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Review

Cellular senescence in cardiac diseases

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ABSTRACT

Replicative capacity of somatic cells is limited. It indicates that aging also develops at the cellular level, and this is described as "cellular senescence". Senescent cells become flattened, enlarged, and irreversibly lose capacity for proliferation. Lack of specific and conclusive markers for cellular senescence makes it difficult to comprehensively define and understand this biological process especially *in vivo*. Molecules including p53, p21, $p16^{Ink4a}$, p38MAPK, and γH2AX, telomere attrition, enhanced signals for SA-β-gal, etc. are widely used to detect senescent cells, but these are indirect indicators of cellular senescence, and biological markers reflecting direct evidence need to be established. Genetic profiles are altered in senescent cells, letting these cells secrete pro-inflammatory molecules. Aging or age-related disorders including heart failure and atherosclerotic diseases link with an accumulation of cells undergoing cellular senescence in cardiovascular systems including heart and vessels. Senescent cells become pathogenic in most cases by mediating chronic sterile inflammation and tissue remodeling. A recent conceptual as well as technical breakthrough in this research area is "senolysis", meaning the specific elimination of senescent cells. Genetic as well as pharmacological models with senolysis contributed to reverse aging phenotypes and ameliorated pathologies in age-related disorders without enhancing the risk of tumorigenesis, and opened a new avenue for aging research. Several compounds are identified as senolytics, and some are already tested in clinical settings. It was recently reported that senolysis reverses aging phenotype in cardiovascular disorders. Generating therapies targeting suppression or elimination of senescent cells would inhibit the progression of undesirable aspects of aging, and become promising therapies for cardiac diseases.

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Introduction

Chronological aging is characterized by changes of intercellular communication, genomic instability, epigenetic alterations, telomere attrition, loss of proteostasis, mitochondrial dysfunction, stem cell exhaustion, deregulation of nutrient sensing, and cellular senescence [1]. All these contribute to deterioration at organ, cellular, and molecular levels, leading to a decline of physiological activity, leaving organisms prone to death. Aging *per se* is a physiological process, however, it associates with several undesirable aspects, and predisposes us to age-related diseases. Aging and age-related disorders progress through an integration of complex biological processes, and do not allow a simple approach to understand the whole picture, however, evidence indicates the central roles of cellular senescence in the pathogenesis of these conditions. Prevalence of age-associated diseases, including atherosclerotic disorders or heart failure increases with chronological aging, and cells positive for senescent markers are now well recognized to have causal roles for the progression of pathologies in these age-related diseases [2–4]. *In vitro* studies showed that exposure of young somatic cells to senescent cells promotes senescence of the young cells, and this is described as the "bystander effect" [5]. Pharmacological or genetic depletion of senescent cells contributed to reverse aging phenotype, and suppressed pathologies in chronological as well as age-related disease models [6–8]. In this review article, we would like to delineate the role of cellular senescence and the related molecules in cardiac diseases, and discuss the potential of therapies targeting senescent cells.

