the study design is case control. Finally, a total of nine datasets remained available for the mega-analysis of expression patterns (**Table 1**). For each dataset, raw data files rather than the reported research outputs were used to perform the analysis.

Mega-Analysis and Partial Mega-Analysis of Expression Datasets

The expression data were normalized and log2-transformed. Mega-analysis allows pooling of individual-level biological endpoint data across datasets by introducing appropriate correction for between-study variations selected by modeling (Seifuddin et al., 2013). Both the fixed-effect model and random-effect model (Borenstein et al., 2010) were employed to study the effect size of senescence-related genes on COPD. For each expression dataset, the log fold change (LFC) was calculated for the COPD samples and used as the index of effect size. Results from both mega-analysis models were compared. In order to study the variance within and between different datasets, the heterogeneity of the mega-analysis was assessed. In case when total variance Q was equal to, or smaller than, the expected between-study variance df, the statistic $ISq = 100\% \times (Q - df)/Q$ was set to 0, and a fixed-effect model was selected for the mega-analysis. Otherwise, a random-effect model was selected. The Q–p represents the probability that the total variance is explained by within-study variance only.

To discover the genes significantly altered in some, but not all studied datasets, we performed a partial mega-analysis, where the top 50% datasets were employed for the mega-analysis of each gene. The "Top datasets" were defined for each gene individually as datasets demonstrating larger absolute value of effect size than the rest of the datasets. Analyses were conducted using MATLAB (R2017a) mega-analysis package.

[1] https://www.ncbi.nlm.nih.gov/geo/

[2] www.pathwaystudio.com

TABLE 1 | Datasets utilized for chronic obstructive pulmonary disease (COPD)-senescence expression mega-analysis.

Study name	Dataset GEO ID	*n* Control	*n* Case	Country	Tissue
Bastos et al., 2016	GSE37768	20	18	Spain	Peripheral lung
Kalko et al., 2014	GSE27543	6	10	United Kingdom	Musculus vastus lateralis
Kalko et al., 2013	GSE27536	24	30	United Kingdom	Musculus vastus lateralis
Ezzie et al., 2012	GSE38974	9	23	United States	Lung
Tilley et al., 2011	GSE11784	135	22	United States	Airway epithelial cells
Poliska et al., 2011	GSE16972	6	6	Hungary	Alveolar macrophage; peripheral blood monocytes
Bosco et al., 2010	GSE19903	10	10	Australia	Induced sputum cells
Boelens et al., 2009	GSE12472	27	36	Netherlands	Large bronchial
Bhattacharya et al., 2008	GSE8581	19	16	United States	Lung

Results from both mega-analysis and partial mega-analysis were compared to identify significant genes according to the following the criteria, $p < 1.00e^{-7}$ and effect size (LFC) > 0.49 or < -0.74. When a gene presented an effect size LFC > 0.49 or < -0.74 in the mega-analysis, it means that the change in the expression level of the gene had increased by more than 40%, or decreased by more than 40%. While we present all the mega-analysis results in the Aging_COPD→Mega-analysis and Aging_COPD→Partial-Meta, the discussion will be focused on those genes that satisfy the significance criteria outlined above.

Gene Set Enrichment Analysis and Shortest Path Analysis

To gain functional insights into the set of genes previously described as involved in senescence and in COPD and, separately, the set of genes showing significance in the mega-analysis of senescence-related genes, which were not yet described as COPD contributors, the GSEA has been conducted in the Pathway Studio environment. The GSEA results were reported with enrichment *p*-values corrected using the Bejnamini–Hochberg false discovery ratio (FDR) procedure (Reiner et al., 2003). In addition to the GSEA, for each gene set, a literature-based functional pathway analysis was conducted using the "Shortest Path" module of the Pathway Studio[3].

Multiple Linear Regression Analysis

A MLR analysis was employed to study the possible influence of the following three factors on the gene expression changes: sample size, population region, and study date. Values of *p* and 95% confidence interval (CI) were reported for each of these factors. The analysis was performed in MATLAB (R 2017a) with the "regress" statistical analysis package.

RESULTS

Many Senescence-Associated Genes Are Not yet Studied in COPD Context

Pathway Studio-guided literature data mining for the genes associated with aging and senescence has yielded 262 genes, while COPD phenotypes were associated with 918 genes. Despite a significant overlap between aging/senescence-related genes and COPD-related genes (89 genes; $p = 7.06e^{-60}$), over half of the set of aging/senescence-related genes ($N = 173$ or 66.03%) have not been previously implicated in COPD. A complete list of these 173 aging/senescence-related genes along with support evidence and references is presented in the following files: Aging_COPD→Aging_alone genes and Ref for Aging_alone genes.

Mega- and Partial Mega-Analysis of Gene Expression Highlights TNFRSF12A and CD38

Only two genes, *TNFRSF12A* and *CD38*, have satisfied the significance criteria outlined in **Table 2**. Specifically, for an increased expression of *TNFRSF12A*, both mega-analysis and partial-meta analysis results were positive, while *CD38* was identified as significantly involved by the partial-meta analysis only.

Analysis of study heterogeneity showed that, for *TNFRSF12A*, there was no significant between-datasets variance for both mega-analysis and partial mega-analysis (ISq = 0, p–$Q > 0.96$), and therefore, a fixed-effect model was selected for this gene. For *CD38*, in mega-analysis, the between-datasets variance had accounted for 17.34% of the overall variance. When the random-effect model was used, outputs were not significant. Therefore, for *CD38*, only partial mega-analysis results were employed. For each gene, effect sizes, 95% confidence intervals, and weights of each study are presented in **Figure 1**.

As shown in **Figure 1**, two studies/datasets – GSE16972 for *TNFRSF12A* and GSE27543 for *CD38* – demonstrated relatively small variances of effect size, leading to high *z*-scores and high weights within the mega-analysis. To determine whether these small variances were specific for the two genes, or were due to the peculiarities of data distribution within the datasets, we studied the distribution of the z-scores for all the genes differentially expressed in COPD patients suing empirical quantile–quantile plots (QQ plot), against the normal distribution, as shown in **Figure 2**. Even if the *z*-scores of these two datasets were not well fit to a normal distribution, their overall distribution was not that wide, with most of the genes demonstrating near-zero values. Therefore, the small variance corresponding to high *z*-scores observed for the *TNFRSF12A* and *CD38* genes was not different

[3]http://www.pathwaystudio.com

TABLE 2 | Novel aging/senescence-related genes as highlighted by mega-analysis (MA) and partial MA expression.

Gene/type of analysis	Significance	Random-effect model	# Study	LFC	*p*-value	ISq (%)	*p*-value–Q
TNFRSF12A/partial MA	Yes	NO	4	0.54	$5.80e^{-10}$	0	1.00
TNFRSF12A/MA	Yes	NO	9	0.51	$1.53e^{-9}$	0	0.96
CD38/partial MA	Yes	NO	4	-0.89	$2.12e^{-8}$	0	0.82
CD38/MA	No	Yes	8	-0.49	$2.19e^{-2}$	17.34	0.29

LFC, log fold change (the effect size); p-value represents the probability that the fold change is equal to 0; ISq = 100% × (Q – df)/Q represents the percentage of between-variance over total variance; p-value–Q represents the probability that the variance is explained by within-study variance only.

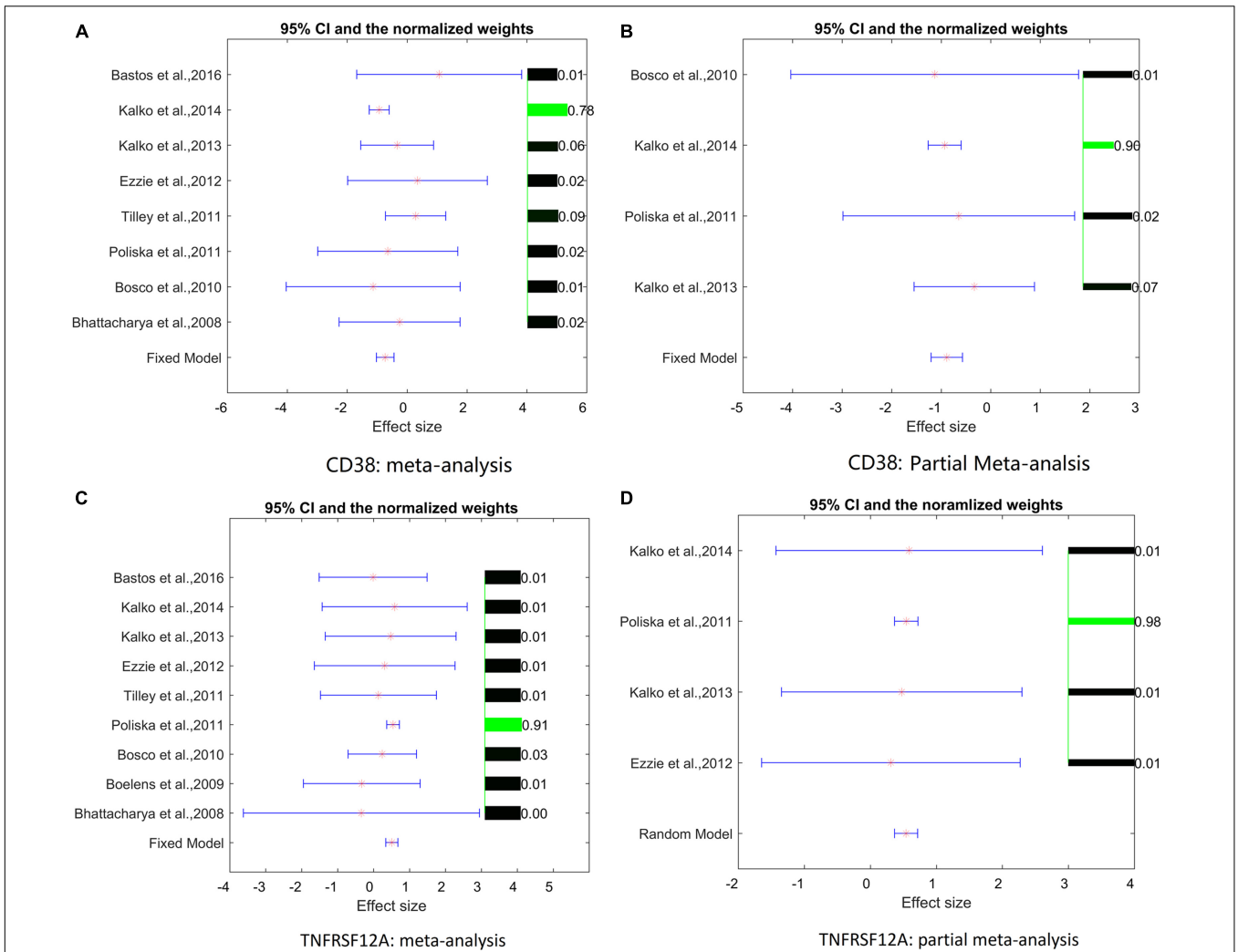

FIGURE 1 | Effect sizes, 95% confidence intervals, and weights for each of two genes. **(A)** Mega-analysis results for *CD38*. **(B)** Partial mega-analysis results for *CD38*. **(C)** Mega-analysis results for *TNFRSF12A*. **(D)** Partial mega-analysis results for *TNFRSF12A*. The bar plot on the right of each figure represents the normalized weights for each dataset/study, ranged within (0, 1); the brighter (green) the color, the larger the relative weight of the study as labeled right next to the bar. For each dataset, the star (in red) and lines (in blue) on the left are the mean of effect size (log fold change), and 95% confidence interval (CI), respectively. References for datasets could be traced by their Gene Expression Omnibus (GEO) numbers.

to distribution expression values for other genes, not highlighted by mega-analysis.

Multiple Linear Regression Analysis

A MLR analysis was conducted to explore the potential influence of three parameters on the expression levels of the genes in the case of COPD. Analysis of the MLR models shows that the region, where each study was performed, exerted significant influence on the expression fold changes detected for *TNFRSF12A* and *CD38* genes with $p < 3.10e^{-4}$ and $2.30 < e^{-3}$, respectively, nor were sample sizes not studying publishing dates were significant influencers.

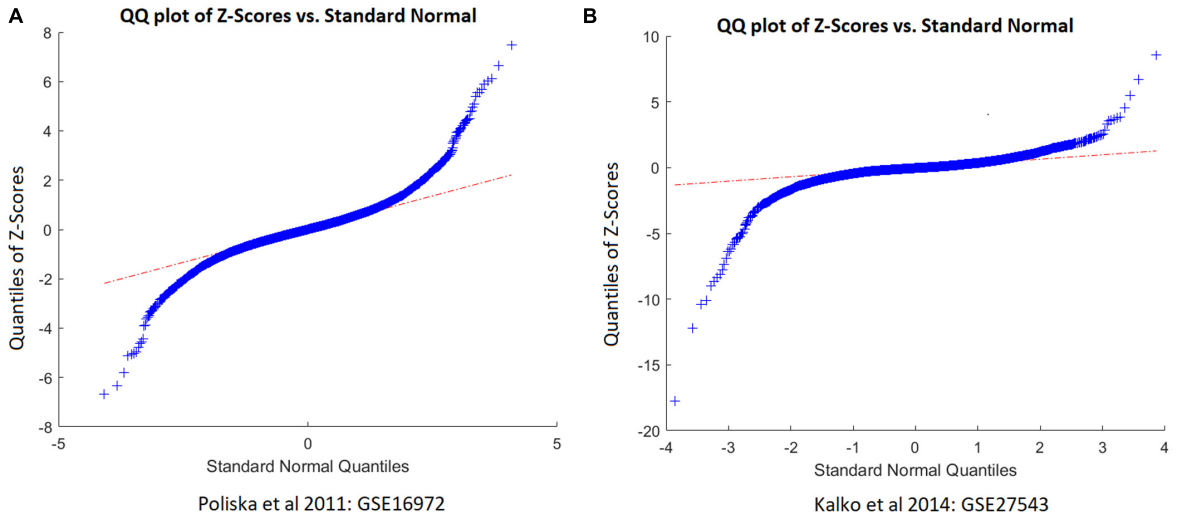

FIGURE 2 | QQ plot of the z-scores of chronic obstructive pulmonary disease (COPD) patients versus standard normal distribution. **(A)** QQ plot built for dataset GSE16972. **(B)** QQ plot built for dataset GSE27543.

GSEA Results

Input list for GSEA analysis is located in Aging_COPD→GSEA. In GSEA analysis of 91 genes implicated both in COPD and in senescence phenotypes (previously described genes, $N = 89$ genes; genes discovered in this study by mega-analysis, $N = 2$), a total of 113 GO terms were significantly enriched ($p < 1e^{-10}$). **Table 3** presents the Top 10 GO terms with enrichment values of $p < 1.19e^{-21}$. Noteworthy, *TNFRSF12A* has been included in 9 of 113 enriched pathways, and in 3 of the Top 10 pathways (**Table 3**). *CD38* was a part of 26 of the 113 enriched pathways, including in 4 of the top 10 pathways (**Table 3**). One of the GO terms, Apoptotic Signaling Pathway, retrieved a gene list with both *TNFRSF12A* and *CD38*.

Pathway Studio-Guided Analysis of Existing Literature

As COPD-related genes, *TNFRSF12A* and *CD38*, were identified *de novo*, after specific exclusion of the genes previously described as involved in COPD, gene-specific PubMed search confirmed that neither *TNFRSF12A* nor *CD38* were previously discussed in the mechanistic context of COPD. However, the Shortest Path analysis performed in the Pathway Studio data mining environment revealed a number of plausible connections between these two genes and COPD, with a set of common interactions (**Figure 3**).

Figure 3A shows that *TNFRSF12A* may influence the pathogenesis of COPD through multiple pathways. As an example, let us trace the connection *TNFRSF12A*→HMGB1→COPD. High-mobility group box-1 (HMGB1) modulates the balance between senescence and apoptosis in response to genotoxic stress, with higher expression levels of this protein profoundly shifting the balance toward senescence (Lee et al., 2019). Increased TWEAK signaling through larger amounts of available TNFRSF12A receptor potently induces HMGB1 expression and secretion (Moreno et al., 2013). Increases in extracellular concentrations of HMGB1 are proportional to the severity of COPD itself (Hou et al., 2011). Outside the cells, HMGB1 associates with numerous other proteins and signals back to the cell through the receptor

TABLE 3 | Top 10 GO terms enriched by 91 genes linked to both senescence/aging and COPD phenotypes.

GO ID	GO name	# of entities	Overlap	*p*-value	Novel gene included
0070482	Response to oxygen levels	544	33	$4.4E^{-27}$	*CD38*
0010942	Positive regulation of cell death	870	37	$1.14E^{-25}$	*TNFRSF12A*
0001666	Response to hypoxia	424	29	$4.49E^{-25}$	*CD38*
0036293	Response to decreased oxygen levels	461	29	$3.94E^{-24}$	*CD38*
0048545	Response to steroid hormone	418	27	$1.21E^{-22}$	*CD38*
0031960	Response to corticosteroid	325	25	$1.22E^{-22}$	No
0043068	Positive regulation of programmed cell death	800	33	$3.06E^{-22}$	*TNFRSF12A*
0051384	Response to glucocorticoid	299	24	$4.27E^{-22}$	No
0019221	Cytokine-mediated signaling pathway	676	31	$4.81E^{-22}$	*TNFRSF12A*
0009636	Response to toxic substance	634	30	$1.19E^{-21}$	No

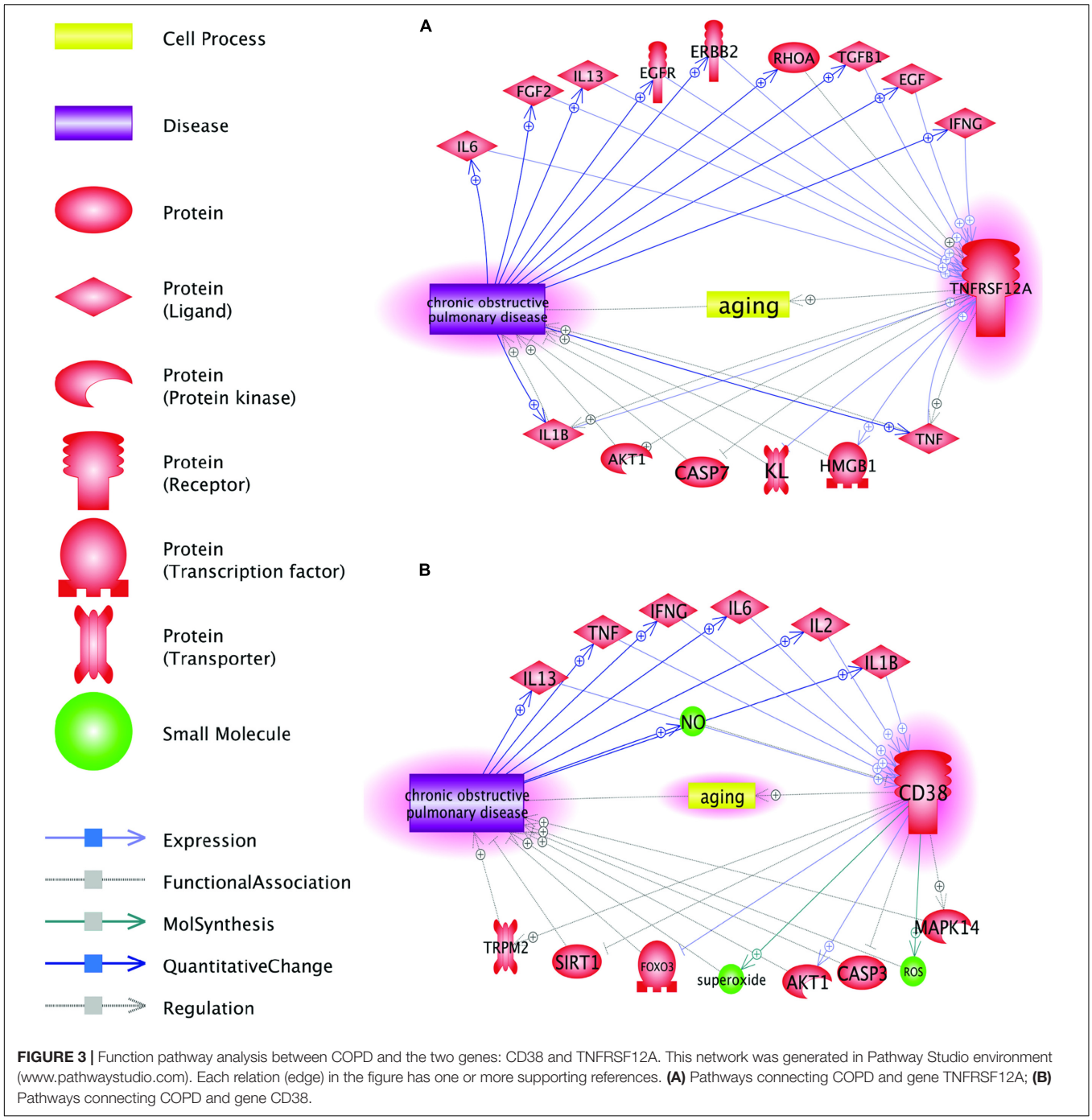

FIGURE 3 | Function pathway analysis between COPD and the two genes: CD38 and TNFRSF12A. This network was generated in Pathway Studio environment (www.pathwaystudio.com). Each relation (edge) in the figure has one or more supporting references. **(A)** Pathways connecting COPD and gene TNFRSF12A; **(B)** Pathways connecting COPD and gene CD38.

for advanced glycation end products (RAGE) and toll-like receptor-4 (TLR-4), thus behaving as a typical damage-associated molecular pattern (DAMP) (Wong et al., 2018; Paudel et al., 2019). The details for all other relations presented in **Figure 3** are described in Aging_COPD→Pathway Analysis. This reference information includes the types of the relationship, amount of underlying supporting references, and related sentences where these relationships have been identified and described. Evidence presented in **Figure 3** also indicates that perpetuated overexpression of *TNFRSF12A* may serve one of the drivers for a vicious circle that keeps COPD patients from resolving inflammation in their lungs.

DISCUSSION

We performed this study in an attempt to identify novel, not yet described molecular pathways, which link the process of tissue and cell aging and the development of COPD. By removing all known intersections between curated gene sets involved in each

of these pathophysiological processes, we ensured that uncovered senescence-related contributors to COPD had not been already described as such. Through the Pathway Studio-guided literature mining, a total of 173 genes involved in the senescence, but not in COPD, were discovered. These genes were investigated for consistent evidence of the changes in their expression in COPD phenotypes across nine mRNA expression datasets acquired from GEO (**Table 1**).

In cross-dataset mega-analysis of gene-level expression patterns, expression levels of seven senescence-related genes were significantly altered in COPD compared to normal lungs ($p < 0.05$, see in Aging_COPD→Meta_Analysis). When across dataset thresholds were lowered by applying partial mega-analysis techniques, a total of 18 senescence-related genes were highlighted as possibly involved in COPD (see in Aging_COPD→Partial Meta). However, only two senescence-related genes, *TNFRSF12A* and *CD38*, have passed pre-selected criterion of the significance of association, which were set at $p < 1e^{-7}$ and LFC of >0.49 or <-0.74 (**Table 2** and **Figure 2**). In particular, in meta- and partial meta-analyses, LFCs of observed *TNFRSF12A* expression levels were at 0.51 and at 0.54, respectively, demonstrating that expression levels of *TNFRSF12A* in COPD samples were consistently increased by more than 40%. When similar criteria were applied to *CD38*, its expression levels were found to be decreased in COPD samples by more than 40% (**Table 2**), with the only difference that the changes in *CD38* expression levels have passed the significance criteria in partial mega-analysis only.

For both molecules, their roles in the senescence and aging-associated diseases are well described, while no apparent connections to COPD have been reported so far. *TNFRSF12A* encodes for an exclusive receptor for tumor necrosis factor-related weak inducer of apoptosis (TWEAK); jointly, this interacting pair of molecules is involved in age-associated pathological changes in skeletal muscle and other organs (Van Kirk et al., 2011; Tajrishi et al., 2014; Hénaut et al., 2016). CD38 is one of the main NAD-degrading enzymes, which increases its expression in aging tissues and is directly responsible to age-related NAD decline (Horenstein et al., 2013; Camacho-Pereira et al., 2016). When the levels of NAD+ are low, a senescence-associated secretory phenotype (SASP) to a certain degree diminishes its damaging power as a part of a fundamental trade-off between aging and energy available to the cells (Mendelsohn and Larrick, 2019). Moreover, adenosine, which is produced in CD38-dependent reaction, further suppresses local immune response (Horenstein et al., 2013). In healthy aging, SASP cytokines promote an expression of CD38 (Chini et al., 2019). In case of lungs affected by COPD, a decrease in CD38 expression was noted in at least some datasets. It should be noted, however, that some COPD datasets do not comply, possibly due to the stage-specific differences in the SASP response of COPD lungs (Parikh et al., 2019). As senescent cells are also capable of inducing surface CD38 expression on macrophages and endothelial cells (Chini et al., 2019), particular balance between CD38-lowering and CD38-enhancing forces may be dependent upon tissue composition and/or disease stage of collected samples and, therefore, may be dataset specific.

Notably, a number of previous studies connected COPD to an increased risk of developing non-small cell lung carcinoma (NSCLC), irrespective of the pack-years history of their smoking (Houghton, 2013; Ng KeeKwong et al., 2017). Both *TNFRSF12A/TWEAK* pair (Whitsett et al., 2014) and CD38, along with its interaction partner CD31 (Malavasi et al., 2008) are involved in multiple cancers, including NSCLC, where they contribute to escape from PD-1/PD-L1 blockade (Chen et al., 2018; Konen et al., 2019) and cytotoxic therapy (Whitsett et al., 2014). Obvious importance of *TNFRSF12A/TWEAK* molecules for the survival of cancers cells prompted the development of the respective targeting agents, which already met initial success in clinical and pre-clinical trials [see Hu et al., 2017, for review]. Our findings point at the potential applicability of the abovementioned therapeutics in COPD context.

The results of the GSEA analysis support the association between senescence and COPD, with 113 pathways uncovered as significantly enriched and connected to both of these phenotypes ($p < 1e^{-10}$; see **Table 3** and Aging_COPD→GSEA). Among them, we have identified 9 and 26 significantly enriched pathways, where either *TNFRSF12A* or *CD38* were listed as shared genes, respectively. Notably, *CD38* plays a role in many enriched oxygen/hypoxia and steroid hormone-related pathways, which both have been linked to COPD previously (Marwick et al., 2013; Wada and Takizawa, 2013). On the other hand, a number of enriched cytokine-mediated signaling pathways, positively regulating cell apoptosis and influencing angiogenesis, include TNFRSF12A as their integral part; these pathways have also been implicated in COPD pathogenesis (Vanella et al., 2017; Kropski et al., 2018).

Employing Pathway Studio-guided "Shortest Path analysis" allowed us to explore plausible connections between these two genes and COPD, simultaneously highlighting a set of common interactors, which includes the signaling molecule and the pro-inflammatory cytokines. Among these, a connection of both TNFRSF12A and CD38 proteins to AKT1 is remarkable, as the stimulation of IGF1/AKT1 axis via the targeting of p16-induced senescence was recently shown to alleviate at least some phenotypic features of COPD (Cottage et al., 2019). Notably, among other connecting molecules are pro-inflammatory cytokines TNF-α, IL-1β, IFN-γ, and IL-6, which are the part of the SASP phenotype (Barnes, 2014; Ohtani, 2019).

Importantly, this study is not free of limitation. In particular, we acknowledge that the study pipeline was executed *in silico*, with no experimental validation of the findings, and that the study set was relatively small, in part, due to the strict quality control of selected datasets. Nevertheless, we believe that the overall conclusion of our study may further our understanding of COPD.

In particular, we noted that CD38/TNFRSF12A and COPD relationships demonstrate the features of bidirectional, self-propagating cycle rather than a unidirectional regulation pathway (**Figure 4**). We speculate that, in COPD, stimulation of TWEAK/TNFRSF12A signaling enhances tissue remodeling by stimulation migration of the cells and the SASP cytokine production [these statements are supported by experiments described in Refs. (Wang et al., 2017; Das et al., 2018;

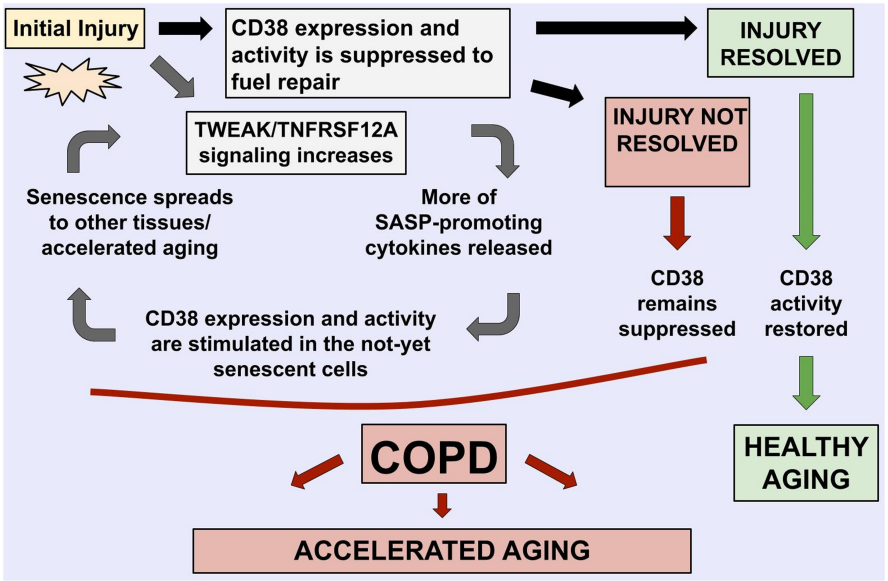

FIGURE 4 | Mechanistic model highlights the roles of *TNFRSF12A* and *CD38* in COPD-related accelerated aging phenotypes, with their balance defining if the lung injury is resolved.

Liu et al., 2019)], which, in turn, induce CD38 mRNA and protein expression, and stimulate activity of this NADase in adjacent non-senescent cells, including populations of macrophages and endothelia [supported by experiments described in Parikh et al., 2019], with the resultant depletion of NAD+ contributing to accelerated aging observed in COPD patients (Triest et al., 2019), and to further increase in TWEAK/TNFRSF12A signaling. The resultant model is described in **Figure 4**, with relative levels of CD38/TWEAK expression establishing whether lung inflammation would resolve or would form a vicious cycle of COPD.

CONCLUSION

Using a large-scale literature data mining and gene-level mega-analysis of multiple gene expression datasets, we specifically searched for novel senescence-associated genes, which may also contribute to COPD. We show that genes *TNFRSF12A* and *CD38* encode factors previously unrecognized as COPD contributors, and have generated the model that explains accelerated aging phenotypes previously observed in COPD patients, and calls for investigation of the balance of TNFRSF12A/ CD38 proteins as the key to establishing vicious cycle of unresolvable tissue remodeling in COPD lungs.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this study can be found in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/). Relation data for genes previously associated either with senescence or with COPD were extracted from existing literature in the Pathway Studio environment (www.pathwaystudio.com) and arranged in the database Aging_COPD, hosted at http://database.gousinfo.com. The downloadable form in Excel is available at http://gousinfo.com/database/Data_Genetic/Aging_COPD.xlsx.

AUTHOR CONTRIBUTIONS

YD, RC, and HC contributed to the conception and design of the study. HC organized the database and performed the Pathway Studio work. RC and YD retrieved the expression data and performed the statistical analysis. RC and HC wrote the first draft of the manuscript. AB provided the interpretation of the data and built the COPD model. AB and HC wrote the final version of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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MicroRNA-221 Modulates Airway Remodeling via the PI3K/AKT Pathway in OVA-Induced Chronic Murine Asthma

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Background: Airway remodeling is one of the most important pathological features of chronic asthma. This study aimed to determine whether microRNA-221 (hereafter, miR-221) can affect airway remodeling in a mouse model of ovalbumin (OVA)-induced chronic asthma.

Methods: Adeno-associated viruses (AAVs) "Bearing miR-221 sponges" were used to downregulate miR-221 in asthmatic mice. Staining with hematoxylin and eosin (H&E), Masson trichrome, and periodic acid–Schiff reagent was used to assess histological changes. The affected signaling pathway in mouse airway smooth muscle cells (ASMCs) was also identified by gene chip technology. A PI3K/AKT-inhibitor (LY294002) was used to confirm the role of the pathway in ASMCs.

Results: The inhibition of miR-221 in a murine asthma model was found to reduce airway hyper-responsiveness, mucus metaplasia, airway inflammation, and airway remodeling ($p < 0.05$). Furthermore, miR-221 was found to regulate collagen deposition in the extracellular matrix (ECM) of ASMCs. Bioinformatics analysis and western blot analysis confirmed that the PI3K-AKT pathway was involved in ECM deposition in ASMCs.

Conclusion: miR-221 might play a crucial role in the mechanism of remodeling via the PI3K/AKT pathway in chronic asthma and it could be considered as a potential target for developing therapeutic strategies.

Keywords: miR-221, asthma, airway inflammation, airway remodeling, ASMCs

Abbreviations: AAV, adeno-associated virus; AHR, airway hyper-responsiveness; ASMCs, airway smooth muscle cells; BALF, Bronchoalveolar lavage fluid; ECM, extracellular matrix; EGF, epidermal growth factor; H&E, hematoxylin and eosin; i.p., intraperitoneal; miR-221, microRNA-221; miRNAs, MicroRNAs; OVA, ovalbumin; PAS, periodic acid–Schiff stain; Pbm, airway basement membrane perimeter; PDGF-BB, platelet-derived growth factor; Re, expiratory resistance; Ri, inspiratory resistance; UTR, untranslated region; Wai, airway wall thickness; Wam, airway smooth muscle area; Wat, airway wall area.

INTRODUCTION

Asthma is a chronic pulmonary inflammatory disease characterized by AHR and the inflammation and remodeling of airways. It is a major public health concern, with approximately 300 million people (especially children) being affected worldwide and over 30 million asthma patients in China alone (GINA) (Bateman et al., 2018). The direct costs and indirect expenses incurred as a result of asthma pose a considerable economic burden on families and society (Chen et al., 2018). Currently, corticosteroids are the most effective way to control asthma attacks (Veeranki et al., 2017). However, the efficacy of steroids is limited to airway remodeling traits such as basement membrane thickness and ECM deposition. Statistically, 5–25% of patients do not respond well to steroid therapy and are considered steroid-resistant (Hansbro et al., 2017).

The long-term recurrent stimulation of chronic inflammatory responses can result in the destruction of epithelial structure, which further leads to asthma airway remodeling (Boulet, 2018). Airway reconstruction is also considered the most important cause of irreversible air flow limitation and continuous AHR. The mechanisms regulating AHR and airway remodeling remain to be elucidated, necessitating the study of the pathogenesis of asthma at the molecular level (Russell and Brightling, 2017).

MicroRNAs are small non-coding RNAs, having important roles in the post-transcriptional regulation of gene expression. MiRNAs bind to the 3'-UTR of a target gene, resulting in translational repression and/or mRNA degradation (Shukla et al., 2011). MiRNAs also play a critical role in the regulation of innate immunity and inflammation in lung diseases such as asthma (Kai et al., 2015; Izabela et al., 2016). As an important effector of allergic diseases, miR-221 has been shown to regulate the cell cycle of mast cells (Mayoral et al., 2009). The contraction and relaxation function of smooth muscle cells is also regulated by miR-221 (Perry et al., 2014). Our previous studies found a significant increase in the expression level of miR-221 in the peripheral blood of children with asthma, and similar results were obtained in the lung tissue of a mouse asthma model (Qin et al., 2012).

To explore the effects of miR-221 *in vivo*, we constructed a mouse model displaying the basic features of chronic asthma and studied the effects of AAV intervention. Furthermore, to elucidate the underlying mechanisms, we examined the changes in signaling pathways in ECM deposition in mouse ASMCs stimulated by miR-221.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the local animal care committee of the National Defense Medical Center (approval number: IACUC-1702005). Six-week-old female BALB/c mice were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, China) and maintained under a specific pathogen-free environment at the Animal Center of Nanjing Medical University. Mice were housed in a light- and temperature-controlled room with free access to deionized drinking water and standard chow. Animals were acclimatized to the laboratory conditions for 1 week prior to the start of the experiments.

Experimental Protocols

Sixty female BALB/c mice were randomly divided into four exposure groups: (1) the control group, (2) shRNA-miR-221 group, (3) OVA group, (4) and OVA + shRNA-miR-221 group. Mice were sensitized via intraperitoneal (i.p.) injection of 20 μg OVA (Grade V, Sigma-Aldrich, United States) absorbed onto 2 mg Imject Alum (Thermo, United States) in 200 μL sterilized 0.9% saline once a week from week 0 to week 2. Mice were then challenged through the respiratory tract with 5% aerosolized OVA in saline for 30 min thrice a week from week 3 to week 10. Mice those received 0.9% saline sensitization and a challenge of the same volume and frequency were used as controls. Twenty-four hours after the last challenge (day 72), mice were anesthetized for the subsequent experiments. BALF and lung tissues were processed for subsequent analysis. The detailed protocol of this study is shown in **Supplementary Figure S2**.

Infection With AAVs

To accurately regulate lung exposure to AAVs at the beginning of the study (day 3), the OVA solutions were instilled intratracheally to the mice. Such a practice greatly alleviated the pain of the mice and satisfied the experimental requirements. Mice were anesthetized by intraperitoneal injection of 4% chloral hydrate. Then, each mouse was suspended with the abdominal side facing outward on a nearly vertical incline by hooking its incisors on a rubber band at the top of the slope, with a light located above the mouse's chest. The tongue of the mouse was gently pulled out with curved tweezers for the airway to be clearly visible by the light penetrating its chest. Then, the mice underwent endotracheal intubation with a venous indwelling needle (22G; BD, China). AAVs (50 μL, over 1×10^{12} vg/mL) were instilled into the lungs of mice through the venous indwelling needle inserted into the airway. The primer design sequences of miR-221-sponge were as follows: mmu-miR-221-3p-sponge-F: acaggatccGAAACCCAGCAGACAATGTAGCTtatacGAAACCCAGCAGACAATGTAGCTacatcGAAACCCAG; mmu-miR-221-3p-sponge-R: acagaattcAGCTACATTGTCTGCTGGGTTTCtgaagaAGCTACATTGTCTGCTGGGTTTCgatgtAGCTACA.

Measurement of AHR

According to the manufacturer's instructions of the AniRes2005 lung function system (Bestlab, version 2.0, China), 24 h after the final aerosol challenge, the mice were tested for AHR to methacholine (MeCh; Sigma-Aldrich). Mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (Urchem, China). The respiratory rate was pre-set at 90/min, and the time ratio of expiration/inspiration was 20:10. AHR was assessed by the indexes of Re, Ri, and the minimum value of Cdyn (Cldyn). Ri and Re R-areas, the graph area between the peak value and baseline, and the valley of Cldyn were recorded for further analysis.

BALF and Cell Counting

The trachea was exposed and intubated by a venous indwelling needle with a 1 mL sterile syringe filter. BAL was performed by the instillation of 0.5 mL saline through the trachea into the lung; the mouse's chest was gently massaged, and the liquid was withdrawn. This process was repeated thrice. The total liquid recovered from different mice was ≥1.2 mL. Then, the BALF samples were centrifuged at 1,200 rpm at 4°C for 10 min. The cell sediment was suspended in saline to facilitate cell counting, placed on microscope slides, and stained with Wright–Giemsa stain. The percentage of eosinophils in the BALF was calculated by counting 100 cells on randomly selected areas of the slide using light microscopy (Olympus, Japan). Supernatants were harvested and stored at −80°C.

Enzyme-Linked Immunosorbent Assay

The concentrations of IL-6 and TNF-α were measured in BALF and serum with the ELISA kit (Ray Biotech, United States). The assay was performed according to the manufacturer's instructions.

Lung Histological Assay

The left lungs of mice were harvested and fixed in 4% paraformaldehyde for 24 h and then embedded in paraffin using a standard protocol after dehydration. Then, 4 μm sections of embedded lung tissue were mounted onto slides and stained with H&E to identify tissue inflammation, with PAS to identify mucus production, and with Masson trichrome to evaluate collagen deposition and smooth muscle hyperplasia. Tissue sections were viewed with a microscope (Olympus) and Image Pro Plus 6.0 software.

Cell Culture and Identification

Primary ASMCs were isolated from the tracheae of Sprague Dawley rats (Animal Core Facility, Nanjing Medical University) according to a previously reported method (Liu D et al., 2018). The culture medium was refreshed every 3 days with 10% FBS, and cells were used for subsequent experiments when they reached passages 6–10.

RNA Isolation and RT-PCR

Total RNA was extracted from lung tissue and cultured ASMCs by TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The cDNA was obtained using total RNA with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, United States). The obtained cDNA was used to determine the expression of miR-221 in the lung tissues and ASMCs by qPCR in an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, United States) with a TaqMan MicroRNA Assay (Applied Biosystems; Thermo Fisher Scientific, Inc). The cDNA was also synthesized from total RNA by M-MLV reserve transcriptase (Takata, Dalian, China), and qPCR was performed to measure the expression of MUC5AC by SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States) in the same system. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Proteins were extracted from cultured cells or lung tissues using RIPA lysis buffer. Proteins were subsequently separated by electrophoresis using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis before being transferred to a PVDF membrane. Membranes were incubated with 5% skim milk for 2 h at 37°C before they were incubated with primary antibodies against COL3A1, COL1A2 (Bioworld Technology, Inc., St Louis Park, MN, United States), and GAPDH (Santa Cruz, Dallas, TX, United States) at 4°C overnight. The next day, the membranes were probed using horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Targeted protein signals were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.), and band intensities were measured using Image Pro Plus 6.0 software.

Statistical Analysis

All data are presented as the mean ± standard error of the mean. Statistical graphs were generated using GraphPad Prism for Windows (ver. 6.00; GraphPad Software, San Diego, CA, United States). One-way ANOVA combined with Fisher's protected *t*-test was used to determine the significance of the differences between groups. Differences at $p < 0.05$ and $p < 0.01$ were considered significant and extremely significant, respectively.

RESULTS

Effects of miR-221 Inhibition on AHR

To investigate the potential role of miR-221 in asthma, AAVs with miR-221-sponge were instilled intratracheally to the mice to downregulate miR-221. As shown in **Figure 1D**, the expression of miR-221 significantly increased in asthmatic mice compared with the control group and decreased in the OVA + shRNA-miR-221 group compared with the OVA group. In short, AAVs with miR-221-sponge could successfully inhibit the expression of miR-221 in the lung tissue of mice. To assess the changes in AHR, we compared airway responses to MeCh in different groups (**Figures 1A–C**). In all experimental groups, both the expiratory and inspiratory resistance increased with an increase in the MeCh dose, while the trough value of Cldyn decreased. At each point, OVA exposure had a significant influence on Ri, Re, and Cldyn ($p < 0.05$ or $p < 0.01$) between the saline group and OVA group. Compared with the OVA group, the inhibition of miR-221 could significantly reduce the changes in lung function ($p < 0.05$ or $p < 0.01$), mainly including reduced Ri and Re and increased dynamic lung compliance. This indicated that the inhibition of miR-221 could effectively reduce the changes in lung function induced by chronic asthma.

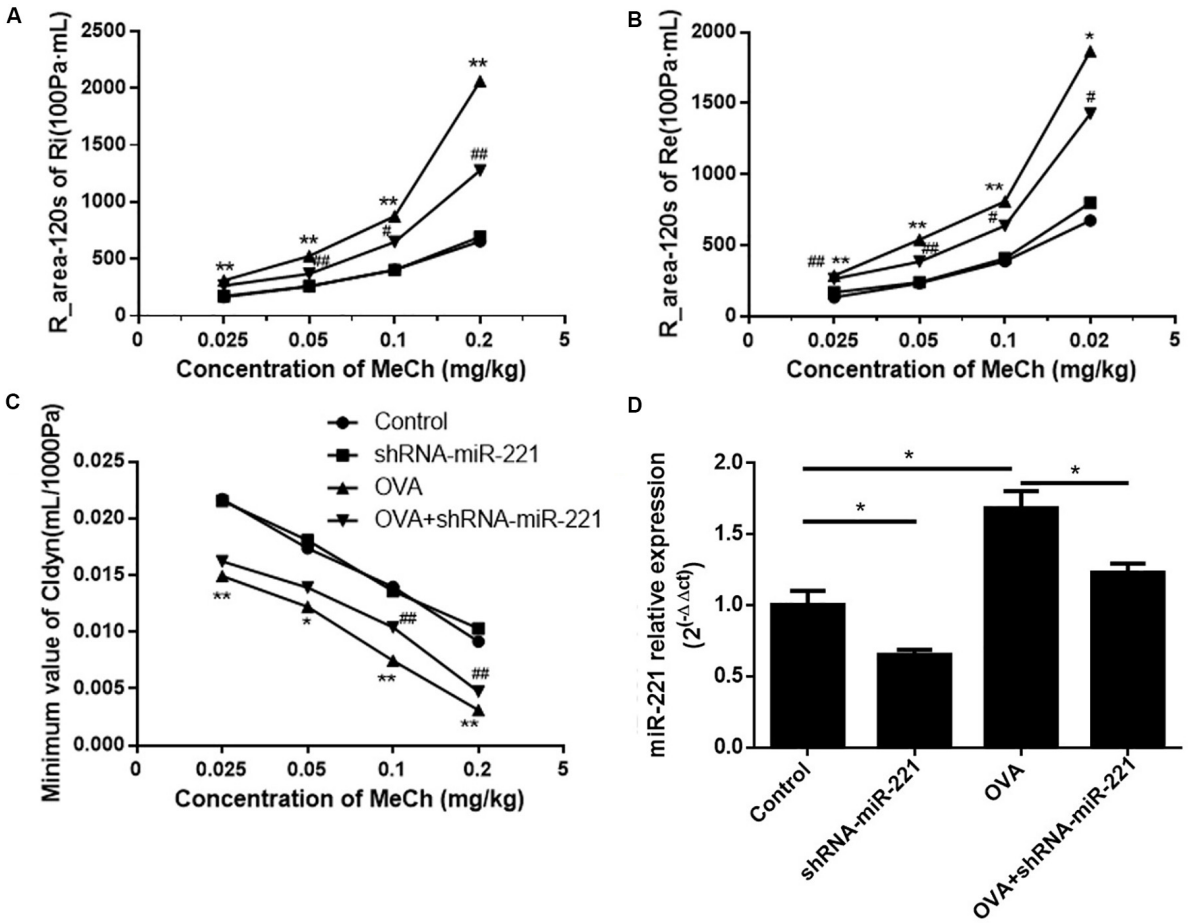

FIGURE 1 | The expression of miR-221 in the lung tissues of mice with AAV infection and airway hyper-responsiveness measurements. **(A)** R-area of Re, **(B)** R-area of Ri, and **(C)** peak value of Cldyn at different doses of MeCh, **(D)** miR-221 expression in the lung tissues of mice with AAV infection. Animal groups (in all panels): $n = 4$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the saline control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the OVA group.

Effects of miR-221 Inhibition on Inflammatory Cell Recruitment and the Level of Cytokines

To examine the persistence of airway inflammation, we compared the levels of total cells, as well as the eosinophil percentages, in BALF. **Figure 2A** shows that the total count of inflammatory cells in the BALF was significantly higher (more than 2 times; $p < 0.01$) in mice that had undergone the conventional chronic protocol than in control mice. The levels of eosinophil percentages in the BALF were also elevated significantly in OVA-challenged mice compared with control mice ($p < 0.01$) (**Figure 2B**). The inhibition of miR-221 also significantly decreased the total cells and eosinophil percentages compared with the OVA group ($p < 0.01$) (**Figures 2A,B**). The cell count findings demonstrated that the downregulation of miR-221 by AAVs (which was significantly higher in the OVA group) effectively alleviated the OVA-exposure effect on elevated total cell counts and on the percentage of eosinophils in the BALF (**Figures 2A,B**) caused directly by OVA sensitization. The cytokines IL-6 and TNF-α in the BALF and serum were detected using ELISA kits to assess the nature of the cytokine response to OVA (**Figure 2**). IL-6 and TNF-α levels in the BALF and serum rose significantly in the OVA group compared to the control group ($p < 0.05$ or $p < 0.01$) (**Figure 2**). In addition, the inhibition of miR-221 resulted in a decline in IL-6 and TNF-α concentrations, showing a significant difference ($p < 0.01$) (**Figure 2**) in the OVA + shRNA-miR-221 group when compared to the OVA group.

Effects of miR-221 Inhibition on Lung Histopathological Changes and on ECM Deposition

Airways from chronic asthmatic mice revealed severe peribronchial inflammatory infiltrate compared with control mice (**Figure 3A**). We measured the Wai, Pbm, Wat, and Wam of bronchi using Image Pro Plus 6.0 software. Airway walls appeared obviously thickened in asthmatic mice compared with control mice (**Figure 3D**). The inhibition of miR-221 could also significantly reduce goblet cell hyperplasia compared to the OVA group (**Figure 3A**). At the same

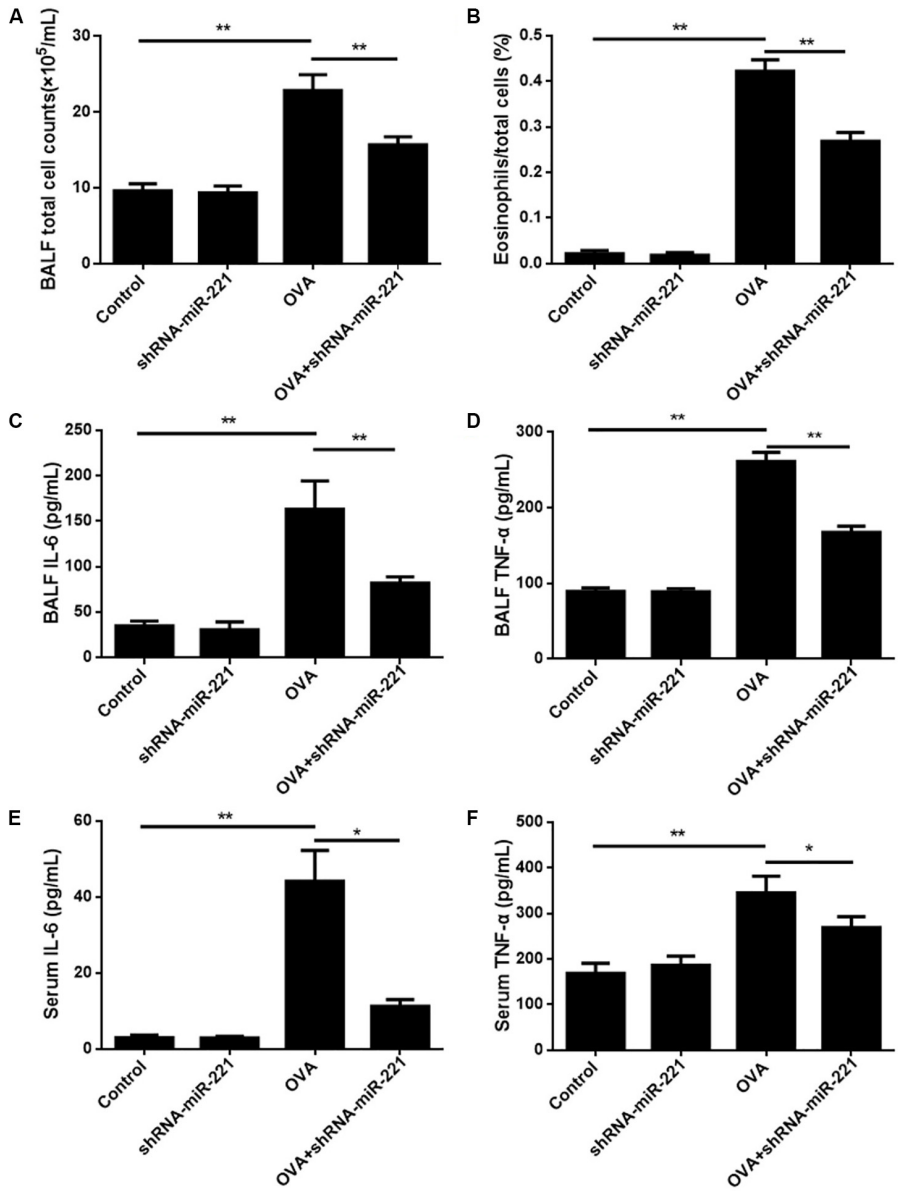

FIGURE 2 | BALF inflammatory cell counts in different experimental groups and levels of cytokines in BALF and serum. **(A)** Total cell counts in BALF. **(B)** Ratios of eosinophil counts in BALF. **(C)** The level of IL-6 in BALF. **(D)** The level of TNF-α in BALF. **(E)** The level of IL-6 in serum. **(F)** The level of TNF-α in serum. Animal groups (in all panels): $n = 6$. $^{**}p < 0.01$.

time, the percentage of PAS-positive epithelial cells also significantly decreased in the OVA + shRNA-miR-221 group compared to the OVA group, consistent with the trend of the mRNA expression levels of MUC5AC in lung tissue (**Figures 3B,C**). Moreover, the downregulation of miR-221 attenuated airway collagen deposition in the lung tissue of mice (**Figure 3A**). Western blot analysis showed that compared to the control group, the expression of type I collagen and type III collagen was higher in the OVA group (**Figures 3E,F**), whereas miR-221 downregulation, induced by OVA, significantly decreased their expression. In summary, the inhibition of miR-221 could alleviate the collagen deposition in the ECM resulting from chronic asthma.

Effects of PDGF-BB and EGF Stimulation on miR-221 Expression and ECM in ASMCs, and Effects of miR-221 Upregulation on the Deposition of the ECM in ASMCs

Studies have shown that PDGF-BB can promote the expression of ECM proteins such as type I collagen and type III collagen. Meanwhile, EGF, a type of cytokine, plays an important role

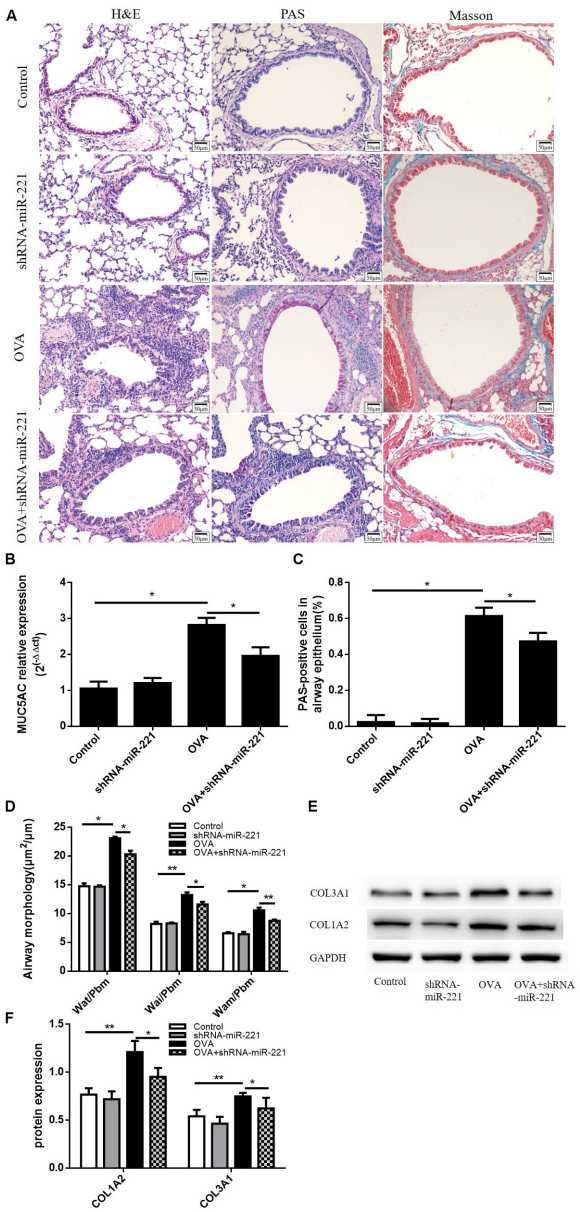

FIGURE 3 | Representative lung sections from different staining methods and the expression levels of ECM proteins in the lung. **(A)** Hematoxylin and eosin (H&E) staining showing the infiltration of inflammatory cells, Periodic acid–Schiff (PAS) staining showing mucus cells (purple stain), Masson trichrome staining showing subepithelial collagen deposition (blue stain). **(B)** The mRNA expression of MUC5AC in lung tissue. **(C)** The percentage of PAS-positive epithelial cells. **(D)** Airway wall thickness was analyzed by HE-stained lung sections. Magnification, 200X; animal groups (in all panels): $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$. **(E,F)** Representative western blots show the levels of type I collagen and type III collagen in the lungs. GAPDH was used as a loading control. Data are means ± SD from three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

in the proliferation and differentiation of various cells. In our study, after transfection with miR-221 mimics, the expression of miR-221 was significantly higher than that of the control group (**Figure 4C**). With the overexpression of miR-221, the levels of COL1A2 and COL3A1 increased compared to the control group (**Figures 4D,F**). As shown in **Figure 4A**, the expression of miR-221 increased with the stimulation of PDGF-BB and EGF in ASMCs. PDGF-BB and EGF could induce the expression of type I collagen and type III collagen (**Figures 4B,E**). Thus, we found that miR-221 downregulation could inhibit airway inflammation and airway remodeling in asthmatic mice.

Effects of miR-221 Upregulation on the Activity of the PI3K/AKT Signaling Pathway

A heat map of differential gene expression in the overexpression control group and the miR-221 overexpression group (miR-221 mimics) is shown in **Figure 5A**. miR-221 overexpression promoted the expression of the PI3K/AKT signaling pathway compared to the overexpressed control group (**Figure 5B**), suggesting that the PI3K/AKT signaling pathway might be involved in the expression of ECM proteins in ASMCs by miR-221. To further verify the role of the PI3K/AKT signaling pathway in the promotion of ECM expression in ASMCs by miR-221, we used LY294002, an inhibitor of the PI3K/AKT signaling pathway, to interfere with ASMCs. Western blot assays showed that the amount of total AKT protein was not significantly changed, but the protein level of phosphorylated (P-AKT) was significantly increased ($p < 0.05$ or $p < 0.01$) (**Figures 5C,D**). Compared with the miR-221 mimic group, the protein expression of COL1A2 and COL3A1 was decreased in the miR-221 mimics + LY294002 (PI3K/AKT inhibitor) group.

DISCUSSION

MicroRNAs have been implicated to have a fundamental role in asthma and in airway remodeling through the regulation of multiple signal transduction pathways involved in the pathogenesis of asthma. There is now increasing evidence that miRNAs (including miR-34/449, let-7, miR-19, miR-21, and miR-455) play potentially important roles in asthma. Simpson revealed that miR-19 is upregulated in asthma by promoting type 2 helper T cytokine production and amplifying inflammatory signaling (Solberg et al., 2012). miR-19a has been found to enhance airway epithelial proliferation as result of the loss of TGFBR2-mediated SMAD3 phosphorylation in ASMCs from patients with severe asthma. A previous study reported that miR-142 inhibits ASMC proliferation and promotes apoptosis during airway remodeling in asthmatic rats by inhibiting TGF-β expression via a mechanism involving the EGFR signaling pathway.

Meanwhile, Mayoral demonstrated that miR-221 can regulate the cell cycle of mast cells and also regulate the contraction and relaxation of ASMCs (Mayoral et al., 2011). The upregulation of miR-221 can also increase mast cell degranulation. Perry MM found that the inhibition of miR-221 can alleviate the proliferation of cells and the release of IL-6 in ASMCs from the asthmatic patients (Perry et al., 2014).

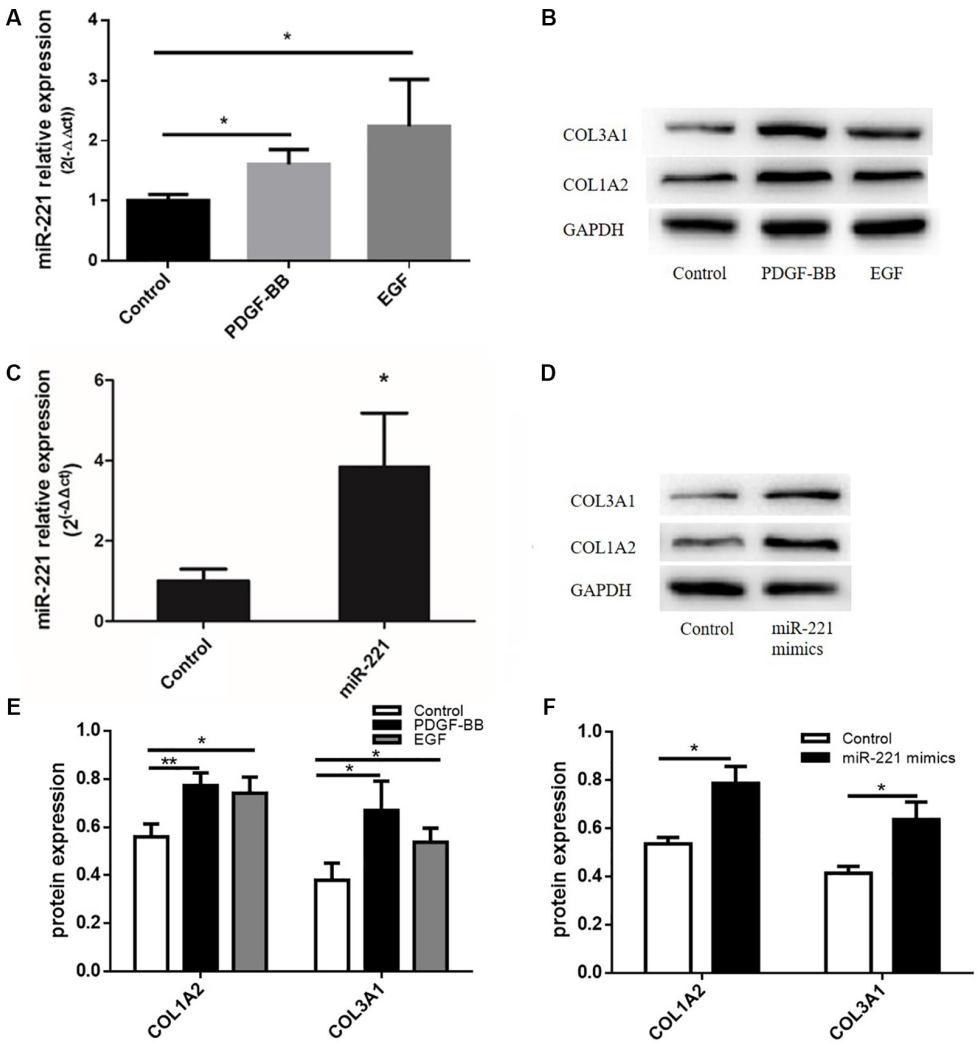

FIGURE 4 | The protein expression levels of ECM after the stimulation of PDGF-BB and EGF in ASMCs and the protein expression levels of ECM after miR-221 overexpression in ASMCs. **(A)** The expression of miR-221 in ASMCs. **(B,E)** The expression of protein in response to the PDGF-BB and EGF stimulation. *$p < 0.05$. **(C)** The expression of miR-221 in ASMCs. **(D,F)** The expression of protein in response to miR-221 upregulation. Data were normalized to GAPDH expression and are presented as expression relative to controls. Data are means ± SD from three experiments. *$p < 0.05$, **$p < 0.01$.

