The Role of Cellular Senescence in Cardiac Pathology

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This thesis is dedicated to:

My dear husband, Mahdi
My precious son, Nickan
My selfless parents
My wonderful brother
My amazing in laws

Abstract

Cellular senescence, a state of stable cell cycle arrest, plays a significant role in various biological processes, like aging, embryogenesis, and wound healing. Senescent cells have a complex senescence-associated secretory phenotype (SASP), involving pro-inflammatory and profibrotic factors that affect cells and tissues through paracrine and autocrine effects. Clinical evidence and experimental studies associate the accumulation of senescent cells and the production of SASP components to age-related cardiac pathologies including heart failure (HF), myocardial infarction (MI), and cardiotoxicity. However, the exact role of senescent cells in these conditions remains unclear. Additionally, the involvement of cellular senescence in other important cardiac conditions, such as atrial fibrillation (AF) remains unknown. Notably, the relative susceptibilities and consequences of senescence for the various types of cardiac cells are very much understudied and poorly understood. Therefore, advancing our understanding of cellular senescence and its effects on the heart is crucial and might lead to therapeutic targets and novel biomarkers. The purpose of this thesis is to gain insight into the role of cellular senescence in cardiac pathology.

Chapter 2 investigates the AF-susceptibility and senescence-marker expression in atrial tissue of rat models of aging (20 month-old) and MI (3 month-old) as well as the effect of reducing atrial senescent-cell burden on AF-susceptibility of post-MI rats with senolytic drug therapy. We show evidence for the accumulation of senescent cells in the atria of aged and MI rats, in association with a pathological AF-substrate. Furthermore, we demonstrate that senolytic therapy suppresses senescence, prevents atrial fibrosis and obviates AF-substrate development in post-MI rats. We provide evidence that senescence-clearance may act selectively via myofibroblasts and endothelial cells in AF pathogenesis.

Due to the potential off-target effects of senolytic drugs, we sought to investigate the role of senescence using the INK-ATTAC transgenic mouse model, in which senescent cells express a 'suicide' gene and undergo apoptosis following the treatment of mice with a dimerizing agent AP. Chapter 3 evaluates the effect of senescent-cell clearance on LV hypertrophy, fibrosis and function in aged INK-ATTAC mice. This evaluation considers the role of various senescent cardiac cell-types in age-related cardiac remodeling. This chapter also assesses potential paracrine effects of senescent fibroblast SASP components. We demonstrate that the clearance of senescent fibroblasts and cardiomyocytes reduces cardiac hypertrophy, fibrosis, and normalizes the prolonged isovolumic relaxation time, indicating restored LV diastolic function, in aged mice. The co-culture of H_2O_2-induced senescent fibroblasts with healthy cardiomyocytes increases the gene expression of hypertrophic markers, suggesting a role of senescent fibroblast SASP components in cardiomyocyte hypertrophy.

Chapter 4 investigates the effect of senescent-cell clearance on cardiac remodeling post-MI in INK-ATTAC mice, showing a marked benefit in terms of survival, MI-size and LV function. This chapter also identifies potential pathways affected by senescence in MI using single nucleus RNA-sequencing (snRNA-seq), specifically implicating immune cells and a specific macrophage subpopulation (Arg1_macrophages).

Overall, this thesis shows a significant contribution of senescent cells to cardiac pathology. This work advances our understanding of the mechanisms linking cellular senescence and cardiac pathology and contributes to the development of innovative therapeutic approaches in cardiac pathology.

Résumé

La sénescence cellulaire, un état d'arrêt stable du cycle cellulaire, joue un rôle important dans divers processus biologiques, tels le vieillissement, l'embryogenèse et la cicatrisation des plaies. Les cellules sénescentes ont un phénotype sécrétoire complexe associé à la sénescence (SASP), impliquant des facteurs pro-inflammatoires et profibrotiques qui affectent les cellules et les tissus par des effets paracrine et autocrine. Des données cliniques et des études expérimentales montrent que l'accumulation de cellules sénescentes et la production de composants du SASP sont associées à des pathologies cardiaques liées à l'âge, notamment l'insuffisance cardiaque (IC), l'infarctus du myocarde (IM) et la cardiotoxicité. Cependant, le rôle exact des cellules sénescentes dans ces conditions reste mal décrit, et leur implication dans d'autres pathologies cardiaques, telles que la fibrillation auriculaire (FA) reste mal connue. Les susceptibilités relatives et les conséquences de la sénescence distinctes pour différents types cellulaires sont encore très peu étudiées. Par conséquent, faire progresser notre compréhension de la sénescence cellulaire et de ses effets sur le cœur est crucial et pourrait conduire à l'identification de nouveaux biomarqueurs et de nouvelles cibles thérapeutiques. L'objectif de cette thèse est de mieux comprendre le rôle de la sénescence cellulaire dans la pathologie cardiaque.

Le chapitre 2 étudie la sensibilité à la FA et l'expression des marqueurs de sénescence dans le tissu auriculaire de modèles de rats vieillissant et d'IM,ainsi que l'effet de la réduction de l'impact des cellules sénescentes sur la susceptibilité à la FA suite à une thérapie médicamenteuse sénolytique de rats post-IM. Nous apportons des preuves de l'accumulation de cellules sénescentes dans les oreillettes de rats âgés et post-IM, en association avec un substrat pathologique pro-FA. En outre, nous démontrons que le traitement aux sénolytiques supprime la sénescence, prévient la fibrose auriculaire et évite le développement du substrat pro-FA chez les rats post-IM. Nous

apportons la preuve que la clairance de cellules sénescentes peut agir de manière sélective via les myofibroblastes et les cellules endothéliales pour prévenir de la pathogenèse de la FA.