Molecular mechanisms of cellular senescence

It was previously reported that replicative capacity of fibroblasts was limited [9]. This indicated that aging also develops at the cellular level, and now this is described as "cellular senescence". Senescent cells become enlarged with a flattened morphology, and show irreversible loss of proliferative potential. Alteration in the expression of genetic profiles in these cells leads to secretion of pro-inflammatory molecules (known as the senescence-associated secretory phenotype (SASP)) [10]. Senescent cells accumulate in various organs of animal models or humans who are elderly and/or have age-related disorders, and thought to become pathogenic through introducing chronic sterile inflammation and tissue remodeling. There are two types of cellular senescence. As Hayflick et al. [9] previously reported, somatic cells have a limited proliferative capacity and eventually enter a state of irreversible growth arrest termed "replicative senescence." Telomeres locate at each end of chromosome, and characterized as a region of repetitive nucleotide sequences, AGGGTT in vertebrates. Telomeres replicate incompletely during cell division, resulting in telomere attrition. Telomeres have critical roles for chromosomal stability and DNA replication. For this reason, when telomere shortening exceeds the physiological range, it is recognized as DNA damage and triggers replicative cellular senescence, mainly via the p53 or $p16^{Ink4a}$ signaling pathways. Another type of cellular senescence is described as "stress-induced premature senescence". Premature senescence develops through various external as well as internal stress signals, and these include oxidative stress or irradiation, constitutive activation of mitogenic stimuli, oncogenic activation, and metabolic stress. The p53 or $p16^{Ink4a}$ signaling pathways also mediate this type of cellular senescence. Preference for p53 or $p16^{Ink4a}$ signaling pathway varies among species, and depends on the cell types [11,12]. In human cells, telomere dysfunction activates either p53 or $p16^{Ink4a}$ signaling, however, in rodent cells p53 signaling seems to be the only activated pathway [13]. p53 signaling is primarily triggered for activation by DNA damage and telomere dysfunction, while $p16^{Ink4a}$ signaling is primarily linked to mitogenic stress, and general cellular stress [11,12,14]. It is now well recognized that the vast majority of stressors that induce cellular senescence activate either or both the p53/p21 or $p16^{Ink4a}$/retinoblastoma protein pathways [4].

Markers of senescent cells

As already described, most senescence-inducing stressors activate either the p53/p21 or $p16^{Ink4a}$/retinoblastoma protein pathways, however, it needs to be noted that activation of these signaling pathways does not provide conclusive evidence that cells are senescent [15]. Some proliferating cancer cells express high levels of $p16^{Ink4a}$ [16]. p21 is also involved in "transient" cell cycle arrest in response to acute cell damage [17]. Phenotype of senescent cells has multiple variations, and cellular senescence needs to be defined through various combinations of markers. To date, several markers are used to detect senescent cells indirectly. Senescence-associated beta-galactosidase (SA-β-gal) activity at pH6 is broadly used to recognize cells as senescent. However, we here note that SA-β-gal activity is also known to increase in fibroblasts cultured under serum starvation [18]. In addition to p53, p21, and $p16^{Ink4a}$, other well-known markers of cellular senescence are high levels of p38 mitogen-activated protein kinase (p38MAPK) or γH2AX, reflecting the activation of DNA damage responses. Senescence-associated heterochromatin foci (SAHF), and senescence-associated distention of satellites (SADs) are also considered to be indicators of cellular senescence [19,20]. It was previously shown that irreparable telomere damage develops in the absence of telomere attrition. Presence of DNA damage at telomeres were described as telomere-associated foci (TAF), and detected as colocalization of γH2AX, p53-binding protein 1 (53BP1) with telomeres. In fibroblasts, irradiation or H_2O_2 administration increased TAF, and this was also shown to increase in liver, gut, and heart with aging [6,21]. Exploring biological markers reflecting direct evidence of cellular senescence continues to be an important research topic (Fig. 1).

Roles of cellular senescence related molecules in cardiac disease

Accumulation of evidence indicates the close connection between pathways involved in cellular senescence with cardiovascular disorders. In humans with end-stage heart failure, p53 level and apoptosis were reported to increase in myocardium [22]. Patients with hypertrophic cardiomyopathy or dilated cardiomyopathy showed higher p53 expression in heart compared to non-failing heart [23,24]. In a murine left ventricular pressure overload model, cardiac-p53 level was increased, and this led to suppression of angiogenesis in the myocardium, promoting tissue hypoxia and cardiac dysfunction [25]. Shorter peripheral blood leukocyte telomere length (LTL) predicted adverse cardiovascular events in coronary artery disease patients [26,27], and recently it was shown that endurance training and interval training, but not resistance training, increased telomerase activity and telomere length in circulating leukocytes [28]. Insulin-like growth factor-binding protein-7 (IGFBP7) inhibits cell proliferation through G1 phase cell cycle arrest, and is considered as a protein member of senescence secretomes. IGFBP7 associates with tissue aging and obesity, and high circulating IGFBP7 was reported to link with poor prognosis in patients with heart failure with preserved ejection fraction (HFpEF) [29]. Interestingly, it was recently reported that neprilysin inhibition reduced circulating levels of this pro-senescent molecule [30]. Several representative cells including cardiomyocytes, endothelial cells, fibroblasts, and progenitor cells