In our previous studies, we found that the expression of miR-221 was higher in asthmatic patients than in control subjects (Zhou et al., 2016). By qRT-PCR, we confirmed that the expression of miR-221 in the peripheral blood of asthmatic children was significantly higher than that of healthy children (Liu et al., 2012). Since miR-221 has been identified to be involved in the development of asthma, we explored whether and how miR-221 affected OVA-induced asthmatic mice in this study.

ASMCs are key players in airway pathologies such as augmented airway inflammation and airway remodeling. It has been recognized that abnormal ASMC proliferation, such as hypertrophy and hyperplasia, can result in the increased thickness of airway walls, contributing to the development of the airway remodeling observed in asthma (Céline et al., 2010; Boulet, 2018). Therefore, it is important to explore the underlying mechanism of ASMC proliferation. Plenty of studies have found that a variety of stimulating factors, including growth factors, hematogenic or inflammatory mediators, cytokines, and ECM proteins, can induce ASMC proliferation (Suojalehto et al., 2014). Growth factor-induced ASMC proliferation is considered the major cause of airway wall thickening in asthma, and increased levels of PDGF-BB have been noted, which contribute to ASMC proliferation (Liu et al., 2015; Dai et al., 2018). In this study as well, we demonstrated that the expression of miR-221 and the protein levels of ECM could be influenced by the simulation of PDGF-BB and EGF, inducing the proliferation of ASMCs. However, the underlying mechanism is not clear. Many types of inflammatory cells play roles in asthma by releasing inflammatory mediators that cause sustained chronic inflammation of the airways. This triggers bronchoconstriction and airway structural changes. We found that the inhibition of miR-221 caused higher inflammatory cell infiltration, goblet

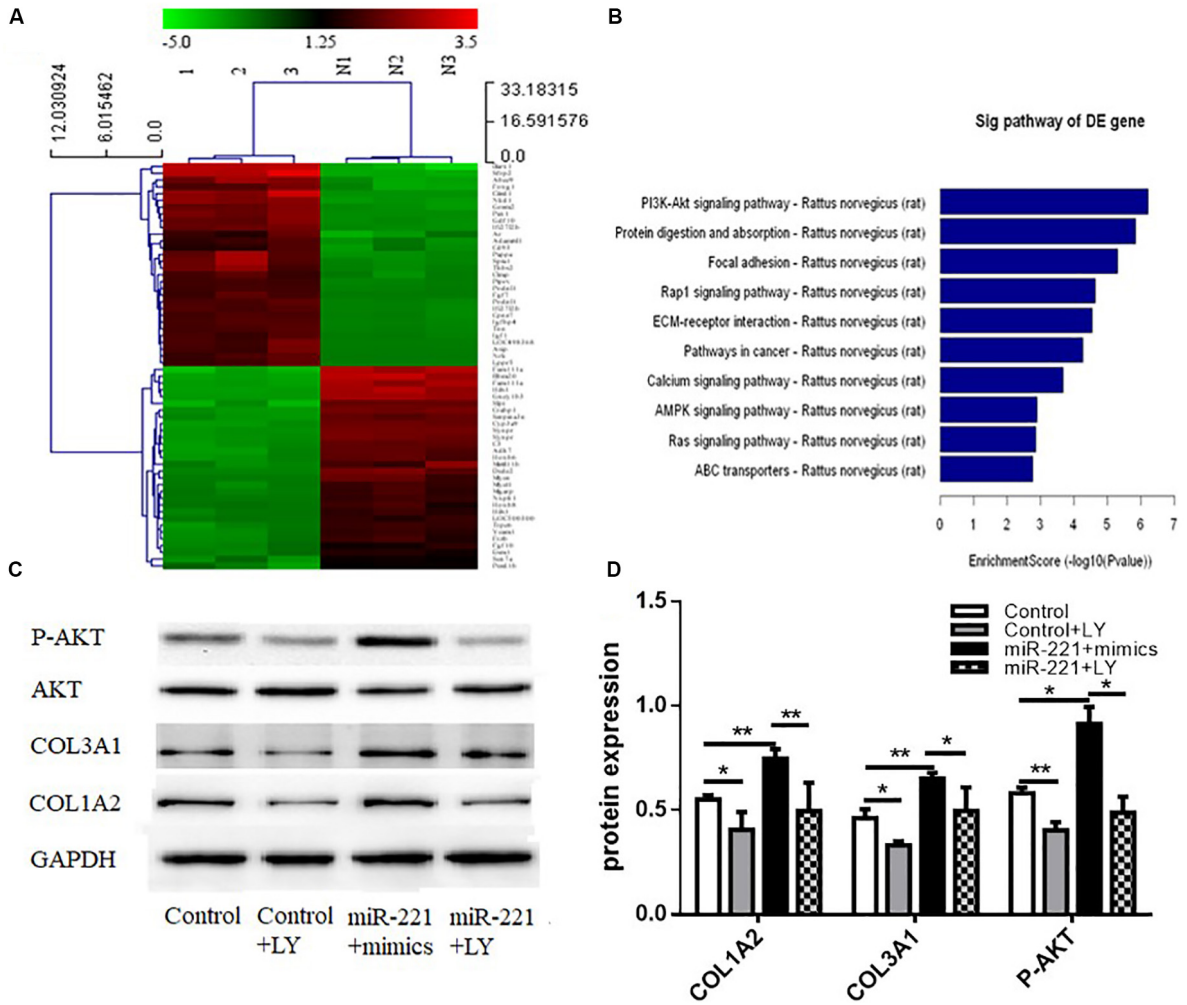

FIGURE 5 | The activity of the PI3K/AKT signaling pathway in response to miR-221 upregulation. **(A)** The heat map of differential gene expression in different groups. **(B)** The changes in the signaling pathway. **(C,D)** The level of protein expression. Data were normalized to GAPDH expression and are presented as expression relative to controls. Data are means ± SD from three experiments. *$p < 0.05$, **$p < 0.01$.

cell hyperplasia, mucus obstruction, and MUC5AC mRNA expression in lung tissue. Goblet cells are simple columnar epithelial cells that secrete gel-forming mucins, like the mucin MUC5AC (Kanoh et al., 2011; Ma et al., 2017). Studies have shown that goblet cell metaplasia and airway hypersecretion can occur in asthma (Chen et al., 2015).

The PI3K/AKT pathway is important for cell growth, differentiation, metabolism, survival, and apoptosis. Numerous studies have demonstrated that the PI3K/AKT pathway participates in the regulation of miRNAs, thereby affecting the proliferation of ASMCs (Liu Y et al., 2018; Wang et al., 2018). The PI3K/AKT pathway is involved in promoting ECM expression. The inhibition of miR-223 has been found to inhibit the deposition of ECM in ASMCs by targeting IGF-1R via the PI3K/AKT pathway (Wang Q. et al., 2015). Wang J reported that miR-29b could regulate the PI3K/AKT signaling pathway by negatively targeting PIK3R1 and AKT3, thereby reducing the expression of ECM proteins such as COL1A2 in mouse hepatic stellate cells (Wang J. et al., 2015). Osaki M also determined that the PI3K pathway could affect the proliferation of ASMCs in asthmatic patients and play an important role in the differentiation, activation, and production of cytokines of T cell receptors and T cells (Osaki et al., 2004). The above research indicates that the PI3K/AKT signaling pathway somehow participates in promoting ECM protein expression. This study also found that the expression of P-AKT in ASMCs increased after the overexpression of miR-221, which further suggests that miR-221 may promote the expression of ECM proteins in ASMCs by activating AKT and phosphorylating it.

Our findings shed new light by providing clues for the prevention and treatment of chronic asthma, especially airway remodeling. However, the limitations of this study should be mentioned. Since we have not identified the corresponding target gene of miR-221 yet (**Supplementary Figure S1**), further studies will need to focus on the exact mechanism of how miR-221 inhibition alleviates asthma.

CONCLUSION

We propose that the downregulation of miR-221 may affect airway remodeling via the PI3K/AKT pathway in murine asthma. These findings highlight a novel aspect of the role of miR-221 in asthma and support the development of a novel therapeutic strategy based on this miRNA.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All animal experiments were approved by the local animal care committee of the National Defense Medical Center (approval number: IACUC-1702005).

AUTHOR CONTRIBUTIONS

FL, DZ, JP, and QY designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. YZo, HD, and YZu performed the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00495/full#supplementary-material

Many studies have confirmed that miR-221 can regulate the biological function of cells by targeting PTEN binding to activate PI3K. Du J showed that miR-221 could target PTEN to reduce the sensitivity of cervical cancer cells to gefitinib through the PI3K/AKT signaling pathway. Meanwhile, we demonstrated that the overexpression of miR-221 promoted the expression of the PI3K/AKT signaling pathway, suggesting that this pathway might be involved in the expression of ECM proteins in ASMCs through miR-221. This led us to wonder if PTEN could reduce airway remodeling by downregulating the expression of miR-221 via PI3K/AKT signaling. We show that the difference in the expression of PTEN with the inhibition of miR-221 was not significantly different between the groups.

FIGURE S1 | (A) Protein expression levels of PTEN in an asthmatic mouse. GAPDH was used as a loading control. **(B)** Relative protein expression of PTEN. There was not significantly different between the inhibition of miR-221 compared to controls. We induced overexpression or inhibition of miR-221 in ASMCs by miR-221 mimics and miR-221 inhibitor.

FIGURE S2 | The study's experimental design and the experimental protocol to develop an OVA-induced mouse model of chronic asthma. BALB/c mice were sensitized by injecting a mixture of OVA with Imject Alum and then challenged with aerosolized OVA (concentration, 5%) for 8 weeks (thrice a week). The groups were as follows: (1) sensitization and challenge with saline along with AAV6-GFP infection (control group); (2) sensitization and challenge with saline along with AAV6-miR-221-sponge infection (shRNA-miR-221 group), (3) sensitization and challenge with OVA along with AAV6-GFP infection (OVA group), (4) sensitization and challenge with OVA along with AAV6-miR-221-sponge infection (OVA + shRNA-miR-221 group). We induced overexpression or inhibition of miR-221 in ASMCs by miR-221 mimics and miR-221 inhibitor. We investigated ASMC viability by using a CCK-8 assay. The results in **Supplementary Figure S3** suggest that miR-221 could not affect ASMC viability.

FIGURE S3 | The effect of miR-221 on ASMC viability. ASMCs were transfected with 50 nM miR-221 mimics and miR-221 inhibitor compared with control. Cell viability was then assessed 48 h post-transfection using a CCK-8 kit. Data are means ± SD from three independent experiments (n = 5).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aging, Cellular Senescence, and Progressive Multiple Sclerosis

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Aging is one of the most important risk factors for the development of several neurodegenerative diseases including progressive multiple sclerosis (MS). Cellular senescence (CS) is a key biological process underlying aging. Several stressors associated with aging and MS pathology, such as oxidative stress, mitochondrial dysfunction, cytokines and replicative exhaustion are known triggers of cellular senescence. Senescent cells exhibit stereotypical metabolic and functional changes, which include cell-cycle arrest and acquiring a pro-inflammatory phenotype secreting cytokines, growth factors, metalloproteinases and reactive oxygen species. They accumulate with aging and can convert neighboring cells to senescence in a paracrine manner. In MS, accelerated cellular senescence may drive disease progression by promoting chronic non-remitting inflammation, loss or altered immune, glial and neuronal function, failure of remyelination, impaired blood-brain barrier integrity and ultimately neurodegeneration. Here we discuss the evidence linking cellular senescence to the pathogenesis of MS and the putative role of senolytic and senomorphic agents as neuroprotective therapies in tackling disease progression.

Keywords: multiple sclerosis, cellular senescence, inflammation, remyelination, neurodegeneration, neuroprotection, senolytics

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INTRODUCTION

Multiple sclerosis (MS) is a chronic, immune mediated disease of unknown etiology characterized by inflammatory demyelination, astrogliosis, neuronal and axonal loss involving the brain and spinal cord. The majority of MS patients follow an initial course with relapses and remissions (RR-MS) followed by a phase of progressive accumulation of disability termed secondary progressive (SP-MS). Ten to 15% of patients with primary progressive MS (PP-MS) exhibit gradual worsening from the start and typically PP-MS presents at an older age than RR-MS (Compston and Coles, 2008). The pathogenesis of the progressive disease courses (P-MS) is poorly understood. However, epidemiological evidence indicates that age is the strongest predictor for the transition from the relapsing phase, which is considered primarily inflammatory to the secondary progressive phase of the disease, which is mainly neurodegenerative (Trapp and Nave, 2008; Scalfari et al., 2011).

Although our understanding of the biological basis of aging remains incomplete, the prevailing hypothesis postulates that it is driven by the accumulation of irreparable molecular and

cellular damage leading to an increased risk of functional decline, disease and ultimately death. Aging exhibits a great diversity of phenotypes and a loose connection between biological and chronological age, probably due to the stochastic nature of molecular damage and the complexity of the interaction between genetic and environmental factors (Kirkwood et al., 2005; Kirkwood and Melov, 2011). Aging is the most important risk factor for the development of neurodegenerative disease (Hou et al., 2019). Cellular senescence (CS) has been recognized as a key biological process underling normal aging (López-Otín et al., 2013; Gorgoulis et al., 2019) and evidence suggest that the accumulation of senescent cells with time may contribute to the pathogenesis of age-related and neurodegenerative disease (Kritsilis et al., 2018). Here, we review the data that support a role for cellular senescence in the pathogenesis of MS.

THE SENESCENCE STATE

Several cell stressors have been identified as triggers of CS, which among others include oxidative stress, mitochondrial dysfunction, replicative stress, cytokines, irradiation, genotoxic agents presented in detail in Gorgoulis et al. (2019). Oxidative stress, mitochondrial dysfunction and cytokines, such as TGF-beta are key features of MS pathology (Gilgun-Sherki et al., 2004; Mahad et al., 2009; Haider et al., 2011; Elkjaer et al., 2019). Most of these triggering factors are associated with DNA damage and activate the signal transduction system of DNA damage response (DDR) (Nakamura et al., 2008). If the damage is irreparable DDR may elicit CS (Rodier and Campisi, 2011).

The senescent phenotype is typically associated with several metabolic and functional changes including stable cell cycle arrest, the expression of a senescence-associated secretory phenotype (SASP) and the accumulation of dysfunctional mitochondria (Coppé et al., 2010; Munoz-Espin and Serrano, 2014; Correia-Melo et al., 2016). SASP consists of pro-inflammatory cytokines, growth factors, cytotoxic mediators, metalloproteinases and reactive oxygen species (ROS). These are capable of affecting neighboring cells and converting them to senescence in a paracrine manner (Kuilman and Peeper, 2009; Acosta et al., 2013). Other changes characteristic of the senescent state are described in greater detail elsewhere (Munoz-Espin and Serrano, 2014; Gorgoulis et al., 2019). The senescence-associated changes reported specifically for CNS cells are summarized in **Table 1**.

Although CS is a homeostatic response aiming to prevent the proliferation and neoplastic conversion of damaged cells (Munoz-Espin and Serrano, 2014) it also has a role in development (Rajagopalan and Long, 2012; Barbouti et al., 2019). Damaged senescent cells remain viable and metabolically active, they accumulate with aging and evidence suggests that their build-up may promote neurodegeneration (Rodier and Campisi, 2011). The detrimental effects of CS on the brain are due to the pro-inflammatory milieu formed by senescent cells that act as sources of inflammatory mediators (Coppé et al., 2010). CS-associated cell-cycle arrest may exhaust the regenerative capacities of adult progenitors, such as oligodendrocyte progenitor cells (OPCs) responsible for myelin repair. In addition, CS along with replication arrest is associated with extensive changes in gene expression, which indicate severe loss or alteration of physiological cell function (Purcell et al., 2014). Finally, endothelial cell senescence may compromise blood-brain barrier (BBB) integrity (Yamazaki et al., 2016), which is essential for preserving brain tissue homeostasis (Berthiaume et al., 2018).

CELLULAR SENESCENCE AND INFLAMMATORY ACTIVITY IN MS

Both innate and adaptive components of the immune response are known to play key roles in the immunopathogenesis of MS (Weissert, 2013; Hemmer et al., 2015). Microglial cells, the resident representative of the innate immune response in the CNS (Ransohoff and Brown, 2012) are known to become senescent under specific circumstances. Cultured microglial cells can become senescent in response to chronic inflammatory stimulation by lipopolysaccharide treatment (Yu et al., 2012). Rat and human microglial cells from AD patients have been shown to undergo replicative senescence due to telomere shortening (Flanary and Streit, 2004; Flanary et al., 2007). With aging, microglial cells exhibit a dystrophic phenotype associated with functional changes, which seem to be distinct from the typical microglial reaction (Streit et al., 2004, 2009; Conde and Streit, 2006). Aged microglia exhibit decreased migratory and phagocytic capacity and secrete constitutively greater amounts of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in culture (Njie et al., 2012; Rawji et al., 2020). The constitutive secretion of proinflammatory cytokines by microglia from aged mice is consistent with the SASP of senescent cells. Myelin clearance is a prerequisite for remyelination to occur (Kotter et al., 2001, 2006; Cantuti-Castelvetri et al., 2018) and impaired phagocytotic capacity of aged microglia and macrophages could hinder myelin repair in older patients. Nevertheless, the presence of senescent microglia and macrophages in MS and its models has not been shown.

With regard to the adaptive component of the immune response in MS, there is evidence of premature immunosenescence with T cell changes resembling those seen in the elderly (Thewissen et al., 2005). An expansion of CD4(+) CD28(–) T cells and a reduction of T-cell receptor excision circles (TREC) has been observed in the peripheral blood of MS patients (Thewissen et al., 2005). Peripheral blood CD4(+) CD28(-) T cells in patients and healthy controls exhibit an effector-memory T cell phenotype with cytotoxic properties, as they secrete cytotoxic granules in response to polyclonal stimuli and MS-related autoantigens. These CD4(+)CD28(-) T cells are attracted by increased levels of fractalkine and IL-15 and accumulate in demyelinated lesions (Broux et al., 2012, 2015). Similarly, a population of IgD(-)$^{-}$CD27(-)CD11c (+)(CD21^{low}) B cells, which have been associated with aging was demonstrated in higher proportions in the CSF and peripheral blood of MS patients than age-matched healthy

TABLE 1 | Observed hallmarks, features, and functional changes associated with CS in CNS cells.

Cell type	Senescence-associated changes	SASP factors	Functional changes	References
Neurons	γH2A.X and 53BP1 upregulation SAHF (macroH2A) SA-β-Gal activity↑ Lipofuscin accumulation	IL-6 protein	?	Sedelnikova et al., 2004; Jurk et al., 2012; Kritsilis et al., 2018
Astrocytes	Cell-cycle arrest SAHF (Hp1γ↑); 53BP1 foci↑ $p16^{INK4A}$ mRNA and protein↑, p21 mRNA and protein↑, p53 mRNA↑ p38MAPK and NF-κB activation SA-β-Gal activity↑ EAAT-1 mRNA and protein↓, Kir4.1 mRNA and protein ↓, GFAP protein↓, S100β, TIMP-1 mRNA ↑; Lamin B1 mRNA↓	CXCL-1 mRNA TGF-β HMGB1 IL-6 mRNA and protein IL-8 mRNA and protein MMP-3 mRNA and protein MMP-9 mRNA	Loss of support of oligodendrocyte differentiation *in vitro* Loss of ability to support neuronal survival *in vitro*	Bitto et al., 2010; Salminen et al., 2011; Bhat et al., 2012; Al-Mashhadi et al., 2015; Görg et al., 2015; Nie et al., 2015; Crowe et al., 2016; Hou et al., 2017, 2018; Chinta et al., 2018; Turnquist et al., 2019; Limbad et al., 2020; Willis et al., 2020; Yabluchanskiy et al., 2020
Oligodendrocytes	γH2A.X upregulation SA-β-Gal upregulation	?	?	Al-Mashhadi et al., 2015
Microglia	Cell-cycle arrest Telomere attrition p38MAPK activation SA-β-Gal activity↑ SAHF	IL-6, IL-1β, TNF-α	Impaired phagocytic capacity *in vitro**	Flanary and Streit, 2004; Flanary et al., 2007; Sierra et al., 2007; Bachstetter et al., 2011; Njie et al., 2012; Yu et al., 2012; Rawji et al., 2020
Oligodendrocyte progenitor cells (OPCs)	SA-β-Gal upregulation Increased DNA damage p21↑ and p16INK4A mRNA and protein↑ mTOR activation	?	Impaired proliferation and differentiation	Kujuro et al., 2010; Choi et al., 2016; Neumann et al., 2019; Zhang et al., 2019
Neural precursor cells (NPCs)	Flattened morphology Telomere attrition p16INK4A SA-β-Gal activity↑	HMGB1 ROS production	Impaired adult neurogenesis *in vivo* Inhibition of oligodendrocyte differentiation *in vitro*	Ferron et al., 2004, 2019; Bose et al., 2010; He et al., 2013; Yang et al., 2017; Nicaise et al., 2019; Willis et al., 2020
Ependymal cells	$p16^{INK4A}$ mRNA ↑ SA-b-Gal activity ↑	?	Impaired BBB function *in vitro*	Yamazaki et al., 2016; Yang et al., 2017
Pericytes	p16INK4A mRNA ↑ SA-β-Gal ↑activity	?	Impaired BBB function *in vitro*	Yamazaki et al., 2016

BBB, blood-brain barrier; 53BP1, p53 binding protein-1; CXCL-1, chemokine C-X-C motif ligand 1; CS, cellular senescence; GFAP, glial fibrillary acidic protein; H2A.X, H2A histone family member X; HMGB1, high mobility group box-1; Hp1γ, heterochromatin protein-1γ; IL, interleukin; MMP, metalloproteinase; mTOR, mammalian target of rapamycin; NPCs, neural progenitor cells; OLG, oligodendrocytes; OPCs, oligodendrocyte progenitor cells; p38MAPK, p38 mitogen-activated protein kinases; ROS, reactive oxygen species, SA-β-Gal, senescence-associated β-galactosidase; SAHF, senescence-associated heterochromatin formation; SASP, senescence-associated secretory phenotype; Timp-1, tissue inhibitor of metalloproteases-1; TNF-α, tumor necrosis factor-α.

** Njie et al. (2012) and Rawji et al. (2020) have provided in vitro evidence of impaired phagocytic capacity of microglia from aged mice but have not provided evidence of CS.*

controls. These IgD(-)$^{-}$CD27(-)CD11c(+)($CD21^{low}$) B cells produced proinflammatory cytokines upon *ex vivo* stimulation and showed MHC class-II expression and costimulatory molecule expression capable to induce proinflammatory T cell responses. Their presence indicates that premature senescence of B cells may promote inflammation and thereby contribute to disease progression in MS (Claes et al., 2016).

Senescent neurons and glia accumulating in the MS brain and secreting SASP-related inflammatory mediators may represent an alternative source of inflammation independent of the immune cells that bring about innate and adaptive immune responses. We have previously provided evidence of senescent glial cells and neurons showing lipofuscin^{+} senescent glial cells in acute and chronic actively demyelinated white matter lesions from SP-MS cases using GL13 histochemistry (Kritsilis et al., 2018). All resident CNS cell types that under some circumstances acquire a senescent phenotype may become sources of parenchymal inflammation. The documented ability of senescent cells to convert neighboring cells to senescence via paracrine action of SASP mediators is consistent with the progressive nature of disability seen in P-MS (Acosta et al., 2013; Chen et al., 2015). Current therapeutic strategies fail to tackle disability progression in P-MS despite being efficacious in preventing MS relapses and new lesion formation thought to be mediated by adaptive immune responses (Pardo and Jones, 2017). This is consistent with the concept of resident glia and neurons secreting SASP-related factors that maintain a low-burning yet persistent and self-enhancing inflammatory environment not affected by immunomodulators and immunosuppressants. The glial cell types, which are prone to senescence in the MS lesions remain to be specified.

AGING, CELLULAR SENESCENCE, AND FAILURE OF MYELIN REPAIR

Endogenous myelin repair (remyelination) of axons surviving inflammatory demyelination is known to occur in MS (Patani et al., 2007). Remyelination contributes to restoration of impulse conduction along axons traversing demyelinating plaques and exerts a neuroprotective effect on remyelinated axons preventing axonal degeneration associated with chronic demyelination (Kornek et al., 2000; Franklin and Goldman, 2015; Mei et al., 2016). Adult oligodendrocyte progenitor cells (OPCs) comprising 5–10% of all CNS cells are primarily responsible for carrying out myelin repair following demyelinating events (Reynolds et al., 2002; Tripathi et al., 2010). The efficiency of myelin repair is known to gradually decline with time and it is shown to be least extensive in the SP-MS phase of the disease (Bramow et al., 2010). This suggests that age-related remyelination failure may contribute to disability progression seen at the advanced stages of MS (Kuhlmann et al., 2008; Goldschmidt et al., 2009). Aging has been shown to reduce the capacity for remyelination in several animal models of demyelination (Shen et al., 2008; Hampton et al., 2012; Cantuti-Castelvetri et al., 2018) in which remyelination inefficacy is associated with impaired recruitment of OPCs into demyelinated lesions and slower differentiation into myelinating oligodendrocytes (Sim et al., 2002). OPCs from aged rats show features of CS with increased levels of DNA damage, mitochondrial dysfunction and p38MAPK mRNA upregulation (Neumann et al., 2019). In addition, recent experimental evidence indicates that murine astrocytes aged in culture develop a pro-inflammatory senescence-like phenotype and lose their ability to support oligodendrocyte differentiation (Willis et al., 2020).

However, OPC senescence may not only be associated with aging. In experimental autoimmune encephalomyelitis (EAE) model of MS in young mice, OPCs exhibited cell-cycle arrest linked to an upregulation of sirtuin 1 (SIRT1) transcription, suggesting that failure of OPC proliferation may be due to CS (Prozorovski et al., 2019). Furthermore, *in vitro* exposure of cultured murine OPCs to Aβ oligomers triggered CS and inhibited myelin sheet formation indicating that toxic factors can elicit senescence in OPCs (Horiuchi et al., 2012; Zhang et al., 2019). In the APP/PS1 model of Alzheimer's disease OPCs expressing upregulated p16, p21, and senescence-associated-β-Galactosidase (SA-β-Gal) markers of CS have been identified in association with Aβ plaques and treatments aiming to remove senescent OPCs (senolytics) attenuated neuroinflammation and cognitive deficits, indicating that OPC SASP promotes neuroinflammation and functional impairment (Zhang et al., 2019).

Other progenitor cells including neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs) also have a role in promoting remyelination and tissue repair (Nicaise et al., 2017; Rivera et al., 2019). It is documented that the adult CNS harbors multipotent neural progenitor cells (NPCs) that can produce neurons, astrocytes, and oligodendrocytes (Weiss et al., 1996; Johansson et al., 1999). They are thought to primarily reside in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011). Most NPCs in the adult brain exist in a quiescent state (Ding et al., 2020) unless CNS injury or specific stimuli elicit their proliferation, migration and differentiation (neurogenesis) (Mothe and Tator, 2005). Evidence supports the functional significance of NPCs as hippocampal neurogenesis is critical for cognition (Suh et al., 2009; Christian et al., 2014) and its disruption is associated with cognitive impairment (Aimone et al., 2014). In addition, studies have demonstrated that adult NPCs from the subventricular zone (SVZ) and the spinal cord contribute to the generation of new oligodendrocytes and myelin repair in models of demyelination (Nait-Oumesmar et al., 1999; Danilov et al., 2006; Menn et al., 2006; Xing et al., 2014; Maeda et al., 2019). Thus, NPCs could provide an alternative source of myelinating oligodendrocytes and probably also a source of neurons in demyelinated MS lesions (Chang et al., 2008).

Aging is associated with progressive reduction in adult neurogenesis (Lugert et al., 2010; Cipriani et al., 2018), which is ascribed to a diminution of the pool of stem cells capable of activation and division (Lugert et al., 2010) and it is associated with functional impairment (Hollands et al., 2017). Accumulating evidence suggests that these NPCs are also prone to senescence. Cultured NPCs exhibit characteristics of senescence, such as enlarged and flattened morphology, increased levels of SA-β-Gal and p16 and decreased level of phospho-Retinoblastoma (pRb) upon long term incubation with Aβ oligomers (Ferron et al., 2004; He et al., 2013; Li et al., 2016). Cell cycle arrest of adult progenitor cells in the context of CS or inhibitory paracrine stimuli by neighboring senescent cells may impair progenitor proliferation, reduce the regenerative capacities of the CNS and render it susceptible to neurodegeneration. This notion is supported by *in vivo* evidence from the BUBR1 KO progeroid mouse model in which adult neurogenesis was impaired in the SGZ and SVZ in an age-dependent manner (Yang et al., 2017). In MS, SOX2+ NPCs from demyelinated white matter lesions of autopsy material and NPCs from induced pluripotent stem cell lines from P-MS patients were found to express markers of CS. These senescent progenitor cells exhibited impaired capacity to support oligodendrocyte maturation *in vitro*, compared to NPCs from age-matched controls. Proteomic and transcriptomic analysis of the P-MS NPC secretome identified high-mobility group box-1 (HMGB1) as a senescence-associated inhibitor of oligodendrocyte differentiation, which induces expression of epigenetic regulators. HMGB1 was found to be expressed by progenitor cells in MS white matter lesions (Nicaise et al., 2019). Failure of spontaneous remyelination in MS may be at least partly due to conversion of OPCs and other neural progenitor cells to a state of CS induced by MS-specific triggers including oxidative stress, chronic inflammation, mitochondrial dysfunction and aging.

NEURODEGENERATION AND DISABILITY PROGRESSION IN MS

Neuroaxonal loss is the pathological correlate of irreversible disability (Trapp et al., 1998; Papadopoulos et al., 2006).

Although axonal loss can be an early feature of MS pathology associated with inflammatory lesion formation, in P-MS new focal inflammatory demyelinating plaques are rare (Trapp and Nave, 2008). Neuroaxonal loss in P-MS is driven by neurodegenerative mechanisms, which are poorly understood (Lassmann et al., 2007, 2012; Trapp and Nave, 2008).

Recently, telomere length of white blood cells (WBCs) used as a measure of WBC biological age was found decreased in P-MS patients compared to aged-matched controls (Habib et al., 2020). Moreover, shorter telomere lengths correlated with greater brain atrophy and higher levels of disability (Krysko et al., 2019), suggesting that biological aging contributes to neuroaxonal loss and disability progression in MS. Total brain atrophy, as assessed by MRI, reflects primarily gray matter atrophy due to neurodegeneration (Filippi et al., 2012) and correlates with long-term disability in MS (Fisniku et al., 2008; Filippi et al., 2013). Combined longitudinal MRI-based brain morphometry and brain age estimation using machine learning, revealed accelerated progressive brain aging in MS patients compared to healthy controls, which was related to brain atrophy and increased white matter lesion load (Høgestøl et al., 2019; Cole et al., 2020). Healthy aging is associated with brain cell loss, which may account up to 0.4% of brain volume per year (De Stefano et al., 2016). Both apoptotic and senescent cells are cleared by the immune system in a highly regulated manner and may contribute to age-related brain volume loss (Hoenicke and Zender, 2012; Ovadya et al., 2018).

Although post-mitotic cells do not fit the strict definition of CS, there is evidence of neurons developing a senescence-like phenotype. Neurons of aged mice have been shown to accumulate hallmarks of cellular senescence including double-strand DNA breaks, heterochromatinization, upregulation of SA-β- Gal, p38MAPK activation and production of SASP-related mediators including ROS and IL-6 (Sedelnikova et al., 2004; Jurk et al., 2012). The demonstration of neuronal granular cytoplasmic lipofuscin deposits in subpial demyelinated cortical lesions and normal appearing cortex from SP-MS cases using GL13 histochemistry supports the notion that human neurons may also acquire a senescence-like phenotype in MS (Kritsilis et al., 2018).

Although the functional state of senescent cells has not been fully elucidated, CS is accompanied by changes in gene expression and phenotypic changes, which constitute serious restrictions in the functionality of cells (Purcell et al., 2014). The number of senescent cells increases with age (Rodier and Campisi, 2011). When the number of dysfunctional senescent cells exceeds a certain threshold in a brain with reduced reserves due to age and MS-related cell loss, brain tissue function is likely to become compromised (Oost et al., 2018).

Neuronal survival strongly depends on the functional integrity of glial cells. Cultured astrocytes from aging rats have been found to upregulate the activity of SA-β-Gal, a marker of CS while they showed a reduced ability to maintain survival of co-cultured neurons, thus associating astrocyte senescence with neurodegeneration (Pertusa et al., 2007). Senescent astrocytes expressing $P16^{INK4A}$ and secreting metalloproteinase-1 (MMP-1) have been found in post-mortem tissues of Alzheimer's disease patients (Bhat et al., 2012). Primary human astrocytes made senescent by X-irradiation were found to downregulate genes encoding glutamate and potassium transporters leading to neuronal death in co-culture assays. These findings indicate that excitotoxicity, a recognized mechanism of neurodegeneration in MS (Werner et al., 2001) may result from impaired homeostatic capacities of senescent astrocytes (Limbad et al., 2020). Notably, dysregulated splicing of several genes from human senescent astrocytes has been demonstrated and an association between peripheral blood GFAPa, TAU3 and p14ARF isoform levels and cognitive decline has been demonstrated, indicating a link between astrocyte senescence and disability (Lye et al., 2019). Although no evidence of astrocyte senescence has been shown in association with MS pathology, their key role in neuron-glial crosstalk, regulation of neuronal metabolic and ion homeostasis and modulation of synaptic transmission via glutamate suggest that age-related astrocytic senescence may promote neuronal dysfunction and degeneration, contributing to MS progression.

Age-related accumulation of senescent endothelial cells is linked to impaired tight junction structure and compromised blood-brain barrier (BBB) function (Farrall and Wardlaw, 2009; Yamazaki et al., 2016; Castellazzi et al., 2020). Several lines of evidence from human studies and experimental animal models support a key role for fibrinogen in neuroinflammation (Davalos and Akassoglou, 2012). Blood-derived fibrinogen has been shown *in vivo* to interact with microglia via the CD11b/CD18 integrin receptor leading to perivascular microglial activation and axonal loss (Davalos et al., 2012). Fibrinogen has been found at the edge of chronic active lesions, which exhibit ongoing inflammatory demyelination, but not in inactive lesions, suggesting that fibrinogen may play a role in sustained inflammation even in the chronic setting. Endothelial senescence leading to a constantly leaky BBB may permit fibrinogen to diffuse into the brain parenchyma and drive axonal damage and loss mediated by persistent microglia activation as well as inhibition of remyelination (Petersen et al., 2018). The sustained nature of age-related BBB leakiness is consistent with the putative role of CS in neuroaxonal loss-mediated disability progression in MS.

CAN SENOLYSIS BE NEUROPROTECTIVE?

Currently, there is an unmet need for neuroprotective treatments that can effectively prevent disability progression in MS. A growing body of evidence implicates CS in the pathogenesis of neurodegeneration in a number of settings (Martínez-Cué and Rueda, 2020), rendering CS a promising target for neuroprotection. Anti-senescent or senotherapeutic approaches may involve the selective death of senescent cells (senolysis) to reduce the load of senescent cells and their detrimental effects on tissues. Alternatively, senotherapy may be based on the modulation of the senescent cell phenotype (senomorphism) in order to block the damaging effects of the SASP or other senescence-associated mediators (Kirkland et al., 2017). Senotherapy in MS would aim at preventing senescence-associated chronic inflammation, loss of cell function and neuroaxonal loss and promoting remyelination. Compounds with senolytic or senomorphic actions have been studied *in*

vitro and *in vivo* with promising results (Myrianthopoulos et al., 2019; Thoppil and Riabowol, 2020). Evidence from genetically modified mice support the neuroprotective potential of senolytic manipulation. Lifelong elimination of p16INK4A cells in BubR1 progeroid mice by activation of an INK-ATTAC transgene substantially delayed age-related disease, whereas late life elimination of p16INK4A cells attenuated these age-related pathologies (Baker et al., 2011). In addition, a senolytic compound (ABT263) was shown to attenuate tau phosphorylation and aggregation and to improve memory deficits in the PS19 transgenic model of tau-dependent neurodegeneration, by removing senescent glial cells (Bussian et al., 2018).

Fasting and metformin treatment could reverse the senescent state of rat OPCs and improve remyelination capacity (Neumann et al., 2019). Furthermore, rapamycin treatment modified the senescent state of progressive MS patient-derived NPCs produced from induced pluripotent stem cells and improved their capacity to promote OPC differentiation *in vitro*, providing evidence that senomorphic treatment can promote remyelination in MS (Nicaise et al., 2019). Simvastatin has shown efficacy in delaying brain atrophy and disability progression in MS trials (Chataway et al., 2014). This neuroprotective effect may be mediated via its senomorphic actions, which include downregulation of p38MAPK activation, SASP markers, TNFa, and GM-CSF as shown in other settings (Liu et al., 2015; Ayad et al., 2018). Many promising compounds with senolytic or senomorphic activity, such as metformin or simvastatin used with different indications could be repurposed and used as neuroprotectants combined with currently available immunomodulators. Immune-mediated physiological clearance of senescent cells could potentially be therapeutically enhanced by medications or vaccines aimed at priming the immune response to remove specific senescent populations (Burton and Stolzing, 2018; Song et al., 2020). Reprogramming of senescent cells may be another approach (Tamanini et al., 2018; Mahmoudi et al., 2019).

On the other hand, treatments aiming at the disease processes that precede and accelerate CS, such as inflammation, oxidative stress and mitochondrial dysfunction at the earliest stages of the disease may delay CS and hinder CS-related neurodegeneration. Furthermore, senescence-inducing practices and medications including exposure to ionizing radiation and DNA-damaging chemotherapeutics should be avoided. Interestingly, approved MS treatments, such as corticosteroids, beta-interferons and mitoxantrone should be re-evaluated for their long-term effects given that they have been shown to promote CS (Moiseeva et al., 2006; Ikeda et al., 2010; Poulsen et al., 2014).

CONCLUDING REMARKS

Aging is an important risk factor for the development of several neurodegenerative diseases including P-MS, where the neurodegenerative component dominates. A primary causative role of CS in MS is highly unlikely given the great diversity which characterizes aging-related neurodegenerative pathologies that have CS as a common feature. However, CS may be a shared mechanism, which substantially contributes to the pathogenesis and impact of neurodegenerative diseases and thereby may determine disease susceptibility, age at disease presentation and rate of progression. In MS, senescence may be responsible for chronic non-remitting inflammation, which is not amenable to immunomodulation, lost or altered glial and neuronal function, failure of remyelination, impaired BBB integrity and neurodegeneration (**Figure 1**).

Nevertheless, current evidence for a role of CS in disability progression in MS is intriguing but limited and indirect. Shedding light on CS and its role in neurodegeneration is essential to safely exploit it therapeutically. To facilitate these efforts a thorough histopathological investigation of post-mortem MS tissue at various disease stages and levels of disability would inform us of the extent, timing, particular cell types converted to senescence and all features of pathology associated with the accumulation of senescent cells. A more concise

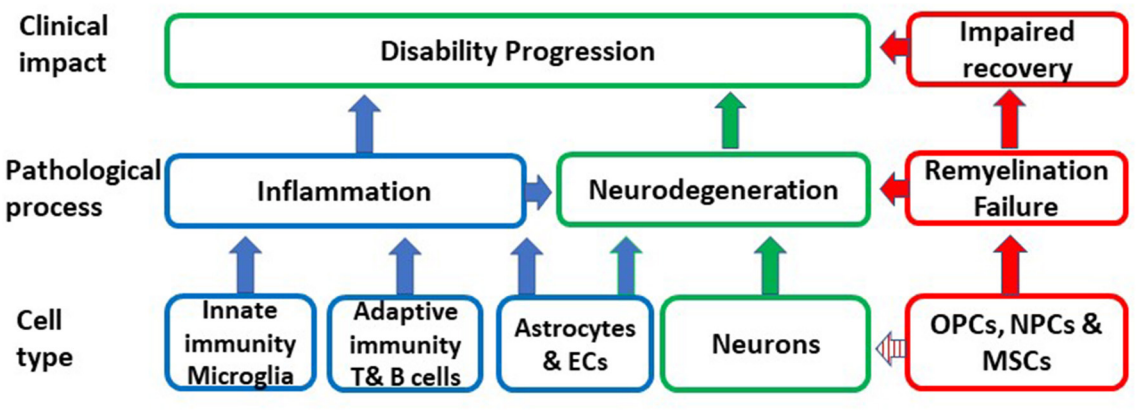

FIGURE 1 | Schematic representation of the putative impact of the conversion of different cell types to senescence on inflammation, remyelination, neurodegeneration and ultimately on disability progression. OPCs, oligodendrocyte progenitors; MSCs, mesenchymal stem cells; NPCs, neural progenitor cells; EC, endothelial cells.

understanding of the biology of CS of neural cells, its triggers and mediators is required. Advanced human 3D and organoid culture techniques (Marton et al., 2019; Yoon et al., 2019) could help identify the specific factors that induce CS and the contribution of each cell type to tissue injury. Transgenic animal models of demyelination could provide mechanistic evidence to disentangle the detrimental effects of senescent cell types and their mediators and become platforms on which to test senotherapeutic agents. The complex physiological and pathophysiological roles of CS, along with the cell-type specific variability in senescence triggers and phenotypes, necessitates a cautious approach to avoid pitfalls when dealing with such a multifaceted biological process. If senescent cells are proven to drive neurodegeneration senotherapy may become the groundbreaking neuroprotective strategy to prevent and potentially reverse progressive disability in MS.

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Telomere Attrition in Neurodegenerative Disorders

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Telomere attrition is increased in various disorders and is therefore a potential biomarker for diagnosis and/or prognosis of these disorders. The contribution of telomere attrition in the pathogenesis of neurodegenerative disorders is yet to be fully elucidated. We are reviewing the current knowledge regarding the telomere biology in two common neurodegenerative disorders, Alzheimer's disease (AD), and Parkinson's disease (PD). Furthermore, we are discussing future prospective of telomere research in these disorders. The majority of studies reported consistent evidence of the accelerated telomere attrition in AD patients, possibly in association with elevated oxidative stress levels. On the other hand in PD, various studies reported contradictory evidence regarding telomere attrition. Consequently, due to the low specificity and sensitivity, the clinical benefit of telomere length as a biomarker of neurodegenerative disease development and progression is not yet recognized. Nevertheless, longitudinal studies in large carefully selected cohorts might provide further elucidation of the complex involvement of the telomeres in the pathogenesis of neurodegenerative diseases. Telomere length maintenance is a complex process characterized by environmental, genetic, and epigenetic determinants. Thus, in addition to the selection of the study cohort, also the selection of analytical methods and types of biological samples for evaluation of the telomere attrition is of utmost importance.

Keywords: telomere, telomere length, telomere attrition, Alzheimer's disease, Parkinson's disease, neurodegenerative disroders

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INTRODUCTION

Telomeres are tandemly repeated nucleotide sequences at the end of the linear chromosomes. Their main function is maintaining genomic stability by protecting chromosomal ends from degradation and end-to-end fusion (Blackburn and Szostak, 1984). The telomere sequence in humans consists of TTAGGG repeats, while the number of the repeats varies between different cells and even between chromosomes (Graakjaer et al., 2003). Due to the inability of DNA polymerase to fully replicate both strands of DNA at the end of linear chromosomes, telomeres become shorter with each cell division leading to replicate senescence (Shay and Wright, 2000; Martínez-Cué and Rueda, 2020). Telomere length (TL) may, therefore, serve as a biological clock indicating cellular age and is

Abbreviations: AD, Alzheimer's disease; LTL, leukocytes telomere length; MCI, mild cognitive impairment; PD; Parkinson's disease; ROS, reactive oxygen species; TL, telomere length; TRF, terminal restriction fragment.

considered as a potential biomarker of aging and age-related diseases (Kong et al., 2013; Turner et al., 2019). We are reviewing current knowledge and discussing future prospective of telomere research in two common neurodegenerative disorders: Alzheimer's disease (AD) and Parkinson's disease (PD).

TELOMERE BIOLOGY IN HUMAN AND MEASUREMENT OF TELOMERE LENGTH

Telomere Length Maintenance

A six-protein complex known as shelterin or telosome plays the main role in maintaining and protecting telomeres integrity and function while its dysfunction results in chromosome end fusion and DNA arrangements (Sfeir and de Lange, 2012). Components and functions of the shelterin complex are described in more detail elsewhere (De Lange, 2005). Also, the single-stranded G-rich overhang can form telomeric G-quadruplex structure (Lipps and Rhodes, 2009) that can inhibit telomere extension by blocking telomerase from accessing telomeres (Tang et al., 2008). Telomerase is a ribonucleoprotein complex that enables the maintenance of the telomere length in continuously dividing cells such as germline, embryonic, and stem cells. It consists of a reverse transcriptase subunit (telomerase reverse transcriptase, TERT) and RNA component (telomerase RNA component, TERC) that serves as a template for telomere extension (Feng et al., 1995; Harrington et al., 1997). TERC is ubiquitously expressed in all human cells while TERT expression is usually undetectable in somatic cells and increased in cancer cells (Feng et al., 1995; Meyerson et al., 1997). Telomerase function is regulated by different genetic factors and epigenetic factors associated with modulation of *TERT* transcription (Cong et al., 2002). Decreased telomerase activity or impaired telomerase recruitment leads to chromosomal instability and accelerated cellular senescence (Garcia et al., 2007; Sarek et al., 2015).

Telomere Attrition in Health and Disease

The telomeres are longest at birth and shorten with increasing age, where the rate of telomere shortening is fastest directly after birth (Frenck et al., 1998). Telomeres in cells lacking telomerase become shorter with each cell division. When telomeres reach a certain critical length the cell exits the cell cycle and becomes senescent (Yu et al., 1990). Senescent cells secrete numerous extracellular factors such as proteases, growth factors, and inflammatory cytokines that act on non-senescent neighboring cells. Senescent cells are then eliminated by the immune system. However, this process of clearance can be impaired, especially in aged tissues, leading to the accumulation of senescent cells which contributes to further tissue dysfunction and tissue aging (Muñoz-Espín and Serrano, 2014). TL may, therefore, serve as a biological clock indicating cellular age and is considered as a potential biomarker of aging and age-related diseases (Sanders and Newman, 2013). It has been estimated that leukocyte telomeres in adult humans shorten at a rate of 24.7 bp per year (Müezzinler et al., 2013). Although, TL is synchronous within organs at the time of birth, it is highly variable in different organs of the same individual later in life (Youngren et al., 1998; Takubo et al., 2002). Besides cellular aging, several other factors can influence TL and the rate of telomeres attrition. TL appears to be longer in adult females (Benetos et al., 2001) and African Americans compared to Caucasians (Hunt et al., 2008). Furthermore, environmental factors and lifestyles such as unhealthy diet, lack of physical activity, obesity, chronic stress, smoking, and alcohol consumption have a negative impact on TL (Valdes et al., 2005; Cherkas et al., 2008; Puterman et al., 2010; Pavanello et al., 2011; Carulli et al., 2016), presumably associated with oxidative stress and inflammation (Chung et al., 2002; von Zglinicki, 2002). Due to an abundance of guanines in the telomeric repeats, telomeres are highly sensitive to damage by oxidative stress (Steenken and Jovanovic, 1997; von Zglinicki, 2002; Kino et al., 2017). Oxidative stress can arise from reactive oxygen species (ROS) or free radicals as a by-product of aerobic metabolism and ATP production in mitochondria (Reichert and Stier, 2017). Although oxidative damage can cause telomere shortening through double-stranded breaks to DNA, most telomere attrition due to oxidative stress occurs during DNA replication as a result of single-strand DNA damage (von Zglinicki, 2002). Chronic inflammation has been suggested to promote overall cell turnover leading to telomere shortening and replicative senescence (Epel, 2009; Eitan et al., 2014), therefore cumulative inflammatory load is associated with increased odds for short TL (O'Donovan et al., 2011). Moreover, inflammation can induce the release of ROS that damages telomeric DNA *via* oxidative stress (Jaiswal et al., 2000) leading to calcium release from the mitochondria, mitochondrial dysfunction, and further elevation of ROS levels (Eitan et al., 2014).

Numerous publications correlate different disorders and diseases with progressive telomere shortening. These are predominantly age-related disorders and chronic diseases such as cardiovascular diseases, hypertension, arthritis, osteoporosis, diabetes, cancer, and neurological disorders (Wentzensen et al., 2011; Kong et al., 2013; Wang et al., 2016). Nevertheless, it remains to be shown, if the observed telomere shortening is a cause for these conditions, a consequence, or both.

Telomere Length Measurement

TL is usually measured in leukocytes considering that blood is easily accessible. Leukocytes telomere length (LTL) is considered as a surrogate marker for other cells as well. In elderly patients, TL in leukocytes is significantly shorter compared to skin, synovial tissue (Friedrich et al., 2000), skeletal muscle, and subcutaneous fat, where these tissues displayed a similar age-dependent TL attrition rate (23–26 bp/year) and the in-between tissues correlation of TL (Daniali et al., 2013). TL and attrition rates vary even among different subpopulations of leukocytes and therefore differences in leukocytes subpopulations can influence high LTL variability among individuals (Lin et al., 2015). Nevertheless, inter-individual variation in LTL distribution was stable over time (Toupance et al., 2019).

There are several methods available for measuring TL. They differ in the measured parameter (average TL or the shortest

telomere), type and amount of sample required, accuracy, reproducibility, robustness, throughput, and technical skills required. Terminal Restriction Fragment (TRF) analysis using Southern blot (Harley et al., 1990) requires a relatively large amount of genomic DNA (3 μg per sample) and is labor-intensive. It is widely used because of its high reproducibility (coefficient of variation <2%; Kimura et al., 2010) and is regarded as the gold standard. Quantitative PCR (qPCR) measures the average TL in genomic DNA in a relative way, while the absolute value of average TL might be calculated using a standard curve. In comparison with TRF analysis, qPCR is easier to perform, less time consuming, requires a smaller amount of genomic DNA (50 ng per sample), and has a higher throughput (Cawthon, 2002, 2009). It is widely used for large population studies, even though there may be large variations among laboratories (Aubert et al., 2012). Pipetting errors can significantly affect the results and were partly overcome with an improved monochrome multiplex qPCR (mmqPCR) allowing the signals from the telomere amplicons and control amplicons to be collected simultaneously from the same well (Cawthon, 2009). Their main disadvantage is high intra- and inter-assay variability with reported coefficients of variation between 2 and 28% (Cawthon, 2002, 2009; Martin-Ruiz et al., 2015; Tarik et al., 2018). Single Telomere Length Analysis (STELA) is a PCR based technique combined with Southern blot that measures TL on individual chromosomes (Baird et al., 2003). It is highly accurate, requires very little starting material, and is appropriate for analysis of rare cell types. It has limited large scale applications as it has low throughput and is labor-intensive (Baird et al., 2003; Aubert et al., 2012). Telomere Shortest Length Assay (TeSLA) has similar limitations. It is a ligation and PCR-based approach for detecting amplified TRFs from all chromosomes. It detects small changes in the shortest telomeres in a certain time interval (Lai et al., 2017). Quantitative fluorescence *in situ* hybridization (q-FISH) method enables measurement of all individual telomeres per metaphase (Lansdorp et al., 1996) and can be combined with flow cytometry (flow-FISH) to measure TL in distinct cell populations (Rufer et al., 1998). Although technically more demanding, they give very reliable and reproducible results (Aubert et al., 2012).

The selection of a method should be made with consideration of their advantages and disadvantages. For larger epidemiology and population studies qPCR is the most practical choice while q-FISH and STELA are more appropriate for studies with fewer samples or when more accurate and specific measurements are required (Aubert et al., 2012). The question that remains unanswered is which parameter is more relevant for the evaluation of cellular senescence—average TL or shortest telomere? Most of the studies use methods that measure average TL rather than the shortest telomeres. While shortest telomeres are the ones that determine cellular survival and cell viability, their length might be crucial for the onset of cellular senescence (Hemann et al., 2001). Therefore, a specific group of chromosomes with the shortest telomeres might be responsible for the induction of cell senescence rather than all or only one or two critically short telomeres.

TELOMERE LENGTH IN BRAIN

During embryonic brain development, most cells express telomerase, while after birth, the activity in most cells quickly declines to undetectable levels (Wright et al., 1996). However, high telomerase activity is present in neuronal stem cells and neuronal progenitor cells in the developing brain (Cai et al., 2002) and adult brain, particularly in the hippocampus, olfactory bulbs and subventricular zone (Martín-Rivera et al., 1998; Caporaso et al., 2003; Zhou et al., 2011) where neuronal stem cells are mainly located (Gross, 2000; Lie et al., 2004), which contribute to the viability and self-renewal potential. Telomerase activity in neuronal stem cells and neuronal progenitor cells is essential for cell proliferation, neuronal differentiation, neuronal survival, and neurogenesis (Liu et al., 2018). However, it is not sufficient to completely preserve TL in lifelong replicating cells and is leading to impaired neurogenesis and abnormal differentiation (Ferrón et al., 2009).

Studies of the relationship between TL and cognitive performance have led to inconsistent results. While TL was reported in cognitive decline in elderly people and suggested to be a possible biomarker of cognitive aging (Kljajevic, 2011; Yaffe et al., 2011; Devore et al., 2011), some studies found no significant association (Hochstrasser et al., 2012; Harris et al., 2016). Also, longer telomeres were associated with better cognitive performance (Hägg et al., 2017). The effect of short telomeres is visible in telomere biology disorders characterized by very short telomeres (Bertuch, 2016). In these patients, a central neuronal system is affected causing abnormal brain development and neuropsychiatric complications such as seizures, psychoses, mental disabilities, anxiety, and progressive memory loss (Savage and Bertuch, 2010; Bhala et al., 2019).

LTL has been proposed as a surrogate marker for the central nervous system TL (Daniali et al., 2013), yet the degree to which these measurements represent the actual state in the brain is still unclear. A *post-mortem* study in neuropsychiatric disorders reported a highly significant difference in TL in different brain regions, where telomere were the longest in *substantia nigra* and the shortest in the dorsolateral prefrontal cortex (Mamdani et al., 2015). A recent study investigated an association of short-term change in LTL and a concomitant change in structural brain plasticity. Leukocyte telomere shortening was associated with a cortical thinning, while lengthening was associated with cortical thickening, implying that short-term LTLs may be a dynamical phenomenon (Puhlmann et al., 2019).

Telomere attrition has been investigated in many neurodegenerative disorders (Eitan et al., 2014; Kota et al., 2015). In this review article, we have focused on two most common neurodegenerative disorders; Alzheimer's and Parkinson's disease.

Telomere Attrition in Alzheimer's Disease

AD is a slowly progressing neurodegenerative disease and a common cause of dementia in the elderly worldwide (Prince et al., 2016). Unfortunately, there is no effective therapeutic intervention for AD prevention (Cummings et al., 2019) while several studies have investigated possible biomarkers that

would enable a diagnosis before irreversible brain damage (Aisen et al., 2017).

Amyloid-beta deposition and advanced age are the major risk factors for AD development, and both have been associated with the activation of microglia (Flanary et al., 2007) consequently leading to extensive research focusing on microglial molecules and pathways as drug targets for AD (Biber et al., 2019). A telomere shortening and reduction of telomerase activity were described in rat microglial cells during normal aging along with a tendency to telomere shortening in humans with AD dementia (Flanary et al., 2007). Amyloid deposits in dementia patients show a higher degree of microglial dystrophy compared to non-demented patients (Flanary et al., 2007). Contradictorily, studies in aged telomerase knockout mice showed that telomere shortening can decrease the progression of amyloid plaque pathology, possibly due to telomere-dependent effects on microglial activation (Rolyan et al., 2011).

Oxidative stress can trigger chronic inflammation and contribute to neuronal dysfunction and cell death in AD (Mhatrea et al., 2004; Maccioni et al., 2009) and is chronically elevated before amyloid-beta plaques and neurofibrillary tangles occur (Chou et al., 2011). Neurons expressing a high level of pathological tau were not expressing TERT. The absence of TERT led to increased damage of neurons by ROS, probably caused by hyperphosphorylated tau (Spilsbury et al., 2015).

Studies of TL in patients with AD are inconsistent. Their major observations along with the characteristics of the study cohorts and methods are summarized in **Table 1**. Scarabino et al., 2017, reported significantly shorter LTL in AD patients and suggested that cognitive decline is correlated with telomere attrition and conversion from mild cognitive impairment (MCI) to AD (Scarabino et al., 2017). However, stable MCI had decreased LTL compared to AD patients and controls (Movérare-Skrtic et al., 2012). Contrarily, several studies noted that TLs of the MCI patients (Zekry et al., 2010; Hochstrasser et al., 2012) or AD patients (Takata et al., 2012; Tedone et al., 2015) did not differ from the controls, but were shorter in ApoE4 homozygous AD patients (Takata et al., 2012). After the patients were classified as slow or fast progressing, the slow-progressing group unexpectedly displayed shorter TLs (Tedone et al., 2015). The increasing severity of AD was associated with a decrease in TLs and an increase in the number and aggregation of telomeres (Mathur et al., 2014). In so far the largest study with more than 300 patients followed-up for up to 16 years, deceased patients and patients who developed dementia had shorter TLs (Honig et al., 2012).

Guan et al. (2012) published shorter LTLs and increased oxidative stress in AD patients of both genders, however, after vitamin E administration oxidative stress lowered (Guan et al., 2012). Interestingly, one year later they found no significant difference between women with AD and controls but a decreased number of the longest telomeres (>9.4 kb) was observed (Guan et al., 2013).

A few studies investigated TLs in brain tissue. *Lukens et al., 2009*, reported a direct correlation between TL in leukocytes and cerebellum, but cerebellum TLs were not significantly different in AD patients (Lukens et al., 2009). Shorter TLs were observed in leukocytes and buccal cells in AD patients, however, their TLs in hippocampal cells were inconsistently reported as longer (Thomas et al., 2008) or shorter (Franco et al., 2006).

The data regarding the involvement of telomere attrition in AD progression is contradictory and needs further elucidation. The common characteristic of all these studies but one

TABLE 1 | Studies on telomere length in Alzheimer's disease.

Study	Sample	TL Method	Sample size (AD/controls)	TL (AD vs. controls)
Nudelman et al. (2019)	Leukocytes	qPCR multiplex	94/223	Shorter
Flanary et al. (2007)	Microglia	Flow-FISH	4/1	Shorter
Garcia et al. (2017)	Buccal cells	3D q-FISH	44/44	Shorter
Scarabino et al. (2017)	Leukocytes	qPCR multiplex	61/56	Shorter
Liu et al. (2016)	PBMCs	qPCR multiplex	68/79	Shorter
Tedone et al. (2015)*	PBMCs	Flow cytometry	31/20	Unchanged
Kota et al. (2015)*	Leukocytes	qPCR multiplex	57/55	Shorter
Mathur et al. (2014)*	Buccal cells	3D q-FISH	41/41	Shorter
Guan et al. (2013)	Leukocytes	TRF	23/29	Unchanged
Guan et al. (2012)*	Leukocytes	TRF	40/59	Shorter
Takata et al. (2012)*	Leukocytes	qPCR multiplex	74/35	Unchanged
Movérare-Skrtic et al. (2012)	Leukocytes	qPCR monoplex	32/20	Unchanged
Hochstrasser et al. (2012)*	Monocytes	TRF	18/14	Shorter
Honig et al. (2012)*	Leukocytes	qPCR monoplex	314/1.469	Shorter
Zekry et al. (2010)	Lymphocytes	Flow cytometry	80/204	Unchanged
Lukens et al. (2009)*	Cerebellar neurons	qPCR monoplex	29/22	Unchanged
Thomas et al. (2008)*	Leukocytes	qPCR monoplex	54/26	Shorter
	Buccal cells		54/26	Shorter
	Hippocampal cells		13/9	Longer
Franco et al. (2006)*	Hippocampal neurons	q-FISH	8/7	Shorter
Honig et al. (2006)	Leukocytes	qPCR monoplex	125/132	Shorter
Panossian et al. (2003)*	PBMCs	TRF	15/15	Shorter

*Abbreviations: TL, telomere length; AD, Alzheimer's disease; q-FISH, quantitative fluorescence in situ hybridization; PBMCs, peripheral blood mononuclear cells; TRF, terminal restriction fragment analysis; qPCR, quantitative polymerase chain reaction. *Included in the meta-analysis (Forero et al., 2016).*

(Honig et al., 2012) is a small cohort of patients (**Table 1**), which is probably the main reason for the inconsistency. The meta-analysis in 2016 included 13 primary studies with altogether 860 AD patients and 2,022 control patients and showed consistent evidence of shorter telomeres in patients with AD, which was more evident in leucocytes (Forero et al., 2016). A recent longitudinal study on Alzheimer's disease Neuroimaging Initiative Cohort focused on the TL dynamics concerning late-onset AD risk and progression. Telomere attrition rate did not differ significantly between MCI and AD group, but the later did have significantly shorter TL (Nudelman et al., 2019). On the other hand, longer TL at baseline was associated with rapid progression in cerebrospinal fluid biomarkers positive patients from the same cohort (Mahoney et al., 2019).