Le chapitre 3 évalue l'effet de la suppression des cellules sénescentes sur l'hypertrophie, la fibrose et la fonction du ventricule gauche (VG) chez les souris transgéniques INK-ATTAC âgées. Cette évaluation tient compte du rôle de divers types de cellules cardiaques sénescentes dans le remodelage cardiaque lié à l'âge. Ce chapitre évalue également les potentiels effets paracrines des composants du SASP issus des fibroblastes sénescents. Nous démontrons que la clairance des fibroblastes et des cardiomyocytes sénescents réduit l'hypertrophie cardiaque, la fibrose et normalise le temps de relaxation isovolumique prolongé, indiquant une fonction diastolique VG restaurée chez les souris âgées. La co-culture de fibroblastes sénescents, induits par H_2O_2, avec des cardiomyocytes sains augmente l'expression génique des marqueurs hypertrophiques de ces derniers, suggérant un rôle des composants du SASP des fibroblastes sénescents dans l'hypertrophie des cardiomyocytes.

Le chapitre 4 étudie l'effet de la clairance des cellules sénescentes sur le remodelage cardiaque post-IM chez des souris INK -ATTAC, montrant un bénéfice marqué en termes de survie, de taille d'infarctus, et de fonction du VG. Ce chapitre identifie également les voies potentiellement affectées par la sénescence dans l'infarctus du myocarde à l'aide du séquençage d'ARN à noyau unique (snRNA-seq), révélant particulièrement des cellules immunitaires et une sous-population spécifique de macrophages (Arg1_macrophages).

Globalement, cette thèse montre une contribution significative des cellules sénescentes dans la pathologie cardiaque, permet d'en comprendre les mécanismes, et aidera au développement d'approches thérapeutiques innovantes.

Translation by: Patrice Naud

Author Contributions

Mozhdeh Mehdizadeh (Mozhdeh M) was the lead investigator and first author of the manuscripts in this thesis. Mozhdeh M designed experiments in collaboration with Dr. Stanley Nattel. Mozhdeh M troubleshooted and optimized protocols, collected data, and analyzed results. Mozhdeh M wrote initial drafts of all manuscripts and generated figures.

Dr. Stanley Nattel designed, funded projects, and supervised all Montreal Heart Institute trainees and technical staff, particularly Mozhdeh M, throughout the projects, and was extensively involved in preparation, writing and finalization of the papers.

Chapter 1. My review paper published in Nature Reviews Cardiology has been widely used within the introduction. However, alongside this review paper, the introduction also incorporates more literature reviews on some topics.

The role of cellular senescence in cardiac disease: basic biology and clinical relevance

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M.A., E.T., G.F. were involved in the paper collection, introduction preparation and they contributed to discussion of the content and reviewed and/or edited the manuscript.

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Chapter 2. The Role of Cellular Senescence in Profibrillatory Atrial Remodeling Associated with Cardiac Pathology

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I.H.A.T. and **A.S.** performed experiments and analyzed data related to Western Blots and dPCR in human samples.

R.H. performed AF inducibility experiments.

F.X. analyzed optical mapping experiments.

M.K. performed human surgeries, provided human atrial tissue, and interpreted clinical data.

N.V. analyzed immunofluorescence data.

J.C.T. supervised experiments and blind analyses of echocardiography.

M.G.S. and J.F.T. co-supervised experiments and blind analyses for histological experiments.

D.D. conceptualized, supervised, and funded human sample experiments.

E.T. assisted with design and data interpretation.

G.F. assisted with design and data interpretation. Supervised immunofluorescence imaging and analysis.

Chapter 3. Clearance of Cardiac Senescent Cells improves Age-related Cardiac Remodeling in Mice

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Martin M was involved in the cardiac cell isolations, performed optical mapping and analyzed part of the optical mapping data.

M.A. assisted with design and data interpretation.

P.N. performed qPCR experiments.

A.O. assisted with animal preparation for echo and hydroxyproline assay.

K.G. performed part of the *in vitro* work.

N.V. analyzed immunofluorescence data.

E.T. assisted with design and data interpretation.

G.F. assisted with design and data interpretation. Supervised immunofluorescence imaging and analysis.

J.C.T. supervised experiments and blind analyses of echocardiography.

M.G.S. and J.F.T. co-supervised experiments and blind analyses for histological experiments.

Chapter 4. Clearing Senescent Cells Prevents Cardiac Dysfunction and Death in Mice with Acute Myocardial Infarction

Mozhdeh Mehdizadeh, Francis Leblanc, Patrice Naud, Yasemin Altuntas, Sandro Ninni, Eric Thorin, Gerardo Ferbeyre, Jean-Claude Tardif, Martin G. Sirois, Jean Francois Tanguay, Guillaume Lettre, Stanley Nattel. *In preparation for submission.*

F.L. prepared library for snRNA-seq, analyzed the data, and prepared Figure 7 and 8.

P.N. performed qPCR experiments, was involved in the library preparation for snRNA-seq, and assisted with data interpretation.

Y.A. assisted with animal preparation for echocardiography.

S.N. assisted with design and data interpretation.

E.T. assisted with design and data interpretation.

G.F. assisted with design and data interpretation.

J.C.T. supervised experiments and blind analyses of echocardiography.

M.G.S. and J.F.T. co-supervised experiments and blind analyses for histological experiments.

G.L. assisted with design and data interpretation for snRNA-seq.

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Contribution to original knowledge

Chapter 2:

There are two human studies that show the expression of senescence markers in the atrial tissue of atrial fibrillation (AF) patients. While these studies suggest an association between senescence markers and AF-risk, they do not provide any direct evidence of causality. In this thesis, to understand the role of senescence in AF, we evaluate AF-susceptibility and senescence-marker expression for the first time in animal models: rat models of aging and myocardial infarction (MI).

The novel findings in this chapter are as follows:

- We found evidence of the accumulation of senescent cells in the atria of aged rats and in the atria of rats with left ventricular (LV)-dysfunction due to MI, in association with a pathological AF-substrate.
- Senolytic therapy suppresses markers of cellular senescence, prevents atrial fibrosis and obviates AF-substrate development in post-MI rats.
- We found evidence for a possible role of senescent myofibroblasts and endothelial cells in the pathogenesis of AF.