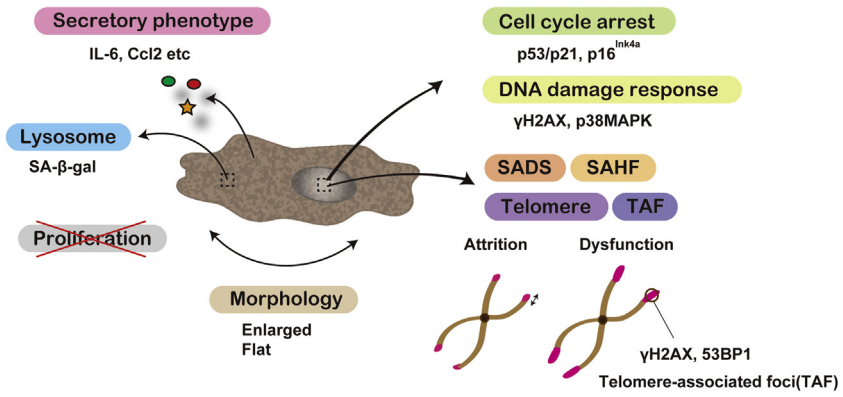

Fig. 1. Markers of senescent cells. Indirect markers characterizing cellular senescence are as follows, and these vary among tissues and cells. (1) Enlarged and flat morphology, (2) cell cycle arrest (p53/p21, $p16^{Ink4a}$ signals become high), (3) DNA damage response (γH2AX, p38 MAPK signals become high), (4) irreversible termination of proliferation, (5) high senescence-associated beta-galactosidase (SA-β-gal) activity, (6) telomere attrition, (7) telomere dysfunction, (8) senescence-associated distention of satellites (SADs), and (9) senescence-associated heterochromatin foci (SAHF).

are involved in the maintenance of cardiac homeostasis, and roles of cellular senescence-related molecules in these cells are discussed next.