Telomere Attrition in Parkinson's Disease

Parkinson's disease is characterized by both motor and non-motor features (Kalia and Lang, 2015) where neurodegenerative cell loss is associated with chronic inflammation (Gao et al., 2003) and oxidative stress (Petrozzi et al., 2001). In PD patients, telomere shortening seemed to be related to the concentrations of carbonyl proteins in plasma (Watfa et al., 2011). It is assumed that the DNA damage leads to progressive hypomethylation in subtelomeres indicting telomeric structural changes (Maeda et al., 2012). In an animal model, telomerase deficient mice showed telomere attrition, but unexpectedly showed no differences in dopamine concentration or oxidative stress (Oeckl et al., 2014), while α-synuclein transgenic mice with shorter telomeres developed an accelerated disease with significantly decreased survival (Scheffold et al., 2016).

Studies of TLs in patients with PD showed contradictory results and are summarized in **Table 2**. Only two studies reported shorter telomeres in PD patients. A study in Japanese women with PD reported shorter telomeres, while length distribution showed a decrease of the long telomeres (>9.4 kb; Maeda et al., 2012). Additionally in PD, TL was reported to be shorter in buccal cells but not in LTLs (Kolyada et al., 2016). Contrary to expectations, a large case-control study in men reported shorter telomeres to be associated with a reduced risk for PD (Schürks et al., 2014). Paradoxically, longer telomeres in PBMC along with no TLs change in *substantia nigra* were also reported in PD patients (Hudson et al., 2011).

The majority of the studies reported no significant telomere attrition in PD (**Table 2**). A Swedish prospective population-based study on idiopathic parkinsonism reported no significant difference between PD patients and controls at baseline and during follow-up while longer telomeres were a risk factor for the development of dementia (Degerman et al., 2014). A study in Japanese men with PD reported no statistical difference in TLs. However, a significant decrease in TL was observed in PD patients in the 50s and 60s (Guan et al., 2008).

Reported studies evaluating TL in PD are very heterogeneous regarding the study population, sample type, TL detection method, and the cohort size. Even though telomere attrition was observed in only two studies with 30 or fewer participants and different sample types (Maeda et al., 2012; Kolyada et al., 2016), while longer TL was reported in two studies investigating significantly larger cohort (Hudson et al., 2011; Schürks et al., 2014), the contradictory observations are still surprising. The meta-analysis performed by Forero et al., 2016, included eight primary studies with altogether 956 PD patients and 1,284 controls showing no consistent evidence of telomere attrition in PD (Forero et al., 2016).

DISCUSSION

Studies of telomere dynamics in AD and PD have provided inconclusive results. Currently, the telomere attrition is believed to be associated with AD (Forero et al., 2016), but there is no consistent evidence of telomere attrition in PD (Forero et al., 2016). TL maintenance is a complex process characterized by environmental, genetic, and epigenetic determinants. Thus, the variability in oxidative stress and inflammation and other confounders in individuals with neurodegenerative diseases listed above may partially explain inconsistent results. Therefore, before the study, exclusion criteria must be carefully chosen to avoid conditions with increased telomere erosion. Since the telomeres progressively shorten with increasing age, different age groups should not be

TABLE 2 | Studies on telomere length in Parkinson's disease.

Study	Sample	TL method	Sample size (PD/controls)	TL (PD vs. controls)
Kolyada et al. (2016)	Buccal cells	qPCR multiplex	30/34	Shorter
	Leukocytes			Unchanged
Degerman et al. (2014)*	Leukocytes	qPCR monoplex	136/30	Unchanged
Schürks et al. (2014)*	Leukocytes	qPCR monoplex	408/809	Longer
Maeda et al. (2012)	Leukocytes	TRF	17/20	Shorter
Hudson et al. (2011)*	PBMCs	qPCR monoplex	109/99	Longer
	Substantia nigra		28/17	Unchanged
Watfa et al. (2011)*	Leukocytes	TRF	20/15	Unchanged
Eerola et al. (2010)*	Leukocytes	qPCR monoplex	131/115	Unchanged
Guan et al. (2008)*	Leukocytes	TRF	28/27	Unchanged
Wang et al. (2008)*	Leukocytes	qPCR monoplex	96/172	Unchanged

*Abbreviations: TL, telomere length; PD, Parkinson's disease; PBMCs, peripheral blood mononuclear cells; TRF, terminal restriction fragment analysis; qPCR, quantitative polymerase chain reaction. *Included in the meta-analysis (Forero et al., 2016).*

compared and the use of the appropriate age-matched controls is crucial.

The clinical benefit of TLs as a biomarker of neurodegenerative diseases is questionable due to its low specificity and sensitivity. Telomere shortening is not present only in neurodegenerative diseases, but also in a vast amount of other diseases and different demographic factors may contribute to telomere attrition. A recent study by *Smith et al.*, using an umbrella review approach where findings were derived by examining the epidemiological credibility of the evidence, pointed out several weaknesses in reporting relationship between TL and different disorders (Smith et al., 2019). They showed no or merely weak association between TL and several health outcomes, however, the association with AD risk was suggestive (Smith et al., 2019). Nevertheless, it is still under discussion if telomere shortening is a cause or consequence in various disorders (Kordinas et al., 2016). Namely, pathological conditions may cause telomere attrition but short telomeres can also by itself induce pathological processes by increasing oxidative stress and inflammation. Therefore, it is of utmost importance that limitations of the individual study approach are considered when interpreting the data and to keep in mind that the TL as a biomarker by it selves might only give insight into the current state of the disease.

Different study designs, methods for TL measurement, and tissues in which TLs are investigated may contribute to the variability between studies. Moreover, although qPCR is the most commonly used method in TL measurement, there are still inconsistencies in recent reports. Recently, Lin et al. carefully evaluated all critical steps in qPCR telomere length assay and provided guidance and recommendations for each step based on current knowledge (Lin et al., 2019). Therefore, further efforts should be made to develop and implement standardized protocols. The experimental approach should be chosen regarding its applicability for a particular purpose of the study and specific disadvantages and advantages. External and internal controls are essential for the reproducibility and precision of the measurements. All reports evaluating TL should include the minimum information necessary to characterize the used method (Lin et al., 2019) including basic quality metrics enabling comparison of results from different laboratories.

One of the most important aspects is the selection of the representative tissue that corresponds to the pathological processes in the affected tissue, such as the brain in neurodegenerative diseases. The discrepancy in TL in different brain regions (Mamdani et al., 2015) is raising the question to what degree LTL is informative in neurodegenerative disorders. Large *post mortem* studies of TLs in different brain cells are needed to fully evaluate the LTL as the appropriate surrogate marker for the central nervous system.

The number of subjects to show statistical significance between two groups is a crucial factor and must be calculated taking into account high inter- and intra-variability in TLs. In most reported studies, the sample size was relatively small, which may be the cause for statistical insignificance. Furthermore, classification of the patients into subgroups, such as progression rate, disease stage, etc. may be beneficial to show statistical significance, but it requires an even larger total sample size. In this view, the longitudinal studies in carefully selected cohorts may provide more reliable results since a smaller sample size is needed (Aviv et al., 2006) and they enable evaluation of the telomere attrition dynamics. Such studies may assist in the clarification of the role of telomere attrition not only in development but also in the progression of the disease. That has proved beneficial in our previous studies focusing on telomere attrition in type 1 diabetes. Our initial study in pediatric patients did not confirm the association between TL and glycemic control (Tesovnik et al., 2015) contrary to the later retrospective longitudinal study (Tesovnik et al., 2018). Furthermore, integration of the clinical, genomic, and biochemical data from longitudinal studies in a multivariable predictive model may be beneficial in an attempt to identify additive biomarkers influencing the development and/or progression of AD and PD.

To conclude, longitudinal studies in carefully selected cohorts and sample types are required to elucidate the complex role the telomeres may have in the pathogenesis of neurodegenerative diseases and possible targets for intervention with a final goal to possibly prevent or delay neurodegenerative diseases. Furthermore, the integration of various experimental and clinical data might improve the strength of such studies. Additionally, standardized protocols for TL measurements addressing sample types and methods might be beneficial since and would enable a more relevant comparison of results from different laboratories.

AUTHOR CONTRIBUTIONS

TL and EK wrote the manuscript. VD revised the manuscript. KT conceptualized, wrote, and revised the manuscript. All authors reviewed and approved the final manuscript.

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Otic Neurogenesis Is Regulated by TGFβ in a Senescence-Independent Manner

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Cellular senescence has classically been associated with aging. Intriguingly, recent studies have also unraveled key roles for senescence in embryonic development, regeneration, and reprogramming. Developmental senescence has been reported during embryonic development in different organisms and structures, such as the endolymphatic duct during inner ear development of mammals and birds. However, there is no study addressing the possible role of senescence on otic neurogenesis. TGFβ/SMAD is the best-known pathway linked to the induction of developmentally programmed cell senescence. Here, we studied if TGFβ2 induces cellular senescence during acoustic-vestibular-ganglion (AVG) formation. Using organotypic cultures of AVG, and characterizing different stages of otic neurogenesis in the presence of TGFβ2 and a selective TGF-β receptor type-I inhibitor, we show that TGFβ2 exerts a powerful action in inner ear neurogenesis but, contrary to what we recently observed during endolymphatic duct development, these actions are independent of cellular senescence. We show that TGFβ2 reduces proliferation, and induces differentiation and neuritogenesis of neuroblasts, without altering cell death. Our studies highlight the roles of TGFβ2 and cellular senescence in the precise regulation of cell fate within the developing inner ear and its different cell types, being their mechanisms of action highly cell-type dependent.

Keywords: senescence, TGFβ2, inner ear development, inner ear neurogenesis, organotypic culture

INTRODUCTION

The vertebrate inner ear is a complex sensory organ responsible for the perceptions of sound and balance. The transmission of these auditory and balance information to higher brain regions relies on the auditory and the vestibular ganglia. These otic neurons extend their processes connecting the sensory epithelium to the brainstem nuclei through the eighth cranial nerve (Fekete and Campero, 2007; Fritzsch et al., 2015). During development, otic neuroblasts firstly

gather together as the acoustic-vestibular ganglion (AVG), by delaminating from the neuro-sensory domain in the epithelium of the otocyst (Camarero et al., 2003; Magariños et al., 2012). In the context of embryonic development, the process of cellular senescence, although classically linked with aging and cancer (Collado et al., 2007), has been recently described to have functional roles from various animal species. Cellular senescence is a form of irreversible cell cycle arrest combined with phenotypic changes and pronounced secretory activity (Gorgoulis et al., 2019). Senescent cells express inhibitors of cyclin-dependent kinases, as p21, p15, and p16, whereas the expression of cell cycle-promoting genes is suppressed. Senescent cells also show increased expression of the lysosomal β-galactosidase enzyme, a feature manifested as stronger senescence-associated β-galactosidase staining (SAβG), the most widely used marker (Hernandez-Segura et al., 2018), and present a secretory profile known as the senescence-associated secretory phenotype (SASP) that include soluble factors that modulate the cellular microenvironment (He and Sharpless, 2017). Developmentally programmed senescence acts together with apoptosis in the elimination of unwanted cells as well as in patterning and morphogenesis (Lorda-Diez et al., 2015). It shares most of the cellular characteristics of replicative and oncogene-induced senescence, such as arrested proliferation, increased SAβG staining, and a secretory phenotype. However, so far only p21 appears to be critical for developmental senescence (Gorgoulis et al., 2019). Although initially developmental senescent cells were reported to lack DNA-damage markers (Muñoz-Espín et al., 2013), it was later reported that the interdigital limb tissue from chicken embryos is associated with phosphorylated -H2AX (Montero et al., 2016). Current data suggest that senescent cells may have multiple functions in the embryo (Da Silva-Álvarez et al., 2019; Rhinn et al., 2019). These development-associated senescent cells arise in very precise patterns in time and space, before subsequently disappearing, pointing to a tightly regulated appearance and removal of these cells (Muñoz-Espín et al., 2013; Storer et al., 2013; Davaapil et al., 2017). Some of the structures that have been associated with programmed cellular senescence are the endolymphatic sac, the mesonephros, the neural tube, the apical ectodermal ridge, the interdigital tissue and the endolymphatic duct (Muñoz-Espín et al., 2013; Storer et al., 2013; Lorda-Diez et al., 2015; Gibaja et al., 2019; Rhinn et al., 2019).

This cellular process constitutes a part of a set of highly organized events that drive embryogenesis. Mechanistically, cellular senescence has been linked to the TGFβ/SMAD and PI3K/FOXO pathways. Particularly, the transforming growth factor-beta (TGFβ) signaling pathway has been described to be key in regulating programmed senescence in the context of the degenerating mesonephros (Muñoz-Espín et al., 2013) and inner ear morphogenesis (Gibaja et al., 2019; Varela-Nieto et al., 2019).

The TGFβ superfamily of growth factors is vital for the development and homeostasis of metazoans (Feng and Derynck, 2005). Members of this superfamily are extremely well conserved throughout evolution and they regulate diverse cellular functions such as migration, programmed cell death, differentiation, growth, and adhesion. Moreover, these actions are extremely well-orchestrated in time and space during development (Wu and Hill, 2009). The general signal transduction pathway for these growth factors consists basically of ligand dimers binding and activating heteromeric complexes of type I and type II transmembrane receptors, which in turn phosphorylate the intracellular mediators (SMADs). These mediators form complexes between each other and with other proteins, to regulate target gene expression (Wharton and Derynck, 2009; Wu and Hill, 2009). TGFβ superfamily has been also described to play central roles in multiple aspects of nervous system development and function. Among the various members of this superfamily, TGFβ2 has been involved in regulating the temporal identity and potency of neural stem cells (Dias et al., 2014), in retinal neuronal survival (Walshe et al., 2011; Braunger et al., 2013), proliferation of granule cells in the cerebellum, neuronal migration of glioma cells and axon elongation in hippocampal neurons, among others (Meyers and Kessler, 2017). Other members of the TGFβ superfamily have been widely studied in the otic vesicle (Kamaid et al., 2010; Ohyama et al., 2010). In this study, we aimed to explore whether TGFβ2 also plays a role in inner ear neurogenesis and if so, whether these effects are also mediated, at least partially, by cellular senescence. Our results show that TGFβ2 exerts a powerful action in the inner ear neurogenesis, but these actions are independent of cellular senescence, contrary to what we recently observed during endolymphatic duct development. We show that TGFβ2 reduces proliferation, and induces differentiation and neuritogenesis of neuroblasts, without altering cell death.

MATERIALS AND METHODS

Chicken Embryos

Chicken embryos were obtained from fertilized eggs from a local farm (Granja Santa Isabel, Cordoba, Spain) and they were incubated in a humidified atmosphere at 37.8°C. Embryos were staged as HH15, HH18, and HH19 according to Hamburger and Hamilton's criteria (Hamburger and Hamilton, 1951).

Embryo and Tissue Preparation for *in situ* Hybridization and Immunofluorescence

Whole embryos or tissues were dissected in phosphate-buffered saline (PBS) and fixed overnight in 4% (w/v) paraformaldehyde (PFA; Merck, Darmstadt, Germany) in PBS at 4°C. Subsequently, embryos were cryoprotected overnight in 15% sucrose/PBS at 4°C and then embedded at 37°C in 15% sucrose/10% gelatine in PBS. Gelatine-embedded tissues were frozen in isopentane at −80°C and then sectioned (20 μm) at −25°C in a cryostat (Cryocut 1900; Leica Microsystems, Deerfield, IL, USA). The sections were obtained from 3–6 embryos and used for *in situ* hybridization or immunofluorescent staining as described Aburto et al., 2012b).

Isolation, Organotypic Culture, and Treatment of Otic Vesicles and Acoustic-Vestibular Ganglia

Otic vesicles were obtained from HH18 embryos (65 h of incubation), by dissecting them from the surrounding

mesenchymal tissue with sharpened tungsten needles. Subsequently, they were transferred into four-well culture-plates (Delta treated for adhesion culture conditions and non-treated for regular culture assays; Nunc Roskilde, Denmark) and then incubated at 37°C in a water-saturated atmosphere containing 5% CO_2 for the times indicated in the text. The standard culture medium consisted of M199 medium with Earle's salts (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 2 mM glutamine (Gibco, Paisley, UK) and antibiotics [50 IU/ml penicillin (Ern, Barcelona, Spain) and 50 mg/ml streptomycin (CEPA, Madrid, Spain)]. For immunostaining and TUNEL labeling, otic vesicles were fixed for 2 h in PFA at 4°C. For SAβG staining the otic vesicles were fixed for 10 min with the fixative solution provided by the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA).

AVG was dissected from HH19 chicken embryos (82 h of incubation) and plated onto glass coverslips that had been previously coated with poly-D-lysine and fibronectin (Davies, 2011). AVG was cultured in 0.25 ml F12/Dulbecco's modified Eagle medium (Gibco) containing 100 μg/ml transferrin, 16 μg/ml putrescine, 6 ng/ml progesterone, 5.2 ng/ml sodium selenite (all from Sigma) and antibiotics as described (Aburto et al., 2012a,b). Explants were treated with TGFβ2 diluted in medium (10 ng/ml, PeproTech, Rocky Hill, NJ, USA). AVG explants cultured in medium and adding the equivalent volume of medium without TGFβ2 was used as experimental controls (0S). Three to six AVG explants per condition were assayed.

Immunohistochemistry and Immunofluorescence

Details of the antibodies used for immunofluorescence are shown in **Supplementary Table S1**. Procedures used have been described elsewhere (Magariños et al., 2010; Aburto et al., 2012a,b). Briefly, samples were washed and permeabilized in 1% PBS/Triton-X-100 (PBS-T) non-specific binding sites were blocked for 1 h in PBS-T with 3% (wt/vol) BSA (Sigma-Aldrich, Saint Louis, MO, USA) and 5% (vol/vol) normal goat or donkey serum. Samples were incubated with the primary antibodies overnight at 4°C, diluted in PBS-Tween20 (0.05%). For immunofluorescent staining of frozen sections, the primary antibodies were used as described above and the secondary antibodies [Alexa Fluor 488 goat anti-mouse (1:200), Alexa Fluor 647 goat anti-rabbit and/or Alexa Fluor 546 goat anti-rabbit secondary antibodies (1:200; all from Molecular Probes, Eugene, OR, USA)] were incubated at room temperature for 1.5 h (frozen sections) or 2.5 h (otic vesicle and AVG explants). The samples were mounted in Prolong Gold with DAPI (Invitrogen, Carlsbad, CA, USA) and visualized by fluorescence (Nikon 90i, Tokyo, Japan) or confocal microscopy (Leica TCS SP2, Wetzlar, Germany).

Levels of SOX2, number of PHH3+ cells, G4 glycoprotein intensity levels and AVG areas were quantified using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda) in compiled confocal microscopy projections as reported (Aburto et al., 2012a,b; Magariños et al., 2010). The color channels of the signals of interest were converted into grayscale images. Subsequently, both the area and the intensity of the signal were measured and normalized to the 0S condition, which was given an arbitrary value of 100. Three to six samples per condition were assayed. The data are presented as the mean ± SEM and the statistical significance was estimated with Student's *t*-test.

TUNEL Assay

Apoptotic cell death patterns were studied by Tdt-mediated dUTP nick-end labeling (TUNEL) of fragmented DNA using the kit Dead-EndTM Fluorometric TUNEL System (Promega, Madison, WI, USA), as described by the manufacturer in frozen sections as reported (Frago et al., 2003), or adapted to whole organ labeling (Camarero et al., 2003; Frago et al., 2003). Otic vesicles were mounted in Prolong Gold/DAPI and visualized by confocal microscopy. TUNEL-positive nuclei were quantified from compiled confocal microscopy projections by FIJI software, and the results are presented as the mean ± SEM of the positive cells per total area. Values were normalized to those of the 0S explants. Three to five AVG per condition were assayed.

In situ Hybridization

In situ hybridization with digoxigenin-labeled antisense RNA probes (1 mg/ml) was performed essentially as described previously with some minor modifications (Sánchez-Calderón et al., 2004). Three embryos of the stages HH15 and HH18 were tested in parallel in at least two independent experiments. The probes utilized in this study are *TGFB* and *TGFBR2*. The specificity of the signal was assessed by hybridization of a sense riboprobe.

Senescence-Associated Beta-Galactosidase Staining

The senescence-associated beta-galactosidase staining (SAβG) was performed by using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA). After culture and fixation, the otic vesicles were washed in PBS and incubated in the X-Gal solution at pH 6 at 37°C protected from light. For SAβG in whole embryos at stages HH18 and HH19, the embryos were fixed for 15 min and incubated in the X-Gal solution. To perform the SAβG in chicken embryo tissue sections, the embryos were obtained, fixed for 15 min, frozen, and sectioned with a cryostat by the Histology Core of the "Centro Nacional de Biotecnología." Sections were post-fixed for 2 min and incubated with the X-Gal solution.

For SAβG staining of otic vesicles, explanted otic vesicles were fixed with the fixative solution provided in the SAβG kit for 8 min and incubated with the X-Gal solution at pH 6 at 37°C. Then, otic vesicles were fixed with 4% PFA, permeabilized with PBS-T, washed with 2% PBS-BSA and incubated with the Click-iT Reaction Cocktail (Invitrogen) with Alexa-488 azide (5 μM, Invitrogen) at RT in darkness for 30 min. Finally, they were washed again with 2% PBS-BSA and mounted in Vectashield with DAPI (Vector, Peterborough, UK).

Image Analysis and Statistical Analysis

At least three to six otic vesicles or AVG per condition were used for each experiment, dissected from at least three different chicken embryos. Fluorescence intensities and areas of interest

were quantified using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda) in compiled confocal microscopy projections as reported (Aburto et al., 2012a,b). Subsequently, both the intensity of the signal and the area were normalized to the 0S condition, which was given an arbitrary value of 100. The data are presented as the mean intensity, or the mean number of positive cells per total area, ± SEM. The statistical significance was estimated using Student's *t*-test.

RESULTS

Expression of the TGFβ Pathway Elements During Inner Ear Neurogenesis

TGFβ factors and their receptors are expressed in the developing inner ear (Gibaja et al., 2019). Here, we studied the expression pattern of the *TGFB2* transcript by *in situ* hybridization during the initial phases of inner ear development (**Figures 1Aa–f**). *TGFB2* mRNA presented a localized expression at both HH15 and HH18 stages in the otic epithelium, where the otic neuroblasts are being differentiated, known as the neurogenic region. This expression pattern partially co-localized with the neural marker Islet-1 (**Figures 1Aa–f**, arrowheads). We also explored the expression of the receptor *TGFBR2* at HH18, which showed a marked presence throughout the AVG (**Figure 1Ag**, arrowhead). This suggests that ganglionic neuroblasts can respond to TGFβ. Moreover, consistent with the activation of the TGFβ2 pathway, we found the typical nuclear immunopositivity for phosphorylated SMAD2 (pSMAD2) effector protein, both in the neurogenic region at the otic epithelium and in the AVG different ganglionic populations: the delaminating neuroblasts (**Figure 1Ah**, arrow), the trans-amplifying neuroblasts (**Figure 1Ah**, arrowhead) and the postmitotic neurons (**Figure 1Ah**, asterisk) of the AVG.

TGFβ Pathway Does Not Induce Senescence in the AVG

As we previously described, TGFβ2 is a potent regulator of cellular senescence during inner ear development, which has been proven to be an essential process for proper morphogenesis of the endolymphatic duct (Gibaja et al., 2019). We, therefore, aimed to investigate whether developing AVG neuroblasts in HH19 stages underwent cellular senescence regulated by TGFβ2. β-galactosidase staining (SAβG) was used to detect senescent cells in the developing AVG, both in whole-mount embryos and cultured otic vesicles (**Figure 1B**). Whilst SAβG stained senescent cells at different areas of the otic epithelium, such as the endolymphatic duct Anlagen (**Figure 1Bb**, black arrowhead), we could not observe any senescent cells at the AVG under any of the culture conditions tested (**Figures 1Ba,b**, white arrowheads): control condition (0S) or in the presence of TGFβ2.

We also investigated the presence of senescent cells in the developing inner ear of HH19 embryos. A positive SAβG signal was evident in the early developing endolymphatic duct (**Figures 1Bc,d**, black arrowheads) as reported. In contrast, we could not find a clear SAβG signal at the AVG (**Figures 1Bc,d**, white arrowheads), following the *ex vivo* observations.

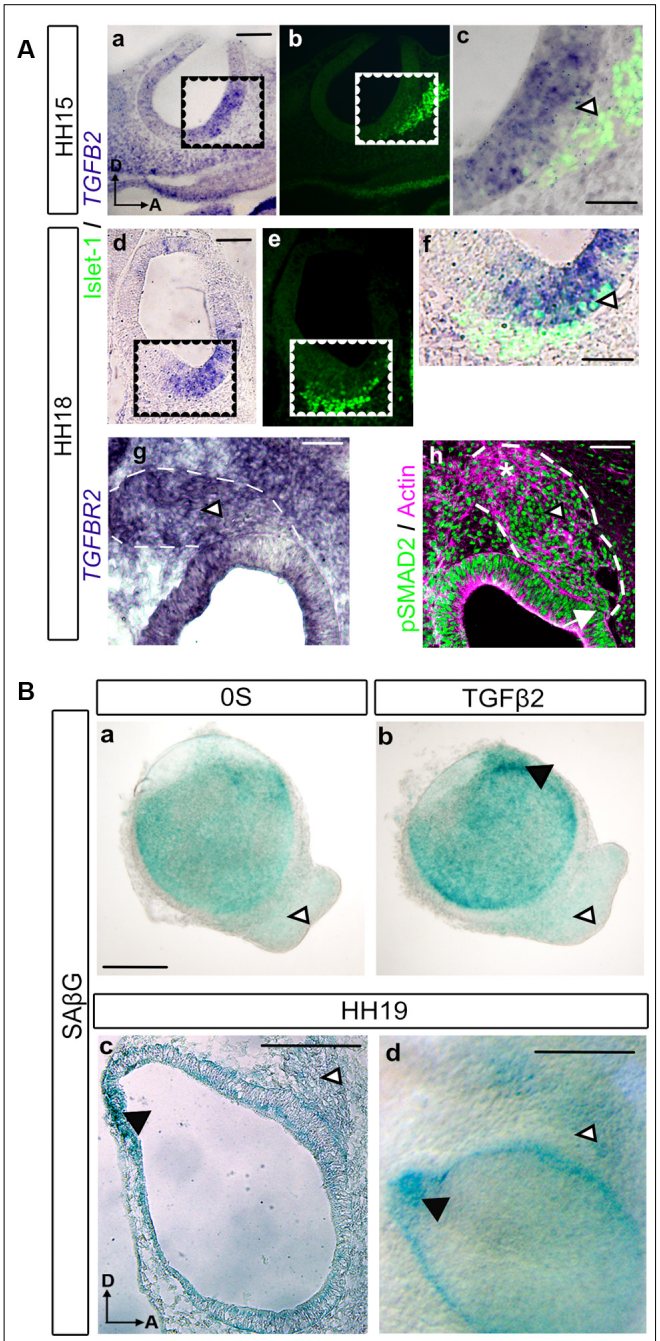

FIGURE 1 | TGFβ pathway expression during early otic neurogenesis. **(A)** TGFβ pathway components (*TGFB2*, *TGFBR2* and pSMAD2) and neural markers expression in the developing inner ear. *TGFB2 in situ* hybridization in transverse sections of chicken embryos. At HH15, *TGFB2* was expressed in the otic neurogenic region (**a**, cropped area in **c**). The associated neuroblasts are labeled with Islet-1 (green in **b,c**). At HH18 *TGFB2* continues to be present in the otic neurogenic region (**d**, cropped area in **f**) associated to the neuroblasts marker Islet-1 (green in **e,f**). Also, at HH18, *TGFBR2* showed a marked presence throughout the acoustic-vestibular-ganglion (AVG; **g**, arrowhead). Immunostaining for activated SMAD (pSMAD) showed nuclear immunopositivity in the otic epithelium and in the acoustic-vestibular ganglion (green in **h**, arrow and arrowhead, respectively). Actin was used as a marker

(*Continued*)

FIGURE 1 | Continued
of general structure in the OV and AVG area (magenta in **h**). Scale bars 75 μm **(a,b,d,e)** or 30 μm **(c,f,g,h)**. **(B)** Otic vesicles were isolated from HH18 chicken embryos and cultured for 20 h in **(a)** serum-free medium (0S) or **(b)** in the presence of TGFβ2 (10 ng/ml). SAβG staining was used to detect cellular senescence. TGFβ2 increases SAβG in the endolymphatic duct primordium (black arrowhead) while no SAβG+ cells were observed in the AVG with or without TGFβ2 treatment (white arrowheads); SAβG staining in a cryosection **(c)** or whole mount **(d)** HH19 chicken embryo is associated to the endolymphatic duct (white arrowheads) whereas no staining is detected in the AVG (black arrowheads). Scale bars 75 μm. Representative microphotographs are shown from at least three embryos and 3–6 isolated otic vesicles per condition. Orientation: A, anterior; D, dorsal.

TFGβ Pathway Is Required for the Differentiation of Acoustic Vestibular Neurons

TGFβ2-treated otocysts in culture exhibited profound alterations in the newly-formed AVG. More precisely, we could observe a stronger attachment and increased migration of the ganglionic cells (**Figures 2a,d**, arrowheads). This was further studied by examining the expression of the neural markers Islet-1 and Tuj-1 in cultured explants treated with or without TGFβ2 (**Figures 2b,c,e,f**). Islet-1 is a transcription factor expressed by proliferative neuroblasts and showed increased expression both in the otic epithelium and AVG. Tuj-1 is a β-III-tubulin constituent of neural fibers of postmitotic neurons. With these markers, we confirmed that the TGFβ2-targeted cells were indeed AVG neuroblasts, which showed a greater spreading from the otocyst. Moreover, TGFβ2-treated AVG showed an increased and extended distribution of Tuj-1 (**Figures 2c,f**, arrowheads), suggesting an accelerated differentiation of proliferating Islet-1 positive neuroblasts to neurons.

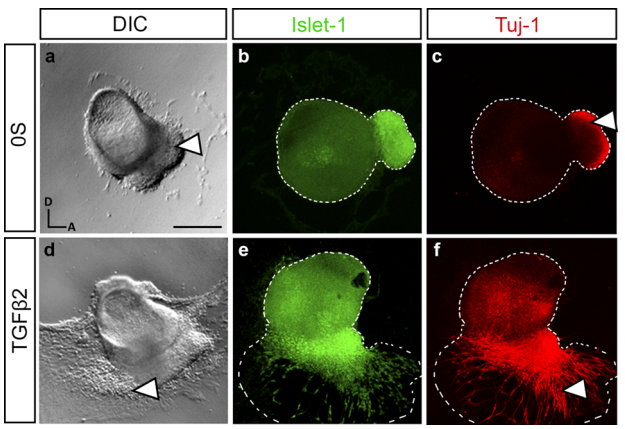

FIGURE 2 | TGFβ2 modulates neurogenesis in cultured otic vesicles. Otic vesicles were isolated from HH18 chicken embryos and cultured for 20 h in serum-free culture medium without additions (0S, **a–c**) or in the presence of TGFβ2 (10 ng/ml, **d–f**). Double immunostaining was carried out for the neuroblast nuclear marker Islet-1 (green) and the post-mitotic marker Tuj-1 (red). Representative images of compiled confocal microscopy projections are shown from at least six otic vesicles per condition. Orientation: A, anterior; D, dorsal. Scale bar: 150 μm.

To further verify if AVG neurite outgrowth was promoted by TGFβ2, we prepared cultures of explanted AVG (HH19) to study the differentiation of neuroblasts (**Figure 3**). Exogenous TGFβ2 enhanced the differentiation and neuritogenesis in ganglionic neuroblasts, as shown by the significant 7% increased levels of the neuron-specific glycoprotein G4 (**Figures 3i,i′,j,j′** quantifications in **m**) and the 33% decreased levels of the transcription factor SOX2 (**Figures 3e,f**; quantifications in **m**). These differentiating effects of exogenous TGFβ2 were abrogated by a selective TGF-β receptor type I inhibitor, LY2157299 (**Figures 3h,l**; quantifications in **m**). The presence of the inhibitor alone did not show any effect compared to the control condition (**Figures 3g,k**).

TGFβ2 treatment showed a 69% significant reduction of proliferating neuroblasts measured by the mitotic marker phospho-Histone H3, PHH3 (**Figures 4a,b**, quantification in i). Furthermore, TGFβ2 inhibition by LY2157299 recovered the number of proliferating neuroblasts (**Figures 4c,d**, quantification in **i**). There was no evident alteration in the number of apoptotic cells (**Figures 4e–h**, quantification in **i**), suggesting that the observed decrease in proliferating neuroblasts was not due to increased cell death but to accelerated differentiation.

DISCUSSION

Cells with features of senescence have been identified in several transient anatomical structures in the developing embryo, and appear to play a role in shaping organogenesis (Muñoz-Espín et al., 2013; Storer et al., 2013; Davaapil et al., 2017; Gibaja et al., 2019; Rhinn et al., 2019; Varela-Nieto et al., 2019). However, to date, there is no report linking cellular senescence with neurogenic processes during embryogenesis. TGFβ2 has been previously described to promote the formation of the AVG (Okano et al., 2005), but a more insightful characterization of these effects was lacking. Thus, we aimed to gain a deeper understanding of the actions mediated by TGFβ2, by using organotypic cultures of AVG and characterizing the different stages of otic neurogenesis in the presence or absence of TGFβ2. We recently described an important role for TGFβ2 in regulating cellular senescence during the formation of the endolymphatic duct in vertebrates (Gibaja et al., 2019). We wondered if TGFβ2-mediated actions during the formation of the AVG could be also mediated, at least partially, by TGFβ2-induced cellular senescence.

In the present study, we offer insight into the key function of TGFβ2 in inner ear neurogenesis during embryonic development. We show that TGFβ2 regulates the differentiation of otic neuroblasts at the AVG by promoting cell cycle arrest, upregulation of neuronal markers such as G4 glycoprotein, and downregulation of the high mobility group (HMG) transcription factor SOX2. SOX2 is required for the initial events of otic neuronal specification, including the expression of the proneural basic helix-loop-helix transcription factor Neurogenin1 (Steevens et al., 2019). Thus, TGFβ2 signaling is necessary for the progression of otic neuroblasts differentiation into mature otic neurons. Otic neuroblasts derive from the neurosensory domain in the anteroventral region of the otic

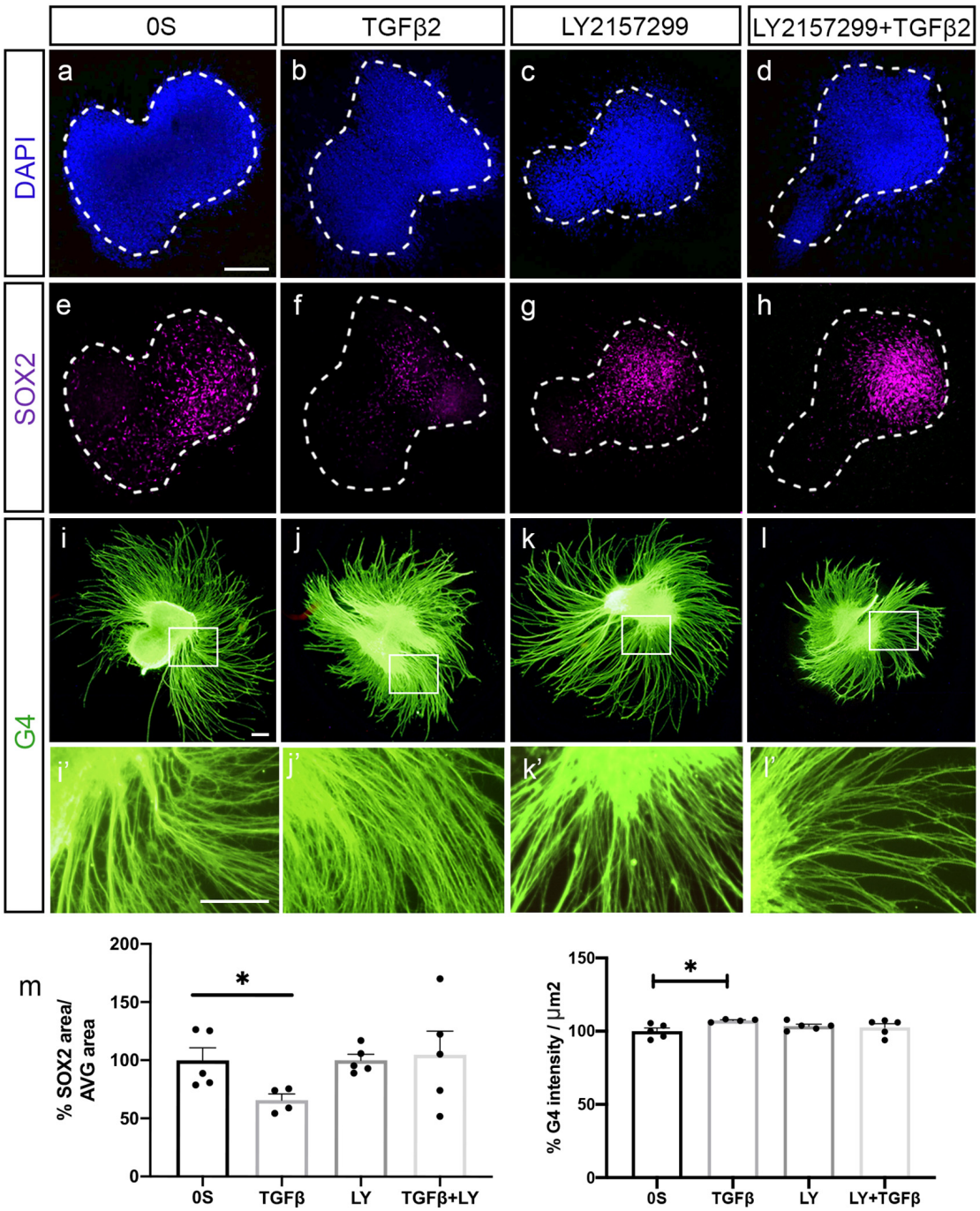

FIGURE 3 | TGFβ2 promotes neural differentiation. **(a–l)** Acoustic-vestibular ganglia (AVG) cultured in serum-free medium (0S), in the presence of TGFβ2 (10 ng/ml), LY (10 μM) or a combination of both. Whole AVG explants were immunostained for the transcription factor SOX2 (magenta) expressed in neuroblasts and for the G4-glycoprotein used as a marker of neuronal processes (G4, green). Representative images of compiled confocal microscopy projections are shown of four to five AVG per condition. Scale bar 100 μm. **(m)** G4-mean intensity and SOX2-area were normalized and quantified as described in "Material and Methods" section, and the results are shown as the mean ± SEM relative to the 0S condition. Statistical significance was estimated with the Student's *t*-test: $^*P < 0.05$ vs. 0S.

epithelium at the otic cup and otic vesicle stages (Magariños et al., 2012; Gálvez et al., 2017), which express SOX2. Interestingly, we show *TGFB2* mRNA expression precisely in the neurosensory domain, in which the otic neuroblasts are being specified and subsequently delaminate to give rise to the ganglionic neuroblasts. These results point to a role of TGFβ2 in promoting the progression of differentiation in neuroblasts. TGFβ2 also significantly reduced the number of proliferating neuroblasts

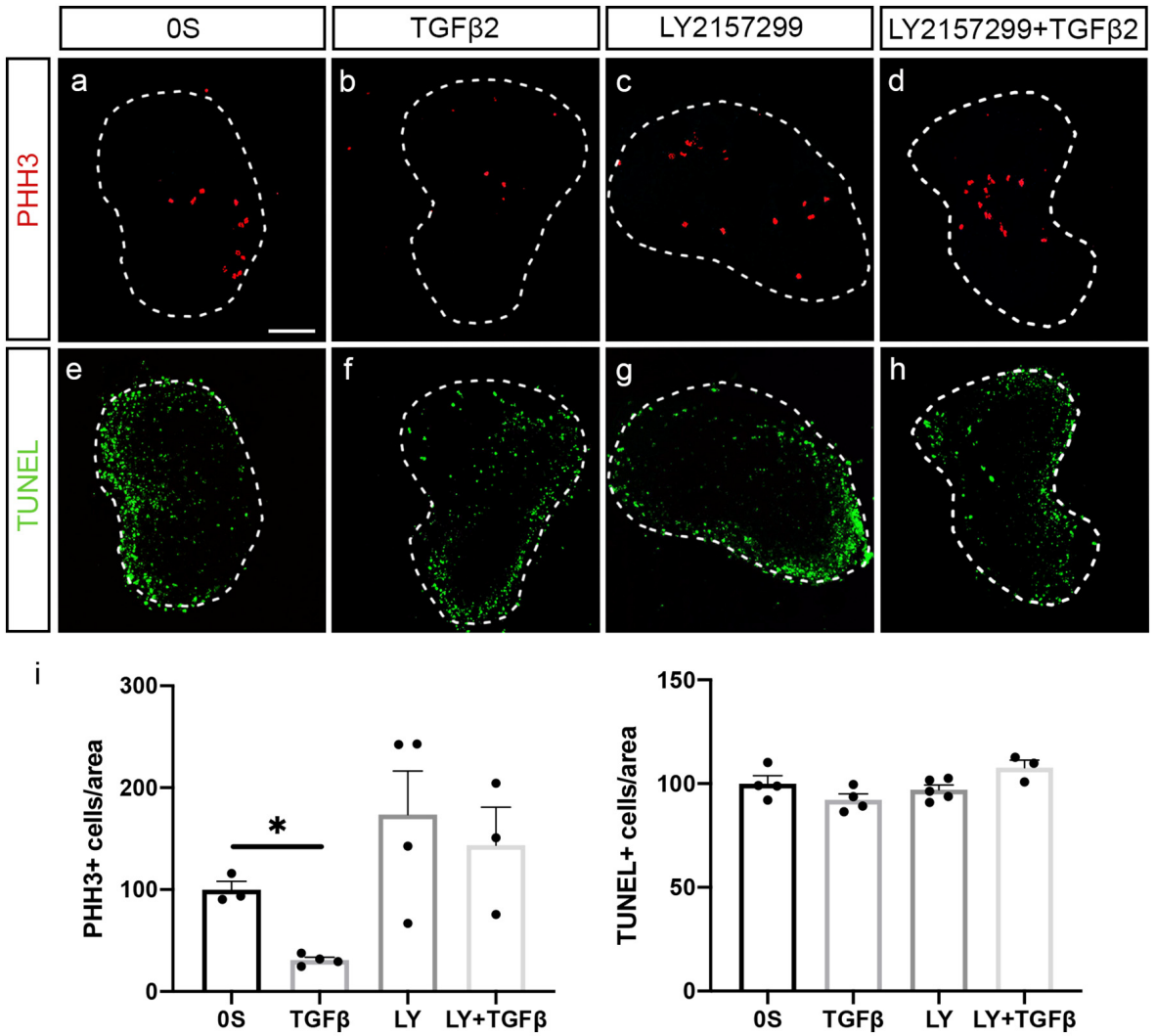

FIGURE 4 | TGFβ2 decreases neural proliferation. **(a–h)** Acoustic-vestibular ganglia cultured in serum-free medium (0S), in the presence of TGFβ2 (10 ng/ml), LY (10 μM), or a combination of both. Apoptotic cell death was visualized by TUNEL (green), and proliferation was detected by immunofluorescence for the mitosis marker phospho-Histone 3 (PHH3, red). Representative images of compiled confocal microscopy projections are shown of three to five AVG per condition. Scale bar: 100 μm. **(i)** TUNEL-positive or proliferative-PHH3-labeled cells were quantified as described in "Materials and Methods" section, and the results are shown as the mean ± SEM relative to the 0S condition. Statistical significance was estimated with the Student's *t*-test: $^*P < 0.05$ vs. 0S.

in cultured AVG, further supporting the pro-differentiating actions of TGFβ2 on otic neuroblasts. According to our data, these actions seem to be independent of programmed cellular senescence, as we could not detect SAβG staining in the AVG from chicken embryos nor cultured otic vesicles. However, it has been also reported that it is possible to have senescent cells that do not stain with SAβG, as demonstrated in cells lacking *Glb1* (Rhinn et al., 2019). Importantly, some studies showed developmentally programmed senescence acting together with apoptosis (Lorda-Diez et al., 2015). Nonetheless, our results show that TGFβ2 does not alter cell death, supporting the idea that this molecule acts independently of programmed cellular senescence in the AVG. Nonetheless, to rule out the mechanistic action of TGFβ2 through cellular senescence in this context, deeper molecular studies, such as p21 expression or the secretory profile (SASP) of otic neuroblasts are needed. On the other hand, we could also detect lower levels of *TGFBR2* in the neurosensory domain, but we cannot discard the possibility that TGFβ2 actions in the neuroblast population are mediated through other receptors of the TGFβ superfamily, and/or that low levels of *TGFBR2* are sufficient to mediate these actions. Accordingly, we detected phosphorylated SMAD2 throughout the otic epithelium, as well as in the developing AVG. The TGFβR2 signal was very intense in the AVG, which may point towards the ability of the ganglionic neuroblasts to activate TGFβ signaling cascade. Alternatively, TGFβ2 secreted in the neuroepithelium could be acting directly on the ganglionic neuroblast population through TGFβ receptors, promoting the

differentiation from trans-amplifying neuroblasts to post-mitotic neurons in the AVG.

Although the effects mediated by TGFβ2 in cultured otic vesicles or AVGs were both pointing to an enhanced maturation of otic neuroblasts, it is worth discussing the differences among the two systems. In otic vesicles, in the presence of TGFβ2, we could observe an enhanced adhesion of the organotypic culture to the substrate. Moreover, ganglionic neuroblasts presented a high migration rate away from the otic epithelium, as we could detect Islet-1-positive nuclei migrating forward the otic vesicle. These neuroblasts also expressed higher levels of Tuj-1. On the other hand, in the case of cultured AVG, there were no obvious effects on the migration rate of neuroblasts, but rather on their differentiation state. These differences can be possibly attributed to the different differentiation state of neuroblasts in the two culture systems tested, being otic vesicle neuroblasts in an earlier differentiation stage than those conforming to the AVG.

TGFβ-signaling has been also described to play key roles in regulating apoptosis during embryonic development. However, it is also clear that the death-inducing capacity of TGFβ is context-dependent, i.e., it is restricted to certain cell types, to a certain state of differentiation, and most notably, to the presence or absence of other growth factors (Schuster and Krieglstein, 2002). Examples of this, are the increased apoptosis in the endocardial cushions during the development of aorticopulmonary septum in mouse embryos upon elevated TGFβ2 levels (Kubalak et al., 2002) vs. the inhibition of apoptosis after TGFβ2 treatment in cultured retinal ganglion cells (Braunger et al., 2013). In our study, cultured AVG did not show alterations in the levels of apoptosis in the presence or absence of TGFβ2 nor in the presence of TGFβ-signaling inhibitor.

Understanding the molecular basis of developmental changes leading to the formation of the vertebrate auditory and vestibular ganglia is paramount to set a solid basis for efforts to regenerate this intricate organ in hearing loss patients (Chen et al., 2009, 2012). Our results add to this knowledge by showing that TGFβ2 promotes otic neuronal differentiation and that this does not involve the activation of cellular senescence. In contrast, previous results in our group showed that TGFβ2 does promote cellular senescence in the endolymphatic duct (Gibaja et al., 2019). Generally, our observations highlight the role of TGFβ2 in the precise regulation of cell fate within the developing inner ear and its different cell types. However, its mechanisms of action are highly cell-type dependent.

DATA AVAILABILITY STATEMENT

The data will be deposited in DIGITAL.CSIC (https://digital.csic.es), the institutional repository of the Spanish National Research Council.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because DIRECTIVE 2010/63/EU does not consider foetal forms before last third of their development.

AUTHOR CONTRIBUTIONS

MA, IV-N, and MM designed the experiments and wrote the manuscript. MM, MA, and RB-A performed the experiments. MA, RB-A, IV-N, and MM analyzed and interpreted the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00217/full#supplementary-material.

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Heat Shock Alters Mesenchymal Stem Cell Identity and Induces Premature Senescence

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Heat stress can have a serious impact on the health of both humans and animals. A major question is how heat stress affects normal development and differentiation at both the cellular and the organism levels. Here we use an *in vitro* experimental system to address how heat shock treatment influences the properties of bovine mesenchymal stem cells (MSCs)—multipotent progenitor cells—which are found in most tissues. Because cattle are sensitive to harsh external temperatures, studying the effects of heat shock on MSCs provides a unique platform to address cellular stress in a physiologically relevant model organism. Following isolation and characterization of MSCs from the cow's umbilical cord, heat shock was induced either as a pulse (1 h) or continuously (3 days), and consequent effects on MSCs were characterized. Heat shock induced extensive phenotypic changes in MSCs and dramatically curtailed their capacity to proliferate and differentiate. These changes were associated with a partial arrest in the G1/S or G2/M checkpoints. Furthermore, MSCs lost their ability to resolve the inflammatory response of RAW macrophages in coculture. A possible explanation for this loss of function is the accumulation of reactive oxygen species and malfunction of the mitochondria in the treated cells. Heat shock treatments resulted in stress-induced premature senescence, affecting the MSCs' ability to proliferate properly for many cell passages to follow. Exposure to elevated external temperatures leads to mitochondrial damage and oxidative stress, which in turn conveys critical changes in the proliferation, differentiation, and immunomodulatory phenotype of heat-stressed MSCs. A better understanding of the effect of heat shock on humans and animals may result in important health and economic benefits.

Keywords: bovine, mesenchymal stem cells, heat shock, oxidative stress, senescence, immunomodulation

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INTRODUCTION

Elevated ambient temperatures are increasing in frequency and can severely affect human and farm animal well-being, especially during the summer period. Heat stress increases the concentration of intracellular reactive oxygen species (ROS) in cells (Slimen et al., 2014) and has detrimental effects on mammalian fertility and well-being (Kitagawa et al., 2004; Agarwal et al., 2014). ROS react with DNA, proteins, and other macromolecules, leading to an accumulation of mutations and

misfolded proteins (Cooke et al., 2003). Moreover, increased ROS levels elicit stem cell depletion and functional defects in several tissues (Burgess et al., 2014). In bovine, the frequency of many chronic inflammation–related diseases is elevated during a hot period (Olde Riekerink et al., 2007; Chen S. et al., 2018), resulting in reduced animal welfare and significant economic losses to the dairy industry (Key et al., 2014). *In utero* heat stress was recently found to reduce the placental weight and blood flow and decrease birth weight of calves, and they impaired innate and cellular immunity (Dado-Senn et al., 2020). However, although heat is a common stressor, the functional connection between elevated temperatures and the higher rates of chronic inflammation is still obscure.

Adult stem cells are the longest living proliferative cells in multicellular organisms (Uccelli et al., 2008). They have an intrinsically increased risk of accumulating metabolic and genetic damage that will eventually lead to their destruction. The accumulation of such damage can be enhanced by extrinsic factors including environmental stress or exposure to toxins, together leading to the functional decline of the stem cells (Ermolaeva et al., 2018).

Mesenchymal stem/stromal cells (MSCs) are nonhematopoietic multipotent cells, most frequently derived from adult tissue sources such as bone marrow and adipose tissue or birth-associated tissue such as endometrial and placental tissues, amnion, and umbilical cord (Hass et al., 2011; Nowakowski et al., 2016). In the body, MSCs regulate bloodstream monocyte frequencies in reaction to inflammation (Mendez-Ferrer et al., 2010; Shi et al., 2011) and are capable of multilineage differentiation into cell types such as adipocytes, osteoblasts, chondrocytes, myocytes, β-pancreatic islets cells, and neuronal cells (Kuroda and Dezawa, 2014; Gao et al., 2016). *In vitro*, MSCs have been reported to exert anti-inflammatory, immunosuppressive, and trophic characteristics (Carrade et al., 2012; Kuroda and Dezawa, 2014; Li Y.W. et al., 2017; Shi et al., 2018). They exert these effects by influencing proliferation, recruitment, function, and fate of both the innate and adaptive immune cells, including T cells, B cells, dendritic cells, natural killer cells, and macrophages, which is likely mediated through both direct cell-to-cell contact and secretion of diverse immunoregulatory mediators (Aggarwal and Pittenger, 2005; Squillaro et al., 2015).

Several studies examining the cross-talk between MSCs and macrophages have shown MSCs to promote the anti-inflammatory M2 phenotype, which contributes to inflammation resolution and tissue repair in a variety of species (Braza et al., 2016; Chiossone et al., 2016; Yin et al., 2017). This results through the secretion of anti-inflammatory cytokines such as transforming growth factor β and TNF-stimulated gene 6 (Braza et al., 2016; Chiossone et al., 2016; Luz-Crawford et al., 2016; Howie et al., 2017; Li Y.W. et al., 2017). MSCs under intrinsic or extrinsic induced damage might become senescent (de Magalhaes and Passos, 2018). Senescent cells stop proliferating, become enlarged, and change their transcriptional pattern. Moreover, senescent cells secrete proinflammatory cytokines such as interleukin 1 (IL-1), IL-6, and IL-8 under control of the transcription factor nuclear factor κB [senescence-associated secretory phenotype (SAPS)] (Coppe et al., 2008). These stabilize senescence in an autocrine fashion and contribute to bystander effects, i.e., induction of DNA damage and senescence in normal surrounding cells (Gorgoulis et al., 2019). The entry into the senescent state is the result of either replicative senescence (cellular aging) or stress-induced premature senescence (SIPS) (Toussaint et al., 2002). SIPS is a state of irreversible division arrest of cells that retain their metabolic activity and is caused by persistent exposure to environmental stress, namely, heat stress, oxidative stress, or DNA-damaging mediators (Glotin et al., 2008; Alekseenko et al., 2012). Although much is known about aging-related stress, the causes and consequences of SIPS, as well as the degree and significance of SIPS in physiological conditions, are not well known and understood.

The objectives of this study are to characterize the immunomodulatory properties of bovine umbilical cord (bUC) MSCs and to examine the effect of high temperature [e.g., heat shock (HS)] on their function. MSCs of bovine origin have been previously isolated and characterized from different tissues (Bosnakovski et al., 2005; Cabezas et al., 2014; Mançanares et al., 2015; Xiong et al., 2016). *In utero* thermoregulation is dependent on the mother's core temperature, and maternal heat stress can impact the fetal temperature through the fetal–placental circulation (Dado-Senn et al., 2020). Therefore, we investigated the properties of bUC-MSCs that survived physiological HS exposure for varying periods of time and under a range of temperatures. We show that while sublethal temperature shock induced SIPS and impaired bUC-MSC capacity for differentiation into multiple cell lineages, the effect on immunomodulatory functions is dependent on the duration of the HS.

MATERIALS AND METHODS

Tissue Processing and Cell Culture

The UC tissue was processed following Toupadakis et al. (2010). Cells were plated in a low-glucose Dulbecco modified eagle medium (Gibco, Carlsbad, CA, United States) containing 10% fetal bovine serum (Gibco) and a penicillin–streptomycin mixture (3%), expanded, and cryopreserved at different passages. For more details, see **Supplementary Material** and Methods.

Population Doubling Time Assessment

Following pulse and constant HS treatments, 100 K cells from each treatment were plated in 10-cm plates. This process was repeated every 4–6 days for 15 passages (over 100 days). Population doubling (PD) and PD time were calculated using the formulas $N = N0 \times 2\ d$ (where N, N0, and d are the final cell numbers, the initial cell number, and the number of cell divisions, respectively) and $N = N0 \times 2t/\tau$ (where N, N0, and τ are the final cell numbers, the initial cell number, and PD time, respectively).

Evaluating Immunomodulation Function

Mesenchymal stem/stromal cells' anti-inflammatory properties were examined through their ability to suppress the immune response, testing their effect on T-cell proliferation using a

mixed lymphocyte reaction [adapted from Arzi et al. (2017)] and examining phenotypic shifts in macrophages toward their anti-inflammatory state (M2) [adapted from Chiossone et al. (2016), Luz-Crawford et al. (2016), Yin et al. (2017)]. For more details, see **Supplementary Material** and Methods.

Oxidative Stress Levels Detection

We used a CellROX Green Reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions, with or without 500 μM H_2O_2 (Sigma, St. Louis, MO, United States) for 30 min at 37°C. Three biological repeats were used for each treatment. For more details, see **Supplementary Material** and Methods.

Mitochondrial Membrane Potential Measurement

We used a JC-1 assay (ENZO Life Sciences International, Farmingdale, NY, United States) as described by Smiley et al. (1991). Mitochondrial membrane potential (MMP) was qualified based on the fluorescence emitted and classified into two main colors: red—high potential, and green—low potential. Three biological repeats were prepared for each treatment, and a 1-h prestaining incubation with 500 μM H_2O_2 was prepared as a positive control. For more details, see **Supplementary Material** and Methods.

Cell Proliferation Assay

We used a CellTrace carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation Kit (Invitrogen). Two to three biological repeats were used for each treatment. For more details, see **Supplementary Material** and Methods.

Cell Cycle Analysis

Following pulse and constant HS treatments, full cell cycle analysis and S phase quantification were carried out using PI/BrdU stainings adapted from Lehner et al. (2011). Immunohistochemistry was performed as described previously (Lichter et al., 1990; Selig et al., 1992). More than 100 cells were counted in each slide. For more details, see **Supplementary Material** and Methods. For detection of S-positive cells during the 43 h prior to the end of HS treatments, cells from 42°C pulse HS and 72-h constant HS were incubated with BrdU for a range of time periods (1–43 h) and taken for BrdU detection protocol on day 3. Three biological repeats were taken for each treatment, and a negative control was taken without BrdU, but with first and secondary antibodies.

Senescence-Associated β-Galactosidase Marker Assay

This was carried out using a Senescence Assay Kit (Abcam, Cambridge, United Kingdom) and measured by flow cytometry. Three biological repeats were used for each treatment.

Statistical Analysis

GraphPad Prism (La Jolla, CA, United States) was used for statistical analysis and visualization. $P < 0.05$ was considered statistically significant.

RESULTS

MSC Culture From Cow Umbilical Cord

Bovine umbilical cord-Mesenchymal stem cells were isolated from bUCs as described in "Materials and Methods" and cultivated for 15 cell passages. The cells exhibited typical fibroblastic morphology (**Figure 1A**) and maintained proliferation potential for at least 15 passages (over 100 days, **Figure 1B**). It should be noted, however, that after the fifth passage the number of populations doubling per day was reduced significantly (**Figure 1B**, pink line and error head). Following isolation, the cells were characterized as bUC-MSCs by marker expression (**Figure 1C**), and chondrogenic, osteogenic, and adipogenic differentiation potential was assessed (**Figure 1D**).

Bovine Umbilical Cord-Mesenchymal Stem Cells Have Immunomodulatory Potential

Following the initial characterization of the bUC cells as MSCs, we examined their immunomodulatory potential as suggested by others (Menard et al., 2013). First, inhibition of T-cell proliferation was examined. PBMCs were isolated from whole blood and cocultured with bUC-MSCs for 4 days at a ratio of 1:5, respectively, with or without activation with concanavalin A (Con-A). T-cell proliferation was assessed by MTT assay, and a fold change between activated and nonactivated cells for T cells alone or cocultured with bUC-MSCs is presented in **Figure 1E**. While activated T cells alone exhibit proliferation, coculturing with bUC-MSCs reduced T-cell proliferation significantly. The effect remained strong even when cells were at passage nine.

Next, because the influence of MSCs on T cells is thought to be modulated at least in part by macrophages, the effect of bUC-MSCs on the balance between proinflammatory M1 macrophages and anti-inflammatory M2 macrophages was assessed (Francois et al., 2012). RAW (mouse macrophages cell line) cells were cocultured with bUC-MSCs for 21 h in normal RPMI media, which was then changed to conditioned RPMI media with LPS for 3 h for macrophage activation. Following the 24-h coculture, the identity of the cells was examined by flow cytometry using CD86 and CD163 antibodies (**Figure 1F**) and by reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR) for cytokine expression levels (**Figure 1G**). As expected, activated RAW cells present a high degree of CD86+ M1 type, but once cocultured with bUC-MSCs, the degree of CD86 expression was significantly reduced, whereas CD163 expression inclined. The coculture with MSCs reduced the expression of proinflammatory cytokines such as IL-1β and tumor necrosis factor α (TNF-α), an effect that the control cells (mouse embryonic fibroblasts-MEFs) did not demonstrate.