Chapter 3:

Recent research suggests the possible therapeutic potential of targeting senescence in the prevention and/or treatment of age-related cardiac remodeling. To our knowledge, no data are available in the literature regarding the effects of senescent-cell clearance on LV hemodynamic parameters, systolic and diastolic function, and electrophysiological properties.

The cell-types mediating the effects of cellular senescence on age-related cardiac remodeling is an important, but often ignored issue.

The novel findings in this chapter are as follows:

- We found evidence of the accumulation of different senescent cardiac cell-types in the LV of aged mice in association with age-related cardiac remodeling.
- We showed that the clearance of senescent fibroblasts and cardiomyocytes in aged mice reduced cardiac hypertrophy, fibrosis, and normalized the prolonged isovolumic relaxation time.
- We showed a potential paracrine effect of senescent fibroblasts on cardiomyocytes, causing them to hypertrophy.

Chapter 4:

The potential role of cellular senescence in MI-related remodeling has been suggested by some studies, however, the precise impact of senescence at the cardiac cellular level as well as its involvement in pathways associated with MI remains unestablished.

The novel findings in this chapter are as follows:

- We obtained evidence for the accumulation of senescent cells in the infarct-zone of MI mice.
- We found that the clearance of senescent cells improves post-MI survival rate, cardiac function, and structure potentially due to altered inflammatory response.
- We identified potential pathways affected by senescence in MI using single nucleus RNA-sequencing; specifically, we found an involvement of immune cells and a specific macrophage subpopulation, arginase1 (Arg1) macrophages.

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List of Abbreviations

AAV9	Adeno-associated virus 9
AC5	adenylate cyclase type 5
AF	atrial fibrillation
AKI	acute kidney injury
AP	AP208017
APD	action potential duration
AMPK	5' adenosine monophosphate-activated protein kinase
APD	action potential duration
Arg1	arginase-1
ATM	ataxia telangiectasia mutated protein
AUC	under the curve
AV	atrioventricular
BCL2	B-cell lymphoma 2
cAF	chronic AF
CaMKII	calcium/calmodulin-kinase type II
CCAC	canadian council on animal care
CCL	C-C Motif Chemokine Ligand 4
Ccl2	chemokine (C-C motif) ligand 2
CCN1	communication network factor 1
CDCs	cardiosphere-derived cells
CDK	cyclin dependent kinase
CKD	chronic kidney disease
CM	cardiomyocytes
Col1a1	collagen 1a1
Col3a1	collagen 3a1
CPCs	cardiac progenitor cells
Csf2	colony stimulating factor 2
CVD	cardiovascular disease
CXCL	chemokine (C-X-C motif) ligands
CYGB	Cytoglobin
CYGB	cytoglobin
DADs	delayed after depolarizations
DAPI	4′,6-diamidino-2- phenylindole
DDR	DNA damage response
DDR2	discoidin domain receptor tyrosine kinase2
DEG	differentially expressed genes
DMEM	Dulbecco's Modified Eagle Medium
ECG	electrocardiogram

ECM	extracellular matrix
EDN3	endothelin -3
EdU	ethynyldeoxyuridine
EMT	epithelial-to-mesenchymal transition
eNOS	endothelial nitric oxide synthase 3
EPS	electrophysiological study
FACS	fluorescence Activated Cell Sorting
FDR	fasle discovery rate
FGF	fibroblast growth factor
FS	fractional shortening
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GDF15	growth/differentiation factor 15
GFP	green fluorescent protein
Glb1	galactosidasw beta 1
HF	heart failure
HFpEF	heart failure with preserved ejection fraction
HPRT1	phosphoribosyltransferase 1
I_{kur}	ultrarapid delayed-rectifier potassium current
I_{sus}	sustained potassium current
I_{to}	transient outward potassium current
$I_{Ca,L}$	L-type Ca^{2+} current
IGF-1	insulin-like growth factor-1
IGFBP	insulin-like growth factor-binding protein
IGFBP	insulin-like growth factor-binding protein
IL	interleukin
I_{KACh}	acetylcholine-activated inward-rectifier potassium current
INK-ATTAC	p16Ink4a apoptosis through targeted activation of caspase
IPF	idiopathic pulmonary fibrosis
JNK	c-Jun N-terminal kinase
LA	left atrium
LAD	left anterior descending
lncRNA-21	long non-coding RNA 21
Lox	lysyl oxidase
LV	left ventricle
LVAWd	LV anterior thickness at the end of diastole
LVDd	LV diameter at end cardiac diastole
LVDs	LV diameter at end cardiac systole
LVEF	LV ejection fraction
MAPK	mitogen activated protein kinase
Max dpdt	maximum rate of increase in pressure during contraction

MI	myocardial infarction
Min dpdt	maximum rate of decrease in pressure during relaxation
miRNA	microRNA
MMPs	matrix metalloproteinases
MSCs	mesenchymal stem cells
mTOR	mechanistic target of rapamycin
Myh	myosin heavy chain
NCX	Na^{+}- Ca^{2+} exchanger
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NIH	National Institute of Health
NLRP3	NLR Family Pyrin Domain Containing 3
NPPA	natriuretic peptide A
NPPB	natriuretic peptide B
PAI-1	plasminogen activator inhibitor-1
Pax8	paired box gene 8
PCA	principal component analysis
PDGF	platelet-derived growth factor
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PH3	phosphor-histone H3
PH3	phosphor-histone H3
PI3K	phosphoinositide 3-kinase
PIM-1	proto-oncogene serine/threonine-protein kinase-1
PKB	protein kinase B
PRDX1	peroxiredoxin
PW	pulsed wave
RAA	right atrial appendage
RAAS	renin angiotensin aldosterone system
RADs	right atrium diameter at end cardiac systole
RB	retinoblastoma
ROS	reactive oxygen species
RyRs	ryanodine receptors
SA	sinoatrial
SA-β-gal	senescence associated beta galactosidase
SASP	senescence associated secretory phenotype
SC	subcutaneously
SERCA2a	SR Ca^{2+} adenosine triphosphatase type 2a
sFRP1	frizzled related protein 1
shRNA	short hairpin RNA
SIRT-1	sirtuin-1
SIS	stress induced senescence