Cardiomyocytes

Cellular senescence was traditionally recognized as a machinery to inhibit uncontrolled replication in proliferative cells, but nowadays it is thought that post-mitotic cells such as Purkinje neurons also develop a senescent-like phenotype. For example, murine cortical and Purkinje neurons were reported to exhibit an increase in γH2AX, IL-6, 4-HNE, p38MAPK, and SA-β-gal levels with aging [31]. Shortly after birth, cardiomyocytes exhibit cell cycle arrest due to the activation of DNA damage response introduced through exposure to higher concentration of oxygen in the postnatal environment [32]. Adult cardiomyocytes were long thought to be terminally differentiated post-mitotic cells, however, accumulating evidence indicates these cells retain proliferative capacity. It was previously reported that in humans, cardiomyocyte turnover was at a rate of <1% per year [33], and this was demonstrated to decline with aging both in humans and mice [34,35]. The underlying machinery of diminished cardiomyocyte turnover with aging is yet to be defined, and whether this is attributable to cardiomyocyte senescence is not clear due to the lack of specific senescence markers. In mice, it was reported that chronological aging links with an increase in cardiomyocyte size, together with reactive oxygen species (ROS) production, telomere attrition, and high level of p53 or $p16^{Ink4a}$ expression. In this study, compared to young mice (4 months of age), aged mice (20–22 months of age) exhibited increased left ventricular weight and cardiomyocyte volume, and showed reduction in cardiomyocyte number, together with reduced ventricular function, indicating the pathological roles of cardiomyocyte senescence in the aged heart [36]. In cardiomyocytes, toxic reagents such as doxorubicin administration was shown to upregulate $p16^{Ink4a}$, p21 and SA-β-gal level [37]. In humans, patients with heart failure demonstrated shorter cardiomyocyte telomeres compared with nonfailing donors. Hypertrophic cardiomyopathy (HCM) patients with reduced systolic function exhibited cardiomyocytes with shortest telomeres in association with resident DNA damage (as analyzed with γH2AX or 53BP1 staining), and patients with ischemic cardiomyopathy (ICM) also showed shorter telomere length. Cardiomyocytes from idiopathic cardiomyopathy (IDCM) patients exhibited a trend to reduced telomere length, but this did not reach statistical significance. Interestingly, telomere attrition did not develop in vascular smooth muscle cells from failing hearts of HCM, ICM, and IDCM patients, and this was comparable with non-failing donors. In this study, it was also shown that telomere shortening in cardiomyocytes did not develop with aging in non-failing donors [38]. Whether cardiomyocyte senescence exists *in vivo* was controversial, but recently, it was reported that length-independent telomere damage drives post-mitotic cardiomyocyte senescence [6]. In a chronologically aged murine model, TAF positive cardiomyocytes increased with aging (comparison of mice 3, 15, and 30 months of age). Interestingly, γH2AX signal did not increase in cardiomyocytes, but increased only in telomeres. Irradiation increased TAF level in mouse embryonic cardiomyocytes, rat neonatal cardiomyocytes, and H9C2 myoblasts. In cardiomyocytes collected through Langendorff heart perfusion, transcripts $p16^{Ink4a}$ and p21 were increased, and staining studies showed p21 and SA-β-gal signal increased with aging in cardiomyocytes in the heart. In right atrial appendage of humans, TAF signal also increased in cardiomyocytes in individuals aged >70 years, compared to <70 years of age. In this study, senescent cardiomyocytes were shown to contribute for non-canonical senescence-associated secretory phenotype, and promoted myofibroblast differentiation in fibroblasts and cardiomyocyte hypertrophy in mice [6]. Presence of senescence-like phenotype in cardiomyocytes was studied in atrium in humans, and whether this develops in human left ventricle remains an open question to be explored. Recently, an interesting paper showed the bidirectional role of p53 in cardiomyocytes. Mak et al. generated cardiomyocyte specific p53 knockout model by crossing MHC-$MerCreMer^{+/-}$ with $p53^{flox/flox}$ mice (here we describe as "CM-p53KO"). CM-p53 KO mice showed enhanced cardiac as well as cardiomyocyte hypertrophy, and this associated with reduced cardiac systolic dysfunction when these mice became 6 months-old. In contrast, CM-p53 KO exhibited improved cardiac function together with reduced cardiac as well as cardiomyocyte hypertrophy in left ventricular pressure overload model. These results indicated that p53 signal in cardiomyocytes contributed for inhibition of pathological cardiac hypertrophy in aging model, but augmented this in left ventricular pressure overload, showing that the roles of p53 in cardiomyocytes is context dependent [39].

Endothelial cells

Endothelial cells in aged-sedentary individuals (around 60 years old) were reported to express higher protein level of p53, p21, and $p16^{Ink4a}$, and this was also shown to reduce in aged exercising adults (around 57 years old) [40]. Preclinical studies indicated the pathological roles of endothelial cell-senescence in age-related

disorders including obesity, diabetes, and heart failure [41,42]. Heart failure can be characterized into two types depending on the level of systolic function. One is described as heart failure with reduced ejection fraction (HFrEF), another is classified as HFpEF, and both types of heart failure are prevalent among elderly persons [43]. In the failing heart, chronic sterile inflammation develops, and this is well recognized to promote cardiac remodeling [44]. In a murine left ventricular (LV) pressure overload model, activation of p53 signaling in vascular endothelial cells was reported to induce cardiac inflammation and remodeling [41]. In response to LV-pressure overload, p53 level increased in endothelial cells of capillaries in left ventricle, and had causal roles for the elevated expression of intercellular adhesion molecule (ICAM)-1 by these cells. This contributed to promoting inflammation in the failing heart in association with reduced systolic function. Another study showed depletion of p53 in endothelial cells ameliorated capillary rarefaction, improved cardiac function, and suppressed cardiac fibrosis and remodeling [45]. HFpEF occurs in approximately half of all patients with heart failure. Inflammation in coronary microvasculature is now thought to have central roles in the pathogenesis of HFpEF [46], and it was recently indicated that cellular senescence in endothelial cells may also be involved. When senescence-accelerated mice were fed a high-fat high-salt diet, endothelial cell senescence developed in cardiac tissues, and this coincided with the typical hemodynamic and structural changes of HFpEF [47]. Administration of palmitate, representing a metabolic stress, led to Sirt1 inactivation and increased senescent markers including p53, $p16^{Ink4a}$, and SA-β-gal in HUVECs [48]. Given that aged and/or obese population has higher prevalence for HFpEF, inhibition of endothelial cell senescence pathway may become a next generation therapy for this untreatable disorder.