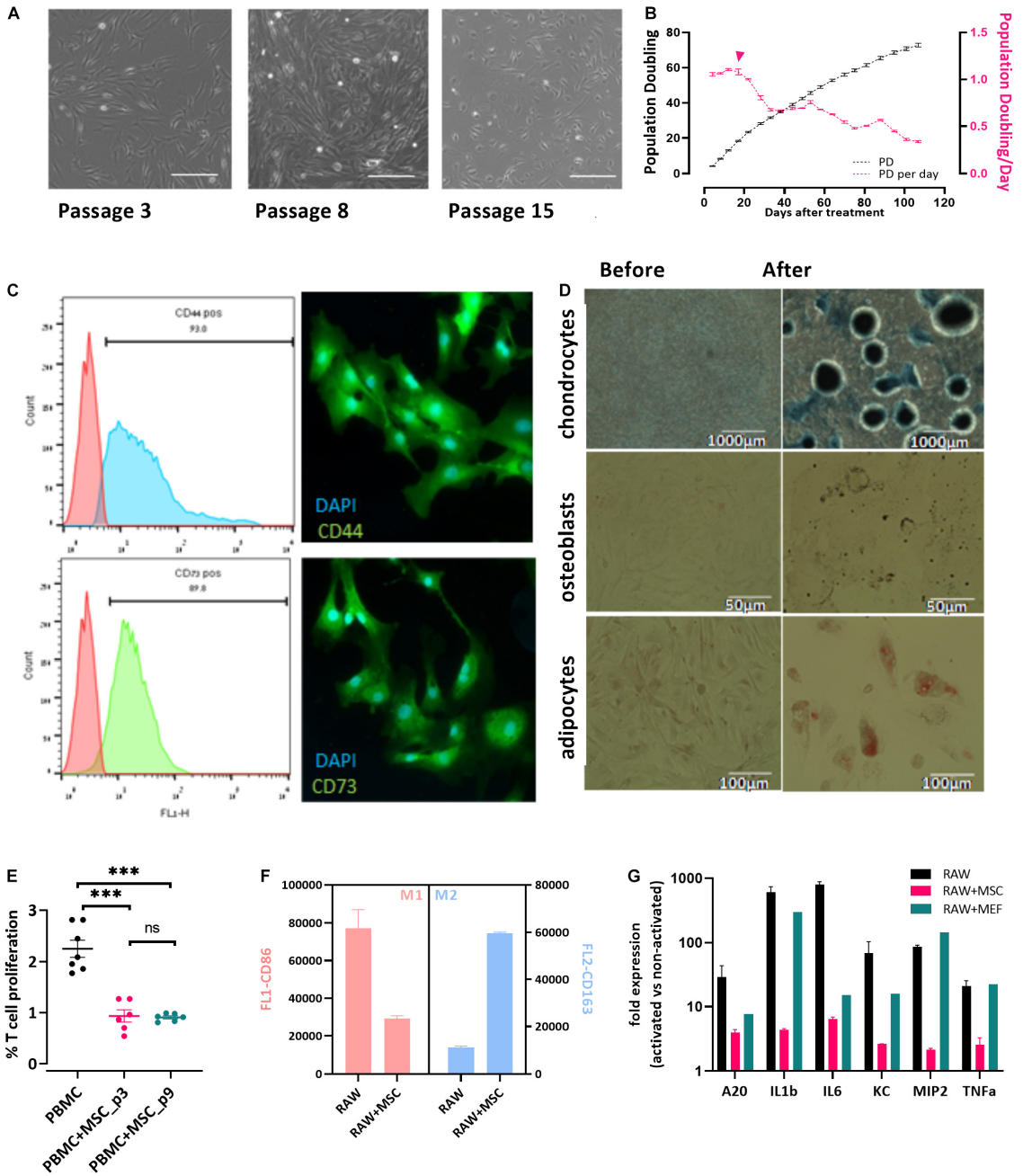

FIGURE 1 | Cells isolated from cow's umbilical cord display known MSC characteristics. **(A)** bUC-MSC displays a spindle-shaped morphology, proliferates, and adheres to plastic surfaces. No changes in cell morphology were observed for up to eight passages, but at passage 15, proliferation slowed. Scale bar = 500 μm. **(B)** bUC-MSC total population doubling (PD, black line) and population doubling time (PDt, pink line) for 25 passages. Population doubling was calculated as described in the "Materials and Methods" section. **(C)** Immunophenotyping (by flow analysis, left panel) and fluorescent microscopy imaging (right panel, with DAPI staining for nucleic acid, scale bars = 50 μm) of CD44 and CD73 MSC markers. **(D)** bUC-MSCs can differentiate into chondrocytes, osteoblasts, and adipocytes (stained by Alcian blue, Alizarin red, and oil red O, respectively). **(E)** T-cell proliferation assays were performed using bovine peripheral blood mononuclear cell (PBMC) activated by Con-A and cocultured with bUC-MSCs for 4 days at a MSC: PBMC ratio of 1:5. Cell proliferation was determined by MTT assay. The results represent the fold change between the Con-A–activated and nonactivated PBMCs, measured by the absorbance at 540 nm, and the activation of PBMC alone was set to 1. Data are mean ± SEM for $n = 6$, and Student t-test was used for comparison of means. $^{***}p < 0.001$. **(F)** Macrophages cell line (RAW) was cocultured with bUC-MSCs for 21 h and then activated by LPS for 3 h. RAW cells without bUC-MSCs were examined as reference cultures. RAW cells were then stained and flow-analyzed with proinflammatory M1 (pink bars, CD86) or anti-inflammatory M2 (blue bars, CD163) distinctive antibody. Mean fluorescence intensity ± SEM is indicated for $n = 3$. All bars are significantly different ($p < 0.01$) by Student t-test. **(G)** The transcriptional profile of five mouse genes was investigated by RT-qPCR on RNA extracted from the activated macrophages with or without cocultured bUC-MSC or MEF negative control. The expression levels of typical proinflammatory genes were reduced in RAW cells cocultured with bUC-MSCs but not with MEFs. Three housekeeping genes were used as reference genes—UBC, EIF5a, and EEF1A1. Data are mean ± SEM for $n = 3$ of RAW, RAW + MSC, and $n = 2$ for RAW + MEFs.

These results establish the immunomodulatory capacity that the bUC-MSCs hold in suppressing inflammation.

Thermal Preconditioning Affects Cell Growth and Morphology

To examine the effect of changing external conditions on the potency of the cells, we evaluated the HS response of bovine MSCs. HS treatment in tissue culture was performed by applying two protocols meant to examine the short- or long-term consequence of exposure to physiological sublethal HS conditions. For pulse HS, MSCs at early passages (P1–P4) were moved from 37°C to either 39°C or 42°C for 1-h HS followed by a 3-day recovery back to 37°C (**Figure 2A**). For the constant HS protocol, cells were moved from 37 to 40.5°C for 24, 48, or 72 h (**Figure 2B**). Constant and pulse HS protocols were performed, and the effect on the cells' survival, growth, clonogenicity, and morphology were examined. No significant death was observed in the MSC culture using trypan blue staining (**Figure 2C**), yet the number of cells was lower following the HS treatment. This behavior, also noted and studied previously in the context of MSCs (Antebi et al., 2018), is not representative of all bovine fibroblasts. When bovine fetal fibroblast cultures were treated with similar HS protocols, the majority of the cells died (**Figure 2C**).

Enhanced ability of proliferation is a well-known trait of multipotent stem cells, and bUC-MSCs have shown the ability to proliferate for multiple passages (**Figure 1B**). However, following severe pulse or constant HS, PD declined from ∼3 PD in the 4 days since plating to 1–2 PD in the cells that suffered the HS (**Figures 2D,E**). Additionally, the reduced clonogenic capacity of the HS-treated cells was evident by significantly lower colony-forming unit fibroblasts (CFU-f) numbers as compared to MSCs grown in normothermia (**Figure 2F**, a graph summarizing triplicate results and representative pictures of the plates). This is in agreement with studies showing that different stresses might cause a decrease in the number of CFU-f of MSC culture (Boyette et al., 2014; Kheirandish et al., 2017). These results indicate that induced HS reduces colony-forming capability and cell division in culture without increased cell death. Furthermore, the treated cells became flattened and larger (morphology typical of senescent cells) as evident under the microscope (**Supplementary Figures S1A,B**) and measured by flow analysis side scatter (**Figures 2G,H**).

To assess whether the change in the cells proliferative capacity is temporal or inherited to the daughter cells, we continued to grow the HS-treated cultures for 80 more days following the treatment (which took place on days 0–4, P2) and measured the cumulative PDs (**Figure 2I**). Although at each passage live cells were seeded at a fixed confluence (1×10^5 cells/9.6 cm^2), the gap between the control and HS-treated cells remained stable for more than 13 passages. By following the attenuation of the number of doublings per day (PDt), we noted that the cells' proliferation capacity almost fully recovered after their immediate reduction following the HS treatments [as in Wiese et al. (2019)]. Approximately three passages later (P7, blue arrowhead in **Figure 2J**, inset), the treated cells started slowing their PDt, whereas the control culture PDt started to decrease only two passages afterward (P9, black arrowhead in **Figure 2J**, inset). This finding suggests that the treated cells initiated cell cycle slow-down and replicative senescence earlier than the untreated MSCs. However, all cells continued proliferation at a slow but stable rate for more than 15 passages (black arrow in **Figure 2J**) before the culture became inhomogeneous and eventually ceased proliferation completely.

Functional Consequences of HS

Together with proliferation capacity, immunophenotypic analysis and three-lineages differentiation are the parameters for the characterization of MSCs (Dominici et al., 2006). We analyzed how the *in vitro* differentiation potential is affected by the HS treatment (**Figure 3A**). Osteogenic, chondrogenic, and adipogenic differentiation potential declined following the severe pulse and constant HS. Osteogenic and chondrogenic differentiation of HS-treated cells were impaired mainly for 42^oC, 48- and 72-h treatments. As fewer differentiation centers were visible, the intensity of staining was weaker, and their size was smaller. HS-treated cells exhibited similar results following adipogenic differentiation. Delayed fat accumulation was visible, the morphologic change took longer to initiate, and fewer cells accumulated lipid droplets that were also smaller as compared to control cells. These results suggest that even short thermal stress has an incremental impact on the overall differentiation potential of MSCs *in vitro*, which might explain some of the impaired physiological functions of animals exposed to heat stress. The immunophenotypic analysis showed that although the composition and expression level of surface markers change during long-term cultivation (Wagner et al., 2008), no effect of the HS treatments on the expression levels of the distinctive surface markers was observed (**Figure 3B**).

The Functional Consequence of HS on MSC Immunomodulatory Capability

The effect of MSCs on the immune system is a major reason for the interest in these cells and an important characteristic for both therapeutic use and physiological homeostasis. We analyzed the capability of MSC coculture to induce the switch of activated macrophages from proinflammatory M1 to anti-inflammatory M2 by comparing the cytokine expression pattern of RAW macrophages cocultured with MSCs before and after HS treatments (as illustrated in **Figure 3C**). While the coculture of RAW with normal MSCs or MSCs after the pulse HS treatment reduces the expression of mouse proinflammatory cytokines such as KClike Cxcl1, IL-1b, and TNF-α, the constant HS treatment eliminates this immunomodulatory capacity of the cells (**Figure 3D**, see also **Supplementary Figure S2**).

Together, these data indicate a dissimilar effect of the two HS protocols, at least in some aspects. While short pulse HS impairs proliferation and differentiation, but not immunomodulation, constant thermal stress of 24 h or longer can reduce the immunomodulation capability of the surviving MSCs. In a physiological context, this may suggest that the lack of regulation

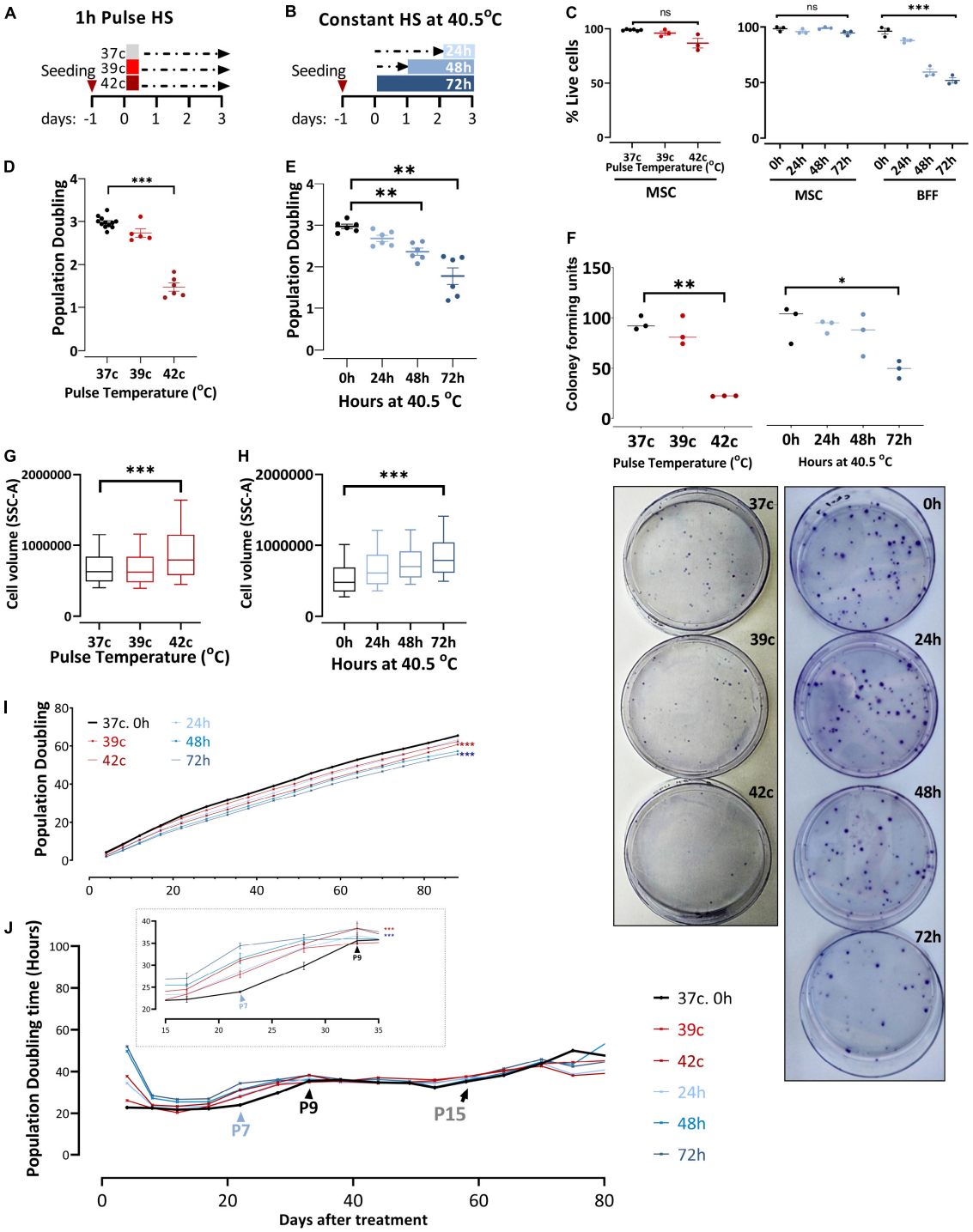

FIGURE 2 | In response to heat shock, bUC-MSCs change their morphology and proliferation rate. **(A)** Pulse-HS: bUC-MSCs are plated and 24 h later are placed at 39 and 42°C for 1 h and then allowed to recover at 37°C for 3 days. **(B)** Constant HS: bUC-MSCs are plated and 24 to 72 h later are moved to grow at 40.5°C for 1–3 days. **(C)** Dead: live MSC ratio as measured using trypan blue does not change dramatically following both HS treatments. In contrast, bovine fetal fibroblast (BFF) shows massive death rates following constant HS treatment. **(D)** bUC-MSC Population doubling declines following pulse and **(E)** constant HS. **(F)** Drop in clonogenicity of cells after pulse or constant HS. Data from C to F are mean ± SEM for $n \geq 3$. Unpaired, two-tailed Student *t*-test was used for comparison of means. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns = nonsignificant. **(G)** Side-scatter geo-mean data of cells after pulse and **(H)** constant HS are shown. One flow analysis of 10,000 cells is shown (representative result, $n = 3$). **(I)** Growth curve of untreated cells (black line) and cells after HS. Each line represents a sample, and each time point represents a passage. The mixed-effect model was used for comparison, $^{***}p < 0.001$. **(J)** Population doubling time measured by hours shows the kinetics of proliferation/passage. Immediately after the HS treatment, the cells have nearly ceased to proliferate, but after 1 passage, they narrow the gap with the control cells. Inset shows a narrower window of 20 days between passages 6 and 9. However, the HS cells never completely closed the gap, and even after about 15 passages, all cells are declining toward replicative senescence and cease proliferating. Mixed-effect model was used for comparison, $^{***}p < 0.001$.

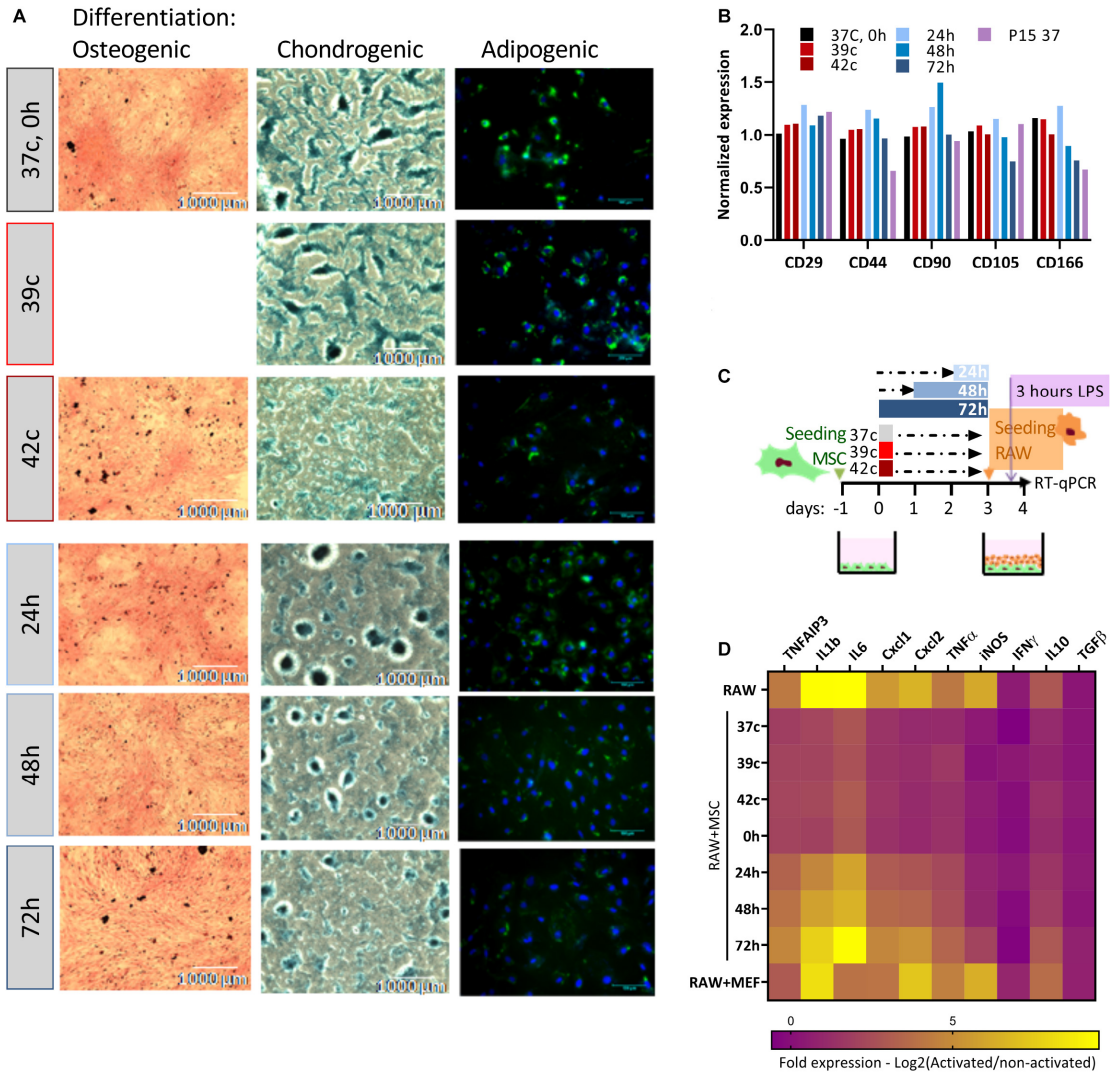

FIGURE 3 | Loss of differentiation and immunomodulation abilities following heat shock. **(A)** bUC-MSCs gradually lose the ability to differentiate into osteocytes (left, stained by Alizarin red), chondrocytes (middle, stained by Alcian blue), and adipocytes (right, stained by Bodipy and DAPI) following pulse and constant HS. Representative pictures from three independent experiments are shown. **(B)** No change in expression levels of typical MSC markers following HS, with all samples normalized to control untreated MSCs. **(C)** Macrophages cell line (RAW, illustrated orange) was cocultured with bUC-MSCs (in green) for 21 h following HS treatment and then activated by LPS (purple) for 3 h. RAW cells without bUC-MSCs were examined as a reference, and coculture with neutral cells (MEFs) was carried out to control for coculture effect. **(D)** The transcriptional profile of 10 mouse genes was examined by RT-qPCR. The expression levels of typical proinflammatory genes were high (yellow) in RAW cells alone and reduced (violet) in RAW cells cocultured with bUC-MSCs but not with MEFs. Long HS treatment at 40.5°C eliminated the reduction, indicating that those cells were malfunctioning. Three housekeeping genes were used as reference genes—UBC, EIF5a, and EEF1A1. Data are the mean for $n = 3$.

on the immune system is the basis for the higher probability of chronic inflammations. Accordingly, we looked to uncover the mechanism for this loss-of-stemness and immunomodulatory functions in the stressed cells.

HS Increases Intracellular ROS Production in Cell Culture

HS has been implicated in promoting oxidative stress either through excessive ROS production or decreased antioxidant defense, as was previously shown in dairy cows (Bernabucci et al., 2002; Abdelnour et al., 2019). To examine whether HS affects the levels of oxidative stress in MSC cell culture, CellROX dye levels were examined at day 3 after HS, with or without the addition of H_2O_2 for the last 30 min. Interestingly, 1 h at up to 39°C did not affect the ROS levels, whereas 42°C for 1 h induced high oxidative stress even after a 3-day recovery (**Figure 4A**). Similarly, a direct positive correlation was found between the number of days at 40.5°C and ROS levels (**Figure 4B**). The addition of H_2O_2 in the last 30 min was made to examine the resistance of the cells to secondary oxidative stress. However, although the net ROS levels were elevated,

the positive correlation between ROS levels and HS remained, indicating that acclimation to HS did not involve resistance to secondary oxidative stress. This behavior is specific to MSCs after HS because the cells did not change their morphology (**Supplementary Figure S3A**) or ROS levels (**Supplementary Figure S3B**) when grown in 1% oxygen—hypoxia—for 3 days.

Expression of some stress response–related genes (FOXO3, GPX1, and PPARγ) was elevated following 24-h HS, but not in other HS-treated cells despite the elevated ROS levels these cells contained (**Figure 4C**). Hypoxia-inducible factor α (HIF1α) was slightly up-regulated 3 days after the 1-h pulse HS but not after the constant HS. Additionally, no significant histone H2AX phosphorylation—a measure of DNA double-strand breaks—could be identified in the cells after the HS treatment by immunofluorescence detection of γH2AX (**Figure 4D**). We hypothesize that the increase in DNA damage loci after 24 h in HS (**Figure 4D**, graph and representative pictures) is resolved in the later constant HS time points—48 and 72 h—by the action of the antioxidant gene product transcribed at 24 h (**Figure 4C**). Thus, although the HS is followed by higher oxidative stress in the cells, no major DNA damage was caused or accumulated. This is in line with the minor proportion of cell death and suggests that the notable drop in the number of cells following treatment is not due to severe damage.

Functioning mitochondria is required for the immunomodulation activity of MSCs (Islam et al., 2012). To examine if the HS treatment impaired the mitochondria, the JC-1 assay for the detection of MMP was used (Smiley et al., 1991). JC-1 indicates high MMP by the formation of red fluorescent aggregates, while at the same time displays MMP depolarization by forming a green fluorescent monomer. Normal untreated cells show a low green/red ratio (25% of the cells contain damaged mitochondria), whereas an average of 62% of the pulse (**Figure 4E**) and 46% of the constant (**Figure 4F**) HS cells present green, depolarized membrane potential. This decline in mitochondrial activity may be the cause of the drop in PD and reduced immunomodulation in the treated cells.

To examine if elevated ROS levels and mitochondrial damage are inherited through passaging, we stained the same stressed cells with CellROX dye after long passaging at P15 (**Supplementary Figure S3C**). At this stage, cells show slower growth (**Figures 2J,I**) and 10 times higher ROS levels than at P3. However, a small difference remains between the previously HS-treated cells and the control cells. In contrast, mitochondrial damage is elevated in all aged cells, but no difference could be observed between the previously HS-treated cells and the control cells (**Supplementary Figure S3D**). Overall, these results show that cells after either long-term culturing or HS show parallel metabolic and functional attenuations.

HS Leads to Decreased Proliferation, Cell Cycle Arrest, and Premature Senescence

A significant decrease in cell growth following elevated ROS levels and mitochondrial dysfunction could indicate either elevated apoptosis levels or changes in the proliferation capacity of the cells due to cell cycle arrest or slow-down. To examine the first option, we measured the percentage of dead and apoptotic cells in the population, staining for propidium iodide (PI) or annexin V, respectively. The two most extreme HS conditions used—42°C for 1 h and the 72-h HS—had a twofold increase in dead cells (from 12 to 21, and 27%, respectively, **Figure 5A** and **Supplementary Figure S4A**). A minor increase in apoptosis from about 5 to 6, or 12% of the cells being positive to annexin V was also detected (**Figure 5B**). This increase is very small and could not explain the reduction in cell numbers after HS. Furthermore, no change in the apoptosis-related gene BAX was observed after any of the HS treatments (**Figure 5C**). Hence, we examined a second option to assess changes in cell proliferation rate.

The expression of HS proteins HSP27 and HSP90 increased significantly following constant HS (**Figure 5C**), which indicates acclimation to heat in these cells (Matozaki et al., 2019).

In order to examine the proliferation rate of bUC-MSCs following HS treatments, a CFSE fluorescent tracing, diluted at each cell division, allowed us to visualize and distinguish between cell generations. CFSE staining was performed on bUC-MSCs on day 0, followed by pulse or constant HS experiments, and the fluorescent signal was measured by flow cytometry on day 3. With each cell doubling, the cell staining became weaker. The control cells, grown at 37°C, doubled three to five times during these 4 days (**Figures 5D,E**—black histogram). After pulse HS at 42°C, a significant decrease in cell doubling was observed, and about 10% of the population did not complete more than one round of doubling (**Figure 5D**). Following constant HS, a significant decrease in cell doubling was observed again, with a high variation in the number of doubling times in the population (**Figure 5E**). These results signify a decrease in proliferation rate after constant HS as well as following a 1-h pulse of high temperature, which affects the cell cycle even after a 3-days recovery in normothermia.

Next, PI-BrdU flow cytometry analysis revealed that following pulse HS, cell cycle was arrested at G2/M (**Figure 5F** and **Supplementary Figure S4B** top panels), whereas constant HS led to G1/S arrest (**Figure 5F** and **Supplementary Figure S4B** bottom panels). To verify the reduction in % S phase, cells were similarly stained with anti-BrdU and S phase BrdU-positive cells were counted under the microscope (**Supplementary Figure S4C**) and by flow cytometer (**Supplementary Figure S4D**). The results show that, indeed, both pulse and constant HS cell populations are less replicating than the control untreated cells (**Figure 5F** and **Supplementary Figures S4C,D**). These results can point to two different situations: either homogenous populations with a longer cell cycle or two subpopulations of cells emerging from the HS treatment, of which is arrested in senescence or quiescence and the other showing normal proliferation capacity. To distinguish between these two options, cells were incubated with BrdU for a range of times before the HS protocol ended (**Supplementary Figure S4E**). Interestingly, the pulse HS treatment displayed a Gompertzian-like BrdU incorporation kinetics, with 5 to 15% fewer cells incorporating BrdU than the untreated population. The constant 72-h HS, however, presented a unique curve shape of BrdU incorporation, which may suggest a population-wide

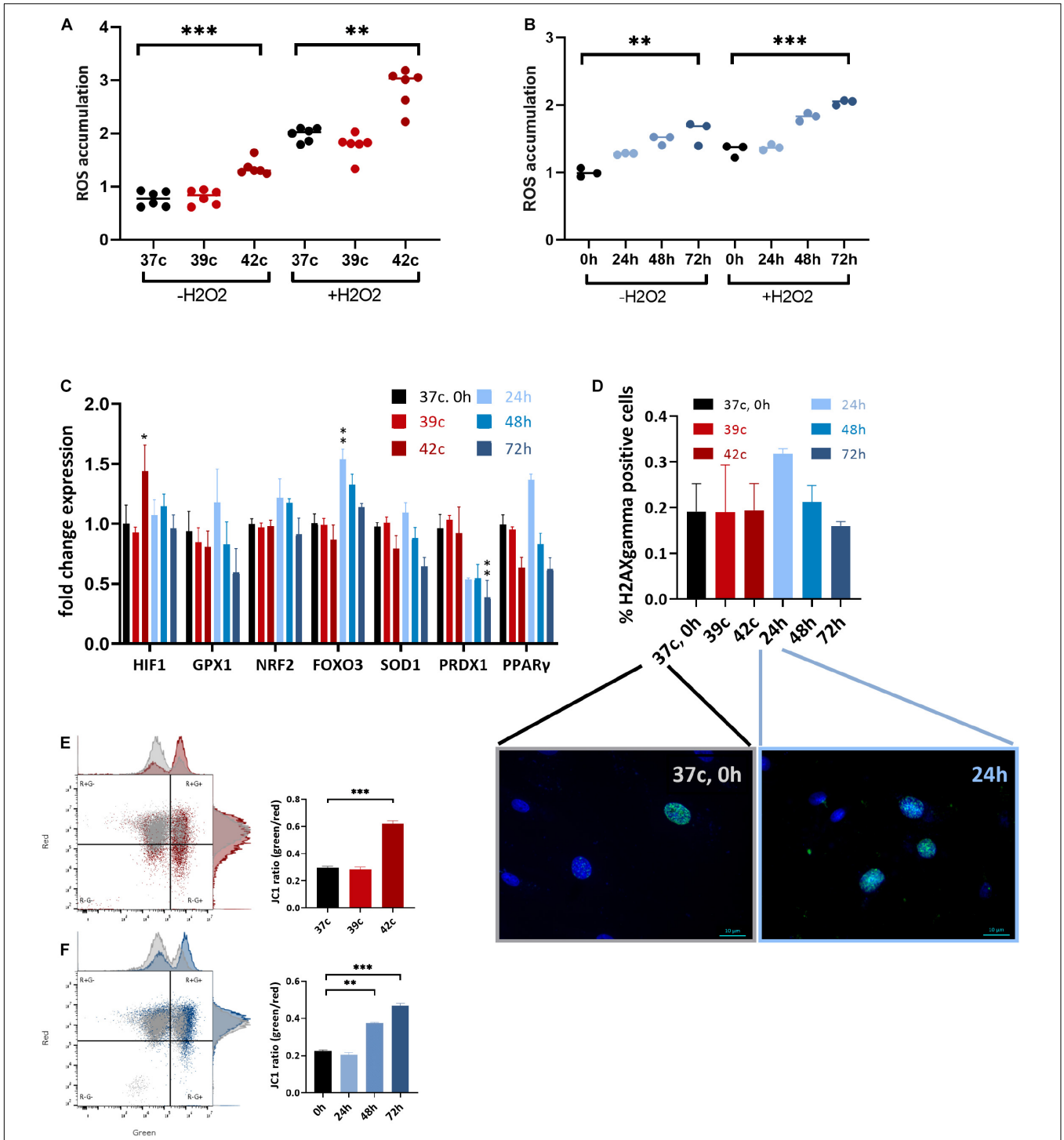

FIGURE 4 | Change in metabolic function following HS in bUC-MSCs. Flow cytometry results of CellROX staining for **(A)** cellular ROS levels after pulse HS without and with 500 μM H_2O_2 for 30 min. **(B)** cellular ROS levels after constant HS without and with 500 μM H_2O_2 for 30 min. Data presented are a mean of green fluorescence, ±SD $n = 6$. **(C)** Change in expression levels of oxidative stress response pathway genes. All genes were normalized to control bUC-MSCs. Statistical significance was determined using the Bonferroni–Sidak method, and results represent the mean ± SEM, $n = 4$. **(D)** Histone H2AX phosphorylation was measured by fluorescent microscopy imaging with anti-γH2AX (green) and DAPI staining for nucleic acid (blue), scale bars = 10 μm. Negative and positive nuclei were counted (∼200 from each slide) from two unrelated experiments, mean ± SEM are shown. **(E)** Membrane potential was measured using JC-1 assay and flow analysis (see example in the left panels). Percent of red cells (upper left, R+G–) declined after pulse HS (red) and **(F)** constant HS (Blue) as compared to the untreated cells (gray), $n = 3$. Statistics: two-way ANOVA with Benjamini, Krieger, and Yekutieli *post hoc* test as compared to 37°C, 0 h, $^{**}p < 0.01$, $^{***}p < 0.001$.

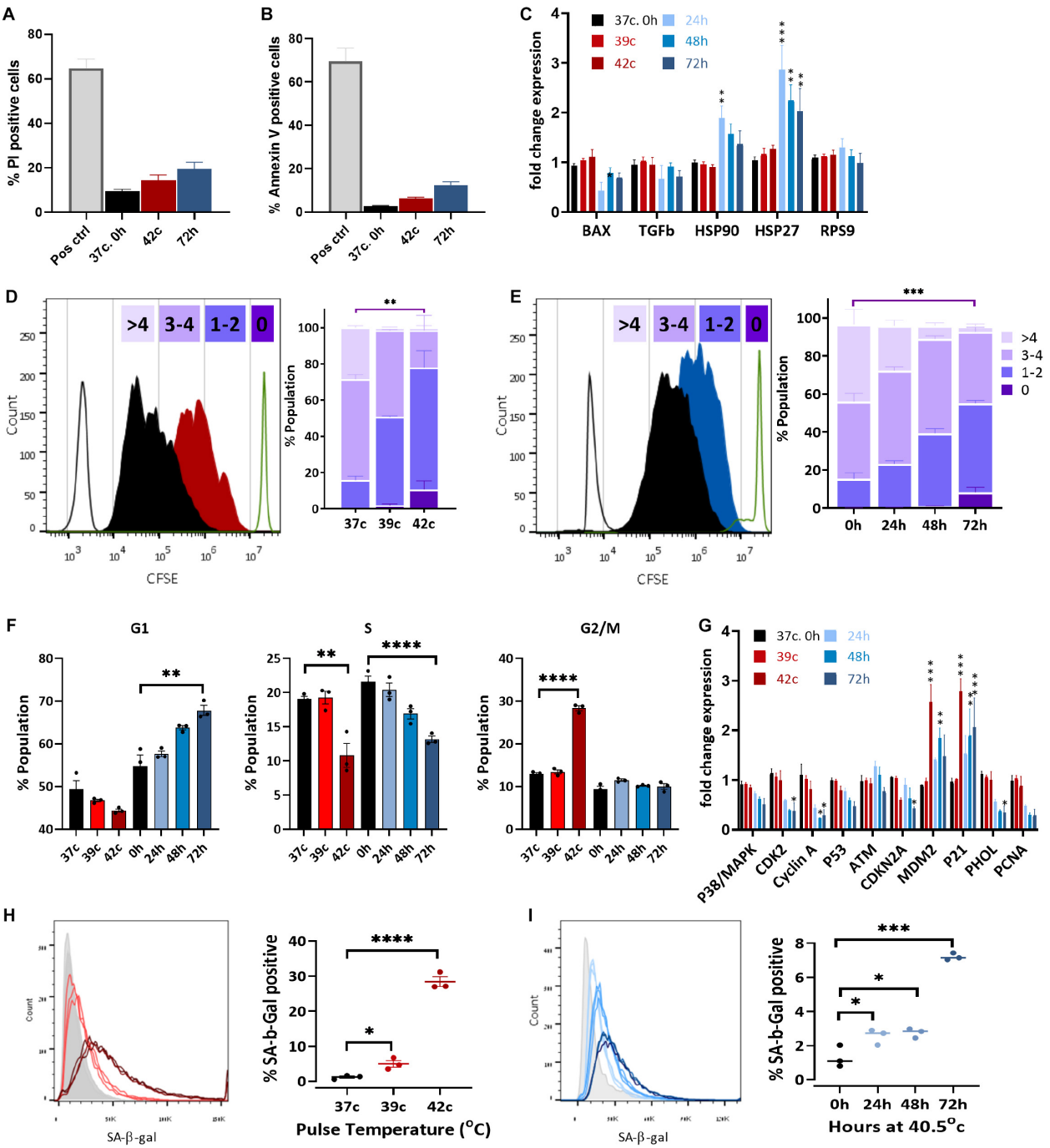

FIGURE 5 | Heat shock induces cell cycle arrest and premature stress-induced senescence (SIPS). Flow cytometry assay of cell death using PI staining **(A)** and apoptosis with annexin V staining **(B)** of pulse HS at 42°C and constant HS for 72 h. **(C)** Change in expression levels of heat shock proteins and apoptotic-related genes. RPS9 was used as a control gene, and data were normalized to WT bUC-MSC expression levels. Statistical significance was determined using the Holm–Sidak method; results represent the mean ± SEM, $n = 4$. **(D,E)** Cell proliferation was assessed by the CFSE dilution method following pulse HS **(D)** or constant HS **(E)**. Each experiment was performed in triplicate; results represent the mean ± SD. Statistics: two-way ANOVA with Benjamini, Krieger, and Yekutieli *post hoc* test as compared to 37°C, 0 h, $^{**}p < 0.01$, $^{***}p < 0.001$. **(F)** PI/BrdU staining flow cytometry analysis of cell cycle phases distribution following constant or pulse HS. Data are presented as mean ± SEM. Statistics: *t*-test with the Holm–Sidak method, $n = 3$–9. **(G)** Detection of BrdU incorporated for 1–43 h into the DNA of pulse HS at 42°C and constant HS for 72 h treated cells by flow cytometry. Data are the mean ± SEM for n = 3–6. **(H)** Change in expression levels of cell cycle–related genes. Data were normalized to WT bUC-MSC expression levels. Statistical significance was determined using the Holm–Sidak method; results represent the mean ± SEM, $n = 4$. **(H,I)** % cells positive for SA-β-gal staining during cell recovery from pulse **(H)** and constant HS **(I)**. Data are mean ± SEM for $n = 6$ and a Student *t*-test was used for comparison of means, $^{***}p < 0.001$, $^{****}p < 0.0001$.

change in the kinetics of entering S phase. Transcriptional analysis of cell cycle–related genes revealed a significant increase in the expression of *CDKN1a* (*p21*), a cell cycle inhibitor, and major regulator of the senescence program (Alekseenko et al., 2014) (**Figure 5G**). In agreement with the absence of detectable DNA damage in the senescent population, *p53* and *ATM* are not up-regulated. The elevated expression of MDM2, which promotes *p53* degradation, might explain the low levels of apoptosis in the stressed populations. No *p16* (*CDKN2A*) upregulation is seen, in agreement with the previous findings (Dulić et al., 2000; Alekseenko et al., 2012; Muñoz-Espín et al., 2013; Zhai et al., 2019), suggesting this is not a requisite marker for senescence. Interestingly, S phase genes such as *PCNA* and *POLH* are down-regulated in the constant HS group, arrested at G1. Correspondingly, these genes are not down-regulated in the pulse HS cells that are arrested in G2/M.

This is the only gene that showed an increase following all the constant HS treatments, as well as in the pulse HS of 42°C, regardless of the recovery period at 37°C.

To dissect the long-term changes in the cells following HS, we compared the stressed cells (at P3) before and after culturing them for >10 additional passages (at P15) (**Supplementary Figure S5A**). JC-1 assay shows reduced mitochondrial potential in all aged cells, with no significant long term effect on the HS-treated cells (**Supplementary Figure S5B**). To examine if elevated ROS levels and mitochondrial damage are inherited through passaging, we stained the same stressed cells with CellROX dye after long passaging—at P15 (**Supplementary Figure S5C**). At this stage, cells show slowed growth (**Figures 2J,I**) and 10 times higher ROS levels than at P3. However, a small difference remains between the previously HS-treated cells and the control cells. In contrast, mitochondrial damage is elevated in all aged cells, but no difference could be observed between the previously HS-treated cells and the control cells (**Supplementary Figure S5D**). Overall, these results show that cells after either long-term culturing or HS show parallel metabolic and functional attenuations. We next compared the transcriptional program to examine the change in genes that are either typical MSC marker-, cell cycle-, or apoptosis-related genes and genes related to stress and inflammation (**Supplementary Figure S5E**). As expected, most genes had major changes in gene expression following long-term passages. *HSP90* presents down-regulation, similar to *PPARγ*, which was shown to decrease during cellular aging (Park et al., 2005; Ye et al., 2006; Li Y. et al., 2017). On the other hand, some genes presented upregulation in the P15 bUC-MSCs (*HIF1*, *GPX1*, *BAX*). However, no effect of past HS treatment was observed in the P15 cells, and all showed uniform transcription patterns. Interestingly, a cluster of cell cycle arrest–related genes [namely, *p38/MAPK*, *p53*, *CDKN2A* (*p16*) and *PRDX1*] with low expression levels in the aged P15 MSCs was similarly down-regulated following 72-h HS treatment and to a lesser extent also in the other constant and pulse HS-treated cells. However, some genes changed after HS but not at P15 (*p21, HSP27*) whereas others were up-regulated at P15 but not following HS (*BAX*, *GPX1*, and *NRF2*). This revealed that despite some similarities, the molecular mechanisms used following HS are not those turned on following replicative senescence.

Last, to examine whether cell cycle arrest following HS is a result of SIPS (Alekseenko et al., 2014), staining was performed with SA-β-Gal, which is a marker for detecting cellular senescence (Dimri et al., 1995). SA-β-Gal staining increased in the HS-treated cells both for the pulse (**Figure 5H**) and the constant (**Figure 5I**) HS protocols. When compared to P15 cells (**Supplementary Figure S5B**), SA-β-Gal staining for P4 cells after the pulse and constant HS showed much higher levels of senescence, confirming that mild HS indeed promotes senescence in bUC-MSCs.

DISCUSSION

We presented the effect of mild heat stress on the potency and function of bovine MSCs from the umbilical cord. Previous studies have shown that exposure to extremely high temperatures (45°C) for a very short time can lead to senescence of MSCs (Alekseenko et al., 2012, 2014) while inside the body the cells can hardly be exposed to this temperature. Other studies have shown an improvement in MSC survival and function following short and mild HS (Chen X. et al., 2018; McClain-Caldwell et al., 2018; Wang et al., 2019). Therefore, we examined the effect of slightly elevated temperatures (constant HS) on the bUC-MSCs to determine if this HS would affect their proliferation, differentiation, and immunomodulation ability. The different time points (24, 48, and 72 h) were meant to follow the development of cellular acclimation following a sublethal exposure to heat (Matozaki et al., 2019). We also examined whether a short elevation of temperature—still within the physiological range—will affect cells 3 days afterward (pulse HS). Although we tried to stay within the physiological range, it is not clear how many cells are exposed to such temperatures in the body and for how long.

We have shown that in many ways the 1-h pulse HS has a similar long-term effect to that of the 72-h constant HS. Although initially there is no substantial death, the cells express *p21* and slow down their cell cycle, and the differentiation efficiency is reduced. This might indicate that the detrimental effects of HS on the cells (elevated ROS, mitochondrial damage) initiate a chain of events that lead to the onset of SIPS, no matter the length of the HS. However, unlike the constant HS, the pulse HS protocol did not impair the immunomodulation ability of the cells. The different stages of cell cycle arrest and gene expression patterns may explain this discrepancy. In both HS treatments, the percentage of senescent cells was never greater than 30%, and the remaining cells displayed almost normal features after passaging. However, even a low percentage of senescent cells in the population was shown to cause adverse consequences, supporting the need to study the effect of small environmental changes on the cellular population.

It could be hypothesized that in a subpopulation of the cells, the HS caused a shift to a state of reversible quiescent and not to irreversible senescence. Supporting this hypothesis, the BrdU long incubation displays the classical Gompertzian-like kinetics for the WT and the pulse HS cells, as expected with equal cell cycle durations, whereas the constant HS curve varies greatly.

Hence, it seems that a subpopulation of the 72-h HS–treated cells present low self-renewal rates, e.g., quiescence. This state might present an advantage to the organism because the cells will be more protected and less metabolically active in times of thermal stress, but retain the opportunity to be activated and function as normal stem cells in times of need. Indeed, to allow time for DNA repair following oxidative damage, the cells activate their cell cycle checkpoints, leading to cell cycle arrest and preventing the replication of damaged and defective DNA (Dizdaroglu, 1992). On the other hand, oxidative stress, as expressed by elevated levels of cellular ROS and decreased MMP, is a well-known implication of heat stress (Bernabucci et al., 2002) and inducer of SIPS in many cells (Dasari et al., 2006; Glotin et al., 2008; Marazita et al., 2016).

We found a positive correlation between the duration of constant HS to the accumulation of oxidative damage and the increase in SA-β-gal senescence marker staining. During SIPS, cells are known to obtain a SAPS, which in turn can cause collateral damage to neighboring cells and tissues (Zhang et al., 2019). This might explain some of the negative effects of HS treatments and link cellular stress with the physiological distress animals experience in a warm climate. Moreover, this finding might prove useful in the clinic where we wish to have better control of the proliferative status of the cells used for therapy. Our data suggest that thermal shock may have physiological consequences on tissue homeostasis, which could further lead to organ damage and the development of inflammatory and age-related diseases.

Based on existing knowledge about MSCs and senescence, the rate and manifestation of replicative senescence in human and bovine MSCs are in remarkable agreement (Wagner et al., 2008; Gnani et al., 2019; Wiese et al., 2019). However, the effect of hypoxia (Antebi et al., 2018) and thermal stress (Choudhery et al., 2015; Andreeva et al., 2016; McClain-Caldwell et al., 2018; Wang et al., 2019) were inverted; i.e., hypoxia was destructive in human but not in bovine MSC, while mild HS delayed replicative senescence in human. This implies intriguing differences between organisms or tissues of origin as most human MSC studies are carried out on bone marrow or adipose MSCs. This might be an important issue for further study since, even though MSCs have been widely used for the treatment of companion animals, little is known about their potential in the livestock industry (Hill et al., 2019).

Although not yet fully understood, MSCs are thought to play an instrumental role in the maintenance of the body homeostasis. Senescent cells accumulate with age or exposure to stress, cause chronic inflammation, and increase the risk of many diseases (Kornienko et al., 2019). Senescence-related MSC failures, including immunomodulatory activity, hematopoiesis, and paracrine regulation, were shown in human (Munoz-Espin and Serrano, 2014; Li Y. et al., 2017). Understanding the effect of common environmental stress on the functioning of these cells will enhance our knowledge of the consequences of stress at the animal level. Presently, when air temperature in many parts of the world is increasing during the summer months, the study of short- and long-term effects of heat stress on physiological conditions is becoming increasingly important.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

ChS conceived and designed the study, designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. MG established the experimental system. IS, IR-C, DN, and CaS collected the experimental data and prepared the manuscript. SS conceived and designed the study, assembled and analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.565970/full#supplementary-material

Supplementary Table 1 | List of all mouse primers used in this study for gene expression analysis by RT-qPCR.

Supplementary Table 2 | List of all bovine primers used in this study for gene expression analysis by RT-qPCR.

Supplementary Table 3 | List of first and secondary antibodies used in this study.

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REVIEW
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Fibroblast Senescence in Idiopathic Pulmonary Fibrosis

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Aging is an inevitable and complex natural phenomenon due to the increase in age. Cellular senescence means a non-proliferative but viable cellular physiological state. It is the basis of aging, and it exists in the body at any time point. Idiopathic pulmonary fibrosis (IPF) is an interstitial fibrous lung disease with unknown etiology, characterized by irreversible destruction of lung structure and function. Aging is one of the most critical risk factors for IPF, and extensive epidemiological data confirms IPF as an aging-related disease. Senescent fibroblasts in IPF show abnormal activation, telomere shortening, metabolic reprogramming, mitochondrial dysfunction, apoptosis resistance, autophagy deficiency, and senescence-associated secretory phenotypes (SASP). These characteristics of senescent fibroblasts establish a close link between cellular senescence and IPF. The treatment of senescence-related molecules and pathways is continually emerging, and using senolytics eliminating senescent fibroblasts is also actively tried as a new therapy for IPF. In this review, we discuss the roles of aging and cellular senescence in IPF. In particular, we summarize the signaling pathways through which senescent fibroblasts influence the occurrence and development of IPF. On this basis, we further talk about the current treatment ideas, hoping this paper can be used as a helpful reference for future researches.

Keywords: cellular senescence, aging, idiopathic pulmonary fibrosis, fibroblast, senolytics

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IDIOPATHIC PULMONARY FIBROSIS IS AN AGING-RELATED DISEASE

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease of unknown etiology, characterized by massive deposition of extracellular matrix (ECM) in the lung interstitium, leading to the irreversible and slowly progressive destruction of lung structure and function (Raghu et al., 2011). Most patients with IPF have a median survival of only 2–4 years, and respiratory failure due to IPF progression is the most common cause of intensive care unit (ICU) admission and death for IPF patients (Saydain et al., 2002; Fernández Pérez et al., 2010; Ley et al., 2011). Multiple risk factors increase IPF disease development risk in a single or coordinated manner (Raghu et al., 2011). The endogenous risk factors of IPF include genetic background, aging, gender, and pulmonary microbiology, while the exogenous risk factors include smoking, environmental exposure, and air pollution, especially the dust or organic solvents exposure in the occupational population (Iwai et al., 1994; Baumgartner et al., 2000). Comorbidities such as gastroesophageal reflux, obstructive

sleep apnea, and diabetes mellitus are also risk factors (Raghu et al., 2011). Among them, aging is one of the most significant risk factors for IPF.

The risk of interstitial lung disease increases with age. The risk of IPF illness in the elderly over 70 is 6.9 times higher than in the people aged over 40 (Choi et al., 2018). With the advancing age, IPF patients also show more interstitial changes in chest high-resolution computed tomography (HRCT) (Fell et al., 2010). Extensive epidemiological data confirms IPF as an aging-related disease (Hutchinson et al., 2014). The prevalence of IPF is 4.0 per 100,000 in people aged 18–34 years, and this rate is 227.2 per 100,000 in people aged 75 years or older, basing on data recorded in a large American health insurance database from 1996 to 2000 (Raghu et al., 2006). In Japan, from 2003 to 2007, the cumulative prevalence and incidence of IPF are 10.0 and 2.23 per 100,000, respectively, and the incidence also increases with age (Natsuizaka et al., 2014). Further, data from IPF patients in the United Kingdom shows a 35% increase in the incidence of IPF during the study period from 2000 to 2008, with a total incidence of 744 cases per 100,000 population (Navaratnam et al., 2011). From 2004 to 2010, the cumulative annual prevalence of IPF in American adults aged 18–64 increases from 13.4 per 100,000 in 2005 to 18.2 per 100,000 in 2010 (Raghu et al., 2016). Thus, the prevalence and incidence of IPF seem to be on the rise in recent years, partly due to the improved diagnostic methods (Raghu et al., 2014).

Researchers have been trying to figure out how the connections between IPF and aging were established. For one thing, the respiratory system itself shows signs of aging with years. Significant increases in peribronchial collagen and progressive fibrosis are observed in the lungs of natural aging mice (Calabresi et al., 2007). In asymptomatic elders, imaging findings associated with interstitial lung disease are more common, rare in younger populations (Copley et al., 2009). On the other hand, aging is involved in the occurrence and development of IPF disease. Previous studies have identified several common characteristics of aging, and cellular senescence is included as a significant one (Hayflick and Moorhead, 1961; Hayflick, 1965). Aging refers to the decline of various physiological functions and the degeneration of tissues and organs in individuals, which is gradually formed with physiological age growth. Along with the timeline getting closer to the end of the lifespan, the individuals inevitably suffer from this complex natural phenomenon. Quite differently, cellular senescence describes the physiological cell state, which is non-proliferative but living. When the damage to the cells is not extreme enough to initiate the death program but still severe to a certain extent, the damaged cells will begin a senescence program and become senescent (Hayflick and Moorhead, 1961). Aging is based on the accumulation of senescent cells, but cellular senescence can be detected no matter the physical age. Apart from aging, cellular senescence is also involved in a wide range of physiological activities varying from embryonic development, tissue renovates, and wound healing to tumor suppression. Perhaps because of this, the role cellular senescence plays in IPF is more uncertain than aging.

When compared with the age-matched control group, the primary fibroblasts isolated from the lung of IPF patients showed more senescent characteristics, which indicates that cellular senescence is persistent and intense in IPF patients (Álvarez et al., 2017). Under normal circumstances, lung fibroblasts only exist as the mesenchymal cells, located between the epithelial cells in the alveoli or trachea and the endothelial cells in the blood vessels. The alveolar epithelial cells can be harmed by pathogenic microorganisms, dust, drugs, chemicals, oxygen-free radicals, and other factors (Iwai et al., 1994; Baumgartner et al., 2000). Once the alveolar epithelial cells are damaged, while type II alveolar epithelial cells (AEC II) proliferate and differentiate into many flattened type I alveolar epithelial cells (AEC I) to repair the injury, lung fibroblasts are also activated. Activated lung fibroblasts proliferate locally and migrate to the injured area, then they differentiate into myofibroblasts, produce a large number of ECM components, and exhibit contractile function. The accumulated myofibroblasts gradually become senescent after the normal repair progress of lung injury, thus reducing fibroblasts' activation and limiting the progression of fibrosis. From this point of view, senescent lung fibroblasts play a protective role, for they stopping the deposition of ECM with the end of the repair process (Desmoulière et al., 1995). However, using senolytics removing senescent fibroblasts leads to decreased pulmonary fibrosis in mice model of IPF (Baker et al., 2011; Schafer et al., 2017). What's more, senolytics effectively improve the lung function in both the IPF mice model and IPF clinical patients, with well tolerance and security (Schafer et al., 2017; Justice et al., 2019). This evidence from the other side indicates the destructive effect of senescent lung fibroblasts, and the decline in the ability of senescent fibroblasts to degrade ECM may be one explanation (Schafer et al., 2017). There are practical reasons for these inconsistent results. In most cases, lung tissues of IPF patients are obtained through percutaneous lung biopsy or pneumonectomy for clinicopathological diagnosis. For ethical reasons, clinicians have to minimize the trauma to patients in invasive procedures or operations, and there are few remaining lung tissues that can be used for scientific researches after meeting diagnostic purposes. Thus in some relevant studies, para-cancer tissue with normal microscopic appearance is used as the control group of IPF lung tissue. It is doubtful whether the concluded results based on those control are actually effective.

SENESCENT FIBROBLASTS IN IDIOPATHIC PULMONARY FIBROSIS

Unlike lung epithelial cells, which are more like the sensor to external injury and stimulation, pulmonary fibroblasts act as the fibrosis process's direct executor during the disease. Senescent fibroblasts in IPF are abnormally activated, accompanied by telomere shortening, metabolic reprogramming, mitochondrial dysfunction, apoptosis resistance, insufficient autophagy, and senescence-associated secretory phenotypes (SASP) (**Figure 1**). Most of these characteristics of IPF senescent fibroblasts, in turn, promote the occurrence and development of IPF. The following

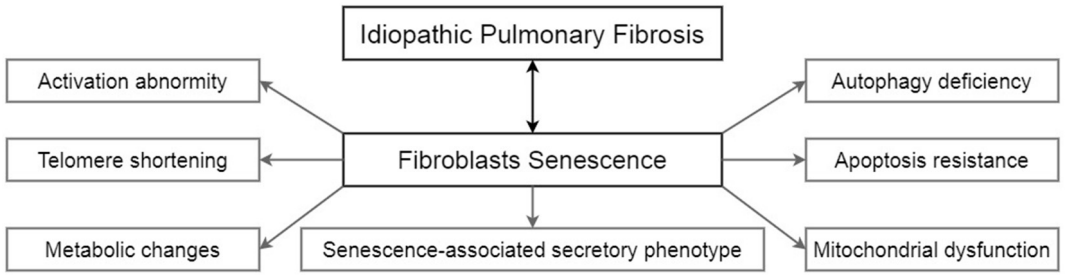

FIGURE 1 | The characteristics of senescent fibroblasts in IPF.

part will clearly illustrate how senescent fibroblasts affect IPF through the specific molecular signaling pathways and piece these fragmented results of experiments together to map a signal regulating network in senescent fibroblasts (**Figure 2**).

Activation Abnormity

The most fundamental feature of IPF is the deposition of ECM, which results from the abnormal activation of lung fibroblasts (Blokland et al., 2020). The activation of lung fibroblasts involves a series of cell behaviors changes such as proliferation, migration, and ECM production. Primary lung fibroblasts isolated from the lung tissue of IPF patients exhibit a difference in cellular senescence degrees compared with age-matched controls. These fibroblasts increase the levels of senescence-associated β-galactosidase (SA-β-gal), P16, P21, P53, and SASP. The morphology of the IPF lung fibroblasts is enlarged and flat, similar to the morphological changes happening in fibroblasts with replication failure. The doubling time of IPF pulmonary fibroblasts is slow during cell passage *in vitro*, demonstrating the declined proliferation capacity (Ramos et al., 2001; Yanai et al., 2015). These IPF lung fibroblasts also rapidly accumulate the activation markers α-smooth muscle actin (α-SMA) in primary culture, suggesting that these cells are also activated fibroblasts, but may not be equivalent to the senescent myofibroblasts (Yanai et al., 2015; Álvarez et al., 2017).

It is still controversial whether senescent fibroblasts in IPF are equal to senescent myofibroblasts. Some studies suggest that this problem is essentially a difference in experimental design, whether the researchers consider the different expressions of α-SMA protein or other specific identification of myofibroblast phenotype. Nevertheless, more importantly, in head and neck squamous cell carcinoma and esophageal adenocarcinoma, the senescent cancer-related fibroblasts show the same molecular expression, ultrastructure, and contractile properties as the typical TGF-β-induced myofibroblasts. Both cells are α-SMA positive, but RNA sequencing shows that there are significant differences in transcriptome between two kinds of fibroblasts, especially genes related to ECM deposition and tissue remodeling. The senescent fibroblasts with positive α-SMA do not mean that they have fibrogenic properties or they are senescent myofibroblasts. In other words, in IPF, the senescent fibroblasts may only be activated in several limited aspects (Mellone et al., 2016; Blokland et al., 2020). The development of omics research and biological big data analysis gives us the confidence to find this answer in the future.

Telomere Shortening

When normal human diploid cells are serially cultured *in vitro*, cells will stop proliferation after a limited number of divisions, and this is the earliest description of cellular senescence (Hayflick and Moorhead, 1961; Hayflick, 1965). Subsequent experiments afterward show that this halt in proliferation is induced by telomere shortening. Telomeres are gradually worn down with the increase of passage times of fibroblasts (Harley et al., 1990), and the loss of telomere protection directly exposes chromosome DNA to danger, which may then lead to cellular senescence through DNA damage checkpoint response (d'Adda di Fagagna et al., 2003; McDonough et al., 2018). The relationship between telomere shortening and cellular senescence is always mentioned in IPF. Lung fibroblasts isolated from IPF patients have shorter telomere lengths than age-matched controls (Álvarez et al., 2017), and these cells exhibit accelerated replicative senescence during the primary culture process (Yanai et al., 2015).

Apart from the number of cell divisions, telomere length is also affected by telomerase. Telomerase consists of three parts: telomerase RNA (hTR), telomerase synergistic protein 1 (hTP1), and telomerase reverse transcriptase (hTRT). Telomerase can make catalytic reverse transcription and provide the needed RNA template. Both functions are essential guarantees of normal telomere length. Multiple mutations of hTRT and hTR gene have been found in IPF patients, and they all lead the way to telomere shortening (Armanios et al., 2007; Tsakiri et al., 2007; Alder et al., 2008; Fingerlin et al., 2013). Among them, V144M, R865C, and R865H mutants of hTRT are more significant, and cell experiments determine that V144 and R865 in TRT are two critical residues required for ensuring normal function of cell telomerase (Tsang et al., 2012). However, further studies have shown that hTRT increases the viability of lung fibroblasts, which is beneficial to fibrosis development (Liu et al., 2007, 2013). Differently, hTRT protects AECII from senescence to ameliorate pulmonary fibrosis, suggesting the cell-type-specific role of hTRT in disease (Liu et al., 2019). The performance of telomerase activator GRN510 in external treatment also elucidates the existence of cell-type specificity. GRN510 only shows telomerase activation and cell lifespan prolongation in

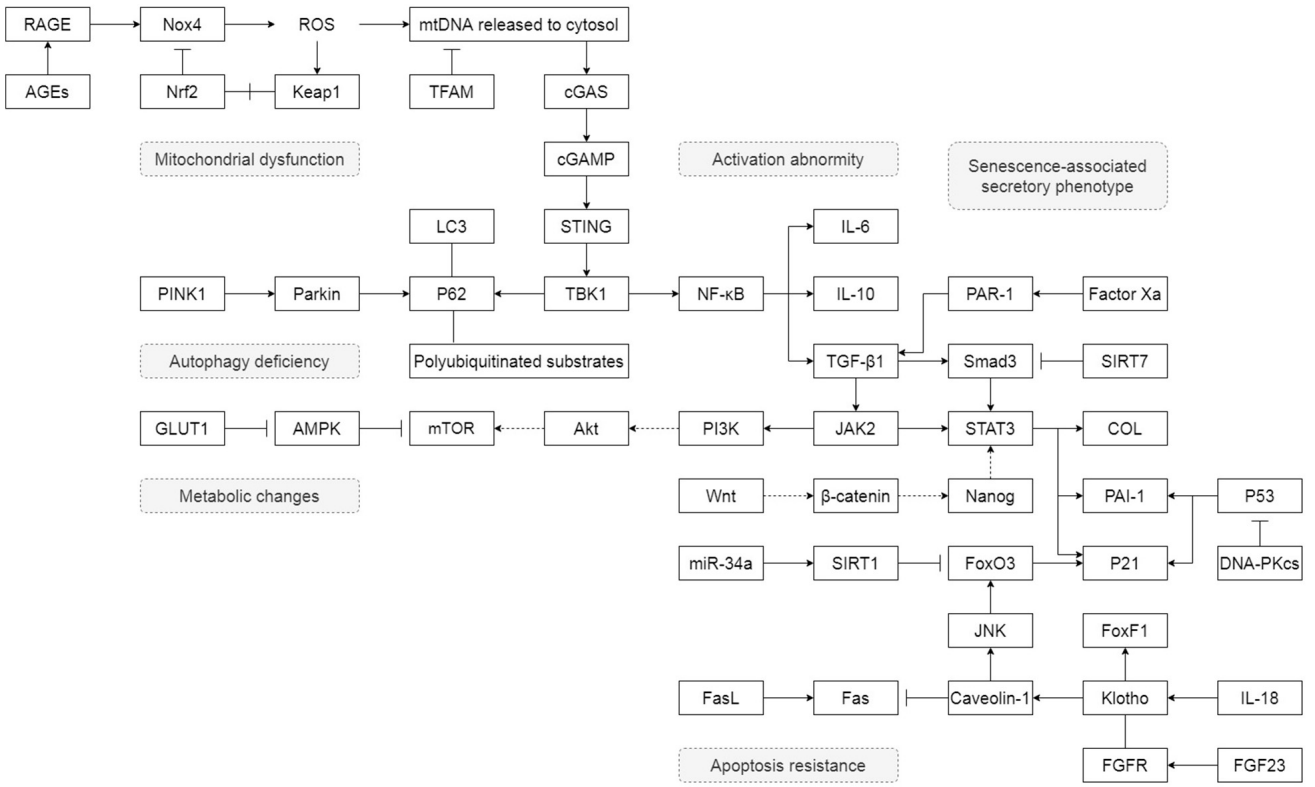

FIGURE 2 | The detail of how senescent fibroblasts affect IPF through the specific molecular signaling pathways. "→" represents "activation"; "⊣" represents "inhibition"; "—" represents "association"; "+" represents "disassociation."

small airway epithelial cells, but not in lung fibroblasts (Le Saux et al., 2013). However, the shorter telomere length in a significant proportion of IPF patients can't be explained by the mutant gene encoding telomerase (Cronkhite et al., 2008). This question may be related to the complex mechanism of telomere maintenance. Telomere elongation requires the assistance of telomere elongation helicases, and mutations in the gene RTEL1, an autosomal dominant trait, have also been detected in IPF patients (Kannengiesser et al., 2015). Besides, DNA needs to be bound to the telomere binding protein during telomere formation; thus, the lack of this protein prevents telomere assembly. In AEC II, the deletion of TRF1, a gene that encodes telomere binding protein, successfully constructs a mice model of IPF, showing lung remodeling and pulmonary fibrosis (Povedano et al., 2015). Nevertheless, the absence of TRF1 in collagen-expressing cells only causes pulmonary edema other than fibrosis (Naikawadi et al., 2016). Briefly, telomere shortening and corresponding senescent phenotypes are shown in IPF fibroblasts, but conversely, it seems that fibroblast senescence induced by short telomere does not cause IPF (Yanai et al., 2015; Naikawadi et al., 2016). The association between telomere shortening and cell senescence may merely occur in AEC II, not in pulmonary fibroblasts, showing cell-type specificity (Povedano et al., 2015; Naikawadi et al., 2016). Interestingly, more telomere-associated foci (TAFs) independent of telomere length are detected from lung fibroblasts in IPF mice models than in normal mice (Schafer et al., 2017). TAFs are the oxidative DNA damage located in telomeric G-reach repeats (Hewitt et al., 2012), and their presence suggests that telomeres are still involved in the fibroblast senescence in IPF through another unknown way.