SMAD	mothers against decapentaplegic
snRNA-seq	single nucleus RNA sequencing
SR	sarcoplasmic reticulum
SRXN1	sulfiredoxin 1
TAC	tranverse aortic constriction
TAPSE	tricuspid annulus plane systolic excursion
TCF21	transcription factor 21
TGFβ	transforming growth factor β
TIMPs	tissue inhibitors of metalloproteinases
TNFα	tumour necrosis factor alpha
TNI	troponin I
VEGF	vascular endothelial growth factor
WGA	wheat germ agglutinin
αSMA	α-smooth muscle actin

1. Chapter 1. Introduction

1.1. The heart, cardiac action potential, and electrocardiogram

The heart functions by rhythmically contracting its muscles to pump blood throughout the body, meeting physiological demands.[1] The rhythm that regulates cardiac muscle contraction is based on the cardiac action potential which is a representation of the membrane potential waveform in cardiac myocytes, reflecting the electrical activity of the cell during the heart's contraction and relaxation.[2] The action potential is characterized by distinct phases, and each phase is influenced by specific ionic currents that contribute to the overall shape and duration of the action potential. The action potential is divided into four distinct phases (Figure 1.1A).[3] During phase 0, there is a rapid upstroke caused by the transient influx of Na^+ ions. The Na^+ channels subsequently become inactivated, and there is a transient efflux of K^+ ions. In phase 2, known as the plateau phase, there is a balance between the efflux of K^+ ions and the influx of Ca^{2+} ions. At the end of the plateau phase, sustained repolarization occurs as the efflux of K^+ ions through the delayed rectifier K^+ channels surpass the influx of Ca^{2+} ions, marking phase 3 of the action potential. Finally, during phase 4, the resting potential of myocytes is maintained.[2]

The cardiac cycle typically begins in the sinoatrial (SA) node, which generates APs spontaneously through its inherent automaticity. These action potentials propagate across the atria, passing through the atrioventricular (AV) node, and then continue into the His-Purkinje system within the ventricle, leading to ventricular contraction (Figure 1.1B).[4] Clinically, this electrical activity can be observed on an electrocardiogram (ECG). The ECG displays distinct waveforms, with the P-wave representing atrial depolarization, the QRS complex indicating ventricular depolarization, and the T-wave representing ventricular repolarization (Figure 1.1C).[4]

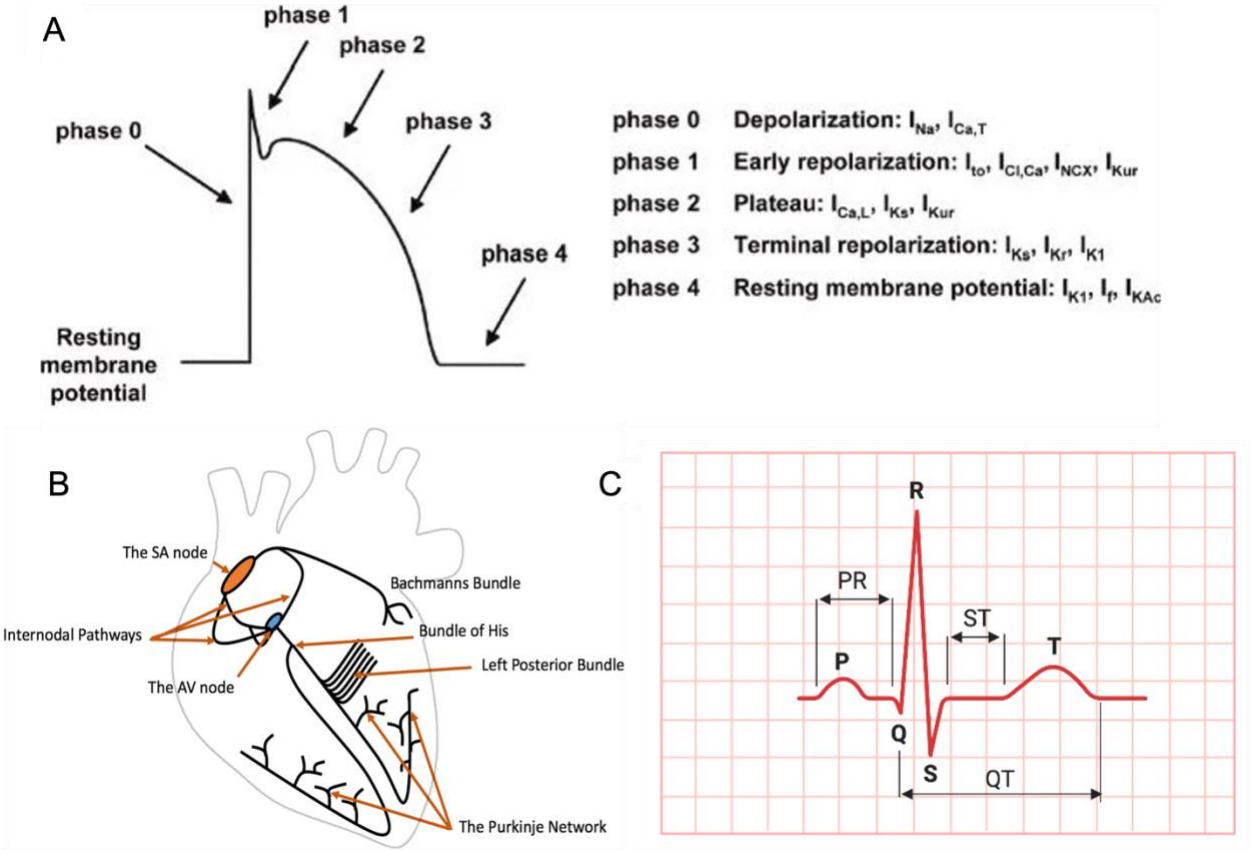

Figure 1.1. The cardiac conduction system, action potential and electrocardiogram.