Fibroblasts

Fibroblasts are considered to represent chief cardiac cell component, accounting for 27% of all cardiac cells in mice and 64% in rats. In humans, non-cardiomyocytes (mainly fibroblasts) are reported to account for 72% of total cells [49,50]. Studies indicate fibroblasts contribute for maintaining homeostasis in heart under physiological as well as stressed conditions. Aging was reported to associate with the presence of fibroblasts containing X-Gal crystals in pericardium [7]. During aging, visceral adipose tissue-derived osteopontin increased in plasma. Systemic genetic depletion of osteopontin, visceral adipose tissue depletion, or pharmacological osteopontin inhibition led to a significant increase in $p16^{Ink4a}$ level in cardiac fibroblasts, and contributed to the suppression of age-induced cardiac fibrosis. This indicated that osteopontin inhibited cellular senescence in cardiac fibroblasts, having causal roles for the augmentation of fibrosis in cardiac tissue. This showed fibroblast senescence associates with healthy cardiac aging [51]. Another study reported that senescence-associated genes including p53, p21, and ataxia telangiectasia mutated (ATM) increased after myocardial infarction in both mouse and human hearts. Heterozygous ATM systemic depletion resulted in reduced SA-β-Gal or p53 signal in cardiac fibroblasts. Suppression of these senescence markers in fibroblasts contributed to enhance fibrosis in non-infarct area (this was comparable between genotypes in infarct area), and reduced systolic function in myocardial infarction model [52]. These preclinical studies indicate that inhibition of cellular senescence in fibroblasts becomes pathological in cardiac tissues. Whether this would be the case in humans is a critical question, and raises potential issues for therapies based on non-targeted delivery of senescence-suppressants which may be developed in the near future.

Progenitor cells

Cardiosphere-derived cells (CDCs) are progenitor cells in cardiac tissue and have a potential to differentiate into three types of cells present in this organ. These are cardiomyocytes, endothelial cells, and smooth muscle cells. CDCs were reported to prevent adverse cardiac remodeling, characterized as reduced fibrosis, ROS, and inflammation. This contributed to suppress the progression of systolic dysfunction, and improved survival in dilated cardiomyopathy mouse model [53]. CDCs were also reported to reverse HFpEF in Dahl salt-sensitive rats by suppression of fibrosis and inflammation in heart [54]. Direct injection of CDCs extracted from young Fisher rats (around 4 months old) into LV cavity of old Fisher rats (around 22 months old) suppressed cardiac fibrosis, improved diastolic function, ameliorated LV hypertrophy in aged rats. In this paper, *in vitro* studies showed young CDCs, extracted from human donors <2 years of age, were reported to secrete exosomes (CDC-XO), contributing to the elongation of telomeres, together with suppression of SA-β-gal signal in CDCs obtained from >55-year old human donors [55]. Cardiac progenitor cells (CPCs) are another type of progenitor cell in the heart [56]. More than half of cardiac CPCs, defined as $Sca\text{-}1^{pos}/c\text{-}kit^{pos}/CD31^{neg}/CD45^{neg}/Tryptase^{neg}$, isolated from aged (>70 years old) subjects showed higher levels of $p16^{Ink4a}$, SA-β-gal, and γH2AX, had telomere attrition, and obtained SASP phenotype. Direct injection of senescent CPCs into left ventricle, led to reduced reparative potential in a murine myocardial infarction model compared to the introduction of non-senescent CPCs injection. This indicated aged human heart contains high proportion of senescent-CPCs, possibly contributing to the progression of cardiac dysfunction [57]. In castaneus (CAST) mice bearing short telomeres from birth, CPCs (defined as $c\text{-}kit^{pos}$) were also shown to develop telomere attrition. CAST mice exhibited activation in p53 and autophagy, and promoted differentiation, senescence, and exhaustion of CPCs [58].