Metabolic Changes

In primary lung fibroblasts obtained from aged mice, glucose transporter protein 1 (GLUT1) dependent glycolysis is activated. By down-regulating the activity of the AMP-activated protein kinase (AMPK), this glycolytic pathway promotes the protein expression of α-SMA in cells (Cho et al., 2017). And in the studies of human metabolomics, lung tissues of IPF patients also show significant differences in energy metabolism compared with healthy controls, especially the up-regulation of the glycolytic pathway (Zhao et al., 2017). This switch is somewhat similar to the Warburg effect in tumor cells. Warburg effect refers to the behavior that tumor cells tend to metabolize glucose into lactic acid through glycolysis under aerobic conditions, unlike normal cells that generate energy through aerobic oxidation of glucose. With this abnormal glucose metabolism behavior, tumor cells evade the normal apoptosis process and enhance their proliferation and migration ability. Moreover, in IPF, both the aberrant activation of fibroblasts and the substantial synthesis of ECM require enhanced energy generation to meet this biosynthetic requirement (Selvarajah et al., 2019).

The increased glycolysis is also the result of multiple signal transduction and interaction within the senescent fibroblasts. Hypoxia resulted from the progress of IPF disease will lead to the disorder of cell energy metabolism, but on the other hand, hypoxia also up-regulates the expression of hypoxia-inducible factor-1α (HIF-1α) protein and mRNA. HIF-1α mediates the overexpression of pyruvate dehydrogenase kinase 1 (PDK1) gene, which in turn activated glycolysis and increased the activation of lung fibroblasts (Goodwin et al., 2018). Consistent with the experimental results obtained from aged mice, up-regulated TGF-β1 signaling in IPF fibroblasts promotes GLUT1 mRNA expression through the typical Smad2/3 pathway, activates GLUT1-dependent glycolysis, and accelerates cell proliferation and production of fibrogenic mediators (Andrianifahanana et al., 2016). Moreover, at the fibrogenic regions in IPF patients and IPF mice models, fibroblasts associated with metabolically active and apoptosis-resistant show lower AMPK activity (Rangarajan et al., 2018). In addition, another product of glycolysis, lactic acid, is also found in the lung tissues of IPF. Lactate activates latent TGF-β1 by changing the pH value of the internal environment and then promotes the occurrence of fibrosis (Kottmann et al., 2012). Conversely, glycolysis inhibition attenuates both lung fibroblast activation and the growth-promoting phenotype of IPF fibroblasts (Dias et al., 2019). Fructose-1, 6-bisphosphate is an intermediate product of glycolysis, which can decrease the proliferation of fibroblasts and the cell's ability to produce ECM, thus halting the occurrence of pulmonary fibrosis (Xie et al., 2015). Increased glycolysis is a consequence of cellular senescence, but in another way, it also promotes pulmonary fibrosis. This may explain the inhibition effect of pulmonary fibrosis by AMPK activators such as metformin (Rangarajan et al., 2018; Kheirollahi et al., 2019).

Apart from glycolysis, metabolic heterogeneity in IPF fibroblasts also includes changes in bile acid, heme, amino acid, and lipid metabolism (Zhao et al., 2017). TGF-β1 increases the production of activated transcription factor 4 (ATF4) in fibroblasts. ATF4 is the central transcriptional regulator of amino acid metabolism and provides glucose-derived glycine to meet the amino acid requirements of cells associated with enhanced collagen production (Selvarajah et al., 2019). There is also a metabolic process related to glutamine, a critical metabolic process in which glutaminase converts glutamine into glutamic acid and then into the TCA cycle metabolite α-ketoglutarate (α-KG), mediating the resistance to apoptosis of fibroblasts (Bai et al., 2019). Also, advanced glycation end products (AGEs), as products of non-enzymatic reactions of fats and proteins with various oxidants during the senescent process, are a group of stable covalent compounds. By acting on the receptor of AGEs (RAGEs), AGEs also affect fibroblast activation (Machahua et al., 2016).

Mitochondrial Dysfunction

The mitochondrial dysfunction triggers include transmembrane potential loss in the mitochondrial inner membrane, down-regulation of electron transport chain (ETC) function, and reduction of key metabolites entering mitochondria from the cytoplasm (Nicolson, 2014). These alterations reduce mitochondrial oxidative phosphorylation and reduce ATP production (Nicolson, 2014). They then switch on several age-related changes, particularly the overproduction of pro-inflammatory and pro-oxidative signals (Correia-Melo et al., 2016). An overall decrease in mitochondrial mass and function are observed in IPF lung fibroblasts than healthy controls (Álvarez et al., 2017; Caporarello et al., 2019). The declining mitochondrial mass is associated with an abnormality in mitochondrial biogenesis and mitophagy. The decreased mitochondrial function is manifested as increased reactive oxygen species (ROS) production, decreased mitochondrial membrane potential, and mitochondrial respiratory chain complex in senescent fibroblasts (Luo et al., 2013). They both ultimately drive the senescence phenotypes of the cells and promote fibrosis progression (Wiley et al., 2016).

Mitophagy, which occurs in normal cells to maintain mitochondrial homeostasis, is down-regulated in senescent fibroblasts, leading to decreased PTEN-induced putative kinase 1 (PINK1) (Sosulski et al., 2015). Inhibited mitophagy activates the downstream pathway of platelet-derived growth factor receptor (PDGFR), further amplifying the mitophagy process (Kobayashi et al., 2016). Besides, levels of peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) and mitochondrial transcription factor A (TFAM) are steadily inhibited in IPF cells (Hecker et al., 2014; Bernard et al., 2015, 2017; Caporarello et al., 2019). Furthermore, dysfunctional mitochondria produce large amounts of ROS, including peroxides, superoxides, and hydroxyl radicals. ROS produced by lung fibroblasts rapidly and sensitively promotes cell proliferation and activation in a dose-dependent manner, and ROS is also endogenous damage to lung epithelial cells (Murrell et al., 1990; Waghray et al., 2005). The production of intracellular ROS is related to the decrease of mitochondrial membrane potential, which could be restored by the deactivation of the mammalian target of rapamycin (mTOR). mTOR activity also affects the signal transduction between mitochondria and nucleus, and the coordinated expression of mitochondrial and nuclear genes is a necessary condition for maintaining the normal function of mitochondria (Lerner et al., 2013). Reactive oxygen-producing enzyme NADPH oxidase 4 (Nox4) is another source of ROS (Jain et al., 2013; Bernard et al., 2017). NOX4 regulates the protein abundance of α-SMA and collagen by controlling the activation of Smad2/3 and regulated PDGF-induced fibroblast migration (Amara et al., 2010). What is worse, the antioxidant capacity of the nuclear factor E2-related factor 2 (Nrf2) is impaired in senescent fibroblasts, resulting in the Nox4-Nrf2 redox imbalance, which ultimately promotes the activation of fibroblasts and the formation of senescence phenotypes (Hecker et al., 2014; Bernard et al., 2015, 2017).

In addition, signal transduction and transcriptional activator factor 3 (STAT3) is necessary for the ETC of mitochondrial complexes I and II to remain activation and is involved in coordinating intracellular homeostasis (Wegrzyn et al., 2009). The elevation of superoxide concentration in senescent lung

fibroblasts continuously promotes the translocation of STAT3 to the nucleus and mitochondria, mediates the metabolic function of mitochondria, and raises the nuclear transcription level of senescence phenotypic related genes (Gough et al., 2009; Waters et al., 2019). Besides, in IPF lung fibroblasts, the promotion effect on cell activation by TGF-β1 signaling is accompanied by a decrease in the expression of silent mating type information regulation2 homolog-3 (SIRT3) gene (Sosulski et al., 2017). SIRT3 plays a deacetylase role in mitochondria to regulate mitochondrial health (Bindu et al., 2017) and plays a key role in repairing mitochondrial DNA damage and protecting mitochondrial integrity, too (Cheng et al., 2013). The depletion of endogenous SIRT3 will increase ROS production and mitochondrial DNA (mtDNA) damage, leading to the progression of pulmonary fibrosis (Sundaresan et al., 2015; Bindu et al., 2017). Damaged mtDNA, which should not be present in the cytoplasm, is released into the cytoplasm and is sensed by the cyclic GMP-AMP synthase (cGAS). cGAS catalyzes the production of cyclic guanosine acid (cGAMP), which activates STING protein and promotes the senescence of lung fibroblasts (Schuliga et al., 2020). What is more, superoxide enhances DNA damage response (DDR), which also amplifies age-related effects in lung fibroblasts as part of a positive feedback process (Schuliga et al., 2018).

Apoptosis Resistance

Apoptosis is an autonomously ordered death controlled by genes and can maintain homeostasis. In the restoration process of lung injury, the activated fibroblasts show changes corresponding to apoptosis and gradually disappear at the end (Desmoulière et al., 1995). Apoptosis of IPF lung fibroblasts is reduced compared with age-matched controls. However, in IPF, apoptosis's stimulation has a higher frequency and level, so the decreased sensitivity of cells to apoptotic signals is a convincing explanation (Álvarez et al., 2017). Apoptosis resistance of senescent fibroblasts is associated with the pathogenesis of IPF (Cha et al., 2010).

Reduced pro-apoptotic proteins Bak and Bax and increased anti-apoptotic protein Bcl-2 family proteins are found in IPF senescent fibroblasts (Moodley et al., 2003; Sanders et al., 2013). The accumulation of Bcl-2 family proteins, which include Bcl-2, Bcl-W, and Bcl-XL, contributes to apoptotic stimuli resistance in senescent cells (Ricci et al., 2013a; Yosef et al., 2016). Alterations in the expression of pro-apoptotic and anti-apoptotic genes are associated with histone modification and DNA methylation (Sanders et al., 2014). In contrast to the Bax gene, the acetylation of histone H4K16 (H4K16Ac) is significantly enriched in the Bcl-2 gene while the trimethylation of histone H4K20 (H4K20Me3) is significantly decreased (Sanders et al., 2013). These site-specific histone modifications regulate the expression of the Bcl-2: Bax gene in senescent fibroblasts, leading to the anti-apoptotic phenotypes (Sanders et al., 2013). Intracellular TGF-β1 signaling increases the Bcl-2 protein level by activating JAK2 and STAT3 (Milara et al., 2018). Both the levels of Bcl-2 and Bax protein in fibroblasts are STAT3-dependent (Moodley et al., 2003), and inhibition of STAT3 signaling can block this resistance to apoptosis (Pechkovsky et al., 2012). Further, the mechanical sensitivity signal transduction pathway up-regulates the expression of the Bcl-2 gene in activated fibroblasts through activation of the Rho/ROCK pathway, reducing fibroblast apoptosis reduction, and contributes to the continuous fibrosis process (Zhou et al., 2013). Cytokines IL-6 and IL-11 increase the expression of Bcl-2 mRNA in IPF lung fibroblasts to inhibit apoptosis (Moodley et al., 2003). The plasminogen activator inhibitor 1 (PAI-1) level in mouse lung fibroblasts increases significantly with age, accompanied by a decrease in apoptosis in fibroblasts (Huang et al., 2015; Marudamuthu et al., 2015).

Also, senescent IPF lung fibroblasts are found to be highly resistant to Fas ligand-induced (FasL) and TNF-associated apoptotic ligand-induced (TRAIL) apoptosis. FasL, TRAIL, and Caveolin-1 (Cav-1) protein abundance is decreased, and AKT activity is increased in these cells (Hohmann et al., 2019). TGF-β1 mediates the down-regulation of Cav-1 in fibroblasts through the MAPK signaling pathway (Sanders et al., 2015), and Cav-1 loss gives fibroblasts anti-apoptotic properties (Shivshankar et al., 2012). The eukaryotic elongation factor 2 kinase (eEF2K) interacts with the MAPK signaling pathway to activate the apoptosis of lung fibroblasts (Wang Y. et al., 2018). Increased AKT activity also appears in a variety of signaling pathways involved in apoptosis resistance. Activation of the PI3K/AKT/mTOR pathway helps IPF lung fibroblasts resist apoptosis (Romero et al., 2016). The low activity of PTEN leads to the inactivation of the transcription activator FoxO3a through the PTEN/Akt-dependent pathway, down-regulates the expression of Cav-1 and Fas gene, and thus gives IPF fibroblasts an obvious anti-apoptotic phenotype (Nho et al., 2013). In addition, activated AKT results in the enhancing decoy receptor-3 (DcR3) level in IPF fibroblasts. Fas is a death receptor in the tumor necrosis factor receptor superfamily that binds to FasL to induce apoptosis (Cha et al., 2010), and DcR3 is another tumor necrosis factor receptor that competitively binds to FasL to protect IPF fibroblasts from FasL-induced apoptosis (Im et al., 2016). P53 is also involved in regulating apoptosis and thus affect the survival advantage of IPF fibroblasts. The p53 protein level increases in senescent fibroblasts and decreases after apoptosis is induced by an inhibitor of anti-apoptotic proteins (Yosef et al., 2016). P53 is responsible for transcription and activation of apoptosis-related genes, such as PUMA (also known as BCL-2-binding component 3) and Bax (He et al., 2013). Human 8-oxoguanine DNA Glycosylase (hOGG1) protects cells from apoptosis induced by oxidative stress ahead of the p53 dependent pathway (Youn et al., 2007). On the other hand, the protein expression of homeodomain interacting protein kinase 2 (HIPK2) is low in fibroblasts from IPF patients, and its interaction with p53 weakens the cells' ability to make decisions between cell cycle withdrawal and apoptosis. Restoring the HIPK2 protein level in IPF cells reduces the chemoresistance (Ricci et al., 2013b). In senescent fibroblasts, the post-translational modification of p53 makes p53 preferentially occupy the promoters of growth inhibition genes, rather than apoptosis regulators

(Jackson and Pereira-Smith, 2006). Further studies are still needed to elucidate the role of p53 protein in apoptosis resistance of senescent cells.

Autophagy Deficiency

Autophagy is responsible for the retrieval of various components by degrading organelles and macromolecules to provide the raw material for reconstructing cellular structures, carefully maintaining the usual fate of fibroblasts (Sosulski et al., 2015). The cystic autophagosome is formed during the autophagy process. Meanwhile, p62 mediates the binding between the ubiquitinated protein and microtubule-associated protein light chain 3 (LC3), participating in autophagosome formation (Patel et al., 2012). Generally, autophagy is non-selective, but in pathological conditions, autophagy can act as a selective barrier to encapsulate certain intracellular abnormal components for isolation (Sosulski et al., 2015). Mitophagy, as a part of selective autophagy, has been discussed in mitochondrial dysfunction. Autophagy plays an essential regulatory role in cellular senescence, and autophagy deficiency is closely related to IPF fibroblast senescence (Araya et al., 2013). Autophagy is reduced in elderly mice after lung injury, accompanied by corresponding increases in oxidative protein and lipofuscin levels. Lung fibroblasts activated by TGF-β1 are characterized by reduced autophagy flux (Sosulski et al., 2015).

Autophagy-related biomarkers in lung fibroblasts consist of apoptotic effector protein Beclin1, LC3, p62, and the autophagosome number (Patel et al., 2012; Xu et al., 2019). All of them imply decreased autophagy activity in lung tissues of IPF patients (Patel et al., 2012). Beclin1, a key regulator of autophagy in IPF lung fibroblasts, is down-regulated compared with normal lung fibroblasts (Patel et al., 2012; Ricci et al., 2013a). IL-37 can enhance the beclin1-dependent autophagy pathway in IPF fibroblasts (Kim et al., 2019). Cellular senescence is conducive to mediating mTOR-related pathways in lung fibroblasts to reduce autophagy as an adaptive response to stress (Patel et al., 2012; Romero et al., 2016). Downstream molecules of mTORC1 include eEF2K and TFEB (Hait et al., 2006; Wang K. et al., 2018). eEF2K can phosphorylate and inactivate eEF2 and regulated the autophagy activity of lung fibroblasts and IPF development through the MAPK signaling pathway (Wang Y. et al., 2018). Inhibition of mTOR activation can stimulate autophagy, which is characterized by increased beclin1 and LC3 levels as well as autophagosome formation (Chitra et al., 2015).

Autophagy is also involved in the regulation of ECM formation. Studies have shown that increasing the autophagy clearance rate to type 1 collagen by lung fibroblasts could reduce the aggressiveness of IPF fibroblasts (Surolia et al., 2019). In IPF, TGF-β1 can induce the overproduction of ECM components such as collagen and fibronectin in lung fibroblasts. Although TGF-β1 also induces LC3B accumulation in parallel, this autophagy marker's content is significantly decreased in IPF lung fibroblasts (Ghavami et al., 2018). Changes in the PTEN-Akt-mTOR axis make IPF fibroblasts to maintain collagen overproduction's pathological phenotype by inhibiting autophagy. Decreased expression of Akt gene direct target FoxO3a inhibits the production of autophagy marker LC3B on the collagen matrix, thereby inhibiting the autophagy response of IPF fibroblasts to collagen (Nho and Hergert, 2014; Im et al., 2015).

Senescence-Associated Secretory Phenotype

Senescent cells typically secrete a complex set of factors known as senescence-associated secretory phenotype (SASP), a unique cellular senescence feature. Elevated transcription levels of these factors can be synchronously detected in the cells (Schafer et al., 2017; Álvarez et al., 2017). SASP in IPF senescent fibroblasts includes proinflammatory cytokines (such as TNF-α, TGF-β, IL-1β, IL-6, IL-8, IL-10, IL-18), chemokines (such as CXCL1, MCP-1), growth regulators (such as FGF, CTGF, GM-CSF, M-CSF, PDGF), matrix metalloproteinases (such as MMP-2, MMP-3, MMP-9, MMP-10, MMP-12), and leukotrienes (LTs, such as LTA4, LTB4, LTC4, LTD4) (Kortlever et al., 2006; Acosta et al., 2008; Rodier et al., 2009; Kojima et al., 2012; Aoshiba et al., 2013; Demaria et al., 2014; Le et al., 2014; Hayakawa et al., 2015; Jun and Lau, 2017; Schafer et al., 2017; Álvarez et al., 2017; Wiley et al., 2019; Zhang et al., 2019; Blokland et al., 2020). Thus, SASP is essentially a concept that belongs to secretomes, which cannot be fully described by several biomarkers' up-regulation or down-regulation. The composition of SASP changes dynamically over time; for example, Notch1 activity controls the transition of secretory components from TGF-β-dependent to inflammatory factor-dependent (Hoare et al., 2016). SASP turns on the switch of cell senescence, promotes the senescence of cell itself through autocrine, and propagates senescence through paracrine.

The SASP activated in senescent cells is a self-amplified secretory network (Acosta et al., 2008). In IPF senescence fibroblasts, mitochondrial dysfunction decreases the NAD+/NADH ratio, while NAD+ metabolism controls the pro-inflammatory SASP production independently of senescence-related growth stagnation (Correia-Melo et al., 2016; Wiley et al., 2016; Nacarelli et al., 2019). IL-1 and TGF-β can induce Nox4 mRNA expression, suggesting a mechanism between SASP and DNA damage signals (Hubackova et al., 2012). In IPF, increased secretion of SASP usually occurs only after persistent DNA damage signals associated with senescence rather than transient DNA damage response (DDR) (Rodier et al., 2009). DDR signal is necessary but not sufficient for the secretion of SASP. The MAPK pathway, activated by multiple stimuli, induces SASP production by increasing the transcriptional activity of NF-κB, which is independent of DDR (Chien et al., 2011; Freund et al., 2011; Aoshiba et al., 2013; Ferrand et al., 2015). In addition, connective tissue growth factor (CTGF) induces fibroblast senescence by mediating ROS accumulation, leading to the activation of p53 and the induction of p16INK4a (Jun and Lau, 2017). Although the secretion of SASP is regulated by the cell cycle arrest associated with the p53 and Rb pathways, inhibition of either p53 or the Rb pathway is not enough to prevent SASP-induced effects (Freund et al., 2011; Ferrand et al., 2015). Both JAK inhibitors and mTOR inhibitors inhibit SASP in fibroblasts (Herranz et al., 2015; Xu et al., 2015).

Besides, IL-18 promotes lung fibroblast senescence and the role of SASP in IPF by blocking the Klotho pathway (Zhang et al., 2019). IL-6 induces normal fibroblast senescence by establishing a senescence induction circuit involving STAT3 and insulin-like growth factor-binding protein 5 (IGFBP5) (Kojima et al., 2012). Inhibition of IL-6 reduces pulmonary fibrosis in mice (Le et al., 2014).

Senescent fibroblasts also influence the local microenvironment and the senescence of adjacent cells through SASP in a paracrine manner and finally aggravate IPF disease (Acosta et al., 2013; Blokland et al., 2020). When the conditioned medium obtained from senescent fibroblasts is co-cultured with alveolar epithelial cells, a higher percent of alveolar epithelial cells are blocked at the G2/M stage than the control group co-cultured with blank medium, resulting in reduced proliferation but increased migration of alveolar epithelial cells (Blokland et al., 2020). Increased senescence of bone marrow mesenchymal stem cells (B-Mscs) as an extrapulmonary manifestation of IPF patients is also associated with this. And senescent B-Mscs in IPF is capable of inducing the senescence of normal senescent lung fibroblasts through their paracrine effects (Cárdenes et al., 2018).

TARGETING SENESCENT FIBROBLASTS IN IDIOPATHIC PULMONARY FIBROSIS

Fibroblast senescence is an important therapeutic target of IPF (**Figure 3**). In the previous studies, a large part of the treatment strategies toward IPF is proposed on the basis of the premise that specific therapies targeting one selected senescence-related molecule or pathway in senescent fibroblasts can reduce the progression of IPF, and there are some experiments indeed confirm the feasibility of this idea. The selective cGAS inhibitor RU.521 eliminates the senescence of IPF fibroblasts induced by ectopic DNA in the cytoplasm (Schuliga et al., 2020). DNase I, which removes the DNA released into the cytoplasm by abnormal pathways, leads to effects similar to RU.521. 5-lipoxygenase (ALOX5) inhibitors BW-B70C and Zileuton block the synthesis of LTs, which are proved as part of the SASP. Inhibition of ALOX5 activity modulates the proinflammatory and profibrotic effect of LTs on senescent IPF fibroblasts (Wiley et al., 2019). In addition, STA-21, a specific inhibitor of STAT3, reduces the stress-induced senescence of lung fibroblasts (Waters et al., 2019). Furthermore, JSI-124, a dual inhibitor of JAK2/STAT3 more effective than a single inhibitor, inhibits the migration, intracellular autophagy, and senescence of lung fibroblasts (Milara et al., 2018). What is more, mTOR inhibitor Rapamycin, antioxidant N-acetylcysteine (NAC), and mitochondrial-targeted superoxide dismutase mito-TEMPO act on the ROS production and DDR response in mitochondria to suppress IPF fibroblast senescence (Schuliga et al., 2018). The non-selective NOX inhibitor diphenyleneiodonium chloride (DPI) and the specific NOX1/4 inhibitor GKT137831 also attenuate the senescence phenotype of lung fibroblasts (Thannickal and Fanburg, 1995; Hecker et al., 2009, 2014).

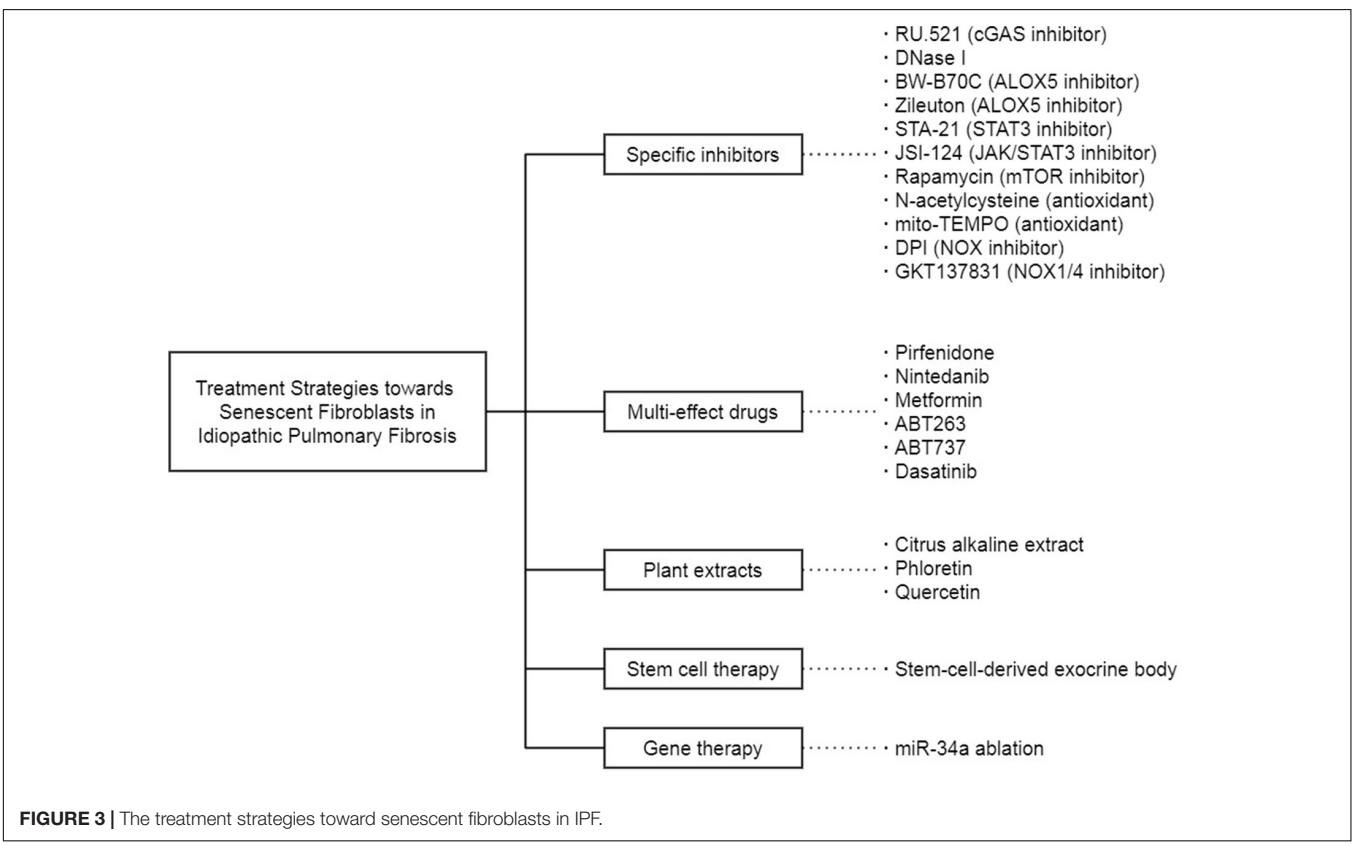

FIGURE 3 | The treatment strategies toward senescent fibroblasts in IPF.

Given that fibroblast senescence in IPF cannot be entirely attributed to a single cause, these targeted drugs are probably incapable of achieving satisfactory results due to their limited effectiveness. Concomitant administration of several drugs is likely to fall into the dilemma between the chaos caused by drug interactions and the inability to cover every discovered signal pathways. Thus, researchers turn to drugs with multiple effects. Pirfenidone is a pleiotropic molecule approved to be one of the antifibrotic drugs to IPF. It inhibits the MUC1 phosphorylation and β-catenin activation induced by TGF-β1 and prevents SMAD binding elements from forming or activating, thereby combating the fibroblast senescence in IPF (Ballester et al., 2020). Nintedanib inhibits TGF-β1 signal transduction intracellularly and induces IPF fibroblasts to autophagy, thereby down-regulating ECM production (Rangarajan et al., 2016). Metformin, a first-line antidiabetic drug, regulated the intracellular metabolic pathways, inhibits the TGF-β1 activated collagen formation, and accelerates the reversal of established fibrosis in an AMPK-dependent manner, thereby acts as an effective antifibrosis agent in the lung (Rangarajan et al., 2018; Kheirollahi et al., 2019). The anti-cancer drugs ABT263 and ABT737 can alleviate the senescence of lung fibroblasts by blocking the up-regulation of Bcl-2 family anti-apoptotic proteins, including Bcl-2, Bcl-XL, and Bcl-W (Chang et al., 2016; Yosef et al., 2016; Zhu et al., 2016). Besides, active substances isolated from edible plants are also research objects. Citrus alkaline extracts (Feng et al., 2019) and phloretin (Cho et al., 2017) affect senescent fibroblasts by activating cyclooxygenase-2 (COX-2) and inhibiting GLUT1, respectively. Quercetin reverses the apoptosis resistance in IPF fibroblasts by promoting the transcription of FasL receptor and Cav-1 gene (Hohmann et al., 2019), while the combination of dasatinib and quercetin (D+Q) selectively kills senescent fibroblasts to ameliorate the progression of pulmonary fibrosis in mice (Schafer et al., 2017). What is even more exciting is that the D+Q performs well in a small-scale, open-labeled pilot clinical trial (Justice et al., 2019). The fourteen stable IPF patients selected for this trial are well tolerated to drugs, and their physical function is significantly and clinically meaningfully improved. Although the pulmonary function is unchanged in this pilot study, the physical elevation achieved by D+Q in IPF patients is still worthwhile in the face of the latest anti-fibrosis drugs, Pirfenidone and Nintedanib.

In addition to these drugs mentioned above, there are also other treatments for senescent fibroblasts in IPF. Stem cell therapy is one of them. The exocrine body derived from human amniotic epithelial cells contains many soluble factors that can regulate inflammation and fibrosis pathways and is considered a potential treatment for IPF. These isolated stem-cell-derived extracellular vesicles reduce lung inflammation and fibrosis in mice by nasal drip (Tan et al., 2018). Gene therapy targeting non-coding RNA is also a new train of thought. A non-coding RNA sequence can link with multiple protein-coding RNA sequences complementarily, thus achieving precise transcriptional regulation as a therapy. For example, miRNA-34a is upregulated in lung fibroblasts from elderly mice (Cui et al., 2017a). Furthermore, in the fibrotic lung of miRNA-34a-deficient mice, the senescence phenotype of primary lung fibroblasts is reduced, and the anti-apoptosis ability is enhanced (Cui et al., 2017b). However, it is not easy to guarantee the safety and convenience of gene therapy targeting non-coding RNA in the human body, and there is still a long way to go from a theoretical proposal to clinical practice.

CONCLUSION AND PERSPECTIVES

Cellular senescence is characterized by cell cycle arrest, macromolecular damage, metabolic disorders, and SASP, which are important aging pathways. IPF is an aging-related interstitial lung disease of unknown etiology. The pathogenesis of IPF itself is difficult to be directly and clearly summarized with several abnormalities. Otherwise, it cannot be called "idiopathic." Although the source of the disease is the key to solve the problem, for IPF patients, intervention in the process of their onset or progression may be a better choice, at least at this stage. We must be clear about the role of cellular senescence in IPF. The related experiments in IPF patients have the problem that the control group is not suitable, while the IPF mice model induced by bleomycin also has the problem of heterogeneity and self-remission. There is no better way to solve the experimental control group's problems, but the improvement in animal model construction can provide more opportunities for senescence-related research in IPF.

Just as cellular senescence is implicitly multifaceted in IPF, how senescent fibroblasts function is also multifaceted. Senescent fibroblasts exhibit abnormal activation, telomere shortening, metabolic reprogramming, mitochondrial dysfunction, apoptosis resistance, autophagy deficiency, and SASP secretion, involving a variety of molecular signaling pathways. However, we have not yet found a sufficiently unique and universal biomarker for cellular senescence. The signaling pathways in senescent fibroblasts are like a dense web, which tightly controls the IPF disease's severity. It may not be enough only to untie a knot on the web.

Under these conditions, the idea of using drugs that precisely clear senescent cells are gaining ground. In the selection of senolytics, compounds from nature undoubtedly perform better for their pleiotropic effects as well as security. When we attempt to discover new senolytics from a vast array of natural and human-made compounds, it is also a beneficial idea in our opinion to find appropriate answers from drugs already on the market or in clinical trials. As the old saying goes, the best way to discover a new drug is to start with an old one. Do drugs that can act on multiple senescence-related pathways at the same time also hide anti-aging properties? In the future, more cell experiments and animal experiments will give us the answer. In addition, just as new pathways are constantly confirmed in IPF, the signaling network in senescent fibroblasts we map will also be enriched. We believe that cellular senescence and senolytics will become new lights in the treatment of IPF.

AUTHOR CONTRIBUTIONS

Both authors read and approved the manuscript for publication.

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REVIEW
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SIRT6 in Senescence and Aging-Related Cardiovascular Diseases

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SIRT6 belongs to the nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases and has established diverse roles in aging, metabolism and disease. Its function is similar to the *Silent Information Regulator 2 (SIR2)*, which prolongs lifespan and regulates genomic stability, telomere integrity, transcription, and DNA repair. It has been demonstrated that increasing the sirtuin level through genetic manipulation extends the lifespan of yeast, nematodes and flies. Deficiency of SIRT6 induces chronic inflammation, autophagy disorder and telomere instability. Also, these cellular processes can lead to the occurrence and progression of cardiovascular diseases (CVDs), such as atherosclerosis, hypertrophic cardiomyopathy and heart failure. Herein, we discuss the implications of SIRT6 regulates multiple cellular processes in cell senescence and aging-related CVDs, and we summarize clinical application of SIRT6 agonists and possible therapeutic interventions in aging-related CVDs.

Keywords: SIRT6, senescence, cardiovascular diseases, autophagy, oxidative stress

INTRODUCTION

Population ageing is a global phenomenon. Virtually every country in the world is experiencing growth in the size and proportion of older persons in their population (United Nations, 2019). With the growing of aged population, the incidence of aging related cardiovascular diseases (CVDs) is increasing. According to a report from the American Heart Association, CVDs (comprising coronary heart disease, heart failure, stroke, and hypertension) currently claims more lives each year than cancer and chronic lung disease combined, and the prevalence of CVD in adults $\geq$ 20 years of age is 48.0% overall and increases with age in both males and females (Virani et al., 2020). Therefore, aging is an independent risk factor associated with the progressive degeneration of the heart, making them more vulnerable to stressors and contributing to increased morbidity and mortality (Chiao and Rabinovitch, 2015).

It has been probably 20 years since the Silent Information Regulator 2 (SIR2) gene was found to extend the lifespan of yeast (Kaeberlein et al., 1999). From that time on, it sparked efforts in many institutions to realize more SIR2-like genes, known as sirtuins, and elucidate their potential to delay the onset of age-related diseases. Sirtuins are a family of histone deacetylases (HDACs) that catalyze deacetylation of both histone and non-histone lysine residues. Their requirement for

nicotinamide adenine dinucleotide distinguishes sirtuins from other HDAC classes and defines them as class III HDACs (Winnik et al., 2015). Mammals contain seven sirtuins (**Figure 1**), SIRT1-7, which are categorized by their different subcellular localization, unique binding substrates and diverse enzymatic activities (Haigis and Sinclair, 2010). These members share a conserved catalytic domain spanning 250 amino acids. The catalytic core comprises an NAD^+-binding domain and four structural zinc-binding domains. Catalysis occurs in a hydrophobic cleft or pocket situated between these two kinds of domains and the hydrophobic cleft or pocket often provides binding sites for modulators. Additionally, sirtuins contain diverse N and C terminal extensions that can direct cellular localization and protein-protein interactions.

The member SIRT6 is widely expressed in all mammalian organs and regulates multiple senescence associated biological processes, including oxidative stress, glucose and fat homeostasis (Mostoslavsky et al., 2006; Zhong et al., 2010; Tao et al., 2013a), inflammatory responses, autophagy, genome integrity, and telomeres homeostasis (Papamichos-Chronakis and Peterson, 2013; Kugel and Mostoslavsky, 2014; Miller and Sadeh, 2014; Lasry and Ben-Neriah, 2015; Tasselli et al., 2017; Abdellatif et al., 2018; De Meyer et al., 2018). Therefore, SIRT6 is involved in many kinds of aging related disease such as neurodegenerative disease, cancer, CVDs. For instance, recent study demonstrated that SIRT6−deficient cynomolgus monkeys exhibit developmental retardation (Zhang W. et al., 2018). However, in Alzheimer's disease (AD) patients, SIRT6 plays AD−protective function via maintaining genomic stability and preventing DNA damage in brain (Jung et al., 2016; Kaluski et al., 2017). It not only reveals a pivotal role in brain development, but also shows a close relationship between the aberration of SIRT6 with human neurodegenerative diseases. In the context of cancer, SIRT6 was considered as a double−edged sword due to its dual role of both tumor suppression and promotion, depending on the type of tumors (Desantis et al., 2017). It protects against tumor growth through the functions of controlling DNA damage repair, genomic stability, cellular metabolic homeostasis, and apoptosis, while it also associated with the poor clinical outcomes by its enzyme activity regulating cancer pathways in cancers such as hepatocellular and colon cancers (Sebastian et al., 2012; Vitiello et al., 2017; Khan R. I. et al., 2018). In the cardiovascular system, SIRT6 plays a protective function by improving vascular endothelial dysfunction to some extent, delaying the formation of atherosclerotic plaques and inhibiting cardiac hypertrophy and heart failure (Sundaresan et al., 2012; Liu et al., 2016). In addition, several studies showed that SIRT6 is a principal regulator of glucose metabolism homeostasis (Zhong et al., 2010; Xiong et al., 2016). Targeting it may be a promising strategy for attenuating diabetic cardiomyopathy (DCM) and reducing myocardial vulnerability to ischemia-reperfusion injury in diabetic patients (Yu et al., 2021).

In this review, we chiefly interrogate the role of SIRT6 in cell senescence, the main CVDs involved in cell senescence induced by SIRT6 dysfunction, and possible

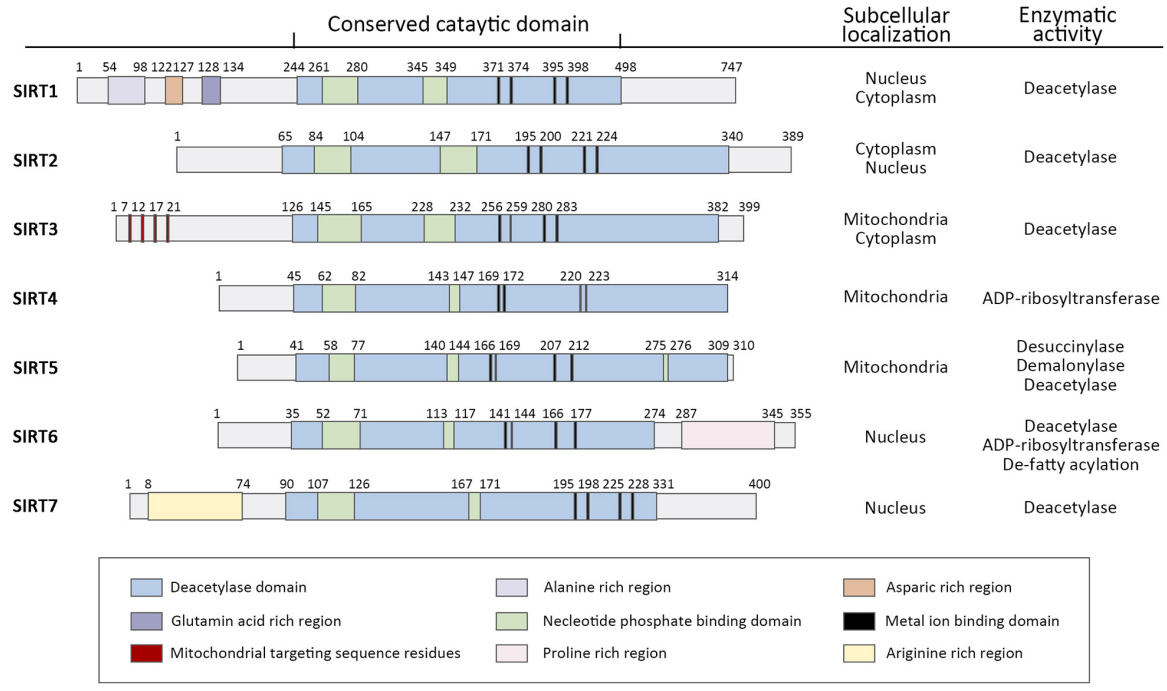

FIGURE 1 | Domain architecture, subcellular localization, and enzymatic activity of human sirtuin family of Class III NAD^+-dependent histone deacetylases. Schematics represent the domain structure of human sirtuins. Amino acid positions are noted above each schematic. The domains are represented in different colors. Adapted from UniProt Universal Protein Resource Database.

clinical application of SIRT6 functional regulatory drugs in CVDs.

SIRT6 AND SENESCENCE

Senescence is a multi-factor process involving the regulation of different age-related molecular and cellular events, including oxidative stress and neurodegeneration, glucose and fat homeostasis (Mostoslavsky et al., 2006; Zhong et al., 2010; Tao et al., 2013a), inflammatory responses, autophagy, genome integrity, and telomeres shorten (Papamichos-Chronakis and Peterson, 2013; Miller and Sadeh, 2014; Lasry and Ben-Neriah, 2015; Abdellatif et al., 2018; De Meyer et al., 2018). The role of sirtuins in senescence was discovered in budding yeast, where overexpression of SIR2 increases replicative lifespan. Subsequently, It was reported that elevated sirtuin levels increase lifespan in the nematode *C. elegans* (Tissenbaum and Guarente, 2001) and the fruitfly Drosophila (Rogina and Helfand, 2004), indicating an evolutionarily ancient role of sirtuins in longevity assurance. However, despite recently there have debates about the direct role of SIR2 in aging and lifespan extension, especially in budding yeast and *C. elegans* (Kaeberlein, 2010; Kenyon, 2010), the overwhelming majority of significant results still support a potential role for SIRT6 in regulating mammalian lifespan (Yuan et al., 2009; Burnett et al., 2011; Kanfi et al., 2012). SIRT6 was shown to extend lifespan in mammals, while deficiency of SIRT6 was associated with progeria, an accelerated aging disorder (Liao and Kennedy, 2012, 2014). Studies have confirmed the important roles for SIRT6 in protecting against aging and disease pathologies: SIRT6-deficient mice are small and have severe metabolic defects, and by 2–3 weeks of age, they develop abnormalities that are usually associated with aging (Mostoslavsky et al., 2006). SIRT6-deficient monkeys die hours after birth and exhibit severe prenatal developmental retardation (Zhang W. et al., 2018). However, SIRT6 overexpression led to an increase in lifespan in male mice (Kanfi et al., 2012). Mechanistically, SIRT6, being a deacetylase at the specific site of histone H3K9 H3K56 H3K18 (Michishita et al., 2008, 2009; Tasselli et al., 2016), inhibits the transcription of transcription factors related to senescence, maintains the structure of telomere chromatin, prevents genomic instability after DNA damage, and protects cells from senescence (Tennen and Chua, 2011; Kugel and Mostoslavsky, 2014). Here, we summarized the function of SIRT6 in age-related cellular events (**Figure 2**).

SIRT6 and Oxidative Stress

Based on the free radical theory, aging is triggered by a long-term cumulative damage of toxic free radicals and reactive oxygen species (ROS) to sensitive targets with biologically significance. Moreover, early studies revealed that the accumulation of ROS is closely related to the poor prognosis of CVDs (Griendling and FitzGerald, 2003). It is known to all that the maintenance of the heart's pumping action requires functional and morphological integrity of mitochondria to ensure an uninterrupted energy supply. Meanwhile, mitochondria, as heart's energy providers, also can generate ROS as a by-product. Recent study indicated that an increase in mitochondrial ROS followed by ultrastructural alterations in the mitochondrial cristae lead to cardiomyocyte damage and, ultimately, cell death (Acin-Perez et al., 2018). Treatment of primary fibroblasts with medium, non-lethal doses of exogenous hydrogen peroxide can activate rapid, senescence-like growth arrest (Chen and Ames, 1994). Analogously, cells grown in the company of high oxygen concentrations exhibit a reduced lifespan and show telomeres shorten (von Zglinicki et al., 1995).

Reports indicate that SIRT6 is highly sensitive to cellular redox state and counteracts the effect of ROS (D'Onofrio et al., 2018). As recently revealed, in response to oxidative stress, SIRT6 is phosphorylated by c-Jun N-terminal kinase (JNK) at residue serine10 and that this modification is necessary for efficient recruitment of poly (ADP-ribose) polymerase 1 (PARP1) to DNA break sites and for efficient repair of DSBs (Van Meter et al., 2016). Moreover, it provides the relationship between oxidative stress and DNA repair that is critical for hormetic response and age-related diseases. Furthermore, SIRT6-deficient human mesenchymal stem cells (hMSCs) exhibited accelerated functional decay, a feature predominately characterized by dysregulated redox metabolism and increased sensitivity to the oxidative stress. In addition, SIRT6 could help assemble nuclear factor erythroid 2-related factor 2 (Nrf2)-RNA polymerase II transcription complex, which was required for the transactivation of Nrf2-regulated antioxidant genes (Pan et al., 2016). SIRT6 has been shown to suppress oxidative stress in the ischemic brain and non-alcoholic fatty liver via regulation of Nrf2 (Ka et al., 2017; Zhang W. et al., 2017).

Nrf2 is an evolutionarily conserved redox-sensitive transcription factor that coordinates antioxidant responses, including enzymes that up-regulate detoxification and repair macromolecular damage induced by ROS (Suh et al., 2004; Ungvari et al., 2011a,b,c). It binds to the antioxidant response elements (AREs) and activates the transcription of many antioxidant genes, including gluthatione S-transferases (GSTs), heme oxygenase 1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), thioredoxin, thioredoxin reductase, as well as proteins involved in scavenging reactive oxygen species (ROS) (Kovac et al., 2015) and glutathione (GSH) biosynthesis and regeneration (Gorrini et al., 2013; Rezazadeh et al., 2019). More importantly, recent advances have identified that the decline in Nrf2-ARE activity is observed in aged cells, which account for that SIRT6 mediated deacetylation of H3K56 is a crucial event safeguarding age-related cells from oxidative stress-associated functional decay (Bailey-Downs et al., 2012; Valcarcel-Ares et al., 2012; Pan et al., 2016). Interestingly, another result indicated that SIRT6 mono-ADP-ribosylation of BAF170, a subunit of BRG/BRM associated factor (BAF) chromatin remodeling complex, is required for activation of a subset Nrf2 responsive genes upon oxidative stress (Rezazadeh et al., 2019). Anyway, these findings showed that SIRT6 serves as an activator of Nrf2-dependent gene transcription. In cardiovascular studies, especially in ischemia/reperfusion injury and vascular endothelial dysfunction, Nrf2 pathway is the major target of SIRT6 to exert antioxidant effects.

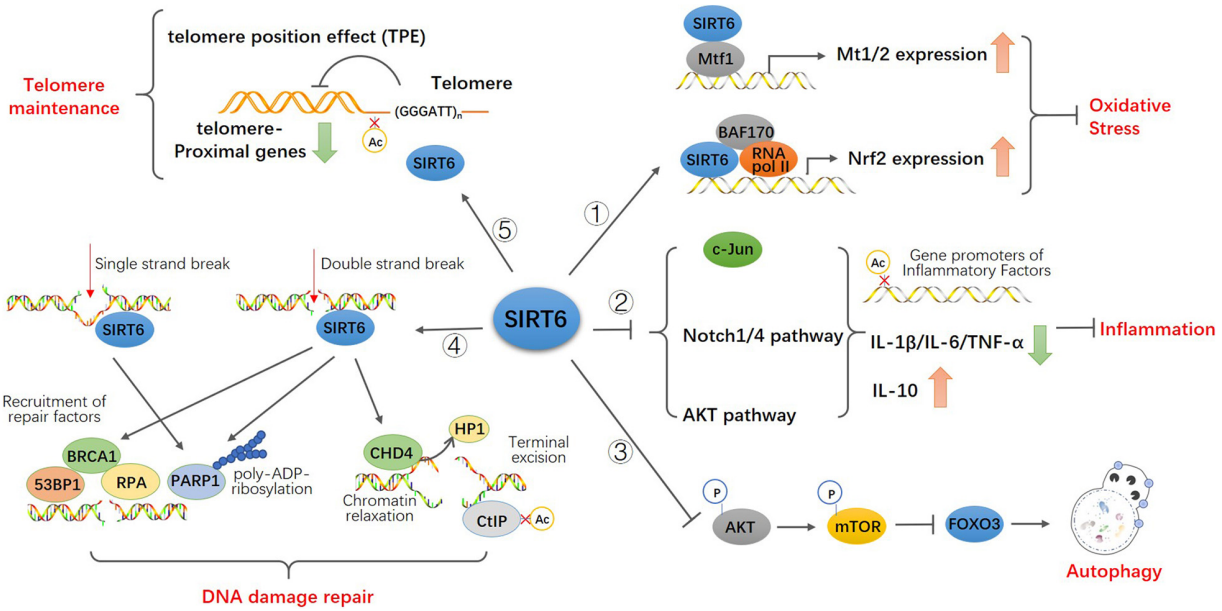

FIGURE 2 | Model for the function of SIRT6 in age-related cellular events. SIRT6 directly binds to Mtf1 to promote the expression of metallothionein Mt1 and Mt2. SIRT6 recruits BAF170 and RNA polymerase II to promote the expression of Nrf2 and downstream genes to participate in antioxidant stress. SIRT6 inhibits the activity of c-JUN, the transcription of Notch1 and Notch4 signals and the phosphorylation of Akt signal via epigenetic regulation. These upregulate the expression of pro-inflammatory cytokines IL-1 β, IL-6, and TNF-α and downregulate anti-inflammatory cytokines IL-10 to attenuates the effect of inflammation. SIRT6 inhibites the activity of Akt-mTOR pathway thus promotes FOXO3-dependent autophagy. Both single and double strand break trigger the recruitment of SIRT6 and activation of PARP1 at the damage sites and promote PARP1 mediated DNA repair. SIRT6 also recruits repair factors 53BP1, BRCA1, and RPA at double-strand breakpoint for damage repair. SIRT6 recruits and interacts with CHD4 to render the relaxation of chromatin required for DNA repair. And CHD4 replaces HP1 in histone H3K9 further promoting homologous recombination. SIRT6 binds to and deacetylate CtIP to promote terminal excision. SIRT6 keeps the low physiological level of H3K9 acetylation and preserves the telomere position effect to maintain normal function of telomere.

In addition, as an adaptive response to the oxidative stress environment, metal transcription factor (MTF) has been previously shown to be the key transcription factor for the induction of metallothionein (Mt) to participate in antioxidant stress (Ghoshal and Jacob, 2001; Laity and Andrews, 2007). The antioxidant stress function of MT in heart prevents cardiomyocytes from diabetic cardiomyopathy and myocardial infarction (Gu et al., 2017; Xue et al., 2019). Recently study revealed that SIRT6 can promote the expression of Mt1 and Mt2. Indeed, both Mt1 and Mt2 promoters were activated by SIRT6. Moreover, SIRT6 can physically interact with MTF1 to have a synergistic effect on those Mt gene promoters (Kim et al., 2019).

Overall, SIRT6 is involved in the regulation of oxidative stress in a variety of tissue cells. And a series of studies have provided compelling evidence demonstrating the pathogenic effect of oxidative stress in CVDs (Zhao et al., 2015; Förstermann et al., 2017; van der Pol et al., 2019). Therefore, targeting SIRT6 to inhibit the generation of ROS and promote the activation of antioxidants represent reasonable therapeutic strategies for CVDs in the future.

SIRT6 and Inflammation

Inflammation is a complex biophysical response of the body to pathogen infection and tissue damage. Although acute inflammation was considered protective, chronic inflammation was linked to numerous diseases (Xiao et al., 2012). For instance, the occurrence of human aging-related diseases is related to chronic low-grade inflammation, which is characterized by increased levels of circulating IL-6 and C-reactive protein (CRP) (Ferrucci et al., 2005; Wikby et al., 2006). A considerable number of elderly people showed the activation of inflammatory bodies and elevated levels of IL-1 β, which are associated with the risk of chronic aging diseases (Franceschi and Campisi, 2014; Furman et al., 2017). This phenomenon has also been confirmed in elderly rodents and primates, where pro-inflammatory changes have occurred in gene expression profiles of vascular endothelial cells and smooth muscle cells, including upregulated expression of inflammatory cytokines [such as IL-6, IL-1 β, tumor necrosis factor-α (TNF-α)], chemokines, adhesion molecules, inducible nitric oxide synthase and other pro-inflammatory mediators. Moreover, it increases the risk of CVDs, including atherosclerotic visceral diseases (Csiszar et al., 2002, 2003, 2004; Ungvari et al., 2007; Song et al., 2012). In addition, according to the data and the hypotheses presented in the study (Ferrucci and Fabbri, 2018), modulating inflammation is a promising approach not only to prevent CVD but also to slow the decline of health that occurs with aging.

Within the past few years, sirtuins have been identified as novel regulators of the immune system (Yang et al., 2007; Csiszar et al., 2008; Yoshizaki et al., 2009), and several studies show that SIRT6 can suppress inflammation in different tissues (Kawahara et al., 2009, 2011; Zhang N. et al., 2016). One of

the master regulators of both adaptive and innate immunity is the NF-κB, which forms complexes with many other proteins, including Rel family members (RelA/p65, c-Rel, and RelB). The NF-κB complexes can translocate from the cytoplasm into the nucleus to trigger expression of target genes that are largely pro-inflammatory. It has been demonstrated that SIRT6, as a potent inhibitor of the NF-κB system, providing a mechanistic link between inflammation and aging (Kawahara et al., 2009; Zhang N. et al., 2016). A study revealed that SIRT6 promoted microRNA-21 expression, this reduced the expression of TGF-β2 and IL-1α and decreased the production of type I collagen and fibroblast proliferation (Fan et al., 2016). Besides, it has been found that SIRT6 restrained TGF-β signaling by deacetylation of H3K9 and H3K56. SIRT6 haploinsufficiency was sufficient for enhancing myofibroblast generation, leading to multiorgan fibrosis and cardiac dysfunction in mice during aging (Maity et al., 2020). Furthermore, recent report unveiled that overexpression of SIRT6 blocked the expression of NF-κB downstream regulators, such as interleukin (IL)-1β, IL-6, and matrix metalloproteinase 9 (MMP-9), all of which promoted fibroblast differentiation in TAC-induced cardiac fibrosis (Zhang et al., 2019).

In mouse liver, SIRT6 deacetylates H3K9 on the promoters of pro-inflammatory gene IL-6 and monocyte chemoattractant protein MCP-1 by inhibiting the transcriptional activity of c-JUN. After SIRT6 gene knockout, the expression of pro-inflammatory cytokines IL-1 β, IL-6, and TNF-α was upregulated significantly, while anti-inflammatory cytokines IL-10 was significantly down-regulated, causing chronic inflammation and fibrosis of the liver (Xiao et al., 2012; Kim et al., 2019). In addition, studies have found that SIRT6 also promotes the production and secretion of inflammatory cytokines (Van Gool et al., 2009; Bauer et al., 2012; Jiang et al., 2013, 2016), leading to chronic inflammation, which is the basis of neuronal death in Parkinson's disease and other neurodegenerative diseases (Nicholatos et al., 2018). Therefore, it is worthy of detailed investigation of the relationship between SIRT6 and other aging-related diseases. In mouse glomerular podocytes, SIRT6 inhibits the transcription of Notch1 and Notch4 signals via epigenetic regulation, lowers the expression of inflammatory cytokines IL-1 β, IL-6, and TNF-α, protects podocytes from inflammatory damage, and effectively reduces the occurrence of chronic proteinuria nephropathy (Liu et al., 2017). In the adventitia inflammation induced by TNF-α, SIRT6 attenuates vascular inflammation by inhibiting the phosphorylation of Akt signal and the expression of monocyte chemoattractant proteins MCP-1 and IL-6 (He Y. et al., 2017).

SIRT6 and Autophagy

Autophagy refers to the cellular process of degradation and recycling of long-lived or damaged organelles and proteins. This includes microautophagy (invagination of lysosomal membrane), molecular chaperone-mediated autophagy (transport of soluble proteins to lysosomes through molecular chaperones and lysosomal membrane receptors) and macroautophagy (where impurities are swallowed by double-membrane autophagosomes before lysosome fusion) (Shirakabe et al., 2016; Delbridge et al., 2017; Nakamura and Yoshimori, 2018; Zhang Y. et al., 2018). Autophagy is initiated by class III phosphatidylinositol-3 kinase (PI-3K) and Beclin-1. Autophagosomes undergo prolongation, microtubule light chain-3 (LC3) recruitment, LC3 proteolysis (lipolysis) to form autophagy lysosomes (fusion of autophagosomes and lysosomes), which regulate cell survival and senescence. Accumulating evidence suggested that autophagy is an essential mechanism for maintenance of tissue homeostasis in the heart during the aging process (Levine and Kroemer, 2008; Rubinsztein et al., 2011; Gatica et al., 2015). Mitophagy is an autophagic response that definitely targets damaged mitochondria. It was critical for the bioenergetics of the cardiovascular system, and mitophagy disorder could develop cardiac dysfunction (Bravo-San Pedro et al., 2017; Nicolas-Avila et al., 2020). Numerous studies have indicated that activate autophagy increase the healthy lifespan of animals, a positive effect that is generally associated with decelerated cardiovascular senescence (Zaglia et al., 2014; Gong et al., 2015; Eisenberg et al., 2016).

The two primary regulatory signal mechanisms implicated in autophagy disorders include inhibition of AMP-dependent protein kinase (AMPK) activation and up-regulation of class I PI-3K/Akt signal, resulting in excessive activation of rapamycin target (mTOR) signal and autophagy disorder (Kennedy and Lamming, 2016). Evidence has emerged that SIRT6 plays critical roles in the process of controlling autophagic degradation (Ng and Tang, 2013). Autophagy could be a harmful process to accelerate aging in some conditions. In a study of human bronchial epithelial cells, it was found that SIRT6 protects human bronchial epithelial cells from senescence by inhibiting insulin-like growth factor signaling-induced autophagy and regulating mTOR signaling (Shao et al., 2016).

However, in cardiovascular cells, autophagy mainly acts as beneficial process to maintain cellular homeostasis and delay aging. In macrophage foam cell from atherosclerosis model, SIRT6 and key autophagy effectors (ATG5, LC3B, and LAMP1) had been observed significantly and the overexpression of SIRT6 markedly reduced foam cell formation by inducing autophagy. The silencing of the key autophagy initiation gene ATG5 reversed the autophagy-promoting effect of SIRT6 with an increase in foam cells, which implied an autophagy-dependent pathway of SIRT6 in protecting against atherosclerosis by reducing foam cell formation (He J. et al., 2017).

Moreover, it has been found that Isoproterenol (ISO)-caused cardiac hypertrophy accompanying with a significant decrease in autophagy activity in primary neonatal rat cardiomyocytes (NRCMs). SIRT6 overexpression enhanced autophagy in NRCMs, whereas knockdown of SIRT6 by RNA interference led to suppression of cardiomyocyte autophagy (Lu et al., 2016). In terms of mechanism, SIRT6 activates FOXO3-dependent autophagy by reducing the level of Akt protein and phosphorylation, thereby enhancing the formation of LC3-II and down-regulating the expression of p62 (Lu et al., 2016). These results are consistent with the previous study that SIRT6 inhibit the transcription of IGF/Akt pathway genes via H3K9 deacetylation, which contributes to suppression of cardiac hypertrophy (Sundaresan et al., 2012).

SIRT6, Genome Stability, and DNA Damage Repair

During the progression of aging, DNA, RNA, and proteins are constantly subjected to chemical alterations that impair their function (Ou and Schumacher, 2018). However, the consequences of DNA damage are much more widespread as DNA contains the information for all RNA and proteins a cell produces. It was considered that thousands of damaging events occur each day in every single one of our cells (De Bont, 2004). Persistent DNA damage can block transcription and replication thus hampering cellular functionality and promoting cellular senescence (Ou and Schumacher, 2018). To counteract the destructive effect of these actions and to maintain genomic integrity, it triggers a DNA damage response (DDR), which ensures efficient repair of all types of damage, including individual DNA base lesions and breaks (Ciccia and Elledge, 2010). The defects in DNA damage response further increases the burden of DNA damage, blocks cell cycle progression and causes the senescence of organs (Niedernhofer et al., 2018). Both endogenous and exogenous factors trigger DNA damage. The endogenous factors are the products of normal cell metabolism, resulting in oxidation, nitrification, and alkylation of DNA (Niedernhofer et al., 2018). On the other hand, exogenous factors, including ionizing radiation, ultraviolet radiation, and alkylating agents trigger DNA single-strand or double-strand breaks, which further causes an increase of inflammatory cytokines and accelerates the aging of the body (Rodier et al., 2009). Many repair mechanisms exist to ensure that nearly all the daily DNA damage is repaired, including base excision repair (BER), nucleotide excision repair (NER), non-homologous end connection (NHEJ), and homologous recombination (HR). Besides, each pathway identifies and repairs specific types of DNA damage to address most DNA damage, but not all DNA damage. It has been discovered that SIRT6 binds closely to chromatin and is an NAD^+-dependent deacetylase of H3K9 and H3K56. Histone deacetylation is related to chromatin conformational closure and decreased chromatin accessibility. Therefore, the discovery of this enzyme activity confirmed the role of SIRT6 in regulating the dynamic binding of DNA damage repair and chromatin and gene expression (Michishita et al., 2008, 2009; Yang et al., 2014).