A) Cardiac action potential. B) Cardiac conduction system. C) Cardiac electrocardiogram. Adapted from Kennedy A et al. The Cardiac Conduction System: Generation and Conduction of the Cardiac Impulse. Crit Care Nurs Clin North Am. 2016 Sep;28(3):269-79. doi: 10.1016/j.cnc.2016.04.001. Epub 2016 Jun 22. PMID: 27484656., with permission from Elsevier.

1.2. Aging and cardiac disease

The global average lifespan of the human population is on the rise primarily due to an increase in longevity.[5] It has been predicted that by 2050, the world's population of individuals aged 60 years and older will increase to 2.1 billion.[6] As the global average lifespan continues to increase, the prevalence of cardiac diseases such as heart failure (HF), myocardial infarction (MI), and atrial fibrillation (AF) also rises significantly in both men and women.[7] Indeed, age is the leading risk factor for cardiac disease in developed nations.[8] Although the phenotype of cardiac aging has been

well characterized, the molecular mechanisms through which aging leads to cardiac disease have not been fully explored. Until recently, the fields of molecular biology of aging and cardiac disease have remained separate; most animal studies of cardiac disease were performed in young rodents, while animal studies of genetic and pharmacological interventions to target aging process rarely assessed cardiac function.[9] Fortunately, this separation has been addressed and recent research has started to explore the underlying mechanisms of aging in cardiac disease.[10-15]

1.3. Characteristics of the aging heart

Cardiac aging is characterized by changes in the heart at four different level including, functional, structural, cellular, and molecular changes.

1.3.1. Functional changes of the aging heart

1.3.1.1. Diastolic function

Left ventricle (LV) diastolic dysfunction is the hallmark of cardiac aging and greatly prevalent in older populations.[16] Diastolic dysfunction may lead to the development of diastolic HF which is referred to as HF with preserved ejection fraction (HFpEF).[17] The characteristics of diastolic dysfunction include prolonged LV relaxation time and increased end diastolic pressure. Determinants of these characteristics are cardiac stiffness, LV wall thickness, chamber geometry, and fibrosis.[17,18]

The left atrium (LA), during early diastole, functions as a conduit, transferring blood passively from the LA to the left LV.[19] Studies have shown that there is a decline in conduit function with aging.[20,21] During late diastole, the LA acts as a pump, contracts actively to augment LV filling.[19] The impact of aging on pumping function of LA is controversial; while some studies indicate an increase in the pumping function with age, others have not shown any significant changes.[20,21]

1.3.1.2. Systolic function

Contrary to diastolic function, systolic function of the heart is relatively preserved with healthy aging.[22] This notion has been confirmed by echocardiogram measurements in aged patients.[23n]However, with exercise, the effects of aging on systolic function are more apparent; ejection fraction (EF)% and maximum heart rate are lower in aged patients indicating a reduction in cardiac reserve.[24,25] This reduction is a result of several factors such as impaired autonomic regulation, reduced intrinsic myocardial contractility, increased vascular afterload, and arterial-ventricular load mismatching.[16]

The LA functions as reservoir during systole, receiving blood from the pulmonary veins and storing energy in the form of pressure. Several studies showed reservoir function of LA declines with age.[26-28]

1.3.1.3. Electrical function

Aging causes a number of changes in the cardiac conduction system. For example, cardiac aging is associated with a reduction in the number of pacemaker cells in the SA node.[29] Furthermore, there are some changes in function of the AV node, the bundle of His, and bundle branches due to tissue remodeling with aging.[17] These changes prolong AP duration (APD) and affect ECG measurements such as an increase in P-wave duration, P–R interval and Q– T interval, a decrease in QRS voltage and T-wave voltage, and a leftward shift of the QRS axis.[25] The alterations of the cardiac electrical system with aging may lead to arrhythmias including AF, paroxysmal supraventricular tachycardia, and ventricular arrhythmias.[17,25,30]

1.3.2. Structural changes of the aging heart

1.3.2.1. Ventricular structure

At the structural level, a major age-related change is an increase in the thickness of LV.[22] This change can be attributed to various biochemical alterations, among which oxidative stress and mitochondrial damage play an important role.[31] As individuals age, oxidative stress and mitochondrial damage contribute to the death of cardiomyocytes.[31] Consequently, with the decline in the number of cardiomyocytes, the remaining cells undergo hypertrophy as a compensatory response, resulting in thickening of the myocardium.[32]

Another notable structural change that occurs in the LV with age is the alteration of LV mass. However, the findings are conflicting.[17,33] Yoneyama et al. conducted a study involving a diverse population of healthy individuals, including Caucasians, African-Americans, Hispanics, and Chinese individuals without evidence of cardiovascular disease (CVD), and found that LV mass increases with age.[34] Similarly, in a 16-year follow-up study by Lieb et al. involving participants from the Framingham Heart Study, it was demonstrated that advancing age strongly correlated with an increase in LV mass over time, primarily observed in individuals with an increased burden of CVD risk factors such as higher body mass index, blood pressure, and diabetes.[35] However, some recent analyses of changes in LV mass suggest that there is no significant effect on mass or a decrease that is specific to male sex with aging.[25,33]