Bone marrow-derived progenitor cells are another type of cell reported to contribute to tissue repair in the failing heart [59]. In ischemic heart disease patients, bone marrow-derived progenitor cells had shorter telomere length, higher level of transcripts for p21 and $p16^{Ink4a}$, and these associated with reduced myeloid differentiation capacity. This indicated the functional decline of BM-derived progenitor cells develops in ischemic heart disease [60]. Evidence shows that suppression of cellular senescence in progenitor cells is critical for maintaining homeostasis in cardiac tissues under stressed conditions.

Senolytics targeting cardiac diseases

Genetic or pharmacological models targeting the selective depletion of senescent cells ("senolysis") were shown to reverse age-related pathological changes [7,61–64]. Aging associates with the accumulation of senescent cells in organs, and these are well accepted to mediate their pathogenic roles by promoting chronic sterile inflammation in obesity, diabetes, heart failure, or atherosclerotic models. INK-ATTAC mice were generated to enable researchers to produce the inducible elimination of $p16^{Ink4a}$-expressing cells by the administration of a compound named AP20187. In a premature aging mouse model, elimination of $p16^{Ink4a}$-positive senescent cells led to inhibition of aging phenotype in white adipose tissues, heart, and kidney. In cardiac tissue at 18 months, elimination of $p16^{Ink4a}$-positive cells did not change heart rate, LV mass, thickness and diameter, ejection fraction, and fractional shortening compared to controls. However, cardiomyocyte cross-sectional area became significantly reduced with senolysis, suggesting that number of cardiomyocytes were increased in this group. Depletion of $p16^{Ink4a}$-positive cells

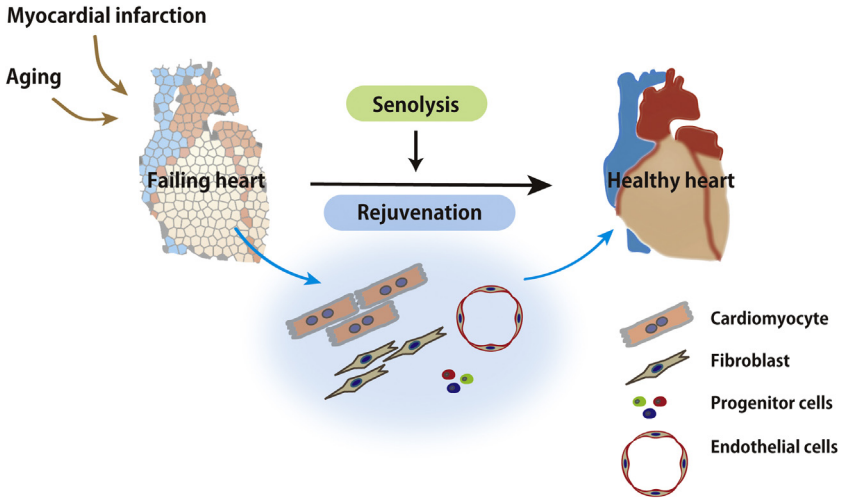

Fig. 2. Emerging concept of therapies targeting failing hearts with senolysis. Rejuvenation of senescent-like cells by senolysis contributes to convert failing heart into healthy heart.