DNA damage is linked to several human diseases, including cancer, neurodegeneration, aging and CVDs (Madabhushi et al., 2014; Ou and Schumacher, 2018; Nakada et al., 2019; Reisländer et al., 2020). In the early stage of DNA damage, SIRT6 recruited SNF2H (an ATP-dependent chromatin remodeling) to DNA double-stranded breakpoint (DSB), to prevent genomic instability via local deacetylation of H3K56, and effectively recruited repair factors 53BP1, BRCA1, and RPA for damage repair (Toiber et al., 2013). In mammalian cells subjected to oxidative stress, SIRT6 was recruited to the DSB to bind to poly (ADP ribose) polymerase 1 (PARP1) and stimulated its activation by catalyzing the ADP ribose glycosylation of the K521 residue of PARP1, thus promoting the repair of DNA damage by connecting with non-homologous ends and HR (Mao et al., 2011). However, overactivation of PARP depletes the level of NAD content (Bürkle, 2001; Jagtap and Szabo, 2005), which is essential for the activity of sirtuins. It is critical to limit the overactivation of PARP1 in the heart to optimize its cardioprotective effect (Liu et al., 2014).

Further, reports indicate that stress-activated protein kinase JNK phosphorylates SIRT6 on serine 10 to promote DNA double-strand break (DSB) repair in response to oxidative stress. This post-translational modification helps to mobilize SIRT6 to the DNA damage site, effectively recruit PARP1 to the DNA cleavage site and promote repair factors 53BP1 and NBS1 to repair effectively (Van Meter et al., 2016). Chromatin relaxation is a prerequisite for the repair of DNA damage. Recent studies suggest that during DNA damage, SIRT6 rapidly shifts to the site of DNA damage, interacts with chromatin remover CHD4, and recruits CHD4, to promote the repair of DNA damage caused by chromatin relaxation. Once the damage site is reached, CHD4 replaces heterochromatin protein 1 (HP1) in histone H3K9 trimethylation (H3K9me3), whereas CHD4-dependent chromatin relaxation and H3K9me3 competition for the release of HP1, in damaged chromatin, are both necessary for precise HR (Hou et al., 2020). When repairing DNA damage via homologous recombination pathway, SIRT6 binds to DSB excision protein CtIP (carboxyl-terminal binding protein acting protein) and deacetylate CtIP to promote terminal excision (Kaidi et al., 2010). Highly unstable genomes were found in patients with multiple myeloma. A high level of SIRT6 promotes the repair of Chk1 DNA damage by triggering ERK2/p90RSK signal inactivation and offering resistance to DNA damage. The deletion of the SIRT6 gene enhances the sensitivity to DNA damage (Cea et al., 2016). It has been found that SIRT6 will be located at the damage site of single-strand breaks in a PARP1-dependent manner, and downstream repair factors will be recruited to promote base excision and repair of BER. In addition, the efficiency of BER decreased significantly with the increase of age, and overexpression of SIRT6 in senescent cells could significantly improve the efficiency of BER (Xu et al., 2015).

SIRT6 and Telomere Homeostasis

Mammalian telomeres are the terminal structures of chromosomes, which comprise TTAGGG tandem repeats and associated protein complexes (protegerins). This complex protects chromosomes from end-to-end fusion and degradation by forming a special tring-like structure to avoid the ends of chromosomes being identified as double-stranded DNA breaks (Griffith et al., 1999; de Lange, 2005; Palm and de Lange, 2008). With each round of cell division, telomeres become shorter and when the shortened telomeres reach the critical length, it would trigger a sustained DDR and cell senescence (Shay, 2016). To escape senescence, cells might active or up-regulate telomerase, a cellular reverse transcriptase that adds new DNA to telomeres at the end of chromosomes. However, most normal human cells lack the telomerase that maintains telomeres (Baur, 2001). Therefore, telomere length is widely considered as a marker of biological aging, although this parameter does not strictly satisfy the criteria of cell senescence by the American

Federation for Aging Research (Mather et al., 2011; De Meyer et al., 2018). The most factors that can modulate telomere length are also cardiovascular risk factors. In clinical studies, an association between short leukocyte telomere length (LTL) and cardiovascular disorders, including atherosclerosis, myocardial infarction, heart failure, and hypertension, has been repeatedly shown (Nowak et al., 2002; Samani and van der Harst, 2008).

In human fibroblasts with specific knockout of the SIRT6 gene, an apparent end-to-end fusion of chromosomes and premature senescence were observed, which could be effectively reversed by ectopic expression of telomerase, suggesting that SIRT6 is implicated in maintaining telomere stability. The lack of SIRT6 caused the excessive acetylation of H3K9, thus leading to telomere dysfunction (Michishita et al., 2008). Furthermore, the function of SIRT6 in maintaining telomere to protect from senescence is also proved in other tissues including vascular smooth muscle cells (VSMCs) (Cardus et al., 2013; Grootaert et al., 2021).

The deficiency of SIRT6 not only causes telomere damage, but also destroys the closed chromatin environment near the telomere and triggers telomere position effect (TPE) dysfunction (Tennen et al., 2011). TPE refers to the epigenetic silencing of proximal telomere genes (Aparicio et al., 1991; Buck and Shore, 1995; Ng et al., 2002; Altaf et al., 2007) and the intensity of telomere silencing enhances with the increase of telomere length (Kyrion et al., 1993; Buck and Shore, 1995). It has been found that the depletion of SIRT6 in human cells elicits TPE dysfunction, while the restoration of SIRT6 expression is sufficient to reconstruct the silencing of the telomere gene (Tennen et al., 2011). Together, these findings establish new roles for SIRT6 in regulating an aging-associated epigenetic silencing process and provide new mechanistic insights into chromatin silencing at telomeres (Baur, 2001; Tennen et al., 2011; Robin et al., 2014).

In addition, telomere repeat binding factor 2 (TRF2), as a significant regulator of telomere integrity, it exerts telomere protection by blocking ATM signal and non-homologous terminal connection to (NHEJ) and promoting telomere replication (van Steensel et al., 1998; Denchi and de Lange, 2007; Ye et al., 2010). Early research confirmed that plaque VSMCs senescence associated with the loss of TRF2 that plays a critical role in process of atherosclerosis (Wang et al., 2015). Interestingly, recent study reveals a novel molecular mechanism that SIRT6 specifically interacts with TRF2 and promotes TRF2 degradation in response to DNA damage (Rizzo et al., 2017).

Taken together, we reviewed the implication of SIRT6 in maintaining the telomere stability. However, recent studies uncover a telomere-dependent control of sirtuins expression and raise the possibility of a feed forward loop whereby damaged telomeres decrease sirtuins expression, which could further impair telomere integrity, leading to a progressive deterioration (Amano et al., 2019). Telomere dysfunction and sirtuins repression, independently, are highly associated with susceptibility to CVDs, accelerated aging, and lifespan reduction, and these two pathways are closely intertwined and cooperate to drive disease.

SIRT6 AND AGING RELATED CARDIOVASCULAR DISEASES

Mounting evidence indicates that activation of SIRT6 can have beneficial effects in CVDs, including atherosclerosis (Zhang Z. et al., 2016; Wang et al., 2020; Grootaert et al., 2021), cardiac hypertrophy (Sundaresan et al., 2012; Lu et al., 2016; Zhang X. et al., 2016), hypertension (Guo et al., 2019), and heart failure (Li et al., 2017; **Table 1**).

It has been established as a significant factor and regulates essential molecular pathways in multiple pathological conditions. In addition, cardiocytes with SIRT6 specific knockout show accumulation of lactate, indicating compromised mitochondrial oxidation. The mechanism involves the activation of FOXO1-mediated transcription of PDK4 to modulate cardiac glucose metabolism (Khan D. et al., 2018). Furthermore, it is important for pancreatic beta cells to improve insulin secretion through the activation of SIRT6. Therefore, pharmacological activation of SIRT6 may be useful to enhance insulin secretion and it has potential for the development of effective drugs to treat diabetic cardiomyopathy (Xiong et al., 2016). When subjected to prolonged hypoxia, cardiomyocytes from transgenic mice with overexpression of SIRT6 showed the improved survival owing to the block of necrosis/apoptosis pathways (Maksin-Matveev et al., 2015).

SIRT6 and Atherosclerosis

Atherosclerosis is the primary trigger of vascular diseases across the globe with ischemic heart disease being one of its major complications (Herrington et al., 2016). Several studies have shown that endothelial cell dysfunction, abnormal lipid metabolism, and other factors are implicated in the occurrence of atherosclerosis (Gimbrone and García-Cardeña, 2016; Musunuru and Kathiresan, 2016). Vascular smooth muscle cells (VSMCs) comprise a major cellular component of the atherosclerotic plaque. VSMCs in human atherosclerotic plaques are characterized by apoptosis, DNA damage, inflammation and an altered energy metabolism (Grootaert et al., 2018). Furthermore, VSMCs from human atherosclerotic plaques undergo senescence and it promotes atherosclerosis and plaque instability (Wang et al., 2015), while removal of senescent cells can reduce atherosclerosis (Childs et al., 2016). Recent study has demonstrated that SIRT6 protein (but not mRNA) expression is declined in VSMCs in human and mouse atherosclerotic plaques (Grootaert et al., 2021). Besides, VSMC-specific overexpression of SIRT6 restrains atherogenesis and decreases tissue markers of cell senescence and inflammation, dependent upon its deacetylase activity. This indicates that endogenous levels of SIRT6 is a critical regulator of VSMC senescence and reveals a therapeutic potential of SIRT6 in atherosclerosis.

SIRT6 and Endothelial Dysfunction

Early study has revealed that vascular endothelial maintains vascular tension, inhibits atherosclerosis, and forms a barrier to control the migration of various substances between blood vessels and tissues (Galley and Webster, 2004). It is an important locus of critical regulatory nodes to retain the homeostasis of the

TABLE 1 | SIRT6 is directly involved in the regulation of cardiovascular diseases.

SIRT6-linked CVD	Targets		SIRT6-linked cellular function	Species	References
Atherosclerosis	H3K9		Telomere homeostasis	Human/mouse	Grootaert et al., 2021
	H3K9/H3K56	NKG2D ligands			Zhang Z. et al., 2016
			Autophagy	Mouse	Wang et al., 2020
	Msr1			Mouse	Arsiwala et al., 2020
			Inflammation	Diabetic patients	Balestrieri et al., 2015
Cardiac fibrosis	H3K9/H3K56	TGF-β		Mouse	Maity et al., 2020
	AMPK-ACE2			Rats	Zhang Z. Z. et al., 2017
	NF-κB			Rats	Tian et al., 2015
Cardiac hypertrophy	H3K9	c-JUNIGF-AKT		Human/mouse	Sundaresan et al., 2012
			Autophagy	Rats	Lu et al., 2016
	NF-κB	PI3K/Akt		Rats	Shen et al., 2016
	STAT3			Rats	Zhang X. et al., 2016
	NF-κB			Rats	Yu et al., 2013
	NFATc4			Rats	Li et al., 2018
Cardiac glucose metabolism	FOXO1/PDK4			Mouse	Khan D. et al., 2018
Cardioprotection against apoptosis	TIP60-GATA4			Mouse	Peng et al., 2020
Cardioprotection against hypoxia	pAMPKα/NF-κB			Mouse	Maksin-Matveev et al., 2015
Heart failure			Telomere homeostasis	Mouse	Li et al., 2017
Hypertension	H3K9	Nkx3.2-GATA5		Mouse	Guo et al., 2019
Coronary artery disease	Two tagSNPs rs352493 and rs3760908 within SIRT6 Gene			Chinese Han population	Tang et al., 2016
Myocardial infarction	Two tagSNPs rs3760905 and rs4359565 within SIRT6 Gene		–	MI patients	Wang et al., 2016

cardiovascular system. Endothelial cell dysfunction encompasses a constellation of various non-adaptive alterations in functional phenotype, which have important implications for the regulation of hemostasis and thrombosis, local vascular tone and redox balance, and the orchestration of acute and chronic inflammatory reactions within the arterial wall (Gimbrone and García-Cardeña, 2016). Therefore, it is significant to point out that endothelial cell dysfunction is involved in many disease processes, including atherosclerosis, pulmonary arterial hypertension and sepsis (Gimbrone and García-Cardeña, 2016; Joffre et al., 2020; Evans et al., 2021). Here we focus on the involvement of SIRT6 in atherosclerosis and endothelial cell dysfunction.

In the process of atherosclerosis, there exist several factors of endothelial cell dysfunction, including endothelial vasodilation damage, endothelial cell injury and repair disorder, abnormal expression of endothelial adhesion molecules as well as cytokines. Endothelial cell dysfunction manifested in lesion-prone areas of the arterial vasculature results in the earliest detectable changes in the life history of an atherosclerotic lesion (Stary, 2000; Virmani et al., 2000).

SIRT6 is expressed in endothelial-rich tissues including the aorta, lung, and brain. In SIRT6 gene knockout mice and endothelium-specific knockout mice, endothelium-dependent vasodilation of aorta to acetylcholine (Ach) was significantly impaired (Xu et al., 2017). To prevent atherosclerosis, maintain the health of endothelial cells, and slow down the aging process of endothelial cells, it is significantly vital to repair damaged endothelial cells (Lappas, 2012). SIRT6 protects endothelial cells from telomere and DNA damage, prevents premature senility, and maintains the ability of cell replication and angiogenesis *in vitro*, all of which are known to inhibit the development of endothelial dysfunction (Cardus et al., 2013). Endothelial cell adhesion molecules, including vascular cell adhesion molecule (VCAM-1), play an important role in atherosclerosis by promoting the adhesion of monocytes to inflammatory endothelium (Libby et al., 2009, 2011). The role of SIRT6 in monocyte adhesion to endothelial cells was evaluated by transfecting SIRT6 into human umbilical vein endothelial cells or interfering with its expression. It has been shown that SIRT6 inhibited monocyte adhesion by lowering the expression of VCAM-1 in endothelial cells induced by TNF-α (Xu et al., 2017).

Also, the abnormal expression of endothelial inflammatory factors regulated by SIRT6 is implicated in the formation of atherosclerosis. Damaged vascular cells (endothelium and smooth muscle) are active in secreting cytokines including IL-1, monocyte chemoattractant protein-1 (MCP-1) and granulocyte-monocyte stimulating factor (GM-CSF). These cytokines produce local intercellular autocrine and paracrine signal rings in the vascular wall to promote the progression of atherosclerosis (Pober and Sessa, 2007; Gimbrone and García-Cardeña, 2016).

In the progression of atherogenesis, NF-κB signal tends to play a central role in the pro-inflammatory activation of endothelial cells by regulating the expression of many downstream molecules such as VCAM-1 and MCP-1 (Collins and Cybulsky, 2001). Noteworthy, SIRT6 interacts with NF-κB RELA subunits and deacetylates H3K9 on the promoter of NF-κB target gene to attenuate NF-κB signal (Kawahara et al., 2009). Furthermore, recent studies found that the activation of NF-κB might trigger fundamental changes in the chromatin structure of endothelial cells via the formation of super-enhancer complexes, hence, regulating the epigenetic level of the phenotype of pro-inflammatory endothelial cells in the process of atherosclerosis (Brown et al., 2014).

Recent reports argued that the epigenetic regulation of NKG2D ligands is also involved in atherosclerosis of SIRT6 heterozygous mice. The down-regulation of SIRT6 up-regulates the expression of NKG2D ligand and causes an increased expression of inflammatory cytokines, which could be nearly completely blocked by NKG2D ligand inhibition (Zhang Z. et al., 2016). Notably, Tumor necrosis factor superfamily member 4 (TNFSF4) is a gene affecting atherosclerosis susceptibility and encodes OX40 ligand. SIRT6 inhibits atherosclerosis by deacetylating H3K9 on the promoter of the TNFSF4 gene (Wang et al., 2005). Studies have found trace cholesterol crystals (CCs) in atherosclerotic plaques, which represent one of the mechanisms causing endothelial dysfunction. In HUVECs, SIRT6 significantly promotes eNOS activity and down-regulates the expression of intercellular adhesion molecules (ICAM-1) and VCAM1 by activating Nrf2, thereby alleviating endothelial dysfunction induced by CCs (Jin et al., 2020). In SIRT6 knockout mice, atherosclerotic plaque was enlarged, plaque vulnerability was enhanced, and the expression of ICAM-1 in aortic endothelial cells was significantly up-regulated, implying that SIRT6 is the primary negative regulator of endothelial dysfunction and atherosclerotic development (Liu et al., 2016).

SIRT6 and Lipid Metabolism

Sources of evidence from clinical studies suggest that low-density lipoprotein-cholesterol causes atherosclerosis-related CVDs (Ference et al., 2017). As such, regulating homeostasis of LDL- cholesterol is significantly critical to body health. Further, additional evidence asserts that proprotein converting enzyme subtilin/Kexin 9 (PCSK9) binds to liver low-density lipoprotein receptor (LDLR) and promotes its degradation in lysosomes, causing a decrease in LDL uptake and an increase in LDL cholesterol concentration (Bergeron et al., 2015). Besides, overexpression of the SIRT6 gene could reduce the level of LDL- cholesterol in hepatocytes of mice fed with a high-fat diet. It has been confirmed that SIRT6 could be recruited by FOXO3 to the promoter region of the PCSK9 gene and inhibit its expression through deacetylation of H3K9 and H3K56, thereby reducing the level of LDL-cholesterol. SIRT6 deficiency can lead to an upregulated expression of the PCSK9 gene and an increase of LDL-cholesterol (Tao et al., 2013a). Also, sterol regulatory element-binding protein (SREBP)-2, which controls the expression of cholesterol biosynthesis rate-limiting enzyme HMG-CoA, is a vital regulator of cholesterol biosynthesis. At the mechanism level, SIRT6 was also recruited to the (SREBP)-2 gene promoter by FOXO3 to inhibit its expression and reduce cholesterol biosynthesis via deacetylation of H3K9 and H3K56 (Tao et al., 2013b).

Additionally, the formation of macrophage foam cells is a typical pathological change of early atherosclerotic (AS), which is primarily due to the imbalance between cholesterol inflow and efflux in mononuclear macrophages and the accumulation of cholesterol ester (CE) in cytoplasmic lipid droplets (LDs) (Moore and Tabas, 2011). Moreover, oxidized low-density lipoprotein cholesterol (ox-LDL), which binds to scavenger receptor (Sr) and accumulates in the cytoplasm has been reported to play a pathogenic role in the occurrence and development of AS (Mitra et al., 2011). Under the condition of ox-LDL, SIRT6 inhibits the expression of miR-33 (an mRNA that negatively regulates ABCA1 and ABCG1), promotes autophagy and cholesterol efflux, and reduces the formation of macrophage foam cells, thereby delaying the progress of AS. SIRT6 gene knockout promotes the formation of macrophage foam cells, hence promoting the formation of atherosclerosis (He J. et al., 2017). Generally, these findings suggest that SIRT6 plays a vital role in low-density lipoprotein cholesterol metabolism, potentially counteracting the formation of atherosclerosis.

SIRT6, Myocardial Hypertrophy, and Heart Failure

After birth, cardiomyocytes withdraw from the cell cycle and become terminally differentiated cells. In adult hearts, compensatory cardiac hypertrophy develops into cardiac hypertrophy by increasing the size of individual cardiomyocytes rather than the number of cardiomyocytes to cope with increased workload. This compensatory mechanism is accompanied by an increase in the size of cardiomyocytes, and the imbalance of fetal genetic programming as well as an increase of protein synthesis (Rohini et al., 2010). Hypertrophy is initially an adaptive response to physiological and pathological stimuli, however, pathological hypertrophy usually progresses to heart failure under the regulation of different cellular signaling pathways (Nakamura and Sadoshima, 2018). The incidence of cardiac hypertrophy sharply increases with age, implying that aging-related mechanisms might play a key role in the molecular regulation of myocardial hypertrophy (Lakatta and Levy, 2003). Reports have confirmed that SIRT6 plays a negative regulatory role in cardiac hypertrophy. SIRT6 knockout mice have cardiac hypertrophy and heart failure, while SIRT6 transgenic mice are not influenced by hypertrophy (Sundaresan et al., 2012). In addition to myocardial hypertrophy, studies have shown that the progression of heart failure is also related to extensive fibrosis, abnormal activation of insulin-like growth factor (IGF)-Akt signal, cardiac hyperstress mediated by β-adrenoceptor, and damage of autophagy (Tian et al., 2015; Lu et al., 2016; Zhang W. et al., 2016).

In the model of hypertrophic cardiomyocytes induced by angiotensin II and coarctation of the abdominal aorta, SIRT6 inhibits the transcriptional activity of NF-κB by deacetylating

H3K9, thereby inhibiting cardiac hypertrophy (Yu et al., 2013). In addition, in cardiac fibroblasts stimulated by angiotensin II and rat myocardium treated with coarctation of the abdominal aorta, it was further confirmed that SIRT6 inhibited the transcriptional activity of NF-κB via deacetylation of H3K9, and inhibited cardiac fibroblasts differentiation into myofibroblasts, thus inhibiting cardiac fibrosis. In SIRT6 knockout cardiac fibroblasts, extracellular matrix deposition and α-SMA increase promote the transformation into myofibroblasts and trigger extensive cardiac fibrosis (Tian et al., 2015).

Additionally, the level of intracellular NAD plays a crucial role in cardiomyocyte hypertrophy. The expression of Nmnat2 (central enzyme of NAD biosynthesis) is down-regulated in hypertrophic cardiomyocytes induced by angiotensin II and constriction of abdominal aorta. Overexpression of Nmnat2 promotes the activation of SIRT6 and blocks angiotensin II-induced cardiac hypertrophy (Cai et al., 2012). Increasing evidence reveals that overactivation of PARP-1 plays a key role in the pathogenesis of cardiac hypertrophy and heart failure. Nonetheless, excessive activation of PARP-1 depletes its substrate NAD and causes cell death. Being a new PARP-1 inhibitor, AG-690/11026014 protects cardiomyocytes from angiotensin II-induced hypertrophy by restoring the NAD level and SIRT6 activity (Liu et al., 2014). The abnormal activation of insulin-like growth factor (IGF)-Akt signal is closely linked to the occurrence and development of numerous diseases including heart failure. Studies on the hearts of mice confirmed that SIRT6 inhibits the activation of the IGF-Akt signal by inhibiting c-Jun transcriptional activity and deacetylation in H3K9, thereby blocking cardiac hypertrophy. Nonetheless, SIRT6 knocked-out mice promotes the over-activation of multiple IGF signal-related genes, leading to cardiac hypertrophy and heart failure (Sundaresan et al., 2012).

Elsewhere, studies found that the activation of signal transducer and activator of transcription 3 (STAT3) is critical in β-adrenergic receptor-mediated pathological remodeling and heart failure. In phenylephrine (PE)-induced hypertrophic cardiomyocyte model and isoproterenol (ISO) induced hypertrophic rat model, the mRNA and protein expression of STAT3 and phosphorylation level (P-STAT3) was significantly upregulated, while the hypertrophic biomarkers including ANF and BNP increased. In contrast, the deacetylase activity of SIRT6 decreased, while the effect of PE-induced hypertrophy could be eliminated by overexpression of the SIRT6 gene. Similarly, the up-regulation of ANP and BNP caused by SIRT6 gene knockout can be reversed by silencing of STAT3. Besides, SIRT6 has been suggested to protect cardiomyocytes from hypertrophy by preventing PE-induced STAT3 activation (Zhang X. et al., 2016).

In the heart, autophagy promotes survival primarily by clearing misfolded protein aggregates and damaged organelles accumulated in cardiomyocytes during cellular stress and nutritional deprivation. While long-term up-regulation of autophagy triggers self-destruction and leads to heart failure (De Meyer and Martinet, 2009). After treatment of primary neonatal rat cardiomyocytes with ISO, apparent hypertrophy and autophagy damage were observed. Also, it was confirmed that SIRT6 could protect cardiomyocytes from hypertrophy by inhibiting the Akt signal, thus, promoting the activation of FOXO3 transcription factor, and enhancing autophagy (Lu et al., 2016).

In addition, compensatory hypertrophy of cardiomyocytes is related to the increase of protein synthesis. One of the master regulators of protein synthesis inside the cell is the nutrient and energy sensor kinase mechanistic target of rapamycin (mTOR) (Laplante and Sabatini, 2009; Saxton and Sabatini, 2017). It has been found that SIRT6 acts as a key regulator of cellular protein synthesis by transcriptionally regulating the mTOR signaling in partnership with the transcription factor Sp1, and the whole process independent of its deacetylase activity (Ravi et al., 2019). Besides, in the hypertrophic heart induced by ISO, the expression of SIRT6 was down-regulated, while the inhibition of mTOR restored cardiac function in muscle-specific SIRT6 knockout mice, which spontaneously developed into cardiac hypertrophy (Saxton and Sabatini, 2017; Ravi et al., 2019). Taken together these data establish a critical connection between SIRT6, mTOR signaling, protein synthesis and cardiac hypertrophy. It will contribute toward understanding and treating diverse pathologies associated with aging.

CLINICAL APPLICATION PROSPECT IN AGING AND CVDs

Given the advantaged effects of SIRT6 in regulating cell senescence and CVDs, targeted activation of SIRT6 and its downstream mechanism signals will be a potential way of delaying aging and treating CVDs. Here, we mainly discuss the activators of SIRT6 in the existing or potential clinical application in aging and CVDs.

Caloric restriction (CR), the significant decrease in calorie intake, is a strategy for improving health and increasing lifespan (Madeo et al., 2019). It has been shown to improve heart function, suppress markers of inflammation and reduce the risk of CVDs and diabetes in humans (Caristia et al., 2020; Kirkham et al., 2020). The beneficial effects of CR occur through an extreme wide range of molecular mechanisms, largely overlapping with epigenetic factors like sirtuins (Gensous et al., 2019) and the promotion of autophagy process (Abdellatif et al., 2018). However, CR has been shown to increase risk of diminishing muscle strength, aerobic capacity, and bone mineral density (Mattison et al., 2012). Therefore, proper exercise in addition to a CR diet is crucial. Resent study found that calorie restriction and physical exercise effectively regulate the activity of sirtuins. For instance, exercise training can effectively regulate the activity of SIRT6 in the skeletal muscle of aged rats and delay the aging process (Koltai et al., 2010). Moreover, caloric restriction significantly improved the renal insufficiency of aged rats, enhanced the expression of SIRT6 and inhibited the transduction of NF-κB signal (Koltai et al., 2010; Zhang N. et al., 2016). These findings suggested that CR was a beneficial life habit that can delay the aging process by regulating SIRT6, which is worthy of the attention of patients with aging-related diseases.

So far, the compounds that can specifically regulate the activity of SIRT6 in CVDs are still limited. The Chinese herbal

medicine, icariin, widely used in eastern countries to treat specific age-related diseases, including CVDs and the improvement of neurological function, has been proved to be an activator of SIRT6. In an *in vitro* cell model, it was discovered that 10^{-16}–10^{-8}mol/L icariin could effectively activate the expression of SIRT6 protein and delay cell senescence by inhibiting NF-κB signal transduction (inhibiting the expression of TNF-α, ICAM-1, IL-2, and IL-6). In the future, we need to supplement the clinical research of icariin in the treatment of CVDs (Chen et al., 2015).

As a water-soluble natural amino acid, ergothioneine (Egt) exists widely in animals and plants. It accumulates a high concentration in some tissues via food chain intake (Halliwell et al., 2018). Several lines of evidence show that it has the effect of anti-oxidation and anti-cell aging, including the protective effect on CVDs and chronic inflammatory injury (D'Onofrio et al., 2016; Servillo et al., 2017). Moreover, it has been found that Egt inhibits the aging process by activating the expression of SIRT1 and SIRT6 protein in endothelial cells, thus reducing the production of ROS and suppressing the downstream NF-κB pathway (Tang et al., 2015). Nonetheless, so far, while acknowledging the absence of toxicity in the range of millimoles of intracellular concentration, the number of clinical studies to evaluate the efficacy and safety of dietary supplementation of Egt is still limited. As such, enriching additional studies on the treatment of CVDs with large samples of Egt is critical.

Other activators of SIRT6 have the biological function of anti-cancer, while the potential effect of these activators in CVDs needs further studies. UBCS039 directly binds to SIRT6 at the hydrophobic pocket and induces H3K9 and H3K56 deacetylation in breast cancer and colorectal cancer cells (Iachettini et al., 2018). Quinoline-4-carboxamides is an excellent selective SIRT6 activator with the function of antiviability and antiproliferation activities in pancreatic ductal adenocarcinoma (PDAC) cells through decreased acetylation leved of H3K9, H3K18, and H3K56 (Chen et al., 2020). Moreover, recent studies have identified allosteric SIRT6 activators, MDL-800, MDL-801, and MDL-811, which bound to the surface allosteric site of SIRT6 and activate SIRT6 deacetylation by promoting the binding affinity of acetylated substrates to cofactor. They also exert a tumor suppressor effect by reducing the acetylation level of H3K9 and H3K56, thus leading to cell cycle arrest in hepatocellular carcinoma, colorectal cancer and non-small cell lung cancer (Huang et al., 2018). We predict that MDL-800 could reduce the Ischemia reperfusion injury in cardiomyocytes, direct evidence of the function of MDL-800 in heart has yet to be reported. Given the importance of deacetylation of histones in CVDs more in-depth studies on these SIRT6 activators in CVDs are essential in the future.

In conclusion, existing studies have shown that SIRT6 is an endogenous regulatory molecule for the inhibition of cell senescence and the prevention and treatment of CVDs. Specifically, SIRT6 performs its different cellular functions via acetyl and long-chain fatty acyl deacetylation as well as ADP-ribosylation, maintains genomic stability by regulating DNA repair and telomere homeostasis. Moreover, it inhibits cell aging by regulating oxidative stress and inflammatory autophagy, plays a profound role in CVDs by regulating triglyceride synthesis and (LDL) cholesterol homeostasis. Therefore, the regulation of SIRT6 activity might influence various human diseases and prolong life. Nonetheless, the molecular mechanism of regulating the activity and function of SIRT6 in the process of anti-aging as well as prevention and treatment of CVDs warrants deeper understanding. With a better understanding of biology, novel clinical treatments can also be designed to activate SIRT6. Additional biological targets are likely to be discovered in the future, laying a basis for understanding the importance of SIRT6 in human aging and CVDs.

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All authors conceptualized and wrote the manuscript.

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P16^{INK4a} Deletion Ameliorates Damage of Intestinal Epithelial Barrier and Microbial Dysbiosis in a Stress-Induced Premature Senescence Model of *Bmi-1* Deficiency

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This study aimed to determine whether *Bmi-1* deficiency leads to intestinal epithelial barrier destruction and microbiota dysfunction, which members of the microbial community alter barrier function with age, and whether *p16^{INK4a}* deletion could reverse the damage of intestinal epithelial barrier and microbial dysbiosis. Intestines from *Bmi-1*–deficient (*Bmi-1$^{-/-}$*), *Bmi-1* and *p16^{INK4a}* double-knockout (*Bmi-1$^{-/-}$p16$^{INK4a-/-}$*), and wild-type mice were observed for aging and inflammation. Duolink Proximity Ligation Assay, immunoprecipitation, and construction of *p16^{INK4a}* overexpressed adenovirus and the overexpressed plasmids of full-length, mutant, or truncated fragments for occludin were used for analyzing the interaction between p16^{INK4a} and occludin. High-throughput sequencing of V4 region amplicon of 16S ribosomal RNA was conducted using intestinal microbiota. We found *Bmi-1* deficiency destructed barrier structure, barrier function, and tight junction (TJ) in intestinal epithelium; decreased the TJ proteins; increased tumor necrosis factor α (TNF-α)–dependent barrier permeability; and up-regulated proinflammatory level of macrophages induced by intestinal microbial dysbiosis. The transplantation of fecal microbiota from wild-type mice ameliorated TJ in intestinal epithelium of *Bmi-1$^{-/-}$* and *Bmi-1$^{-/-}$p16$^{INK4a-/-}$* mice. Harmful bacteria including *Desulfovibrio*, *Helicobacter*, and *Oscillibacter* were at a higher level in *Bmi-1$^{-/-}$* mice. More harmful bacteria *Desulfovibrio* entered the epithelium and promoted macrophages-secreted TNF-α and caused TNF-α–dependent barrier permeability and aging. Accumulated p16^{INK4a} combined with occludin at the 1st–160th residue in cytoplasm of intestinal epithelium cells from *Bmi-1$^{-/-}$* mice, which blocked formation of TJ and the repair of intestinal epithelium barrier. *P16^{INK4a}* deletion could maintain barrier

function and microbiota balance in *Bmi-1*$^{-/-}$ mice through strengthening formation of TJ and decreasing macrophages-secreted TNF-α induced by *Desulfovibrio* entering the intestinal epithelium. Thus, Bmi-1 maintained intestinal TJ, epithelial barrier function, and microbiota balance through preventing senescence characterized by p16^{INK4a} accumulation. The clearance of p16^{INK4a}-positive cells in aging intestinal epithelium would be a new method for maintaining barrier function and microbiota balance. The residues 1–160 of occludin could be a novel therapeutic target for identifying small molecular antagonistic peptides to prevent the combination of p16^{INK4a} with occludin for protecting TJ.

Keywords: inflammaging, TNF-α, *Desulfovibrio*, occludin, p16^{INK4a}, Bmi-1

INTRODUCTION

Mammalian intestine is the critical site of digestion, absorption and assimilation, and a highly immune-active ecosystem that harbors and preserves a large abundance of commensal microorganisms. Intestinal epithelium is the first defensive barrier against environmental and microbial attacks by maintaining a tight physical barrier and executing several critical innate immune functions (Patankar and Becker, 2020).

Aging is an intrinsic physiological process characterized by a gradual function decline in the organs, including intestine and its microbiota (Lin et al., 2019). With aging-dependent decrease in the intestinal barrier function, microbial productions including proinflammatory factors enter the bloodstream, triggering systemic inflammation (Thevaranjan et al., 2017). Besides, aging-related microbial imbalance increases intestinal permeability. Age-related microbial imbalance and intestinal barrier dysfunction have been associated with various senescence-associated intestinal and systemic diseases including inflammatory bowel diseases, celiac disease, type 1 diabetes, obesity, and Alzheimer disease (Lee, 2015). Previous study shows that tumor necrosis factor α (TNF-α) is the main proinflammatory cytokine that causes the destruction of epithelial tight junction (TJ), increases intestinal epithelial permeability, and aggravates senescence-associated systemic inflammation; however, which members of the microbial community alter barrier function with age have not been yet identified (Thevaranjan et al., 2017). The tumor suppressor protein p16^{INK4a} (hereafter referred to as p16), which is encoded by *INK4a* locus, is often transcriptionally activated in senescent cells and seen as a classical aging marker. P16 is up-regulated in multiple tissues during aging and contributes to senescence-associated decline in tissue function and regenerative capacity (Helman et al., 2016). However, whether accumulated p16 plays a critical role in damaging intestinal epithelial barrier and microbial homeostasis in inflammaging process is unclear.

B-cell–specific Moloney murine leukemia virus insertion region 1 (Bmi-1) is associated with senescence and cell cycle regulation. It regulates cell cycle and delays cell aging by inhibiting *INK4a/ARF* gene locus (Chen et al., 2020). Previous study on pig suggests that Bmi-1 is one of the markers of fast-cycling and quiescent intestinal stem cells (ISCs) that drives self-renewal of intestinal epithelial cells, increasing intestinal epithelial cell proliferation by stimulating WNT/β-catenin signaling (Li et al., 2018). Bmi-1–positive ISCs are critical for intestinal epithelium to maintain its function as a primary barrier (Smith et al., 2018). Loss of *Bmi-1* in mice reduces proliferation in the ISC compartment accompanied by p16 accumulation (Lopez-Arribillaga et al., 2015). Several lines of evidence demonstrate that *Bmi-1*–deficient mouse is a stress-induced premature senescence (SIPS) model that appears frail with malnutrition and shortened life span (Liu et al., 2009; Zhang et al., 2010; Jin et al., 2014, 2017; Xie et al., 2015; Chen et al., 2020). However, whether *Bmi-1* deficiency could cause damage of intestinal epithelial barrier and microbial dysbiosis is unclear. It is also unknown if *p16* deletion could ameliorate the damage of intestinal epithelial barrier and microbial dysbiosis in *Bmi-1*$^{-/-}$ mice.

Herein we report that Bmi-1 maintained intestinal TJ, epithelial barrier function, and microbiota balance through preventing senescence characterized by p16 accumulation. Accumulated p16 combined with occludin at the 1st to 160th residue in the cytoplasm of aging intestinal epithelium cells, which blocked the repair of intestinal epithelium barrier. *P16* deletion could maintain barrier function and microbiota balance in *Bmi-1*$^{-/-}$ mice through strengthening formation of TJ and decreasing macrophages-secreted TNF-α induced by *Desulfovibrio* entering the intestinal epithelium.

RESULTS

Damage of Barrier Structure and Dysfunction in Intestinal Epithelium Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

Bmi-1 and p16 were widely expressed in epithelial cells of jejunum, ileum, and colon (**Supplementary Figures 1**, **2**). To examine if *p16* deletion ameliorated the damage of barrier structure and dysfunction in intestinal epithelium in *Bmi-1*$^{-/-}$ mice, body size and weight, and intestinal length, histological structure and secretory function were observed in 7-week-old

Abbreviations: *Bmi-1*$^{-/-}$, *Bmi-1*–deficient; *Bmi-1*$^{-/-}$*p16*$^{INK4a-/-}$, *Bmi-1* and *p16*^{INK4a} double-knockout; TJ, tight junction; SIPS, stress-induced premature senescence: WT-FB, fecal mixed bacteria from WT mice; Des-B, *Desulfovibrio* bacteria; ISCs, intestinal stem cells.

Bmi-1$^{-/-}$ mice, *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice, and wild-type (WT) mice. Results showed that besides the smaller body size and weight, *Bmi-1*$^{-/-}$ mice also showed shorter intestinal length (especially the ileum) in comparison with WT mice. Deletion of *p16* significantly rescued the body size, weight, and intestinal length caused by *Bmi-1* deficiency (**Figures 1A–D**). Significant decreases were observed in the villus length, the ratio of villus length to crypt, the number of Paneth cells, and the number of acid mucin and glycoprotein in *Bmi-1*$^{-/-}$ mice compared with WT mice. *P16* deletion significantly rescued the abnormalities in the villus length, the ratio of villus length to crypt, the number of Paneth cells, and the number of acid mucin and glycoprotein observed in *Bmi-1*$^{-/-}$ mice (**Figures 1E–K**). Ki67-positive cells significantly decreased after *Bmi-1* deletion, which was rescued by *p16* knockout (**Supplementary Figure 3**). P53 significantly increased in jejunum, ileum, and colon of *Bmi-1*$^{-/-}$ mice and then down-regulated by *p16* deletion (**Figures 1L–N**). These results demonstrated that *p16* deletion ameliorated the damage of barrier structure and dysfunction in intestinal epithelium caused by *Bmi-1* deficiency.

Destruction of Tight Junction and Increase in Barrier Permeability in Intestinal Epithelium Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

To investigate if *p16* deletion ameliorated the destruction of TJ and the increase of barrier permeability in *Bmi-1*$^{-/-}$ mice, the key TJ protein ZO-1, tight junctional structure, and several primary TJ proteins including claudin-1, occludin, and claudin-2 in the epithelia of jejunum, ileum, and colon were detected and analyzed. Moreover, peripheral blood was collected and detected fluorescein isothiocyanate (FITC) fluorescence for evaluating intestinal permeability at the fourth hour after oral gavage of FITC–dextran. In *Bmi-1*$^{-/-}$ mice compared with WT mice, significant decreases were observed in ZO-1– and occludin-positive areas (**Figures 2A–C** and **Supplementary Figures 4A,B**) and protein levels of claudin-1, occludin, and claudin-2 in epithelia (**Figures 2F,G**); meanwhile, a significant increase was observed in FITC fluorescence for evaluating intestinal permeability (**Figure 2D**). It was also observed in electron micrographs that the bottom of the epithelial TJ was cracked in epithelia of jejunum, ileum, and colon (**Figure 2E**). *P16* deletion significantly increased the expressions of TJ proteins, maintained the tight junctional structure, and decreased the intestinal permeability (**Figure 2**). These results demonstrated that *p16* deletion improved the destruction of TJ and decreased barrier permeability in intestinal epithelium caused by *Bmi-1* deficiency.

Accumulated *p16* in *Bmi-1*$^{-/-}$ Mice Combined With Occludin in the Cytoplasm of Intestinal Epithelium Cells for Blocking the Repair of Tight Junction

To investigate how *p16* deletion ameliorated intestinal epithelium barrier function in *Bmi-1*$^{-/-}$ mice, we conducted immunoprecipitation of p16 and occludin, and validated p16 could combine with occludin (**Figure 3A**). P16 expression in *Bmi-1*$^{-/-}$ ileum was higher than WT ileum; however, occludin expression in *Bmi-1*$^{-/-}$ ileum was lower than WT ileum in input and Immunoprecipitation (IP) samples (**Figure 3A**). The results of immunofluorescence colocalization and Duolink Proximity Ligation Assay showed p16 bound to occludin in the cytoplasm of intestinal epithelium cells in *Bmi-1*$^{-/-}$ mice (**Figures 3B,C**). Our results showed that p16 expressed in the nucleus and cytoplasm, especially more concentrated in the cytoplasm, and interacted with occludin in the cytoplasm (**Figures 3B,C**). Compared with the *Bmi-1*$^{-/-}$ mice, p16 expression was obviously decreased in epithelium cells of *Bmi-1*$^{-/-}$*p16*$^{-/-}$ and WT mice (**Figure 3B** and **Supplementary Figure 2**). It has been reported that N-terminal of *p16* bound to the N-terminal region of JNK1 (also known as MAPK8) or the N-terminal region of JNK3 (also known as MAPK10), which contain the glycine-rich site (Choi et al., 2005). Analysis with https://www.uniprot.org/align showed an extremely similar domain among human occludin (residues 107–139), MAPK8 (residues 16–49), and MAPK10 (residues 54–100) and also showed an extremely similar domain among mouse occludin (residues 107–139), MAPK8 (residues 24–72), and MAPK10 (residues 62–110). Besides, the 107th–139th residues are also a glycine-rich site of occludin (**Figure 3D** and **Supplementary Information 2**, **3**).

To determine which domain of occludin combined with p16, 293T cells were transfected with green fluorescent protein (GFP)-labeled *Flag-p16* overexpressed adenovirus and then with vehicle–His-tagged vector (negative control), His-tagged overexpressed plasmids of full-length, mutant, or truncated fragments for occludin, respectively. Cell extracts were immunoprecipitated with anti-Flag antibody, and the precipitated proteins were detected by immunoblotting with anti-His or anti-Flag antibody. As was shown from the results of immunoprecipitation, p16 combined with occludin at the residues 1–160 rather than limited to residues 107–139. Because the 266th–522nd mutant plasmid did not express protein, we constructed the 266th–522nd truncated fragment and found that p16 did not combine with this domain (**Figures 3E,F**). Thus, these results suggested that accumulated p16 combined with occludin at the residues 1–160 in the cytoplasm of aging intestinal epithelium cells in *Bmi-1*$^{-/-}$ mice for blocking the repair of intestinal epithelium barrier.

Accumulated p16 in Cacao-2 Cells Blocked the Repair of Tight Junction After Tight Junction Was Damaged by TNF-α

To further determine if the p16–occludin interaction blocks the repair of TJ, Cacao-2 cells were transfected with GFP-labeled *Flag-p16* overexpressed adenovirus and then were given TNF-α stimulation to damage the TJ. Immunofluorescence staining and Western blot were used to detect the expression levels of occludin and ZO-1. Immunofluorescence staining was also used to observe the interaction of p16 and occludin. Our results showed that in comparison with vehicle treatment, TNF-α treatment or *p16* overexpression damaged TJ and decreased the expressions of

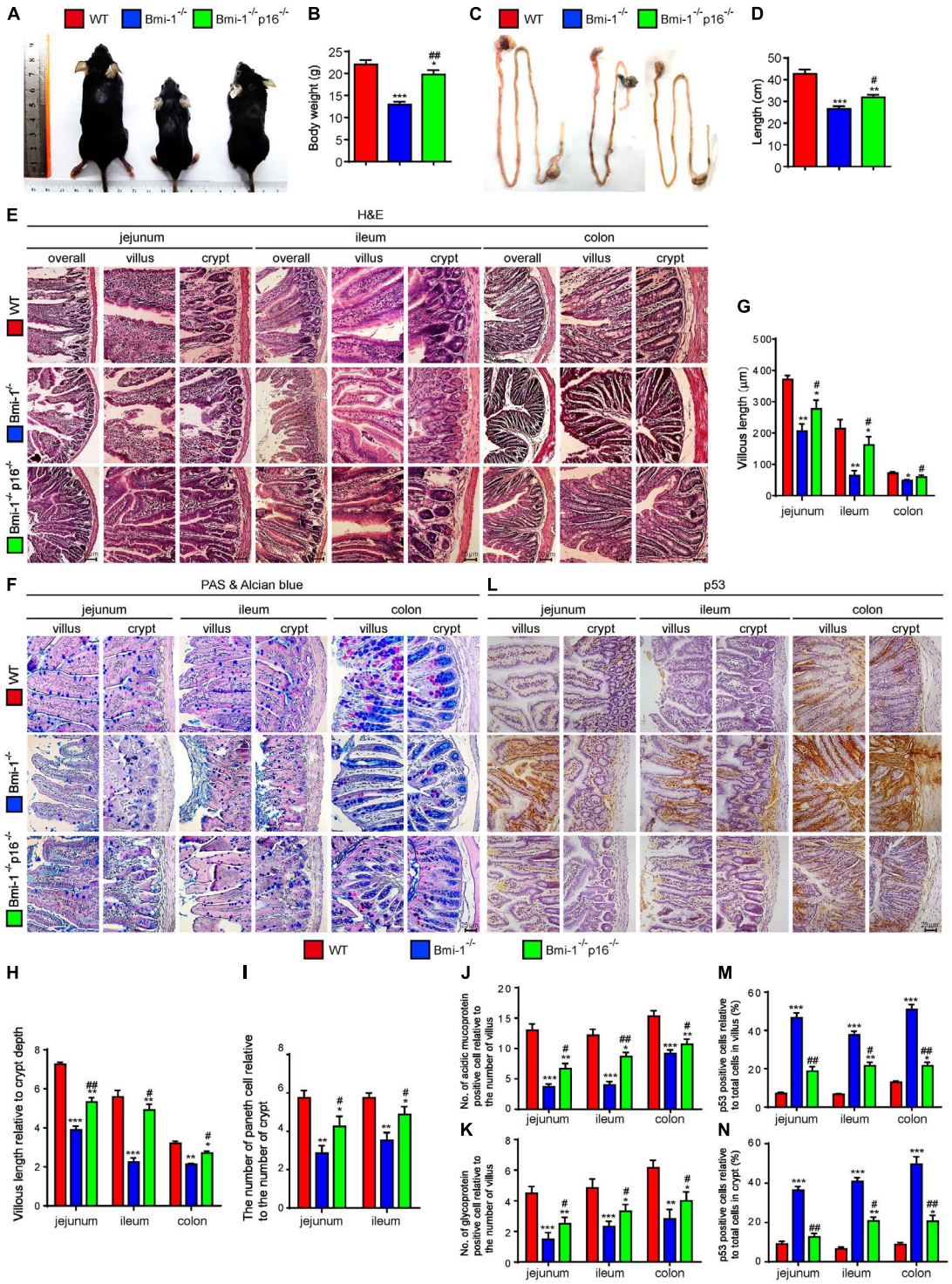

FIGURE 1 | *P16* deletion improved damage of barrier structure and dysfunction in intestinal epithelium in *Bmi-1*$^{-/-}$ mice. The experiments were carried out on the 7-week-old *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. **(A)** Representative appearances. **(B)** Body weight (g). **(C)** Whole view of the intestine. **(D)** Intestinal length (cm). **(E,F)** H&E and AB-PAS staining of jejunum, ileum, and colon. **(G)** Villous length (μm). **(H)** Villous length relative to crypt depth. **(I)** The number of Paneth cells relative to the number of crypts. **(J)** The number of acidic mucoprotein positive cells. **(K)** Glycoprotein-positive cells relative to the number of villus. **(L)** Representative micrographs of paraffin-embedded intestinal sections immunohistochemical staining for p53, with hematoxylin staining the nucleus. **(M,N)** Percentage of p53-positive cells relative to the total cells. Six mice per group were used for experiments. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the WT group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the *Bmi-1*$^{-/-}$ group.

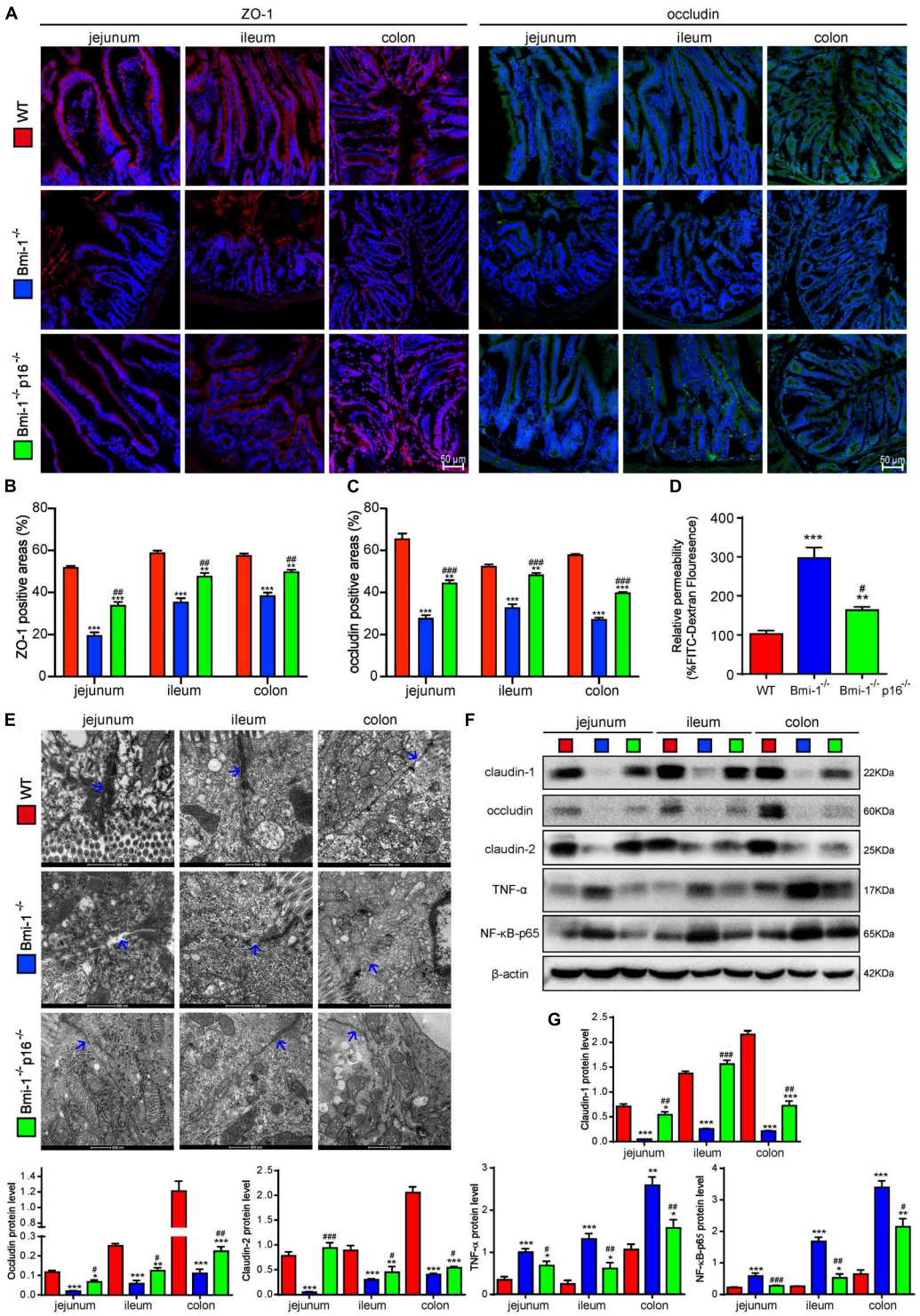

FIGURE 2 | *P16* deletion improved destruction of TJ and increasing barrier permeability in intestinal epithelium in *Bmi-1*$^{-/-}$ mice. The experiments were carried out on the 7-week-old *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. **(A)** Representative micrographs showing immunofluorescence for ZO-1 and occludin, with DAPI staining the nuclei. **(B)** Percentage of ZO-1–positive areas relative to the total area. **(C)** Percentage of occludin-positive areas relative to the total area. **(D)** Intestinal FITC–dextran transmittance showing intestinal permeability. **(E)** Representative micrographs showing transmission electron microscope for the epithelial cells of jejunum, ileum, and colon, with blue arrow showing the bottom of the epithelial TJ. **(F)** Western blots for claudin-1, occludin, claudin-2, TNF-α, and NF-κB–p65 in the epithelial cells of jejunum, ileum, and colon; β-actin was used as the loading control. **(G)** Protein levels relative to β-actin were assessed by densitometric analysis. Six mice per group were used for experiments. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the WT group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$compared with the *Bmi-1*$^{-/-}$ group.

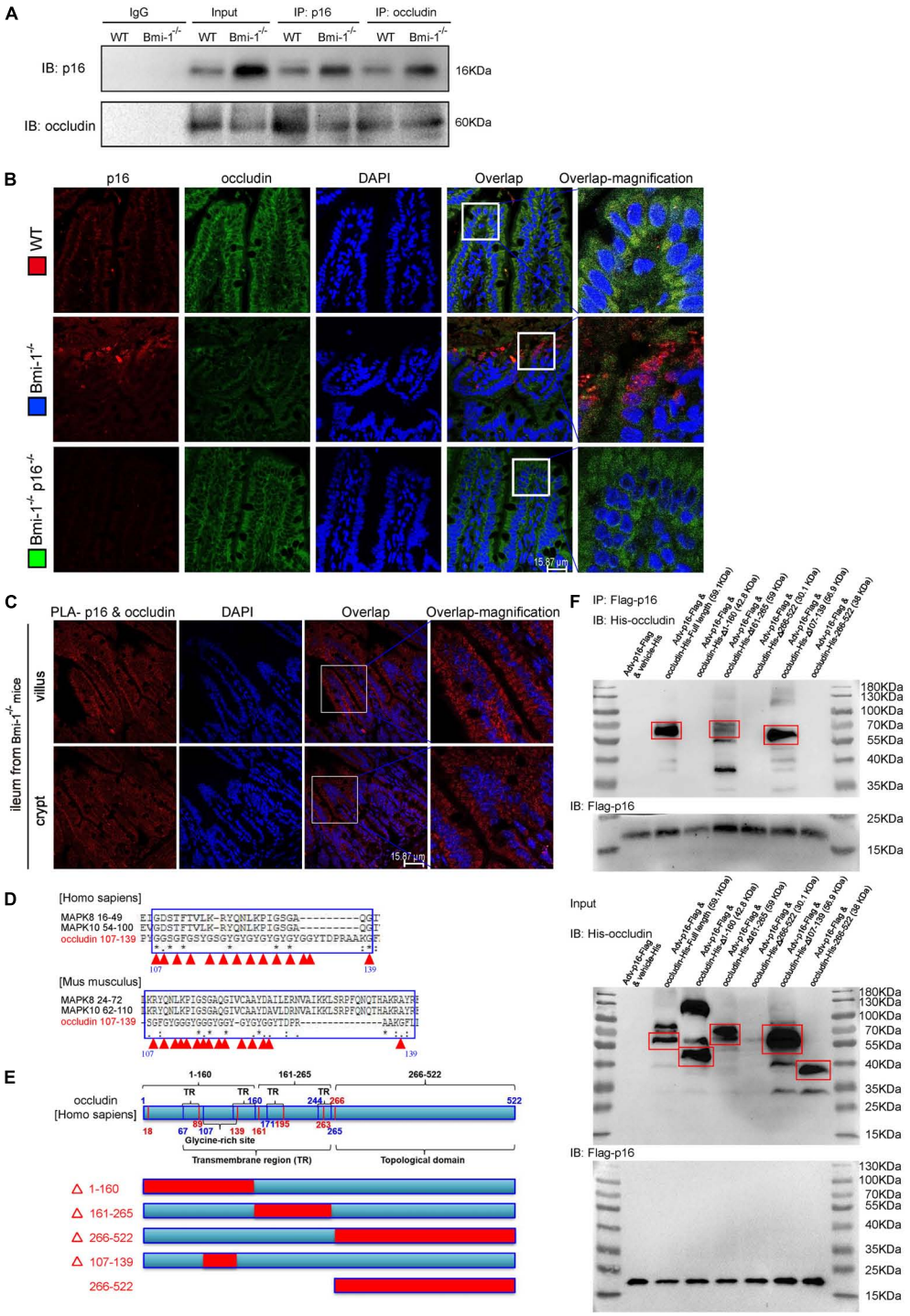

FIGURE 3 | *P16* combined with occludin in the cytoplasm of intestinal epithelium cells in *Bmi-1*$^{-/-}$ mice for blocking reparation of TJ. The experiments were carried out on the 7-week-old *Bmi-1*$^{-/-}$, and WT mice. **(A)** Intestinal epithelial proteins from WT and *Bmi-1*$^{-/-}$ mice were extracted for anti-p16 or anti-occludin immunoprecipitation. Western blots were used for detecting p16 and occludin. **(B)** Representative micrographs showing immunofluorescence double-labeling of p16 and occludin in ileum from 7-week-old *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$ and WT mice. **(C)** Representative micrographs of Duolink PLA for interaction between p16 and occludin in villus and crypt of ileum from *Bmi-1*$^{-/-}$ mice, with DAPI staining nuclei. **(D)** Analysis with https://www.uniprot.org/align showed an extremely similar domain among occludin, MAPK8, and MAPK10 in human and mouse, with red triangle showing glycine site, "*" indicating a single and fully conserved residue, ":" indicating residue with very similar properties, and "." indicating residue that is weakly similar. **(E)** Protein domain of human occludin. **(F)** The 239T cells were transfected with *Flag-p16* (human) overexpressed adenovirus and the His-tagged plasmid of full length, 1–160–mutant (△1–160), 161–265–mutant (△161–265), 266–522–mutant (△266–522), 107–139–mutant (△107–139), or 266–522–truncated fragment for human *occludin*. Cell proteins were extracted for anti–Flag-Tag immunoprecipitation and detected anti-Flag and anti-His antibodies with Western blots. The input proteins also detected anti-Flag and anti-His antibodies with Western blots.

occludin and ZO-1 (**Figures 4A–F**). Compared with vehicle–adenovirus and TNF-α treatment, *p16* overexpressed adenovirus, and TNF-α treatment further damaged TJ and decreased the expressions of occludin and ZO-1 (**Figures 4A–F**). We also observed that when the TJ between cells was destroyed, more occludin expression was found in cytoplasm and colocalized with p16 (**Figure 4G**).

TNF-α–Dependent Epithelial Barrier Destruction Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

To determine if *p16* deletion ameliorated the TNF-α–dependent epithelial barrier destruction in *Bmi-1*$^{-/-}$ mice, TNF-α–positive cells or areas; TNF-α and F4/80 double-positive macrophages; TNF-α protein levels in jejunum, ileum, and colon; and serous TNF-α protein levels of mice were detected and analyzed. Results showed that TNF-α–positive cells; TNF-α and F4/80 double-positive macrophages; TNF-α protein levels in jejunum, ileum, and colon; and serous TNF-α protein levels were significantly increased in *Bmi-1*$^{-/-}$ mice compared with WT mice; however, they were significantly reduced in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice compared with the *Bmi-1*$^{-/-}$ mice (**Figures 5A–E**). Then, nuclear factor κB (NF-κB)–p65–positive cells and NF-κB–p65 and F4/80 double-positive macrophages were detected in jejunum, ileum, and colon, and results showed that they were significantly increased in *Bmi-1*$^{-/-}$ mice compared with WT mice, while significantly reduced in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice compared with the *Bmi-1*$^{-/-}$ mice (**Figures 5F–H**). It suggested that the proinflammatory level of macrophages in intestines was up-regulated in *Bmi-1*$^{-/-}$ mice compared with WT mice and ameliorated by *p16* deletion.

Up-Regulated Proinflammatory Level of Macrophages Induced by Change of Intestinal Microbiota Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

To investigate if *Bmi-1* deficiency and/or *p16* deletion changed the phagocytosis of macrophages, DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)–labeled fecal mixed bacteria from WT mice (WT-FB) were used to infect bone marrow–derived macrophages (BMDMs) from *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. Immunofluorescence staining of macrophage marker F4/80 proved that there was no difference in phagocytosis of BMDMs from WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice (**Figures 6A,B**). This result suggested that Bmi-1 and/or p16 did not affect the phagocytosis of macrophages.

To further make clear if the up-regulated proinflammatory level of macrophages was associated with the change of intestinal microbiota in *Bmi-1*$^{-/-}$ mice, WT–fecal microbiota (FM), *Bmi-1*$^{-/-}$–FM, and vehicle were administered to BMDMs from WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice. In comparison with the WT-FM–treated BMDMs of the same genotype, an increase was observed in the mRNA levels of *TNF-α*, interleukin (*IL*)-*1β*, *IL-6*, NACHT, LRR, and PYD domain–containing protein 3 (*NLRP3*), monocyte chemoattractant protein 1 (*MCP1*), and engulfment and cell motility protein1 (*ELMO1*) and in the protein levels of TNF-α, NF-κB–p65, IκBα, and p-IκBα (Ser32) in *Bmi-1*$^{-/-}$–FM treated BMDMs, especially in *Bmi-1*–null BMDMs. Compared with vehicle of the same genotype, WT-FM and *Bmi-1*$^{-/-}$–FM treatments significantly increased the above mRNA levels and protein levels, except the *ELMO1* mRNA level and IκBα and p-IκBα (Ser32) protein level (**Figures 6C–K**). These results suggested that the up-regulated proinflammatory level of macrophages was associated with the change of intestinal microbiota in *Bmi-1*$^{-/-}$ mice.