1.3.2.2. Atrial structure

Compared to younger individuals, the role of atrial contraction in filling the LV during diastole becomes significantly important in elderlys.[17] This change in function is accompanied by the development of atrial hypertrophy and dilation.[17] In the 16-year follow-up study involving participants from the Framingham offspring study, age was positively correlated with the diameter of the LA among individuals with varying degrees of other risk factors.[36] The increased size of the

LA has been associated with two age-related diseases: AF and HFpEF.[37] These two pathologies often coexist, with approximately two-thirds of HFpEF patients experiencing atrial fibrillation at some point in their lives.[37] Another notable structural change that occurs with aging in the atria is atrial fibrosis, shown by both clinical and animal studies. [38] In addition to atrial dilation and fibrosis, senile amyloidosis is another change in aging-related atrial structure that is implicated as a potential risk factor for AF.[39]

1.3.3. Cellular and molecular changes of the aging heart

Recent research indicates the involvement of various cellular and molecular mechanisms in the aging heart. These mechanisms include, but are not limited to, calcium mishandling, neurohormonal signaling, inflammaging, fibrosis, growth signaling and cellular senescence (Figure 1.2).

1.3.3.1. Calcium mishandling

Ca^{2+} ion is the signaling molecule of excitation-contraction coupling, a well-organized process transforming the electrical stimulation of the sarcolemma into muscle contraction. [40] This process starts with the opening of L type calcium channels during phase 2 of action potential. A small Ca^{2+} current enters cardiomyocyte which subsequently triggers the ryanodine receptors (RyRs) to release a large current of Ca^{2+} from the sarcoplasmic reticulum (SR).[41] This Ca^{2+} binds and activates troponin C within the myofilaments, which is then followed by myocyte contraction. During relaxation, Ca^{2+} ions dissociate from myofilaments and are reuptaken into SR through the SR Ca^{2+} adenosine triphosphatase type 2a (SERCA2a) or extruded outside the cardiomyocyte through the Na^{+}- Ca^{2+} exchanger (NCX).[42]

Ca^{2+} reuptake during relaxation was shown to be impaired during the process of aging, which leads to prolonged relaxation time, diastolic dysfunction and heart failure (HF).[42] This could be

explained by increased spontaneous diastolic Ca^{2+} release from the SR through leaky RyRs, the downregulation of SERCA2 protein levels, or impaired SERCA2 protein function.[43]

In the atria, age-related calcium mishandling plays a significant role in the pathogenesis of arrhythmia, particularly AF.[38]

1.3.3.2. Activated neurohormonal signaling

Activated neurohormonal signaling is another feature of cardiac aging.[44] Two players of this signaling are the renin angiotensin aldosterone system (RAAS) and β-adrenergic signaling.[45]

In the RAAS, renin stimulates the production of angiotensin I and II, which both stimulate aldosterone release from adrenal glands. RAAS has a key role in regulating blood volume, systemic resistance, and cardiac hypertrophy.[46] Several studies have shown that angiotensin II concentration significantly increased in the aged rodent heart due to the upregulation of the anio-converting enzyme.[47]Some studies show similarities such as cardiac hypertrophy, fibrosis and diastolic dysfunction between angiotensin II–treated heart and the aging heart.[16,48] Moreover, several studies have shown that RAAS activation might lead to changes in ion channels by increasing oxidative stress[49-51] leading to arrhythmias, particularly AF.[50,52,53]

The neurohormonal system also involves β-adrenergic signaling, the activation of which increases heart rate, myocardial contractility wall stress, blood pressure and metabolic demand on the heart.[54] The level of circulating catecholamines (noradrenaline and adrenaline) increases with aging, leading to a downregulation of β-adrenergic receptor density at the cell membrane.[55] Yan et al. showed that in mice, the knockout of adenylate cyclase type 5 (AC5) gene, which is a key enzyme downstream from β-adrenergic signaling, increased median lifespan and improved age-dependent cardiac fibrosis and hypertrophy.[56] In aged rabbits, testosterone replacement has been shown to increase arrhythmogenesis in the LA through the enhancement of β-adrenergic activity.[57]

Furthermore, chronic β-adrenergic stimulation may induce reactive oxygen species (ROS) production leading to several cardiac conditions including AF and HF.[16,58]

1.3.3.3. Inflammaging

Inflammation is a determinant of aging and plays a key role in most age-related diseases including cardiac disease.[59] Despite the essential role of inflammation as a defense mechanism against infections, sustained and prolonged inflammation can be detrimental to health.[60] In the heart, chronic inflammation leads to mitochondrial damage and results in an increased free radical production.[61-63] Franceschi et al. introduced the term "inflammaging," which is referred to as chronic, low-grade, systemic inflammation that develops with age in the absence of overt infections.[64]

Inflammaging is characterized by the upregulation of proinflammatory markers such as interlukin-6 (IL-6), IL-1, transforming growth factor-β (TGFβ) family, tumour necrosis factor alpha (TNFα) as well as inflammasomes which are macromolecular complexes triggering the production of inflammatory markers. [64] Recent research points towards inflammaging as a key contributor of age-related cardiac disease.[68,69,70] In old mice, the genetic ablation of NLR Family Pyrin Domain Containing 3 (Nlrp3), an important and well-studied inflammasome, has been found to alleviate age-related cardiac impairment and telomere shortening.[65] Importantly, patients with chronic inflammatory diseases such as psoriatic arthritis or rheumatoid arthritis have higher risks of cardiovascular disease, such as MI, and HF. [66]