utilizing INK-ATTAC genetic system also contributed to enhanced capability of handling isoproterenol-induced stress, indicating that accumulation of $p16^{Ink4a}$-positive cells has pathogenic roles to accelerate cardiac aging [7]. In another genetic model, $p16^{Ink4a}$-3MR (3MR) mouse, a transgenic mouse that kills $p16^{Ink4a}$ positive cells upon administration of ganciclovir (GCV), elimination of $p16^{Ink4a}$ positive cells from plaques led to the regression of atherosclerotic lesions in low-density lipoprotein receptor-deficient mice [8]. Nowadays, several compounds are reported to mediate senolysis, and these are described as "senolytics" (reviewed in Katsuumi et al. [65]). Among them, dasatinib and quercetin (D + Q), and an anticancer agent (ABT263) are extensively tested. ABT263 was reported to have a senolytic effect, contributing to the selective clearance of $p16^{Ink4a}$-positive senescent cells in bone marrow by inducing apoptosis in these cells. This led to rejuvenation of hematopoietic stem cells during aging [62]. D + Q administration improved vasomotor function in aged mice, and this was associated with reduced aortic calcification and osteogenic signaling in a murine model with hypercholesterolemia [66]. Recently, therapeutic potential of senolysis was also tested in cardiac tissues. D + Q administration significantly improved systolic cardiac function, together with reduced end-systolic left ventricular dimension in 24-month-old mice [64]. Global elimination of senescent cells in aged mice in pharmacological (D + Q) or genetic (INK-ATTAC) senolytic models led to the activation of resident CPCs in aged heart, and increased number of proliferative cardiomyocytes [57]. In another report, depletion of $p16^{Ink4a}$ positive cells in 27-month-old INK-ATTAC mice resulted in reduction of TAF positive cardiomyocytes, decline in cardiomyocyte size, and inhibited fibrotic area in heart. In a pharmacological senolytic model, ABT263 administration also reproduced these findings as observed in INK-ATTAC mice, and in both models, cardiomyocytes became positive for proliferative markers [6]. Another report also showed ABT263 administration eliminated senescent cardiomyocytes and attenuated profibrotic protein expression in aged mice. In this study, authors also tested roles of senolytic compound in myocardial infarction model, and showed clearance of senescent cells led to amelioration of myocardial remodeling, diastolic function, and improved survival rate in this ischemic heart failure model [67]. These indicated that elimination of senescent cells has a potential to reverse age-related dysfunction in cardiac tissues through promotion of regenerative capacity in this organ. Selective elimination of senescent cells with senolytics would become a next generation therapy for cardiac disorders [57,65,67–69] (Fig. 2).

Conclusion and future directions

In this review article, we delineated the role of cellular senescence and related molecules in cardiac tissue. In addition to molecules including p53, p21, $p16^{Ink4a}$, p38MAPK, and γH2AX, telomere attrition, enhanced signals for SA-β-gal, SAHF, TAF, SADs are also considered to be indicators of cellular senescence. Due to the lack of specific and conclusive senescence markers, it is especially challenging to conclude the presence of classically defined cellular senescent cells in organs. In many cases, if we follow traditional definitions, it would be appropriate to describe cells showing senescent markers as "senescent-like cells", and studies exploring conclusive senescent markers continue to be an important research topic. We also need to note that pathological implications of cellular senescence are context dependent. Endothelial cell senescence seems to be always detrimental in age-related disorders including heart failure or obesity. However, suppression of cellular senescence in fibroblasts was reported to promote cardiac fibrosis and remodeling through the activation of myofibroblasts. Considering that endothelial cells and fibroblasts share similar pro-senescent stressors including saturated fatty acids, outcomes of therapies targeting the inhibition of cellular senescence would be obtained through the summation of complex biological processes. In addition to the consideration for the concern of tumorigenesis, it would be important to generate tissue and/or cell specific anti-senescence therapy. Senolysis, *i.e.* targeted elimination of senescent cells, is now highlighted worldwide, because this approach was shown to reverse pathological features of aging in genetic as well as pharmacological models. Importantly, so far, this therapy does not seem to promote tumorigenesis. In addition to careful observation to find possible side effects involved, it would also be critically important to establish cell-specific senolytic therapy for age-related cardiac diseases.

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