To determine if *p16* deletion ameliorated proinflammatory level of macrophages activated by aging-associated intestinal microbiota in *Bmi-1*$^{-/-}$ mice, WT-FM, *Bmi-1*$^{-/-}$–FM, and vehicle were administered to BMDMs from *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. Results showed that whatever the treatment was with WT-FM or *Bmi-1*$^{-/-}$–FM, an increase was observed in the mRNA levels of *TNF-α*, *IL-1β*, *IL-6*, *NLRP3*, *MCP1*, and *ELMO1* and in the protein levels of TNF-α and NF-κB–p65 in *Bmi-1*$^{-/-}$ BMDMs compared with WT BMDMs; however, they were significantly reduced in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice compared with the *Bmi-1*$^{-/-}$ mice (**Figures 6C–K**). These results suggested that the proinflammatory level of macrophages activated by intestinal microbiota was ameliorated by *p16* deletion in *Bmi-1*$^{-/-}$ mice.

Fecal Microbiota Transplantation From Wild-Type Mice Ameliorates Tight Junction in Intestinal Epithelium of *Bmi-1*$^{-/-}$ and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ Mice

To determine if the damage of TJ in intestinal epithelium was associated with microbial dysbiosis, FM from 3- to 4-week-old WT mice were transplanted (WT-FMT) to WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice by gavage every other day and lasted for 21 days. Results showed that compared with the WT mice without or with FMT, ZO-1 and occludin expression in ileum epithelium were obviously decreased in *Bmi-1*$^{-/-}$ mice without or with WT-FMT; however, they were significantly increased in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice without or with WT-FMT compared with the *Bmi-1*$^{-/-}$ mice without or with WT-FMT, respectively (**Supplementary Figures 4A–D**). Compared with the *Bmi-1*$^{-/-}$ mice or *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice without WT-FMT, ZO-1 and occludin expressions in ileum epithelium were obviously increased in *Bmi-1*$^{-/-}$ mice or *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice with WT-FMT, respectively (**Supplementary Figures 4A–D**). These results suggested that the destructive degree of TJ was regulated by intestinal microbiota.

Intestinal Microbial Dysbiosis Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

To investigate if *p16* deletion could ameliorate the dysbiosis in the species and abundance of intestinal microbiota in *Bmi-1*$^{-/-}$ mice, principal coordinates analysis of microbial DNA encoding 16S ribosomal RNA (rRNA) was used to detect intestinal microbiota changes. The analysis was carried out at the level of the phylum. Differences were observed between WT and *Bmi-1*$^{-/-}$ mice, and the abundance of Bacteroidetes decreased in

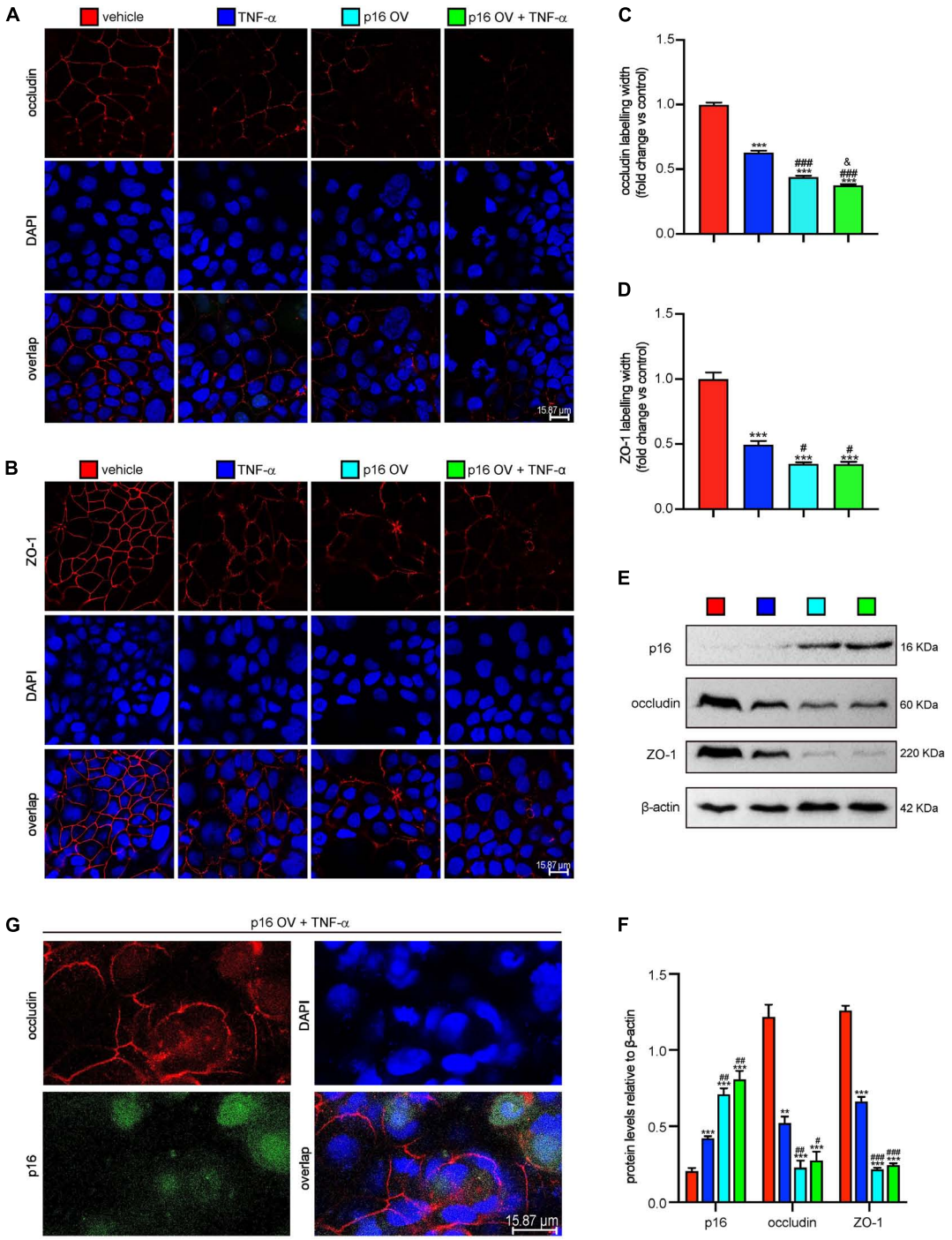

FIGURE 4 | Accumulated p16 in Cacao-2 cells blocked the repair of TJ after TJ was damaged by TNF-α. The Cacao-2 cells were transfected with GFP-labeled *Flag-p16*–overexpressed (*p16*-OV) or vehicle adenovirus and then treated with or without 10 ng/mL TNF-α for 24 h. Immunofluorescence staining was introduced to detect the expressions of occludin and ZO-1. **(A)** Representative micrographs showing immunofluorescence for occludin, with DAPI staining the nuclei. **(B)** Representative micrographs showing immunofluorescence for ZO-1, with DAPI staining the nuclei. **(C)** Occludin positive width relative to control group. **(D)** ZO-1 positive width relative to control group. **(E)** Western blots for p16, occludin, and ZO-1 in Cacao-2 cells, and β-actin was used as the loading control. **(F)** Protein levels relative to β-actin were assessed by densitometric analysis. Cell experiments were performed with three biological repetitions per group. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the vehicle group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with TNF-α–treated group, $^{\&}P < 0.05$ compared with the *p16*-OV group. **(G)** Representative micrographs showing occludin and GFP-labeled p16 in *p16*-OV and TNF-α–treated cells, with DAPI staining the nuclei.

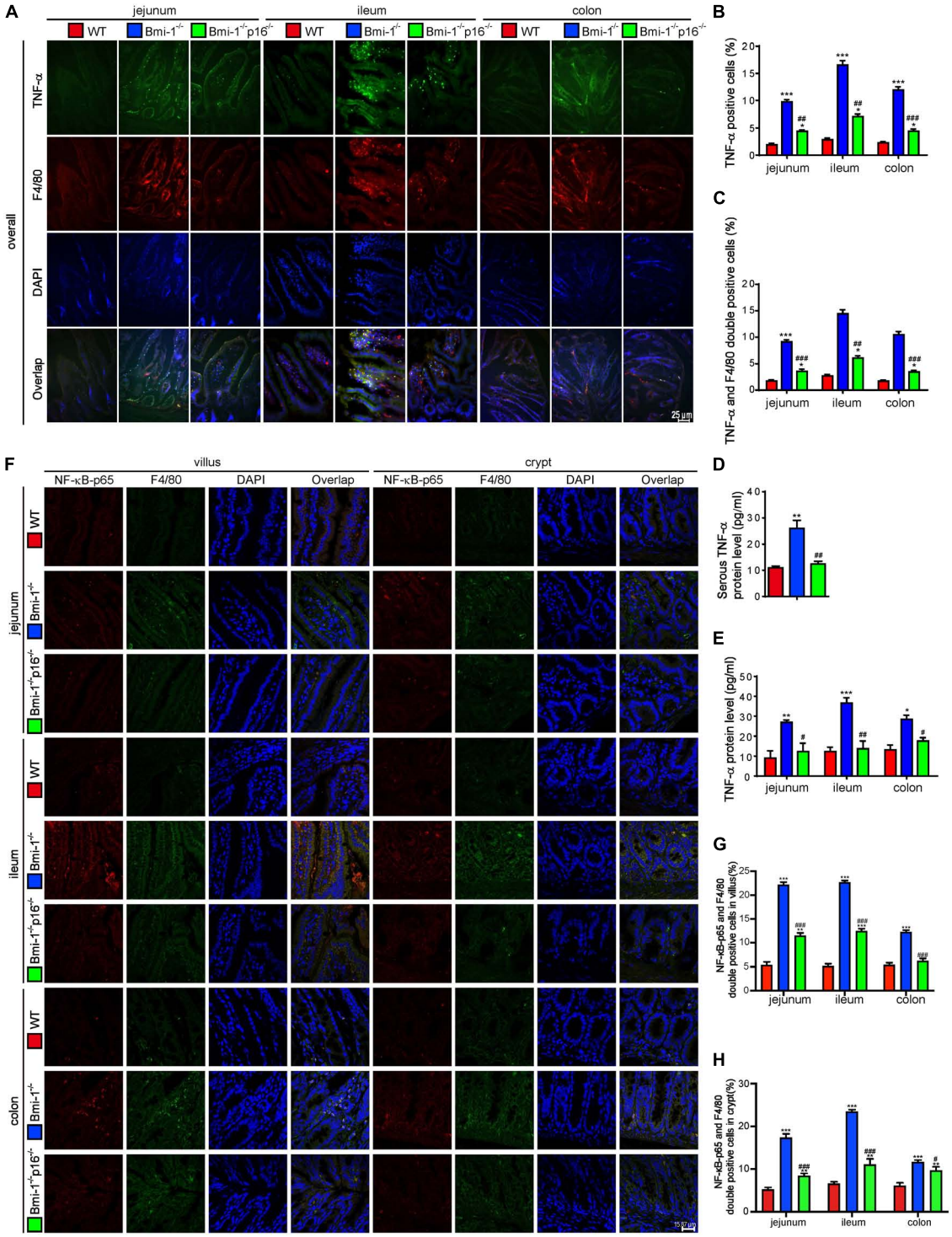

FIGURE 5 | *P16* deletion improved TNF-α–dependent epithelial barrier destruction in *Bmi-1*$^{-/-}$ mice. The experiments were carried out on the 7-week-old *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. **(A)** Representative micrographs showing immunofluorescence for TNF-α and F4/80, with DAPI staining the nuclei. **(B)** Percentage of TNF-α–positive cells. **(C)** Percentage of TNF-α and F4/80 double-positive cells. **(D)** Serous TNF-α protein level (pg/mL) was detected with ELISA assay. **(E)** TNF-α protein levels in jejunum, ileum, and colon (pg/mL) were detected with ELISA assay. **(F)** Representative micrographs showing immunofluorescence for NF-κB–p65 and F4/80, with DAPI staining the nuclei. **(G,H)** Percentage of NF-κB–p65 and F4/80 double-positive cells or areas in villus and crypt. Six mice per group were used for experiments. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the WT group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the *Bmi-1*$^{-/-}$ group.

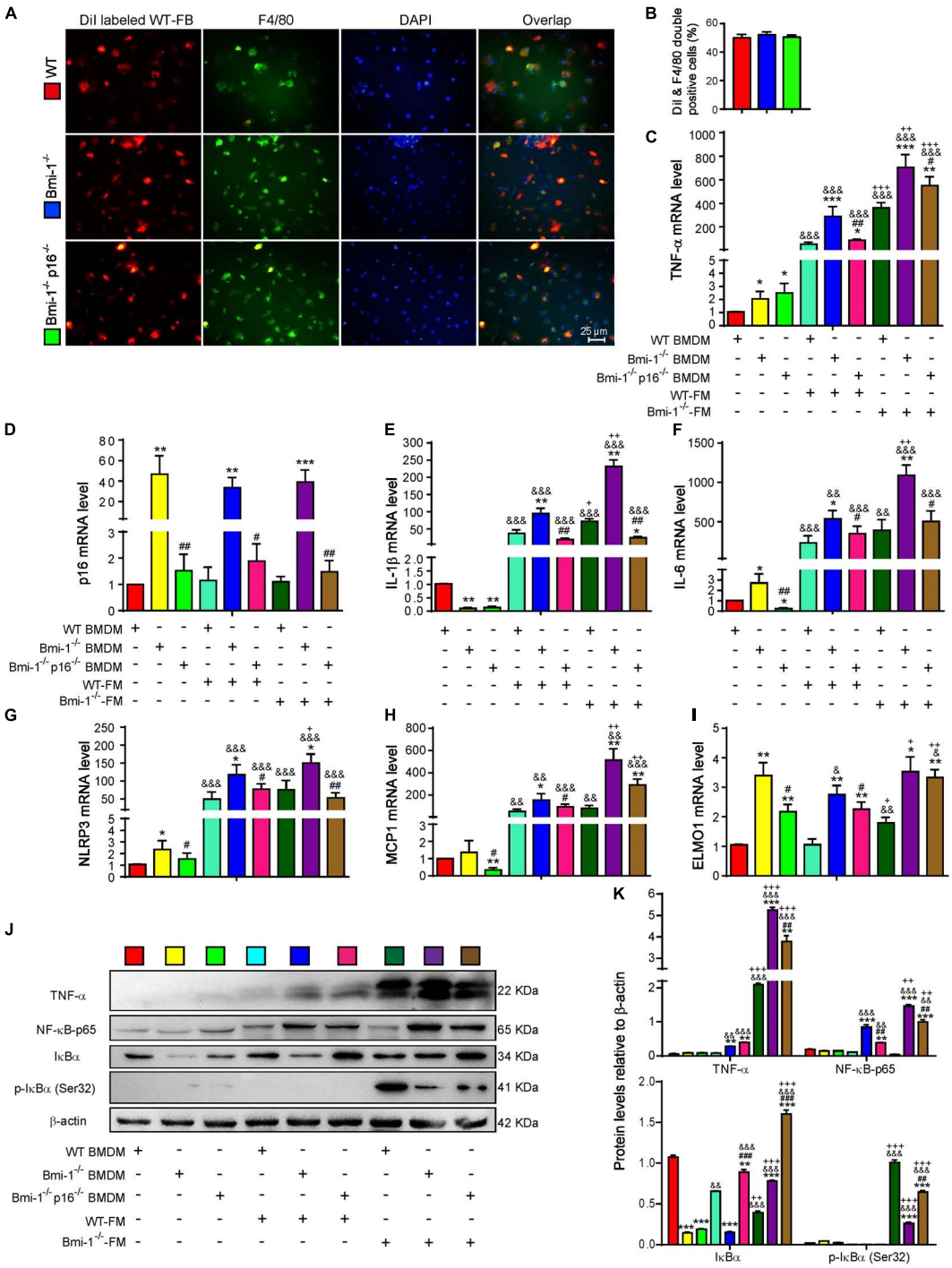

FIGURE 6 | *P16* deletion improved up-regulated proinflammatory level of macrophages induced by change of intestinal microbiota ameliorated in *Bmi-1*$^{-/-}$ mice. Fecal mixed bacteria (FB) were prepared using the fecal of 7-week-old WT and *Bmi-1*$^{-/-}$ mice to infect BMDMs from WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice. **(A)** Representative micrographs showing immunofluorescence for F4/80 after DiI-labeled WT-FM–infected BMDMs, with DAPI staining the nuclei, red dots showing DiI-labeled WT-FB, and green dots showing F4/80. **(B)** Percentage of DiI and F4/80 double-positive cells. **(C–I)** Tumor necrosis factor (*TNF-α*), *p16*, interleukin (*IL-1β*), *IL-6*, NACHT, LRR, and PYD domain–containing protein 3 (*NLRP3*) and monocyte chemoattractant protein 1 (*MCP1*) and engulfment and cell motility protein 1 (*ELMO1*) mRNA levels in BMDMs by real-time RT-PCR, calculated as the ratio to β-*actin* mRNA and expressed relative to WT. **(J)** Western blots of BMDM extracts showing TNF-α, NF-κB–p65, IκBα, and p-IκBα (Ser32); β-actin was used as the loading control. **(K)** Protein levels relative to β-actin were assessed by densitometric analysis. Cell experiments were performed with three biological repetitions per group. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the WT group at the same treatment; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the *Bmi-1*$^{-/-}$ group at the same treatment; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared with the same genotyped BMDMs of vehicle-FM group; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with the same genotyped BMDMs of the WT-FM group.

Bmi-1$^{-/-}$ mice when compared with those in WT mice, leading to intestinal microbiota ratio imbalance (**Figures 7A,B**). *P16* deletion obviously decreased the abundance of Firmicutes and Proteobacteria and increased the abundance of Bacteroidetes in *Bmi-1*$^{-/-}$ mice. After validating that the sequencing sample size meets the requirements of diversity (**Figures 7C,D**), α-diversity was then assessed to reflect within-sample diversity. The richness of species at the phylum level was lower in the *Bmi-1*$^{-/-}$ group than that in the WT group (**Figure 7E**). Venn diagram displayed 329 same operational taxonomic units (OTUs) share between the WT and DKO groups, whereas there were only 110 same OTUs between the WT and KO groups (**Figure 7F**). The results indicated that *p16* deletion could rescue intestinal microbial dysbiosis induced by *Bmi-1* deficiency.

To further clarify the specific changes of intestinal microbiota among *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice, bioinformatics analysis was conducted. Compared with WT mice, *Bmi-1*$^{-/-}$ mice had less diversity of intestinal dominant microbiota, most of which affiliated to Firmicutes including Anaerobtruncus, Lachnospiraceae, Roseburia, and Ruminiclostridium, together with Blautia and Ruminococcaceae, which were closely related to intestinal inflammation. Moreover, the abundance of some intestinal harmful bacteria including *Desulfovibrio*, *Helicobacter*, and *Oscillibacter* was at a higher level in *Bmi-1*$^{-/-}$ mice, whereas the abundance of probiotics such as *Bifidobacterium* was at a lower level. In comparison with *Bmi-1*$^{-/-}$ mice, the abundance of the intestinal harmful bacteria such as *Desulfovibrio* and *Helicobacter* was at a lower level, whereas the diversity of dominant genus elevated, and the abundance of intestinal probiotics including *Bifidobacterium* and *Lactobacillus* was at a higher level in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice (**Figure 7G**). These results demonstrated that *p16* deletion ameliorated intestinal microbial dysbiosis including increasing intestinal microbiota diversity and probiotic abundance and decreasing the abundance of harmful microbiota in *Bmi-1*$^{-/-}$ mice.

Intestinal Microbial Function Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

To determine if *p16* deletion could ameliorate intestinal microbial function in *Bmi-1* mice, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) sequencing analysis was carried out based on principal coordinates analysis of microbial DNA encoding 16S rRNA. Linear discriminant analysis (LDA) sequence was used to make clear species with significant differences among samples. The results illustrated that there were 5, 11, and 9 bacterial biomarkers, respectively, in the intestinal microbiota of WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice (**Figure 8A**). Distribution of marker bacteria in three genotypes of mice is shown in the cladogram (**Figure 8B**). These results could help understand the biomarkers of intestinal microbial changes in WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice.

To further analyze the function of intestinal microbiota, we carried out the PICRUST sequencing analysis and functional enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway on the microbiota with significant difference between WT and *Bmi-1*$^{-/-}$ groups. The results showed that the number of genes related to human functional diseases, including 502,983 functional genes related to human immune system diseases, 3,638 functional genes related to cardiovascular disease, and 148,016 functional genes related to the circulatory system. Environmental information processing, genetic information processing, and metabolism were found as the three aspects with the highest gene abundance (**Figure 8C**).

Function prediction heat map was drawn based on the functional annotation and abundance information of all intestinal microbial samples in KEGG, showing the top 35 bacteria in abundance ranking. An obvious decline was shown in intestinal microbiota energy metabolism; cell growth and death regulation; replication and repair; carbohydrate, glycan, and nucleotide metabolism; terpenoid and polyketide metabolism; protein folding sequencing degradation; and translation function in *Bmi-1*$^{-/-}$ mice compared with WT mice. Among them, the metabolic function mainly focuses on cell motility, membrane transport, and transcription. In comparison with *Bmi-1*$^{-/-}$ mice, the lipid and xenobiotics metabolism functions were enhanced in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice (**Figure 8D**).

Moreover, the genetic information function of intestinal microbiota was predicted and analyzed. In *Bmi-1*$^{-/-}$ mice compared with WT mice, a significant decrease was shown in recombination proteins, peptidase, DNA replication proteins, and amino acid–related enzymes of the intestine; a significant decline was shown in DNA repair, the function of amino sugar and nucleic acid sugar metabolism, cystine and methionine metabolism, pyrimidine metabolism, and oxidative phosphorylation. The function of purine metabolism, fructose, and mannose metabolism, pyruvate metabolism, aminoacyl tRNA biosynthesis, secretion system, and glycolysis were enhanced in intestinal microbiota of *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice compared with that of *Bmi-1*$^{-/-}$ mice (**Figure 8E**).

These results demonstrated that metabolism and biosynthesis of intestinal microbiota were ameliorated by *p16* deletion in *Bmi-1*$^{-/-}$ mice. It suggested that the disorder and dysfunction of intestinal microbiota mentioned above might help explain how *Bmi-1*$^{-/-}$ mice developed premature aging and aging-related diseases.

Desulfovibrio Promoting TNF-α Secretion and NF-κB Signaling Activation in Macrophages Ameliorated by *p16* Deletion in *Bmi-1*$^{-/-}$ Mice

To determine if *p16* deletion ameliorated the effect of *Desulfovibrio* on promoting TNF-α secretion in intestines of *Bmi-1*$^{-/-}$ mice, DiI-labeled *Desulfovibrio* was administered by oral gavage into *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. The number of DiI-positive dots per area, TNF-α–positive cells, and DiI- and TNF-α double-positive cells were analyzed after immunofluorescence staining for TNF-α in intestines. In *Bmi-1*$^{-/-}$ mice compared with WT mice, a significant increase was shown in the number of DiI-positive dots per area, TNF-α–positive cells, and DiI and TNF-α double-positive cells in jejunum, ileum, and colon; however, they were significantly

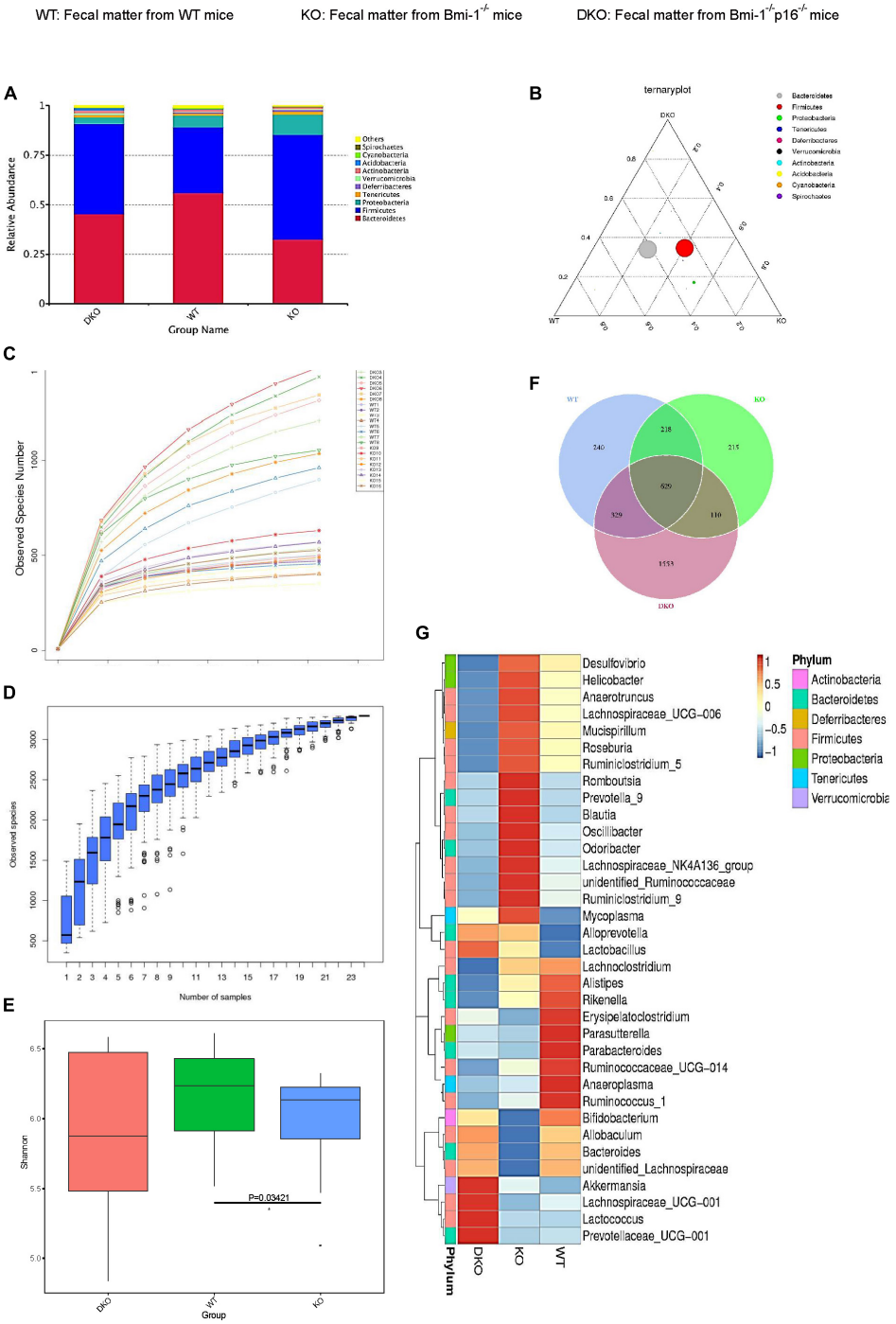

FIGURE 7 | *P16* deletion improved intestinal microbial dysbiosis in *Bmi-1*$^{-/-}$ mice. High-throughput sequencing of V4 region amplicon of 16S rRNA from intestinal microbiota was conducted using the fecal of 7-week-old WT, *Bmi-1*$^{-/-}$ (KO), and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ (DKO) mice. **(A)** Principal components at the phylum level in the intestinal microbiota of WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice. Relative contribution of the top 10 phylum of each sample was shown; "others" represents the sum of abundance except the top 10 phylum, $n = 8$. **(B)** Proportion of intestinal microbiota in 7-week-old WT, KO, and DKO mice at the phylum level. Circle represents a taxonomy at the level of phylum; the size of the circle represents the abundance of the microbiota at the phylum level. **(C)** Abundance display curve of intestinal microbiota in WT, KO, and DKO mice. Abscissa represents sequencing depth; ordinate represents the number of species found. **(D)** Box plot showing abundance dilution. The end of the curve is close to gentle, indicating that the sample size of sequencing meets the requirements of diversity. **(E)** The observed species number and α-diversity index of intestinal bacteria were examined. Significant differences are indicated: Wilcoxon rank sum test, $n = 8$ per group, $^{*}p < 0.05$, compared with the WT group. **(F)** Venn diagrams demonstrate the number of species shared among WT, KO, and DKO groups. **(G)** Heat map showing distribution of intestinal microbiota at genus level from 7-week-old WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice; the phylum of each genus is listed in the note, $n = 8$. The heat map is colored based on row *Z* scores. The mice with the highest and lowest bacterial levels are in red and blue, respectively.

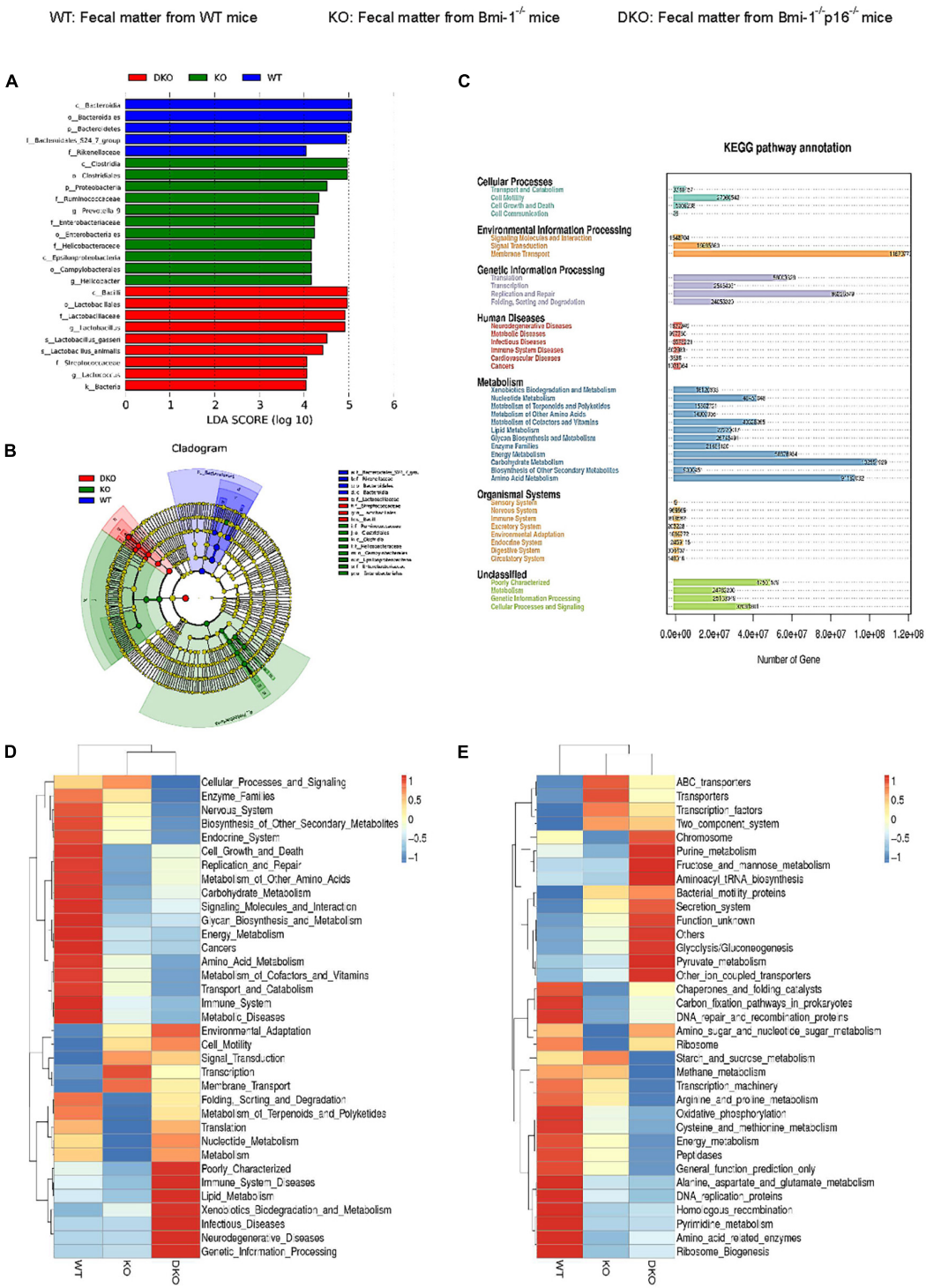

FIGURE 8 | *P16* deletion improved intestinal microbial function ameliorated in *Bmi-1*$^{-/-}$ mice. High-throughput sequencing of V4 region amplicon of 16S rRNA from intestinal microbiota was conducted using the fecal of 7-week-old WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice. **(A)** LEfSe results represented significant differences in bacterial taxa enriched in the WT, KO, and DKO groups. Only taxa with LDA > 4.0 are shown. **(B)** The evolutionary branch map obtained from LEfSe sequence of WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice. Radiation circle represents the classification level from phylum to genus. Yellow circle indicates no difference; red, green, and blue represent species with significant difference in each group. **(C)** Functional enrichment analysis of KEGG pathway. **(D,E)** Heat map of functional analysis based on the average abundance of KEGG pathway in the three groups, $n = 8$. The heat map is colored based on row Z scores. The mice with the highest and lowest functional enrichment are in red and blue, respectively.

reduced in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice compared with the *Bmi-1*$^{-/-}$ mice (**Figures 9A–D**). These results demonstrated that more *Desulfovibrio* entered the intestinal epithelium and produced lots of TNF-α in *Bmi-1*$^{-/-}$ mice, which also confirmed the serious destruction of epithelial barrier for this mouse.

To further investigate if *p16* deletion ameliorated the effect of *Desulfovibrio* on promoting macrophages-secreted TNF-α in *Bmi-1*$^{-/-}$ mice, *Desulfovibrio* or vehicle was administered to BMDMs of *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. Secreted TNF-α level was detected in the supernatant of BMDMs with enzyme-linked immunosorbent assay (ELISA). Results showed that an obvious increase was observed in secreted TNF-α level in *Desulfovibrio*-treated BMDMs compared with vehicle-treated BMDMs at the same genotype. After *Desulfovibrio* treatment, the elevation of secreted TNF-α level was obviously more than WT BMDMs in *Bmi-1*$^{-/-}$ BMDMs; however, it was significantly reduced in *Bmi-1*$^{-/-}$*p16*$^{-/-}$ BMDMs compared with the *Bmi-1*$^{-/-}$ BMDMs (**Figure 9E**).

To further investigate if *Desulfovibrio* promoted the TNF-α secretion and activated the NF-κB signaling in BMDMs, primary BMDM cells from WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice were treated with *Desulfovibrio* (10 μg/mL for 12 h). Immunofluorescence staining was also used to observe the expression level of TNF-α. Western blot was used to detect the expression levels of NF-κB-p65, p-p65 (Ser536), IκBα, and p-IκBα (Ser32). Our results showed that *Bmi-1* deficiency caused BMDMs to increase the secretion of TNF-α, and the expressions of NF-κB-p65, p-p65 (Ser536), IκBα, and p-IκBα (Ser32); however, *p16* deletion partially corrected this process (**Figures 10A–D**). *Desulfovibrio* treatment promoted the secretion of TNF-α (**Figures 10A,B**), and the expressions of NF-κB-p65, p-p65 (Ser536), IκBα, and p-IκBα (Ser32) in WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ BMDMs compared with the same genotype BMDMs without *Desulfovibrio* treatment (**Figures 10C,D**).

These results demonstrated that *Desulfovibrio* promoted the TNF-α secretion and activated the NF-κB signaling in BMDMs, and *p16* deletion ameliorated the effect of *Desulfovibrio* on promoting macrophages-secreted TNF-α in *Bmi-1*$^{-/-}$ mice.

DISCUSSION

This study demonstrated that in an SIPS model of *Bmi-1* deficiency, barrier structure and function in intestinal epithelium were damaged; TNF-α–induced destruction of TJ increased barrier permeability; up-regulated proinflammatory level of macrophages was induced by intestinal microbial dysbiosis and dysfunction; and more *Desulfovibrio* entered the intestinal epithelium to promote TNF-α secretion and NF-κB signaling activation in macrophages. P16 protein was accumulated in aging intestinal epithelium from *Bmi-1*–deficient mice, combined with occludin in the cytoplasm, and finally prevented the form and repair of TJ. *P16* deletion ameliorated damage of intestinal epithelial barrier and microbial dysbiosis caused by *Bmi-1* deficiency. FM transplantation from WT mice ameliorates TJ in intestinal epithelium of *Bmi-1* deficiency and *Bmi-1* and *p16* double-deficient mice (**Figure 10E**).

Bmi-1 is a member of the polycomb repressor complex 1 that mediates gene silencing by regulating chromatin structure and is indispensable for self-renewal of both normal and cancer stem cells (Bhattacharya et al., 2015). It has been reported that Bmi-1 also plays a crucial role in maintaining self-renewal of ISCs in the crypt of epithelium. When the stem cells responsible for daily maintenance of intestinal epithelium are destroyed, *Bmi-1*–expressing cells increase in number, acting as a compensatory mechanism (Tian et al., 2011). Several lines of evidence demonstrate that aging leads to the dysfunction of ISCs in intestinal crypts, driving rapid renewal of intestinal epithelium, generating progenitor cells, and differentiating several cell types such as Paneth cells and goblet cells, which are critical to maintain intestinal epithelial function and homeostasis (Henning and von Furstenberg, 2016; Moorefield et al., 2017). Our previous reports demonstrate that *Bmi-1*–deficient mouse is an SIPS model, induced by imbalanced redox and DNA damage (Zhang et al., 2010; Jin et al., 2014, 2017; Xie et al., 2015; Chen et al., 2020; Sun et al., 2020). We observed that besides the smaller body size and shorter intestinal length, *Bmi-1*–deficient mice also showed abnormal intestinal morphology, such as shorter villus and down-regulation of glycoprotein and mucin, which made up a relatively significant share of the intestinal mucus. Length of villi influences the absorptive surface involved in digestion (Rao and Fritz-Niggli, 1988). Mucus in small intestine is important for efficient nutritional uptake, whereas the mucus in colon helps keep bacteria away from the epithelium (Johansson and Hansson, 2016). In this study, our results showed TJ destruction in intestinal epithelial cells from *Bmi-1*–null mice. TJ integrity, closely associated with intestinal epithelium function, contributes to the function of physical intestinal barrier by regulating paracellular transportation (Lee et al., 2018). Previous report finds that intestinal *Bmi-1* deficiency inhibits epithelium proliferation and hampers self-renewal of the ISCs accompanied by p16 accumulation (Lopez-Arribillaga et al., 2015). In this study, we found that aging epithelium cells characterized by p16 accumulation inhibited proliferation and hampered repair of intestinal epithelium and further disrupted microbial homeostasis. Moreover, the up-regulated proinflammatory level of intestinal macrophages was associated with the change of intestinal microbiota in *Bmi-1*–deficient mice. *Bmi-1* deficiency resulted in significant decreases in the body weight, villus length, the ratio of villus length to crypt depth, the number of Paneth cells, and the number of acid mucin and glycoprotein. Thus, we speculated that all changes of the intestine caused by *Bmi-1* deficiency weakened intestinal immune barrier function, nutrient absorption, and intestinal immunity in mice, which finally contributed to the smaller body size and increased inflammation level of mice.

TJ barrier of the intestine is formed by claudins, occludin, and ZO-1, helping to maintain the selective barrier function of intestinal epithelium (Lee et al., 2018). Among these, occludin is important in the assembly and maintenance of TJ (Raleigh et al., 2011). Occludin that is phosphorylated locates mainly in the membrane, while the less phosphorylated occludin is

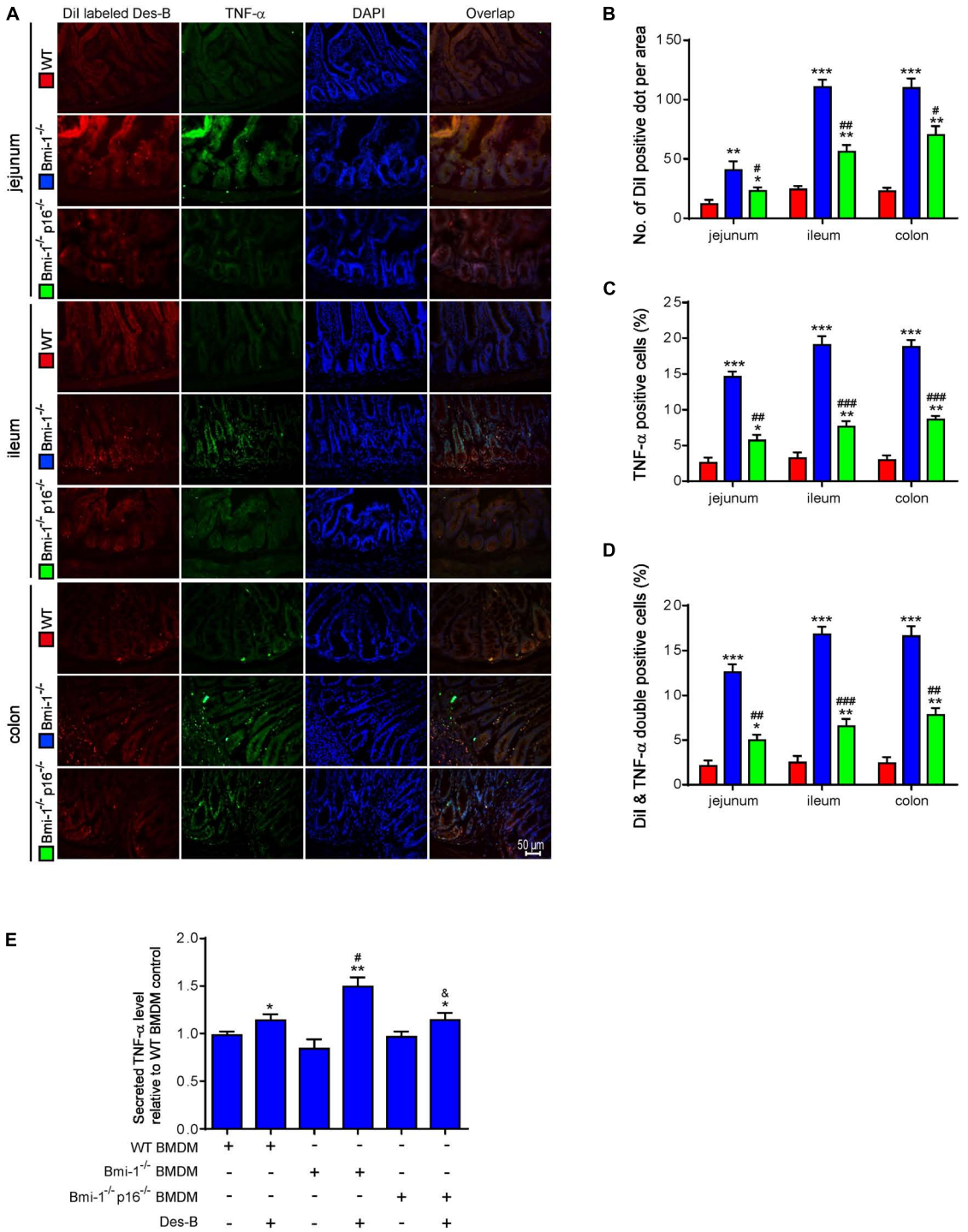

FIGURE 9 | *P16* deletion ameliorated the increase in macrophages-secreted TNF-α resulting from *Bmi-1* deficiency. DiI-labeled *Desulfovibrio* (Des-B) was administered by oral gavage into 7-week-old *Bmi-1*$^{-/-}$, *Bmi-1*$^{-/-}$*p16*$^{-/-}$, and WT mice. **(A)** Representative micrographs showing immunofluorescence for TNF-α in jejunum, ileum, and colon, with DAPI staining the nuclei. **(B)** Number of DiI-positive dot per area. **(C)** TNF-α–positive cells. **(D)** DiI-labeled Des-B and TNF-α double-positive cells. Six mice per group were used for experiments. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the WT group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the *Bmi-1*$^{-/-}$ group. BMDMs was isolated and cultured from bone marrow of WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice. **(E)** Secreted TNF-α level was detected in the supernatant of BMDMs treated with Des-B or vehicle using ELISA. Cell experiments were performed with three biological repetitions per group. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the Des-B vehicle group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the same treatment of WT-BMDM group; $^{\&}p < 0.05$ compared with the same treatment of *Bmi-1*$^{-/-}$–BMDMs.

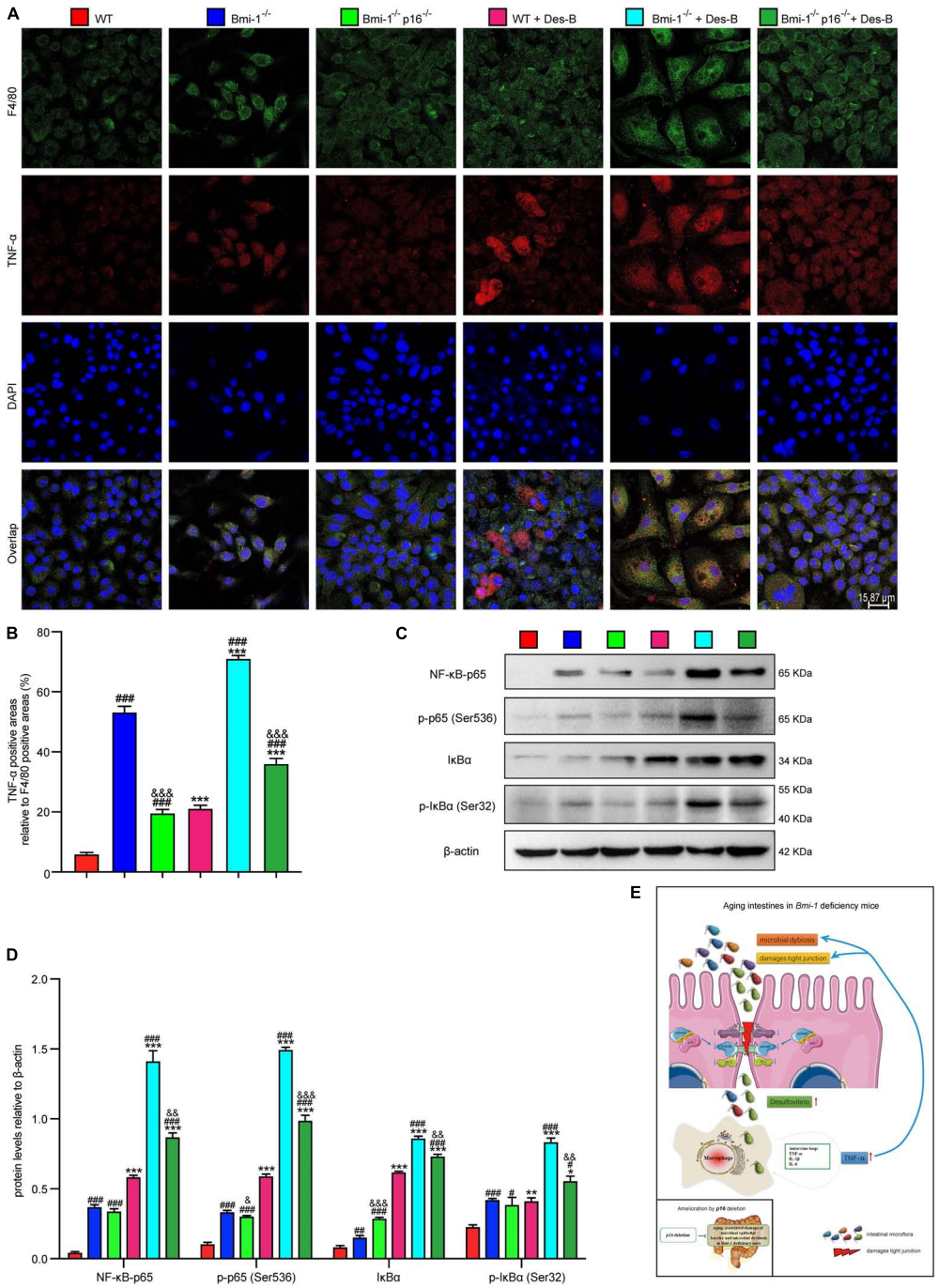

FIGURE 10 | *Desulfovibrio* promoted the TNF-α secretion and activated the NF-κB signaling in BMDMs. BMDMs from WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice were pretreated with *Desulfovibrio* (10 μg/mL) for 12 h. **(A)** Representative micrographs showing immunofluorescence for F4/80 and TNF-α, with DAPI staining the nuclei. **(B)** Percentage of TNF-α–positive areas relative to F4/80 (%). **(C)** Western blots for NF-κB–p65, p-p65 (Ser536), IκB-α, and p-IκBα (Ser32) in BMDM cells from WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice; β-actin was used as the loading control. **(D)** Protein levels relative to β-actin were assessed by densitometric analysis. Statistical analysis was performed with one-way ANOVA test. Values are mean ± SEM from six determinations per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the same genotype without *Desulfovibrio* treatment group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with WT-BMDM group at the same treatment; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared with the *Bmi-1*$^{-/-}$–BMDM group at the same treatment. **(E)** Graphical abstract about aging intestines in *Bmi-1*–deficient mice. Macrophages-secreted TNF-α resulting from *Bmi-1* deficiency not only stimulated macrophages to secrete more inflammatory factors, but also accelerated the intestinal microbiota disorders and destruction of TJ. Harmful bacteria including *Desulfovibrio* cross TJ to further trigger systemic inflammations. Up-regulated p16 in the intestine of *Bmi-1*–deficient mice prevented occludin from forming and repairing intercellular TJ by combining with occludin protein at the 1st–160th residue in the cytoplasm, which exacerbated destruction of TJ. However, *p16* deletion improved intestinal microbial function and maintained the intestinal epithelial barrier in *Bmi-1*–deficient mice.

found in the cytoplasm (Lee et al., 2018). In our study, we observed breakage of cell–cell TJ in the intestinal epithelium of *Bmi-1*–deficient mice, locating exactly at the occludin section. Also, the occludin is down-regulated in intestinal epithelium of *Bmi-1*–deficient mice. These conditions were then reversed by *p16* deletion. We then investigated how *p16* deletion rescues intestinal epithelial barrier dysfunction. Former experiments discovered that N-terminal of p16 (residues 1–80) bound to the N-terminal region of JNK1 (also known as MAPK8) (residues 1–60) or the N-terminal region of JNK3 (also known as MAPK10) (residues 75–100), which contain the glycine-rich site (Choi et al., 2005). Analysis with https://www.uniprot.org/align showed an extremely similar domain between occludin (residues 107–139, which is a glycine-rich site) and MAPK8 (residues 16–38) or MAPK10 (residues 54–76) in human. In mice, an extremely similar domain showed between occludin (residues 107–139) and MAPK8 (residues 24–72) or MAPK10 (residues 62–110). Besides, the 107th-139th residue is also a glycine-rich site of occludin for mouse. As a transmembrane protein, the 90th-135th residue of occludin locates at the extracellular domain. Thus, we speculated that p16 could bind with occludin in cytoplasm, blocking occludin in participating in the formation of intercellular TJ, which further aggravated the destruction of TJ in *Bmi-1*–deficient mice. Then, we found that p16 and occludin were colocalized and combined in the cytoplasm of intestinal epithelial cells. Accumulated p16 combined with occludin at the 1st–160th residue rather than limited to the 107th–139th residue in the cytoplasm of aging intestinal epithelium cells in *Bmi-1*$^{-/-}$ mice, inhibiting its translocation from cytoplasm to cell membrane for blocking the TJ formation and repair of intestinal epithelium barrier. Moreover, the premature senescence induced by *Bmi-1* deficiency caused destruction of intestinal TJ by TNF-α and reduced levels of TJ proteins, exacerbating the destruction of intercellular TJ and increasing intestinal epithelial permeability.

Several lines of evidence demonstrate that TNF-α stimulates permeability and disrupts integrity by up-regulating myosin light chain kinase (MLCK) aggravating intestinal epithelial barrier dysfunction and immune-mediated colitis, and/or reduces TJ protein expressions through activating NF-κB signaling pathway via TNFR1 and ERK1/2 signaling pathway (Ye et al., 2011; Hall et al., 2017; Drolia et al., 2018). Our results confirmed that TNF-α treatment damaged TJ and decreased the expressions of occludin and ZO-1. It has been reported that TNFR2 plays an important role in stimulating epithelial long MLCK expression, aggravating intestinal epithelial barrier dysfunction and immune-mediated colitis. TNFR2 interferes with programmed cell death by activating NF-κB and JNK pathways or by inhibiting TRAF-2 (Su et al., 2009, 2013). In this study, we detected TNF-α and other related proinflammatory factor levels in ileum, jejunum, and colon from *Bmi-1*–deficient mice and observed the up-regulation of proinflammatory factor and activation of NF-κB pathway. Besides, we observed no difference in phagocytosis of macrophage between *Bmi-1* deficiency and WT mice. However, how Bmi-1 and/or p16 regulates the subtype and function of macrophages remains unknown. Previous observation finds that the expression of p16 and senescence-associated β-galactosidase (SA-β-gal) are markers of their physiological programs of polarization in response to immunomodulatory stimuli in macrophages. This characterization is reversible and p53-independent in macrophages and obviously different from cellular senescence characterized by the constitutive expression of these biomarkers following p53-dependent proliferation arrest in rodent cells at least. Thus, p16 and/or SA-β-gal–positive phases are non-senescent phases in macrophages (Hall et al., 2017). In this study, BMDMs in *Bmi-1*–null conditions, especially in *Bmi-1*–null plus *Desulfovibrio* treatment, showed a bigger size and irregular shapes. We think that this shape might be determined by its physiological programs of polarization in response to immunomodulatory stimuli in macrophages, although not because they are senescent. We will investigate whether Bmi-1 determines the polarization and response of macrophages through inhibiting p16 in the follow-up study. It has been reported that chronic exposure to inflammation alters proinflammatory function of macrophage, thus secreting more proinflammatory factors (Thevaranjan et al., 2017). In this study, we found that the up-regulated proinflammatory level of macrophages was associated with the change of aging associated intestinal microbiota in *Bmi-1*–null mice.

A former study has reported that intestinal microbiota dysbiosis not only triggers inflammatory bowel disease (Larabi et al., 2020), but also leads to systemic inflammation, obesity, type 2 diabetes, and chronic kidney disease (Sabatino et al., 2017; Thevaranjan et al., 2017; Virtue et al., 2019). Thus, we conducted high-throughput sequencing of V4 region amplicon of 16S rRNA to detect intestinal microbiota changes, which showed the intestinal microbiota ratio imbalance. Besides the diversity of intestinal bacterium, the number of intestinal probiotics also decreased after *Bmi-1* deletion, whereas the pathogenic bacteria such as *Desulfovibrio*, *Helicobacter*, and *Oscillibacter* increased, leading to the dysbiosis of microbiota. Moreover, the function of carbohydrate, glycan and nucleotide metabolism, cell growth and death regulation, replication, and repair were all impaired in *Bmi-1*–deficient mice, which further damaged the intestinal function and nutrition absorption. Not only the pathogenic bacteria itself, but also the harmful substances it secreted can also lead to intestinal diseases and even systemic diseases (Moreto and Perez-Bosque, 2009). Then, DiI-labeled *Desulfovibrio*, a kind of pathogenic bacteria, was administered by oral gavage into *Bmi-1* knockout, *Bmi-1* and *p16* double-knockout, and WT mice. TNF-α level and the number of *Desulfovibrio* were increased in the intestinal epithelium of *Bmi-1*–deficient mice. To further confirm whether up-regulation of TNF-α is secreted by macrophages through activating NF-κB signaling after *Desulfovibrio* inducing, TNF-α protein level was up-regulated in the supernatant, and NF-κB signaling was activated in BMDMs from the mice, illustrating that *Desulfovibrio* could induce macrophages secreting more TNF-α and further aggravate the damage of intestinal barrier. We also observed that *p16* deletion could ameliorate the microbiota dysbiosis and decrease the inflammation level in *Bmi-1*–deficient mice, which provides potential targets for the

treatment of age-associated intestinal diseases and intestinal microbiota dysfunction.

Taken together, our results demonstrated that Bmi-1 maintained intestinal TJ, epithelial barrier function, and microbiota balance through preventing senescence characterized by p16 accumulation. Clearance of p16-positive cells in aging intestinal epithelium would be a new method for maintaining barrier function and microbiota balance. The 1st to 160th residues of occludin could be a novel therapeutic target for identifying small molecular antagonistic peptides to prevent the interaction of p16 with occludin for protecting TJ.

MATERIALS AND METHODS

Mice and Genotyping

Bmi-1$^{-/-}$*p16*$^{-/-}$, *Bmi-1*$^{-/-}$, and WT SPF mice were prepared as described previously (Jin et al., 2014, 2017). All of them were housed in pathogen-free conditions. For studies of the microbiota, mice were selected from multiple cages and multiple breeding pairs to minimize cage effects or familial transfer of the microbiota as previously described (Thevaranjan et al., 2017). No evidence of cage effects was found in the studies of the microbiota. This study was carried out in strict accordance with the guidelines of the Institute for Laboratory Animal Research of Nanjing Medical University in Nanjing of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University (permit number IACUC-1706001).

Cell Cultures

Seven-week-old mice were anesthetized with 3% pentobarbital sodium (40 mg/kg). The leg bones were separated, and bone marrow was rushed out with phosphate-buffered saline (PBS). BMDMs were then isolated and cultured in Dulbecco modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies Inc., NY, United States) and 50 ng/mL recombinant mouse macrophage colony-stimulating factor (Novoprotein Scientific Inc., Shanghai, China) at 37°C as previously described (Assouvie et al., 2018).

The Cacao-2 cells (#ZQ0056) (Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China) were cultured in 1640 medium with 10% FBS according to the manufacturer's instructions.

Preparation of Fecal Microbiota

Feces were collected daily from 3- to 4-week-old WT or *Bmi-1*$^{-/-}$ mice in sterile conditions. Stools from each group were pooled 100 mg and then resuspended in 1 mL of sterile saline. The solution of FM was vigorously mixed before centrifugation at 800 *g* for 3 min as previously described (Chang et al., 2015). The supernatant bacterial concentration of FM was detected with microplate reader at 600-nm excitation wavelength and should be more than 0.6; 0.6–0.8 is the logarithmic phase of bacterial growth, and 0.8–1.0 is the platform phase of bacterial growth. The concentration of FM was used in both phases.

The supernatant from the WT group was collected and labeled with DiI (Sigma-Aldrich, St. Louis, MO, United States) for tracking according to the manufacturer's protocol before infecting BMDMs as previously described methods (Zhang et al., 2011; Nagyova et al., 2014; Xie et al., 2015). Then, the DiI-labeled FM was incubated with BMDMs for immunofluorescent staining of cells. The supernatant from WT or *Bmi-1*$^{-/-}$ group was collected and incubated with BMDMs for real-time reverse transcriptase–polymerase chain reaction (RT-PCR) detection.

Fecal Microbiota Transplantation

Mice were deprived of food 4 h before an oral gavage. FM from 3- to 4-week-old WT mice (fecal samples 100 μL, 100 mg/mL) were transplanted to WT, *Bmi-1*$^{-/-}$, and *Bmi-1*$^{-/-}$*p16*$^{-/-}$ mice by gavage every other day and lasted for 21 days.

Preparation of the Intestine Sections

Mice were anesthetized with 3% pentobarbital sodium (40 mg/kg) at 7 weeks of age. Jejunum, ileum, and colon were separated and washed with PBS. The intestines were cut into several pieces following the annular surface. Then, the intestine samples were fixed with periodate-lysine-paraformaldehyde (PLP) solution for 24 h at 4°C (Zhang et al., 2010). For hematoxylin–eosin (H&E), Alcian blue and periodic acid–Schiff (AB-PAS) staining, immunohistochemistry, and immunofluorescence, the samples were dehydrated in a series of graded ethanol solutions, embedded in paraffin, and cut into 5-μm sections using a rotary microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) as previously described (Jin et al., 2017). For immunofluorescence staining, the samples were dehydrated in 15 and 30% sucrose solutions (prepared in 1 × PBS) in turn until the tissue settled to the bottom of the tube. Then, the samples were cut into 5-μm sections using a freezing microtome (Thermo Scientific Cryotome FSE Cryostats, Loughborough, Leicestershire, England) as previously described (Chen et al., 2020).

Histology Staining

For H&E, AB-PAS staining, or immunofluorescence, serial paraffin sections were deparaffinized and rehydrated.

Hematoxylin–Eosin Staining

Serial paraffin sections of jejunum, ileum, and colon were stained as previously described (Jin et al., 2017).

Alcian Blue and Periodic Acid–Schiff Staining

AB-PAS staining kit (#G1285, Solarbio Life Sciences, Beijing, China) was used according to the manufacturer's instructions and following methods as previously described (Li et al., 2019).

Immunohistochemical Staining

Serial paraffin sections were generated for antigen retrieval, steamed for 20 min in sodium citrate buffer (10 mM sodium citrate acid, 0.05% Tween-20, pH 6.0) followed by blocking of endogenous peroxidase (3% H_2O_2) and preincubation with

serum (Jin et al., 2014; Xie et al., 2015). Primary antibodies were against Bmi-1 (#5856, Cell Signaling Technology, United States), p16 (#ab211542, Abcam, Cambridge, MA, United States), p53 (#2524, Cell Signaling Technology, United States), and Ki67 (#BS1679, Bioworld Technology Inc., MN, United States). Biotin-labeled second antibody was used. Nuclei were stained with hematoxylin.

Immunofluorescent Staining

Primary antibodies against ZO-1 (sc33725, Santa Cruz Biotechnology Inc., Dallas, TX, United States), TNF-α (sc-52746, Santa Cruz Biotechnology Inc., United States), F4/80 (sc-377009, Santa Cruz Biotechnology Inc., United States), occludin (sc133256, Santa Cruz Biotechnology Inc., United States), and p16 (10883-1-AP, Proteintech Group, Inc., IL, United States) were used, and affinity-purified Alexa Fluor 488–conjugated secondary antibody and 594-conjugated secondary antibody (Life Technologies Corporation, Carlsbad, CA, United States) were used. Nuclei were labeled with DAPI (Sigma-Aldrich, United States) and mounted with medium to prevent quenching (Vector Laboratories Inc., United States) following previously described methods (Jin et al., 2017).