1.3.3.4. Growth signaling

The dysregulation of growth signaling pathways, such as insulin-like growth factor-1 (IGF-1) and mechanistic target of rapamycin (mTOR), is associated with cardiac hypertrophy and aging.[67] Caloric deficiency, which leads to decreased levels of growth factors including IGF-1, has been

linked to the protection of several age-related diseases such as diabetes and cancers.[68] The role of IGF-1 in the aging heart, however, is controversial and both detrimental and beneficial effects have been reported.[69-73] Wessells et al. showed that deficiency in insulin/IGF-1 signaling improves cardiac performance in old drosophila and old mice.[70] On the other hand, an age-dependent reduction in serum IGF-1 in humans has been associated with the higher risk of heart failure in old patients.[72] Similarly, some studies suggest that enhancing IGF-1 signaling could potentially prevent heart failure.[74,75] Thus, further studies are needed to elucidate the link between IGF-1 signaling and cardiac aging. The IGF1 is also implicated in the atrial fibrosis during AF by activating the phosphoinositide 3-kinase (PI3K)-AKT pathway resulting in the expression of connective tissue growth factor (CTGF) and angiotensin II receptor type 1 (AT1R).[76]

1.3.3.5. Cardiac fibrosis

The cardiac extracellular matrix (ECM) is a complex collection of structural and non-structural proteins outside the cells, providing strength and plasticity.[77] The fibrotic tissue in the heart is dynamic, metabolically active, and contractile, capable of adapting to changing conditions.[78] Myofibroblasts are the primary cells involved in the formation of cardiac fibrosis.[79] These cells are activated form of fibroblasts expressing alpha-smooth muscle actin microfilaments, which confer them with contractile properties.[82]

Collagen synthesis in myofibroblasts induced by growth factors such as TGFβ, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and cytokines like IL-1, IL-4, and TNFα.[78] The degradation of extracellular matrix collagens is controlled by matrix metalloproteinases (MMPs), expressed by cardiac myocytes, fibroblasts, myofibroblasts, smooth muscle cells, endothelial cells, and macrophages.[80] MMP activation leads to collagen degradation, increased chamber dimensions, and reduced stroke volume.[81] Endogenous tissue inhibitors of

metalloproteinases (TIMP 1-4) help regulate the balance between collagen degradation and synthesis.[81]

Both clinical and preclinical studies indicate cardiac aging is associated with dysregulation of ECM synthesis and degradation and cardiac fibrosis in ventricles and atria.[79] In the LV, accumulation of collagen in aged hearts leads to stiffness and diastolic dysfunction.[18] In the atria, fibrosis is usually associated with cardiac arrhythmias such as AF.[79]

1.3.3.6. Cellular senescence

Cellular senescence, a stress- or age-related response, is a biological process that manifests as stable cell cycle arrest in association with morphological cellular changes and a distinctive secretome referred to as senescence- associated secretory phenotype (SASP).[82,83] The SASP involves growth factors, lipids, matrix remodeling proteases, and proinflammatory factors, such as cytokines, chemokines, has potential significance in the pathophysiology of age-related diseases.[84]

Characterizing cells as senescent is complex and challenging.[85] There is no single specific biomarker that can reliably identify senescent cells; thus, combinations of markers are typically employed to confirm a senescence phenotype including cyclin- dependent kinase inhibitors (CDK) inhibitors (p16, p21, p53), senescence- associated β- galactosidase (SA- β- gal), and phosphorylated histone H2AX (γH2AX). [85] Senescent cells tend to accumulate with age, such that cellular senescence is often equated with aging and studied in age- related diseases including bone loss, neurodegenerative brain disease, vascular disease, metabolic disorders, and physical dysfunction or frailty, among others.[82,86] However, senescence also plays a key role in some normal physiological processes, including wound healing and embryonic development, and protection against cancer.[87]

Our understanding of the complex relationship between ageing, cardiac conditions and cellular senescence has only been recognized in the last ten years.[88] Based on observational clinical evidence, there is a link between the components of cellular senescence and worse cardiac outcomes.[89] For example, drugs known to have cardiotoxic effects, such as doxorubicin, upregulate SASP components and senescence markers, suggesting a potential mechanistic connection between cardiac disease and cellular senescence.[89] Despite this link, the potential of targeting senescence to prevent and/or treat chronic cardiac pathology, as well as the safety of modulating these intricate pathways, remains uncertain. Therefore, gaining a deeper understanding of the role of cellular senescence in cardiac pathology is of significant potential value, both for mechanistic understanding and for the identification of potential new therapeutic targets. I therefore chose to pursue a study of the pathophysiological role of cellular senescence in heart disease as the major theme of this thesis. A more detailed background description of what is known about cellular senescence follows.

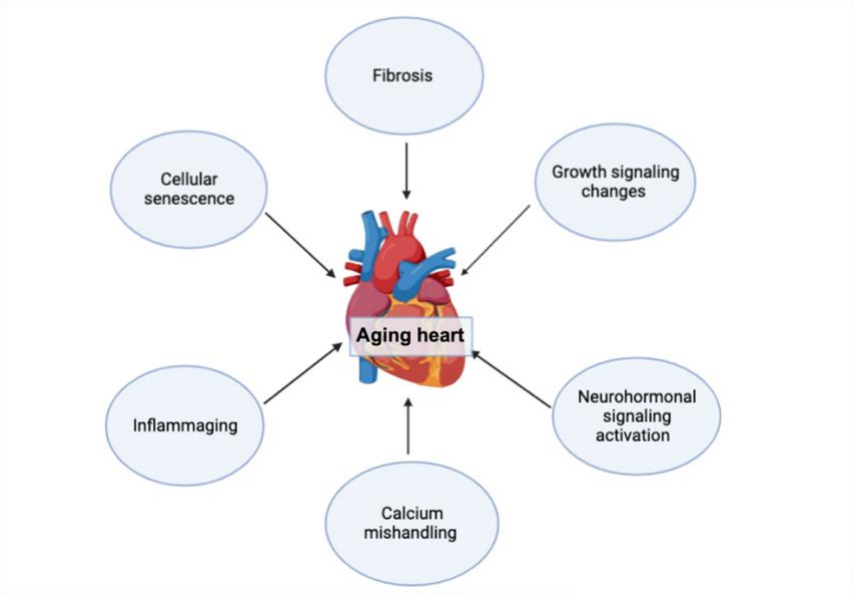

Figure 1.2. Cellular and molecular changes happen in the aging heart.