Construction and Transfection of Overexpressed Adenovirus or Plasmid of Full-Length, Mutant, or Truncated Fragments

GFP-labeled p16 full-length overexpression adenovirus carrying the Flag-tag was designed and synthesized by Genechem Co., Ltd., in Shanghai, China.

Considering the structural features of occludin as a transmembrane protein, we generated four mutant plasmids based on its intracellular and extracellular domain, which contains mutation of 1–160 residues, 161–265 residues, 266–522 residues, and 107–139 residues and a full-length or a truncated fragment (residues 266–522) of occludin overexpression plasmid all carrying the His-tag with pcDNA3.1 vector plasmid. Production of the above plasmids was by TranSheep Bio Co., Ltd., Shanghai, China.

The above adenovirus was transfected in 293T cells incubated with antibiotic-free complete medium at 40–50% fluence for 8 h. After 24 h, plasmids were transfected into these 293T cells using X-tremeGENE HP DNA Transfection Reagent (cat. no.06366236001, Roche Diagnostics GmbH, Mannheim, Germany). Cells were incubated with antibiotic-free complete medium including the above transfection mixture for 48 h, and protein was harvested. Specific details followed the instruction of X-tremeGENE HP DNA Transfection Reagent.

Immunofluorescent Staining of Cells

When the Cacao-2 cell density reached 60%, they were transfected with GFP-labeled *Flag-p16*–overexpressed (*p16*-OV) or vehicle adenovirus and then treated with or without 10 ng/mL TNF-α (#C008, Novoprotein Scientific Inc., Shanghai, China) for 24 h. Then, Cacao-2 cells were fixed with PLP for 10 min and washed three times with PBST (PBS with 0.05–0.1% Tween-20) for 10 min each and blocked with 5% bovine serum albumin at room temperature for 1 h to prevent non-specific reactions. Primary antibodies against F4/80 (sc-377009, Santa Cruz Biotechnology Inc., United States), ZO-1 (#21773-1-AP, Proteintech Group, Inc., IL, United States), and occludin (sc133256, Santa Cruz Biotechnology Inc., United States) were used. Affinity-purified Alexa Fluor 488–conjugated secondary antibody (Life Technologies Corporation, United States) was used. Nuclei were labeled with DAPI.

BMDMs were pretreated with *Desulfovibrio* (10 μg/mL) for 12 h or cultured with DiI-labeled FM preincubated with serum. Cells were reacted with primary antibody after being fixed and blocked. Primary antibodies against F4/80 (sc-377009, Santa Cruz Biotechnology Inc., United States) and TNF-α (NBP1-19532, Novus Biologicals, Centennial, Co., United States) were used, and affinity-purified Alexa Fluor 488–conjugated secondary antibody (Life Technologies Corporation, United States) was used. Nuclei were labeled with DAPI (Sigma-Aldrich, United States) and mounted with medium to prevent quenching (Vector Laboratories Inc., Burlingame, CA, United States) (Gu et al., 2016).

Duolink Proximity Ligation Assay

After routinely dewaxing and hydration, serial paraffin sections of ileum were blocked with sheep serum and detected with antibodies against p16 (#ab211542, Abcam, Cambridge, MA, United States) and occludin (#sc-133256, Santa Cruz Biotechnology Inc., United States). Then, Duolink Proximity Ligation Assay (PLA) *in situ* fluorescence (Sigma-Aldrich, United States) was performed following the manufacturer's instructions with Duolink *in situ* PLA probe anti-mouse PLUS (#DUO92001), Duolink *in situ* PLA probe anti-rabbit MINUS (#DUO92005), Duolink *in situ* detection reagents Red (#DUO92008), and Duolink *in situ* wash buffer fluorescence (#DUO82049). The PLA signal (λex 594 nm, λem 624 nm; Texas Red) was analyzed (Chen et al., 2020).

Transmission Electron Microscopy

Intestinal samples of mice were fixed with 1% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4, 4°C, 2 h), postfixed in 2% osmium tetroxide in a 0.1 M phosphate buffer (4°C, 1.5 h), and dehydrated in a graded series of concentrations of ethanol (50, 70, 90, 95, and 4 × 100% each for 15 min) and acetone (15 min). Eventually, the material was embedded in epoxy resin. Sections were cut to stain with uranyl acetate and lead citrate (Jin et al., 2014; Wang et al., 2019). JEOL 1200EX TEMSCAN electron microscope was used to observe the TJ of intestinal epithelial cells.

Intestinal Permeability

Tracer FITC–labeled dextran (#46944; Sigma-Aldrich) was used to assess *in vivo* intestinal permeability as previously described (Thevaranjan et al., 2017). Mice were deprived of food 4 h before an oral gavage using FITC–dextran (600 mg/kg body weight, 125 mg/mL). Blood was collected from heart after 6 h, and fluorescence intensity was measured on fluorescence plates using

an excitation wavelength of 493 nm and an emission wavelength of 518 nm (Thevaranjan et al., 2017).

Western Blots

Intestinal epithelium of 7-week-old mice or BMDM cells was dissected and immediately placed into RIPA lysis buffer containing a cocktail of proteinase inhibitors and phosphatase inhibitors (#4906845001, Roche Diagnostics Corp., Basel, Switzerland) and phenylmethanesulfonyl fluoride (#ST506, Beyotime Institute of Biotechnology, Shanghai, China) for protein extraction. Western blots were performed as previously described (Smith et al., 2003; Jin et al., 2017). Primary antibodies against claudin-1 (sc-81796, Santa Cruz Biotechnology Inc., United States), occludin (sc133256, Santa Cruz Biotechnology Inc., United States), claudin-2 (sc-293233, Santa Cruz Biotechnology Inc., United States), TNF-α (NBP1-19532, Novus Biologicals, Centennial, Co., United States), NF-κB–p65 (#8242, Cell Signaling Technology, Beverly, MA, United States), p-p65 (Ser536) (ab76302, Abcam, United States), IκBα (AF1282, Beyotime Biotechnology, China) and p-IκBα (Ser32) (sc-8404, Santa Cruz Biotechnology Inc., United States), ZO-1 (sc33725, Santa Cruz Biotechnology Inc., United States), p16 (#ab211542, Abcam, United States), DYKDDDDK Tag (binds the same epitope as Sigma's anti-FLAG M2 antibody, #14793, Cell Signaling Technology, United States), and His-Tag (#12698, Cell Signaling Technology, United States) were used. β-Actin (BS6007M, Bioworld Technology, St. Louis Park, MN, United States) was the loading control for the cytoplasmic fraction and total cell protein.

RNA Extraction and Real Time Reverse Transcriptase–Polymerase Chain Reaction

RNA was extracted from BMDMs untreated or treated with fecal bacteria using TRIzol reagent (#15596, Invitrogen Inc., United States) according to the manufacturer's protocol. Levels of mRNA in cell samples were quantified by real-time RT-PCR as previously described (Jin et al., 2011; Jin et al., 2017). Primers were in **Supplementary Table 1**.

Immunoprecipitation Analysis

After extracting total protein from mouse ileum tissues, immunoprecipitation assay was performed using Pierce Co-Immunoprecipitation (Co-IP) Kit (#26149, Pierce Co-IP Kit, Thermo Fisher Scientific, IL, United States) as the manufacturer instructed. Followed by preclearing lysate with the control agarose resin, tissue proteins were mixed with 1 μg of antibody and then incubated overnight, anti–immunoglobulin G (IgG) antibody as a control. The bound antigens were eluted from the agarose resin using elution buffer. Eluted samples were carried out with sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel electrophoresis. Immunoblotting was carried out as previously described (Chen et al., 2018). Clean-Blot^{TM} IP Detection Reagent (horseradish peroxidase) (#21230, Thermo Fisher Scientific) was used as secondary antibody to eliminate IgG bands. Primary antibodies against p16 (#ab211542, Abcam, United States), occludin (#sc-133256, Santa Cruz Biotechnology Inc., United States), and DYKDDDDK Tag (binds the same epitope as Sigma's Anti-FLAG M2 antibody, #14793, Cell Signaling Technology, United States) were used. The immunoreactive bands were visualized by ECL chemiluminescence (Amersham Pharmacia Biotech, NJ, United States) and analyzed by the Scion image Beta 4.02 (Scion, National Institutes of Health, Bethesda, MD, United States).

Protein Sequence Alignment

The amino acid sequences of occludin, MAPK8, and MAPK10 protein from mouse or human were aligned using online tools (Uniprot/Align)[1] (**Supplementary Information 4**, **5** alignment of occludin, MAPK8 and MAPK10 in human or mouse).

Sample Collection and DNA Extraction

Eight mice were selected from each genotype. Mice feces were collected and then stored at −80°C before use. Total genome DNA was extracted from the samples using DNA magnetic bead extraction kit following the manufacturer's instructions. After extraction, DNA was treated with DNase-free RNase to remove contaminating RNA. DNA concentration and quality were assessed (Pandit et al., 2018; Zhao et al., 2019). Then, the DNA was stored at −20°C for use.

16S rRNA Gene-Based Sequencing Analysis

The V4 region of the 16S rRNA gene was amplified with primers and sequenced as previously described (Caporaso et al., 2010, 2012). Amplified DNA was sequenced using QIIME (Caporaso et al., 2010) software package (Quantitative Insights Into Microbial Ecology, V1.7.0).[2]

Raw data were analyzed by Novogene Bioinformatic Technology Co., Ltd., In-house Perl scripts were used to analyze α- (within samples) and β- (among samples) diversity. Sequences with $\geq$ 97% similarity were assigned to the same OTUs. We pick representative sequences for each OTU and use the RDP classifier (Wang et al., 2007) to annotate taxonomic information for each representative sequence. In order to compute α-diversity, we rarify the OTU table and calculate three metrics: Chao1 estimates the species abundance; Observed Species estimates the amount of unique OTUs found in each sample, and Shannon index. Based on Unifrac distance, principal component analysis was conducted using QIIME to analyze β-diversity (Hamady et al., 2010). Biomarkers were selected with LEfSe (LDA effect size) (Segata et al., 2011). In total, species with significant differences were analyzed using Wilcoxon rank sum test. LDA score was used to assess the impact of significantly different species.

PICRUST was used to predict the metabolic pathways from 16S rRNA gene-based microbiota of the intestine. The predicted functions were then collapsed into hierarchical KEGG pathways using the categorize-by-function step in the PICRUST pipeline (Garcia-Amado et al., 2018). Heat maps of function categories

[1] https://www.uniprot.org/align/

[2] http://qiime.org/index.html

based on KEGG pathways and abundance changes of the differential bacteria were generated by R software.

Infection Experiments *in vivo* and *in vitro*

Desulfovibrio freeze-dried powder (#BNCC173631) was obtained from Beijing Beina Chuanglian Biotechnology Research Institute in Beijing of China. According to the manufacturer's instructions, certain amount of germ-free calf serum was added into the *Desulfovibrio* freeze-dried powder and mixed. The bacterial solution was dropped into Colombian blood agar plate with triangle coating rod spreading evenly. It was then cultured in anaerobic culture zone at 37°C. After the colony was formed, its shape was confirmed under the microscope. The *Desulfovibrio* colony was inoculated into LB liquid medium and amplified after passaging two to three times (the whole process was completed in anaerobic culture zone). Every 100 μL of bacterial solution was added into 900 μL of 10% glycerin and stored at −20°C for use.

In vivo, before the oral gavage, the concentration of *Desulfovibrio* solution was detected with microplate reader at 600-nm excitation wavelength and should be more than 0.6. *Desulfovibrio* solution was labeled with DiI (Sigma-Aldrich, United States) for tracking according to the manufacturer's protocol as previously described methods (Xie et al., 2015). After depriving of food 4 h, mice were administered 10^8 DiI-labeled *Desulfovibrio* by an oral gavage. Then, after 6 h, intestine was taken for immunofluorescence.

In vitro, for the infection experiment, the *Desulfovibrio* solution (10 μg/mL) was incubated with BMDMs in α-MEM medium supplemented with 15% FBS (Gibco, United States) at 37°C for 12 h.

Enzyme-Linked Immunosorbent Assay

BMDMs untreated or infected with *Desulfovibrio* bacteria (Des-B) were cultured in DMEM (without phenol red; Gibco) without FBS for 6 h. Supernatants were collected and filtered with MILLEX-GP 0.22-μm filters (Merck Millipore Ltd., Co., Cork, Munster, Ireland) to remove cell debris, concentrated to 1% volume with Amicon Ultra-4 centrifugal ultrafiltration tubes (NMWL 3KDa) (Merck Millipore Ltd., Co.) (Xie et al., 2015; Jin et al., 2017; Chen et al., 2020), and detected the concentrations of mouse-derived TNF-α with ELISA kit (#YFXEM00031, Yifeixue Biotechnology, Nanjing, China).

Statistical Analysis

All analyses were performed using GraphPad Prism software (version 6.07; GraphPad Software Inc., San Diego, CA, United States) as previously described (Schafer et al., 2017). Measurement data were described as mean ± SEM fold-change over control and analyzed by Student *t*-test and one-way analysis of variance (ANOVA) to compare differences among groups. Qualitative data were described as percentages and analyzed using χ^2-tests as indicated (Jin et al., 2014, 2017; Chen et al., 2020). *p*-values were two-sided, and differences were considered significant at $p < 0.05$.

As in previously described statistical methods (Zhang et al., 2020), to identify differential abundance of phyla, genera, and species between any two groups, Wilcoxon rank-sum test was used, *p*-value was corrected as false discovery rate with the Benjamini–Hochberg method. *p*-values were considered significant at $p < 0.05$. To identify features (taxa and functional modules) differentially represented between any two groups, differentially abundant taxa or functional modules were selected using the LEfSe. Only taxa with LDA score > 4.0 are shown.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://www.ncbi.nlm.nih.gov/bioproject/704896. The BioProject ID is PRJNA704896.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

AUTHOR CONTRIBUTIONS

JJ and DM: conceptualization and funding acquisition. JWZ, CH, HC, ZQ, ZM, JYZ, QW, MC, CX, RW, QL, GZ, DM, and JJ: methodology. JWZ, CH, HC, ZQ, ZM, CX, RW, QL, and JJ: software. JWZ, CH, HC, ZQ, ZM, and JJ: validation. JWZ, CH, HC, ZQ, ZM, JYZ, QW, MC, CX, RW, QL, GZ, and JJ: data analysis. JWZ, JJ, and DM: writing—original draft with help from the other authors. JJ, HC, and DM: writing—review, and editing with help from the other authors. RW, JJ, and DM: project administration and supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cell Cycle Dysregulation and Renal Fibrosis

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Precise regulation of cell cycle is essential for tissue homeostasis and development, while cell cycle dysregulation is associated with many human diseases including renal fibrosis, a common process of various chronic kidney diseases progressing to end-stage renal disease. Under normal physiological conditions, most of the renal cells are post-mitotic quiescent cells arrested in the G0 phase of cell cycle and renal cells turnover is very low. Injuries induced by toxins, hypoxia, and metabolic disorders can stimulate renal cells to enter the cell cycle, which is essential for kidney regeneration and renal function restoration. However, more severe or repeated injuries will lead to maladaptive repair, manifesting as cell cycle arrest or overproliferation of renal cells, both of which are closely related to renal fibrosis. Thus, cell cycle dysregulation of renal cells is a potential therapeutic target for the treatment of renal fibrosis. In this review, we focus on cell cycle regulation of renal cells in healthy and diseased kidney, discussing the role of cell cycle dysregulation of renal cells in renal fibrosis. Better understanding of the function of cell cycle dysregulation in renal fibrosis is essential for the development of therapeutics to halt renal fibrosis progression or promote regression.

Keywords: cell cycle arrest, over proliferation, cell senescence, senescence associated secretory phenotype, renal fibrosis

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INTRODUCTION

Tight regulation of cell cycle is essential for mammalian tissue homeostasis and development, whereas cell cycle dysregulation leads to many human diseases such as cancer, cardiovascular disease, inflammation, and neurodegenerative diseases (Wiman and Zhivotovsky, 2017). Renal fibrosis is a common process of almost all chronic kidney diseases (CKDs) progressing to end-stage renal disease (ESRD). More than a decade of studies have found that cell cycle dysregulation of the renal tubular epithelial cells (TECs) could promote injured kidneys caused by toxins, hypoxia, and metabolic disorders to progress to CKD (Susnik et al., 2015; Moonen et al., 2018).

Under normal physiological conditions, adult mammalian renal cell turnover is very low; most of the renal cells are arrested in G0 phase of the cell cycle (Thomasova and Anders, 2015). Injuries, such as ischemic, toxic, and obstructive injuries, could promote the activation of cell cycle and initial cell proliferation of renal cells, which is an important compensatory mechanism to restore renal function. Mild injuries could be repaired through cell proliferation of renal cells; therefore, renal function could be fully recovered and most renal cells re-enter the G0 phase. However, when the injury is more severe or repeated, cell cycle of renal cells is dysregulated, manifesting as cell cycle arrest or overproliferation, both of which are closely related to renal fibrosis (Canaud and Bonventre, 2015; Yan et al., 2016).

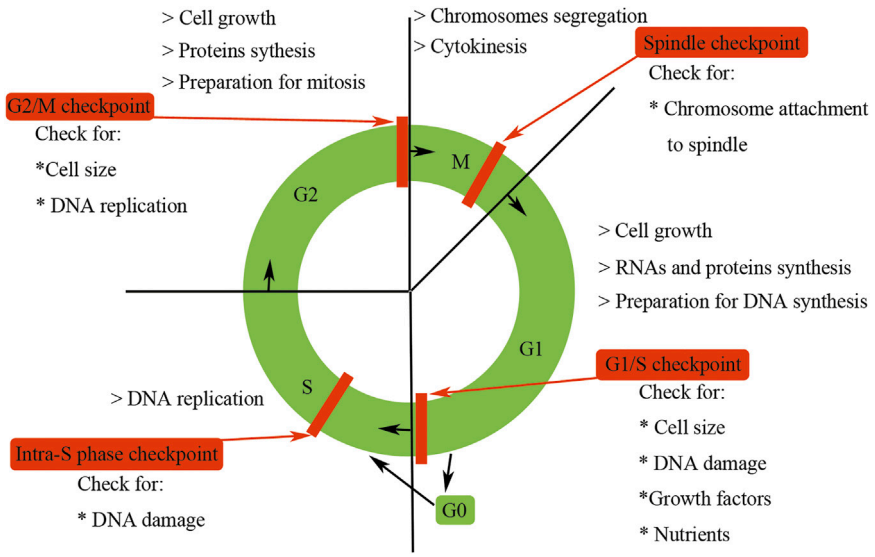

FIGURE 1 | Features of the mammalian cell cycle. Mammalian cell cycle is tightly regulated and can be artificially divided into four distinct phases (G1, S, G2, and M) according to their specific characteristics. G0 phase is usually used to describe cells that have exited the cell cycle and become quiescent. The progression of the mammalian cell cycle is precisely controlled by CDKs, cyclins, and CKIs. Checkpoints could ensure the processes at each phase of the cell cycle have been accurately completed before entering into the next phase.

To address the complex needs of kidney to keep homeostasis and repair, a delicate system to regulate cell cycle progression of renal cells is needed, that is, cell cycle control system (Harashima et al., 2013; Pack et al., 2019). However, this system could be disturbed by severe or repeated injuries, leading to cell cycle dysregulation and renal fibrosis. Recent works have improved our understanding of how cell cycle dysregulation of renal cells regulates the progression of renal fibrosis (Lovisa et al., 2015; Li et al., 2016; Liu et al., 2019b; Liu T. et al., 2019; Koyano et al., 2019; Zhao et al., 2020; Hanai et al., 2021). In this review, we focus on cell cycle regulation of renal cells in mammalian healthy and fibrotic kidney, discussing the relationships between cell cycle dysregulation of different renal cells and renal fibrosis, and finally putting some open problems about cell cycle modulation in renal fibrosis. The better understanding of the function of cell cycle dysregulation of renal cells in renal fibrosis is essential for developing strategies to halt or reverse renal fibrosis progression.

FEATURES OF THE MAMMALIAN CELL CYCLE

Cell cycle begins from the completion of one division to the end of the next, leading to the generation of two daughter cells. Mammalian cell cycle is tightly regulated and can be artificially divided into four distinct phases (G1, S, G2, and M) according to their specific characteristics (Liu et al., 2019a; Martínez-Alonso and Malumbres, 2020) (**Figure 1**). G1 phase is the gap phase, which is characterized by cell growth in size and the synthesis of RNAs and proteins required for DNA duplication. S phase is the synthesis phase during which DNA is synthesized. G2 phase is another gap phase, in which stage cells are characterized by rapid growth in cell size, more protein synthesis and preparation for division. M phase is the mitosis phase, during which the replicated chromosomes are segregated into separate nuclei and cytokinesis promoting the formation of two daughter cells. At the end of the M phase, 1 cell divides into two daughter cells, each of which contains one copy genomic DNA of the mother cell, and a cell cycle is accomplished.

Besides G1, S, G2, and M phases, the term G0 phase is usually used to describe cells that have exited the cell cycle and become quiescent. For example, under normal physiological conditions, most of the mammalian renal cells are arrested in G0 phase. However, Vogetseder et al. have found that a large number of rat epithelial cells in the proximal tubule were not in G0 phase but in G1 phase of the cell cycle (Vogetseder et al., 2008; Witzgall, 2008). Cells in G0 phase could be activated by internal or external stimuli and then re-enter the G1 phase. Some highly differentiated cells, such as neurons or cardiomyocytes, need to exit from the cell cycle permanently so as to satisfy the demands of functional requirements.

The progression of the mammalian cell cycle is tightly regulated by cyclin-dependent kinases (CDKs), cyclins, and cyclin-dependent kinase inhibitors (CKIs) (Morgan, 1997). CDKs drive the events of the mammalian cell cycle and control the rhythm of mammalian cell cycle procession; besides, they also integrate extracellular and intracellular signals to ensure the fine coordination of cell cycle events (Morgan, 1997). CDKs function as cell cycle event drivers, which are completely dependent on the association with cyclins, being first found in sea urchin eggs by their cyclic oscillations during the cleavage division in the early 1980s (Evans et al., 1983). Oscillating synthesis of cyclins controls the stage-specific timing of CDK activity. The association of

cyclins is the primary determinant of CDK activity. Besides cyclins, other additional regulatory subunits—CKIs—are needed to modulate CDK activity, substrate recognition, and subcellular location. CDKs, cyclins, and CKIs form a finely tuned regulatory network to ensure precise progression of the cell cycle. Besides their well-established function in cell cycle control, increasing studies have found that mammalian cell cycle regulators also play an essential role in other biological processes such as transcription, epigenetic regulation, metabolism, stem cell self-renewal, neuronal functions, and spermatogenesis (Lim and Kaldis, 2013).

To ensure genomic integrity and the faithful transmission of correct replicated DNA during cell division, mammals have evolved a quality control system called checkpoint, which presents in different phases of the cell cycle (Johnson and Walker, 1999) (**Figure 1**). The presence of these checkpoints ensures that the processes at each phase of the cell cycle are accurately completed before entering the next phase. The first checkpoint of the mammalian cell cycle is the G1/S checkpoint, which checks for cell size, nutrients, growth factors, and DNA damage, suspending cell cycle for DNA repair and maintaining the integrity of the genome (Johnson and Walker, 1999). The next checkpoint is the intra-S phase checkpoint, which can be activated by the DNA damage escaping from the G1/S checkpoint or occurring during the S phase, and halts the cell cycle in S phase (Johnson and Walker, 1999). The third checkpoint is G2/M checkpoint, which determines whether or not the cell continues to complete mitosis. Specifically, G2/M checkpoint ensures three important things: DNA has been well replicated, all replication errors have been rectified, and the cell size is big enough to divide (Johnson and Walker, 1999). The final checkpoint is the metaphase or spindle checkpoint, which ensures that the chromosomes have been well aligned on the spindle and are sufficient for mitosis (Johnson and Walker, 1999). These four checkpoints to some degree are redundant, but each of them has somehow relative specificity. Checkpoints are activated by incomplete DNA replication due to stalled replication forks, and damaged DNA induced by both internal and external sources such as UV light, ionizing radiation, reactive oxygen species, or DNA-damaging chemotherapeutic agents (Reinhardt and Yaffe, 2009). Checkpoint activation prevents further cell cycle progression of the damaged cells. Besides implementing cell cycle arrest, checkpoint signaling also triggers DNA repair pathways. If the DNA damage exceeds repair capacity, additional signaling cascades are triggered to eliminate these impaired cells.

CELL CYCLE REGULATION AFTER DNA DAMAGE

After DNA damage, mammalian cells will activate two major canonical kinase signaling pathways, that is, ataxia telangiectasia mutated/checkpoint kinase 2 (ATM/Chk2) and Rad3-related protein/checkpoint kinase1 (ATR/Chk1) signaling, to impede mammalian cell cycle progression and start DNA repair (Reinhardt and Yaffe, 2009). The ATM/Chk2 complex is activated by the DNA double-strand fracture, whereas the ATR/Chk1 pathway is activated primarily by DNA single-strand breaks. The ATM/ATR kinases regulate the G1/S, intra-S, and G2/M checkpoints by activating their downstream effector checkpoint kinases Chk2 and Chk1, respectively (Reinhardt and Yaffe, 2009). ATM/ATR could also phosphate p53 (**Figure 2**). In mammalian cells, p53-dependent signaling regulates G1/S arrest mainly through upregulation of p21 expression (**Figure 2**). p21 blocks cell cycle progression by inhibiting the Cdk2/cyclin E complex and therefore inhibiting the dissociation of Rb protein with transcription factor E2F (Vogelstein et al., 2000). Moreover, p21 can also inhibit the cell cycle progression at G2/M phase by the G2/M checkpoint after γ-irradiation or transforming growth factor beta (TGF-β) stimulation in renal epithelial cells (Bunz et al., 1998; Wu et al., 2013). In addition, p53-dependent pathway can also promote the impaired cells to initiate cell death program when the DNA damage is consistently accumulated (Vousden and Lu, 2002).

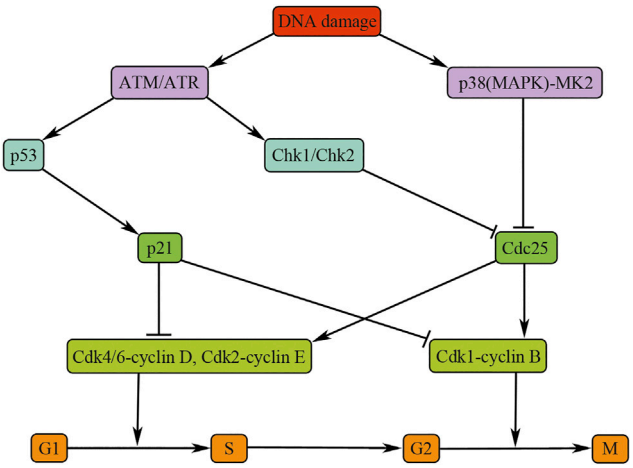

FIGURE 2 | Cell cycle regulation after DNA damage. The figure illustrates the pathways and molecules that regulate the cell cycle upon DNA damage. Inhibition of Cdk4/6-cyclin D and CDK2-cyclin E is essential for G1/S arrest and blocking CDK1-cyclin B is necessary for G2/M arrest. ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Chk, checkpoint kinase; Cdc25, cell division cycle 25; CDK, cyclin-dependent kinase.

Both the ATM/Chk2 and the ATR/Chk1 pathways play their roles mainly through inactivating Cdc25 phosphatases, the positive regulators of cell cycle progression. The p38 (MAPK)/MK2 is a novel cell cycle checkpoint kinase pathway that integrates total stress responses with DNA damage (Reinhardt and Yaffe, 2009). This pathway responds to various intracellular and extracellular stimuli, including cytokines, hyperosmolarity, and UV irradiation and halts the progression of cell cycle in G2/M phase by inactivating Cdc25 (Roux and Blenis, 2004) (**Figure 2**).

CELL CYCLE DYSREGULATION IN KIDNEY FIBROSIS

Renal fibrosis is a common process of almost all CKDs progressing to ESRD and is a failure of wound healing process

initiated by all kinds of injuries, such as toxins, hypoxia, and metabolic disorders (Liu, 2006; Wynn, 2008). Wound healing process is an evolutionary conserved defense program by which the injured tissue could be repaired and recovered. Leukocyte recruitment, angiogenesis, vascular leak, and the appearance of myofibroblasts are all involved in this process (Gabbiani and Majno, 1972). Originally, myofibroblasts are believed to be beneficial for the wound healing process, but more severe or consistently persistent injury leads to persistent presence of leukocytes and myofibroblasts, causing the maladaptive repair and finally resulting in tissue fibrosis (Dulauroy et al., 2012). Renal fibrosis is characterized by excessive deposition of extracellular matrix, which disrupts and replaces the functional parenchyma leading to organ failure. Besides, it affects all three main compartments of kidney, glomerulosclerosis in glomeruli, interstitial fibrosis in tubulointerstitium, and arteriosclerosis and perivascular fibrosis in vasculature (Djudjaj and Boor, 2019). During the process of renal fibrosis, almost all the cell types in the kidney, including fibroblasts, tubular epithelial cells, mesangial cells, and podocytes, are involved in this process, suggesting that it is a very complicated process (Boor et al., 2010; Zeisberg and Neilson, 2010). An increasing body of evidences have suggested that cell cycle dysregulation of these renal cells is closely related to renal fibrosis, especially the TECs (Canaud and Bonventre, 2015; Thomasova and Anders, 2015). In ischemic, toxic, and obstructive mice models of acute kidney injury (AKI), Yang et al. first found that cell cycle G2/M arrest of TECs could induce renal fibrosis through promoting profibrotic cytokine production by TECs (Susnik et al., 2015). They also found that administration p53 inhibitor or removal of the contralateral kidney could promote TECs to bypass the G2/M arrest, alleviating renal fibrosis in the unilateral ischemic injured kidney, suggesting cell cycle dysregulation of renal cells is indeed involved in renal fibrosis. Further studies showed that TECs in the G2-M phase formed a special structure, target of rapamycin (TOR)–autophagy spatial coupling compartments (TASCCs), which could promote the production and secretion of profibrotic cytokines (Canaud et al., 2019).

Cell Cycle Dysregulation of Podocytes and Renal Fibrosis

Podocytes are the highly specialized cells whose foot processes cover the basement membrane of the glomerulus and comprise the filtration slit diaphragms, therefore regulating blood filtration (Pavenstadt et al., 2003). Most human chronic kidney diseases exist with podocyte injury or podocyte loss (Mundel and Shankland, 2002; Nagata, 2016). The loss of podocytes and the inability to renew a damaged glomerulus with functional podocytes will ultimately result in glomerulosclerosis or scarring of the glomerulus (Barisoni et al., 1999). Diabetes and other systemic disease states can lead to podocyte injury and loss, which in turn results in ESRD (Kriz, 2002; Welsh et al., 2010).

Podocytes express cyclin A, cyclin B1, and cyclin D1 and CDK inhibitors (such as p21, p27, and p57). In the early stage of kidney development, Ki-67, which is a marker of the proliferated cells, was highly expressed in immature podocytes, whereas cyclin D1 and CKIs were dramatically downregulated; in the capillary loop stage, CKIs and cyclin D1 were intensely increased, whereas Ki-67, cyclin A, and cyclin B1 were not detectable (Nagata et al., 1998; Barisoni et al., 2000b). The expression changes of cyclins, CDKs, and CKIs were associated with podocytes exiting the cell cycle and differentiate into mature podocytes expressing the podocyte markers, such as WT-1 or podocalyxin (Nagata et al., 1998; Barisoni et al., 2000b). Under normal physiological conditions, mature podocytes are arrested in G0 quiescent phase and express high levels of CDK inhibitors. The constitutive and intense production of CKIs is necessary to maintain the function of the differentiated quiescent podocytes (Nagata et al., 1998). The high level of CKIs probably leads to mature podocytes lacking the ability to renew during adult life.

Severe injuries induce cell death and promote the proliferation of survival cells so as to compensate for the cell loss. As postmitotic and quiescent cells, podocytes do not readily proliferate after injuries; however, in some diseased situations, such as collapsing focal segmental glomerulosclerosis (FSGS), the podocytes were stained positive for proliferating cellular markers and some podocytes even existed as binuclear (Barisoni et al., 1999). Cyclins, CDKs, and CKI expression were also changed in collapsing FSGS and human immunodeficiency virus-associated nephropathy (HIVAN); in these diseased situations, p27, p57, and cyclin D disappeared in podocytes, whereas the p21, cyclin A, and Ki-67 were highly expressed (Barisoni et al., 2000a; Barisoni et al., 2000b; Shankland et al., 2000). These podocytes bypassed cell cycle restriction points and entered the cell cycle, but they were unable to complete cell cycle and finally causing podocytes loss via podocyte mitosis (mitotic catastrophe) (Liapis et al., 2013). In the setting of adriamycin-induced podocyte injury, the presence of p21 has a protective effect on the podocytes in this model of toxic podocytopathy (Marshall et al., 2010). In other glomerular diseases, such as membranous nephropathy, immune-mediated injury led to cyclin A and Cdk2 upregulation in podocytes, mitosis entry, and DNA synthesis. Although these podocytes entered mitosis, they were unable to successfully complete it, and podocytes manifested as multinucleated and absence of cytoplasmic division (cytokinesis).

Diabetic nephropathy is characterized by podocyte hypertrophy. In various experimental models of diabetic nephropathy, such as Zucker diabetic rats and db/db mice, both models of type II diabetes, or type I model, induced by streptozotocin administration, the increasing expression of p27 and p21 was identified (Kuan et al., 1998; Hoshi et al., 2002; Baba et al., 2005). Although diabetic p21 or p27 knockout mice were protected from glomerular hypertrophy and the development of progressive renal failure, the specific mechanism of podocyte hypertrophy and its role in renal fibrosis remain unknown (Kuan et al., 1998; Awazu et al., 2003). As the terminal consequence of podocyte injury, glomerulosclerosis is characterized by segmental obliteration of glomerular capillaries with the extracellular matrix and has been believed to be a process to the complete sclerosis without regression (D'Agati, 2012). As a typical feature of kidney disease, proteinuria is induced by the podocyte injury because slit membrane molecules, the actin cytoskeleton, and cell adhesion molecules have formed a tight network so as to maintain filtration

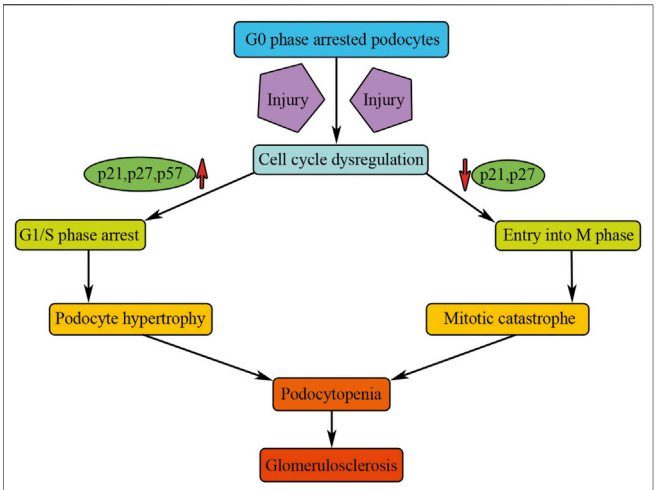

FIGURE 3 | Cell cycle dysregulation of podocytes causes renal fibrosis. Injury could activate podocyte cell cycle entry and results in cell cycle dysregulation of podocytes, causing podocyte hypertrophy or mitotic catastrophe, which will result in podocytopenia and finally result in glomerulosclerosis.

barrier function, and defection of these components leads to proteinuria (D'Agati et al., 2011). Persistent or severe podocyte injuries lead to cell detachment, which is probably caused by mitotic catastrophe (Kriz and Lehir, 2005). As normal function of podocytes requires specific arrangement of the cellular actin cytoskeleton, this may lead to podocytes unable to further form the actin contractile ring required by cytokinesis, resulting in mature podocytes that are unable to complete cytokinesis. Binucleated podocytes are frequently seen in the urine, suggesting podocyte loss caused by mitotic catastrophe is involved in podocyte detachment (Liapis et al., 2013). Moreover, podocyte loss in adriamycin-induced nephropathy was alleviated through administration of an inhibitor of p53-dependent cell cycle arrest, MDM-2, further strengthening the hypothesis (Mulay et al., 2013).

In summary, under normal physiological conditions, mammalian podocytes are arrested in G0 quiescent phase and express high levels of CDK inhibitors. Injuries can induce podocyte death, whereas the remaining podocytes are unable to undergo regenerative proliferation to compensate for the loss of podocytes. Although podocytes can enter the cell cycle and can even undergo nuclear division in a variety of glomerular diseases, they were unable to complete normal cell division. The expression of CKIs, such as p21, p27, and p57, could lead to podocyte G1/S phase arrest, causing the abundant podocyte hypertrophy seen in progressive renal failure (**Figure 3**). When mature podocytes are forced to override cell cycle restriction point, they fulfill an aberrant mitosis followed by detachment and death through mitotic catastrophe (**Figure 3**). Such podocytes appeared multinucleated with aberrant mitotic spindles or micronuclei and were often found in several human and experimental glomerular diseases, such as HIVAN, FSGS, minimal change disease, immunoglobulin A (IgA) nephropathy, or adriamycin-induced nephropathy (Liapis et al., 2013; Mulay et al., 2013); all of these diseases exist with different degree of renal fibrosis.

Cell Cycle Dysregulation of Renal Tubular Epithelial Cells and Renal Fibrosis

Under normal physiological conditions, mammalian mature TECs proliferate at a very low rate, which could be proved by PCNA and Ki-67 immunostaining (Nadasdy et al., 1994; Witzgall et al., 1994). Through this low rate proliferation, kidney can remedy the loss of TECs into the urine, which is few under normal conditions, probably one TEC per human nephron daily (Prescott, 1966). However, the rate of dividing cells remarkably increases after AKI so as to remedy TEC loss (Humphreys et al., 2008). If the injury is mild, the surviving TECs could cover the exposed basal membrane and restore cell number by proliferation (Prescott, 1966; Witzgall et al., 1994; Lin et al., 2005). In addition to proliferation, surviving TECs can also differentiate and express the embryologic markers such as vimentin (Witzgall et al., 1994; Lin et al., 2005), and then redifferentiate into specialized TECs resulting in the recovery of the nephron (Humphreys et al., 2011).

However, when the damage is more severe or repeated, the repair process can be maladaptive, which will lead to incomplete structural and functional recovery of kidney tissue with persistent inflammation, activation, and proliferation of myofibroblasts, vascular rarefaction, increased production of interstitial matrix, and finally resulting in the progression of fibrosis (Grgic et al., 2012). Upon severe injuries, some TECs will arrest in the G2/M

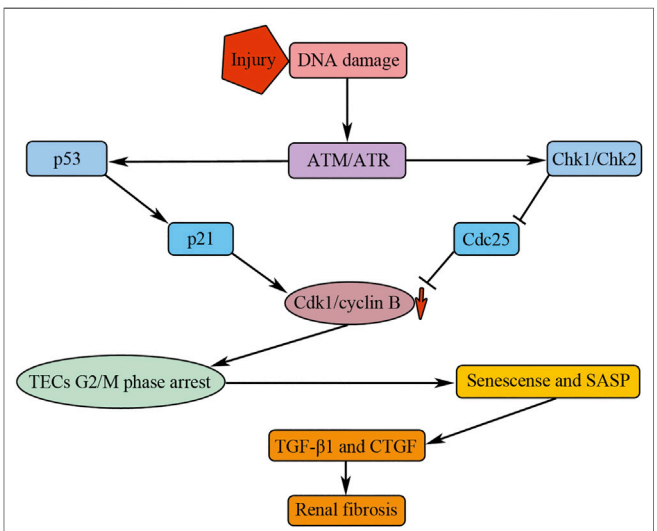

FIGURE 4 | G2/M phase arrest of TECs mediates renal fibrosis by secreting profibrotic cytokines. Injuries result in the activation of ATM/ATR, which could promote the activation of Chk1/Chk2 and p53. p53 and Chk1/Chk2 could induce downregulation of Cdk1/cyclin B kinase activity by affecting p21 and Cdc25, respectively. The downregulation of Cdk1/cyclin B kinase activity results in TECs G2/M phase arrest; the arrested cells undergo senescence and manifest senescence-associated secretory phenotype (SASP), causing renal fibrosis by secreting profibrotic cytokines, such as TGF-β1 and CTGF.

phase of cell cycle and mediate renal fibrosis by secreting profibrotic cytokines such as CTGF and TGF-β1 (Yang et al., 2010; Cosentino et al., 2013; Tang et al., 2013; Zhao et al., 2020) (**Figure 4**). Drug intervention causing an increasing TECs G2/M phase arrest after AKI could aggravate kidney fibrosis, whereas interventions which reduce TECs G2/M phase arrest result in less renal fibrosis (Yang et al., 2010; Cosentino et al., 2013; Gasparitsch et al., 2013; Tang et al., 2013; Zhao et al., 2020). Hence, TECs G2/M phase arrest properly is a novel histologic biomarker of renal fibrosis.

Many factors could influence TECs G2/M phase arrest and therefore affect renal fibrosis outcome. During the process of renal fibrosis, TECs undergo a partial EMT program; during this process, TECs still keep associated with their basement membrane but can express cellular markers of both epithelial and mesenchymal cells (Lovisa et al., 2015). During fibrotic injury, the partial EMT program led to a TEC G2/M phase arrest of the cell cycle; inhibition of partial EMT program can alleviate TEC G2/M phase arrest and attenuate interstitial fibrosis (Lovisa et al., 2015). Specific knockout of Atg5 gene in mouse TECs can destroy TEC autophagy and aggravate the TEC G2/M phase arrest, leading to aggravation of renal fibrosis upon kidney injury (Li et al., 2016). Specific knockout Numb in mouse TECs can alleviate TEC G2/M phase arrest and renal fibrosis induced by unilateral ureteral obstruction or unilateral ischemic renal injury (Zhu et al., 2016). Conventional knockout of Cyclin G1 can alleviate TEC G2/M phase arrest and renal fibrosis induced by severe kidney injury in mice (Canaud et al., 2019). Inhibition phosphorylation of 4E-BP1, a downstream effector molecule of mTORC1 pathway, can alleviate the TECs G2/M phase arrest and renal fibrosis (Sun et al., 2019). Therefore, TECs G2/M phase arrest is a common characteristic of renal fibrosis induced by various injures; however, the specific mechanism causing TECs G2/M phase arrest is still unclear.

Cell cycle regulators probably involve in the process of TECs G2/M phase arrest. Injuries can induce DNA damage of TECs, causing the activation of ATM/ATR (Kishi et al., 2019), which can further activate their downstream target genes Chk1/Chk2 and p53 (Reinhardt and Yaffe, 2009). Chk1/Chk2 inhibits Cdc25, the activator of Cdk1/cyclin B, causing G2/M phase arrest by downregulating the Cdk1/cyclin B kinase activity (**Figure 4**). p53 could activate its downstream target gene p21, causing G2/M phase arrest by downregulating the Cdk1/cyclin B kinase activity (Vogelstein et al., 2000) (**Figure 4**). Roscovitine, an inhibitor of Cdks, have been found to have the anti-fibrosis function (Steinman et al., 2012). In high glucose cultured HK-2 cells, a human proximal renal tubular epithelial cell line, roscovitine, can successfully reduce α-SMA expression and increase E-cadherin expression, suggesting that it can inhibit the EMT process of TECs (Wang et al., 2019). Further studies showed that roscovitine inhibited TECs EMT by inhibiting the upregulation of TGF-β1/p38MAPK pathway in HK-2 cells cultured with high glucose (Wang et al., 2019). In diabetic mice, administration of roscovitine can remarkably alleviate renal functional and histological injuries through inhibiting the expression of collagen, α-SMA, and TGF-β1 (Wang et al., 2019).

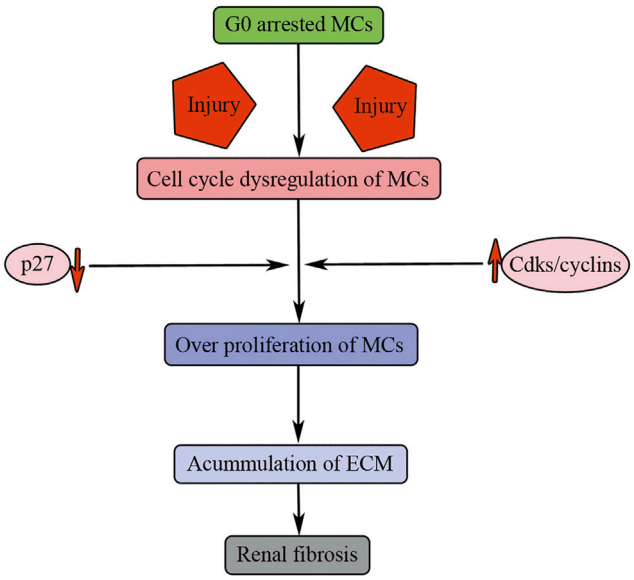

FIGURE 5 | Cell cycle dysregulation of MCs results in renal fibrosis. Injuries result in G0 arrested MC entry into cell cycle. p27 downregulation and Cdks/cyclin upregulation induce overproliferation of MCs, accumulation of ECM, and finally causing renal fibrosis.

Consequently, although the TECs do not abundantly transdifferentiate into myofibroblasts, the G2/M phase arrested TECs could mediate renal fibrosis through paracrine pathway that is reinforced by a state of senescence characterized by the production of profibrotic cellular factors (Canaud et al., 2019) (**Figure 4**). In addition, p21 overexpression could induce the senescence of TECs which is involved in early stage of diabetic nephropathy in streptozotocin-induced diabetes 1 model (Kitada et al., 2014). In contrast to protective effect of p21 in AKI, the continued p21 activation may result in renal fibrosis, as the p21 knockout mice did not develop chronic kidney failure after 5/6 nephrectomy (Megyesi et al., 1999). It has been suggested that deletion of p21 allows hyperplastic compensatory proliferation of residual kidney tissue and prevents maladaptive hypertrophy (Megyesi et al., 1999).

Cell Cycle Dysregulation of Mesangial Cells and Renal Fibrosis

Mesangial cells (MCs) offer structural support for the glomerular tuft partially through the secretion and maintenance of the extracellular matrix. There is less MC proliferation in the adult mammalian healthy kidney, probably because under normal conditions MCs are either not exposed to mitogens or protected from them by inhibitory factors. Mature MCs remain in the G0 quiescent state by upregulation of the cell cycle inhibitor p27 (Combs et al., 1998). The initial of MC division could be stimulated by mitogen of injuries accompanied by a decrease in the expression of p27 (**Figure 5**).

In response to all kinds of injury stimulation, the quiescent MCs were stimulated to proliferation, resulting in an increasing

number of MCs and persistent cellular matrix accumulation, and finally causing glomerulosclerosis. These characteristics could be found in diseases such as IgA nephropathy, lupus nephritis, membranoproliferative glomerulonephritis, and diabetic nephropathy. In the model of mesangial proliferative glomerulonephritis, Thy1 nephritis, the expression of cyclin D, cyclin E, cyclin A, CDK2, and CDK4 were increased during the phase of marked mesangial proliferation (Schöcklmann et al., 1999).

MC proliferation and the resulting matrix formation are the major characteristics of glomerular injury and fibrosis (Johnson, 1994; Shankland et al., 1996). Roscovitine, which can block the activity of CDK2, has been studied in experimental glomerulonephritis (Pippin et al., 1997b). It can decrease MC proliferation and the resulting matrix production (such as collagen type IV, laminin, and fibronectin), leading to alleviated renal fibrosis and improved renal function, suggesting that inhibiting MC overproliferation may be a useful therapeutic targeting for renal fibrosis (Pippin et al., 1997a). In the Thy1 model, accompanied by the onset of MC proliferation, the expression of p27 strikingly decreased. If experimental nephritis is induced in p27 knockout mice, the MC proliferation initial earlier and the proliferative response is bigger, accompanied by prominent extracellular matrix (ECM) accumulation (Marshall and Shankland, 2006). T-type calcium channels play an essential role in MC proliferation by targeting the G1/S checkpoint. Blocking of these channels by pharmacological drugs could inhibit MC proliferation by arresting them in G1 phase and alleviates glomerular damage in Thy1 model (Cove-Smith et al., 2013).

Statins, the cornerstone hormone drugs to manage dyslipidemia, have been found to have lipid-independent benefits against renal injury and fibrosis (Kostapanos et al., 2009; Chen et al., 2019). For example, statins can inhibit mesangial expansion, and the resulting extracellular matrix accumulation in the glomeruli of diabetic animal kidneys, and therefore attenuate renal fibrosis (KIM et al., 2000; Fujii et al., 2007). Moreover, *in vitro* studies had found that statins can inhibit the proliferation of cultured MC, which focal or diffuse proliferation is a typical characteristic of glomerular pathology (O'Donnell et al., 1993; Terada et al., 1998; Danesh et al., 2002). Lovastatin can dose-dependently inhibit DNA replication and proliferation of rat MCs, which can be reversible by added mevalonate (O'Donnell et al., 1993). Further study showed that the effect of lovastatin to inhibit MC proliferation through upregulation of a CDK inhibitor, p27Kip1, protein levels, as knockdown of p27Kip1 showed strikingly decreasing lovastatin-induced cell cycle arrest (Terada et al., 1998). Another study found that the proliferation of MCs induced by high glucose was accompanied by the decrease in p21 protein expression and the increase in CDK4 and CDK2 kinase activities. Simvastatin can increase p21 protein expression and downregulate CDK4 and CDK2 kinase activities (Danesh et al., 2002). These studies suggest that statins can inhibit renal injury and renal fibrosis independently of their cholesterol-lowering effect, such as inhibition of MC proliferation.

Therefore, MC overproliferation after injuries can contribute to renal fibrosis through persistent accumulation of ECM. Under normal physiological conditions, mammalian mature MCs are arrested in the G0 phase of cell cycle through upregulation of the CDK inhibitor p27 (**Figure 5**). However, upon injuries, the quiescent MCs are stimulated to overproliferation causing persistent cellular matrix accumulation, and finally causing renal fibrosis (**Figure 5**).

Cell Cycle Dysregulation of Fibroblasts and Renal Fibrosis

Renal fibrosis is characterized by deposition of extracellular matrix in the potential space between tubules and peritubular capillaries. It is generally believed that myofibroblasts are the primary extracellular matrix-producing cells that produce a fair amount of interstitial matrix components, such as fibronectin and type I and type III collagens. Considering this, one of the key problems in the field is to study the origin of these matrix-producing myofibroblasts (Grande and Lopez-Novoa, 2009; Meran and Steadman, 2011; Schrimpf and Duffield, 2011).

It has been supposed that myofibroblasts have at least five different sources in mammalian fibrotic kidney, including activation of interstitial fibroblasts, differentiation of pericytes, translation of tubular epithelial cells and endothelial cells and recruitment of circulating fibrocytes (Barnes and Gorin, 2011). It has been believed that matrix-producing myofibroblasts mostly derive from resident fibroblasts through activation after kidney injury (Hewitson, 2009). Although this perception has recently been challenged, it is generally accurate (Strutz and Zeisberg, 2006; Grande and Lopez-Novoa, 2009). Recently, Kuppe et al. have found that distinct subpopulations of pericytes and fibroblasts were the main sources of myofibroblasts during human kidney fibrosis through the single-cell RNA sequencing technology (Kuppe et al., 2021). Moreover, they also showed that NKD2 may be a myofibroblast-specific target in human kidney fibrosis, as overexpression of NKD2 in human fibroblast cell line promoted the expression of ECM molecules, whereas knockout of NKD2 markedly downregulated the expression of ECM molecules (Kuppe et al., 2021). Blocking fibroblast to myofibroblast transformation can effectively inhibit renal fibrosis (Gerarduzzi et al., 2017; Li N. et al., 2020), suggesting that fibroblast is the major source of myofibroblast.

Under normal physiological conditions, renal fibroblasts are located in the interstitial space between the capillaries and the tubular epithelia and take shape a network in the whole renal parenchyma, so as to stabilize tissue structure (Kaissling and Le Hir, 2008). These cells are stellate shaped and contain abundant rough endoplasmic reticulum, collagen-secreting granules and actin filaments. They involve multiple cell processes, which keep them in contact with the tubular and capillary basement membranes (Kaissling and Le Hir, 2008). Under normal physiological conditions, renal fibroblasts stay in quiescent G0 phase of cell cycle and express CD73 (also known as ecto-5′-nucleotidase) in their plasma membrane and produce erythropoietin (Kaissling and Le Hir, 2008; Paliege et al., 2010). PDGFRβ and FSP1 were also expressed in fibroblasts

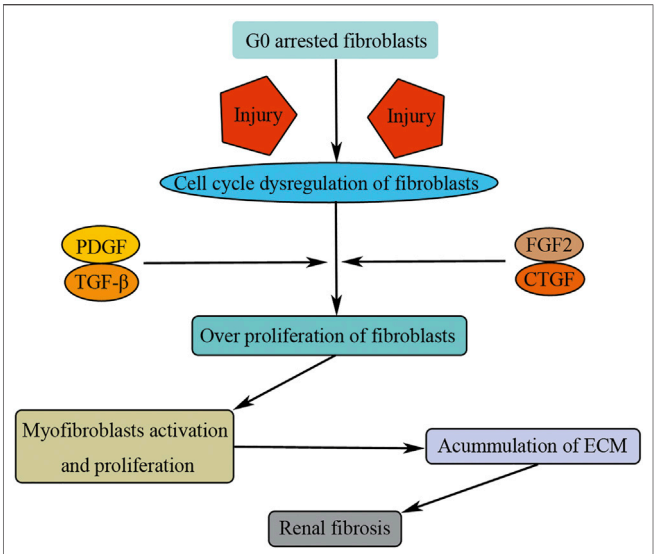

FIGURE 6 | Cell cycle dysregulation of fibroblasts induces renal fibrosis. Injuries result in G0 arrested fibroblast activation and entry into cell cycle. Growth factors (PDGF, TGF-β, FGF2, and CTGF) promote fibroblast overproliferation and translate to myofibroblasts, causing renal fibrosis by consistent accumulation of ECM.

(Floege et al., 2008; Grigorian et al., 2008; Boye and Mælandsmo, 2010; Boor and Floege, 2011). Fibroblasts control interstitial matrix physiological homeostasis by producing a few ECM components in normal conditions. However, after injury, fibroblasts are activated and acquiring the ability to proliferate and translating to myofibroblast that expressing α-SMA, generating a huge amount of ECM components. Myofibroblasts also retain FSP1 and PDGFRβ expression and express vimentin *de novo*.

Activated fibroblasts possess two typical characteristics, that is, proliferation and myofibroblastic activation. The latter manifests as the expression of α-SMA and the production of extracellular matrix. Fibroblasts and myofibroblasts are overproliferated under the stimulus of cytokines, which results in the increasing number of myofibroblasts and accumulation of ECM in injured kidney. Growth factors such as PDGF, TGF-β, FGF2, and CTGF are well-known mitogens promoting fibroblast overproliferation (Strutz et al., 2000; Böttinger, 2007; Phanish et al., 2010; Ostendorf et al., 2012) (**Figure 6**). Besides these well-known cytokines, tissue-type plasminogen activator can also promote fibroblast overproliferation and myofibroblastic activation by recruitment of β1 integrin (Hu et al., 2007; Hu et al., 2008; Hao et al., 2010; Lin et al., 2010).

Therefore, fibroblasts are the main source of matrix-producing myofibroblasts. Under normal physiological condition, fibroblasts stay in quiescent state of G0 phase. Upon injuries, fibroblasts are activated and overproliferated, translating to myofibroblasts, causing renal fibrosis by consistent accumulation of ECM (**Figure 6**). However, the specific mechanism as to how the fibroblast is activated and overproliferated upon injuries remains unclear, and other types of renal cells, such as TECs, play an essential role during this process (Li X. et al., 2020). For example, tubule-derived exosomes can promote renal fibrosis through promoting fibroblast activation and proliferation (Liu et al., 2020).

CONCLUSIONS AND PERSPECTIVES

Mammalian cell cycle is tightly regulated by cell cycle regulators, such as CDKs, cyclins, and CKIs. These cell cycle regulators make sure that cell cycle is regulated precisely, which is essential for mammalian renal cell homeostasis and keeping normal renal function. However, severe or repeated injuries could induce dysregulation of cell cycle manifesting as cell cycle arrest or overproliferation, both of which are closely related to renal fibrosis.

Under normal physiological conditions, most of the renal cells are quiescent cells, arresting in G0 stage; the turnover of renal cells is very low. On one hand, mild injuries could stimulate the proliferation of renal cells so as to compensate the renal cells loss and restore renal function; on the other hand, more severe or repeated injuries lead to cell cycle dysregulation of renal cells, promoting renal fibrosis (**Figure 7**). Cell cycle dysregulation of podocytes manifests as cell cycle entry but could not finish mitosis; cells may be arrested in M phase of cell cycle, causing podocyte loss through mitosis catastrophe, and finally resulting in renal fibrosis. Cell cycle dysregulation of TECs manifests as TECs G2/M phase arrest; the arrested cell undergoes senescence, promoting renal fibrosis by secreting profibrotic cytokines. Cell cycle dysregulation of MCs manifests as overproliferation resulting in persistent cellular matrix accumulation, and finally causing glomerulosclerosis. Cell cycle dysregulation of fibroblasts manifests as overproliferation and activation resulting in renal fibrosis by increasing the number of myofibroblasts and accumulation of ECM. Therefore, cell cycle dysregulation of renal cells may be a perfect target for the treatment of renal fibrosis.

Although cell cycle regulators play essential roles in renal fibrosis, very few cell cycle regulators have been researched in renal fibrosis. Indeed, most of the researched regulators were CKIs, such as p53 and its target protein p21. p21 was upregulated in the kidney after injury (Megyesi et al., 1996). Compared with wild-type mice, p21 knockout mice manifested as more severe kidney dysfunction, more severe kidney damage, and higher rate of mortality rate after AKI (Megyesi et al., 1998; Nowak et al., 2003). In contrast, p21 knockout mice had less histologic lesions after sub-total nephrectomy with enhanced tubular proliferation compared with wild-type mice (Megyesi et al., 1999). p53 was also found to be upregulated in the kidney after injury and its inhibition or gene deletion reduced kidney lesions and renal fibrosis (Wei et al., 2007; Molitoris et al., 2009; Yang et al., 2010; Zhou et al., 2010; Ying et al., 2014; Liu et al., 2019c; Tang et al., 2019). Besides the p53 and p21, there is very little information available about other cell cycle regulators in renal fibrosis. The precise role of each CDK, cyclin, and CKI in different renal cells during renal fibrosis deserves a good deal more investigation.

ACE inhibitors, statins, anticoagulants, glucocorticoids, cyclophosphamide, azathioprine, and mTOR inhibitors are the common medicines used in kidney diseases; most of them can affect cell proliferation in some way. However, the role of these

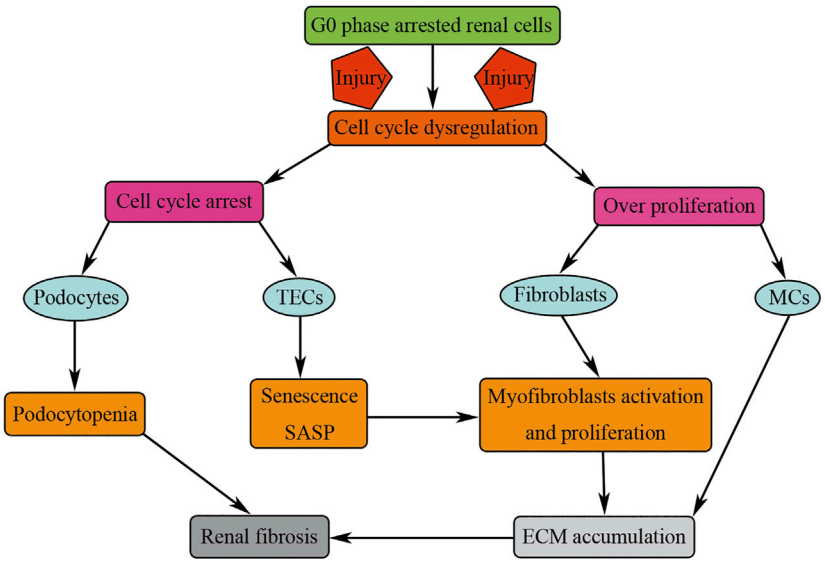

FIGURE 7 | Cell cycle dysregulation of renal cells results in renal fibrosis. Injuries result in cell cycle dysregulation manifested as cell cycle arrest (podocytes and TECs) or overproliferation (fibroblasts and MCs). Cell cycle arrest or overproliferation of renal cells results in renal fibrosis by podocytopenia and consistent accumulation of ECM.

medicines in cell cycle progression of specific renal cells is largely unknown. Statins can inhibit MC proliferation by suppression of the Rho and Ras pathway (Kostapanos et al., 2009). Rapamycin, the inhibitor of mTOR, can mitigate the hypertrophy in diabetes model through downregulation of p70S6 kinase pathway (Sakaguchi et al., 2006). ACE inhibitors can decrease abnormal division of renal progenitor cells by deactivation of NCAM+ and thus alleviate lesions of hyperplastic in podocytopathies; however, this drug can also promote regeneration of glomeruli by the transcription factor C/EBPδ (Benigni et al., 2011; Rizzo et al., 2016). Upon severe injuries, MCs and fibroblast overproliferation promote renal fibrosis; therefore, inhibiting MCs and fibroblast proliferation by drugs is beneficial for the prevention of renal fibrosis. However, these drugs can also inhibit TECs' appropriate proliferation, therefore inhibiting the kidney function restoration. In other words, drugs that inhibit cell proliferation could be harmful for some type of renal cells and aggravate renal fibrosis. Therefore, more additional researches should be conducted to elucidate the function of these drugs in cell cycle progression of specific renal cells and in renal fibrosis.

In conclusion, recent progression in the pathophysiology of renal fibrosis has emphasized the important roles of cell cycle dysregulation of renal cells in renal fibrosis. Although there are a lot of questions to be clarified, these findings open new avenues to better understand, prevent, and slow down renal fibrosis.

AUTHOR CONTRIBUTIONS

J-XT and H-FL formulated and conceived of this study. Y-SW, SL, D-YL, J-HW, J-XT, and H-FL wrote the manuscript. All authors helped to interpret results and approved the final version of the manuscript.

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