The changes include calcium mishandling, activation of neurohormonal signaling, growth signaling changes, fibrosis, inflammaging and cellular senescence. Created by Biorender.

1.4. Overview of cellular senescence

1.4.1. Features of cellular senescence: Historical overview

In 1891, August Weissman suggested that "death takes place because a worn- out tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting but finite".[90] This idea introduced two important concepts: first, the basis of organ failure is an age-related "worn-out tissue"; second, the capacity for repair through cell division is finite and eventually leads to organ failure due to continuous wear and tear. These two concepts are closely linked, as evidenced by the relationship between resting heart rate and life expectancy in mammals, where animals with slower heart rates have longer lifespans than those with faster heart rates.[91] Two typical cardiovascular processes related to aging include a decrease in cardiac inotropy, which is associated with a decline in the cardiomyocyte volume density, and an increase in aortic stiffness that results in an increase in pulse pressure.[92] The discovery of replicative senescence by Hayflick & Moorhead in 1961 verified the concept that physiological cell proliferation is limited.[93] Following the discovery of telomerase by Greider and Blackburn, the Greider laboratory associated telomere shortening to aging in human fibroblasts.[94,95] They also demonstrated that telomere shortening in human vessels is age-dependent and faster in arteries than veins[96]; is accelerated in the human arterial endothelium by risk factors for cardiovascular diseases[97]; and, finally, has a profound impact on healthy aging.[98] Cellular senescence, however, is not limited to replication. Lowe's team discovered that the oncogene Ras can induce premature cellular senescence (Figure 1.3).[99] Protein misfolding, damage to the DNA, or mitochondrial dysfunction trigger stress-induced senescence (SIS) and play a role in tumour suppression.[100-102] Telomere DNA damage, in the absence of telomere shortening, was found to trigger senescence in cardiomyocytes.[12]

Cellular senescence can be endogenously programmed and be an essential step toward organogenesis and wound healing, including cardiac repair.[103-105] Therefore, programmed senescence is a critical contributor to health.[106] Thus, cellular senescence has two faces: 1) *programmed* cellular senescence responding in early life to a need for organogenesis, wound healing and repair, and a safeguard to tumorigenesis; and 2) *unprogrammed* cellular senescence as a consequence of telomere dysfunction, damage or toxic stress in old age associated with the accumulation of senescent cells due to immuno-senescence (senescence of the immune cells responsible for recognizing and eliminating senescent cells), and/or exponential senescent-cell accumulation that overcomes immune-cell clearance capacities.[107] Despite the fact that unprogrammed cellular senescence has been linked mostly to detrimental effects in many cardiac conditions (such as cardiac hypertrophy, toxicity, and failure), in some contexts, such as MI, data suggest that unprogrammed cellular senescence might also have beneficial roles.[105,108]

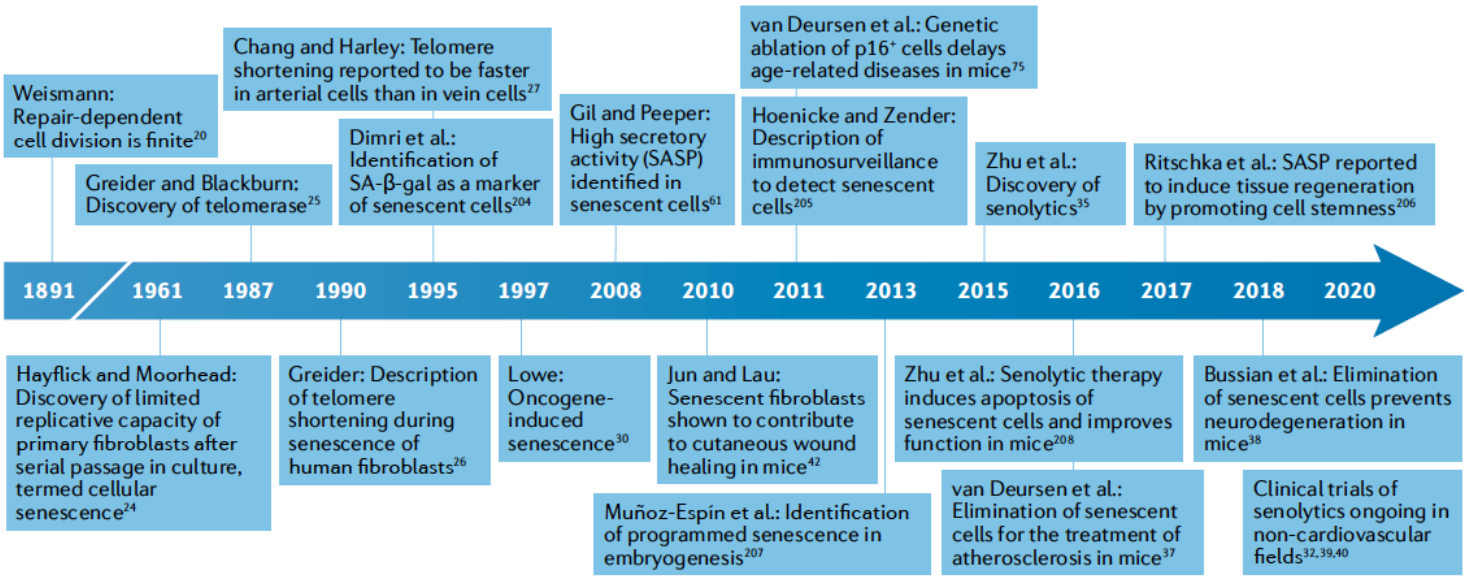

Figure 1.3. Milestones in cellular senescence research. The idea that tissues cannot forever renew themselves was proposed in 1891 by Weismann.

In 1961, Hayflick and Moorhead observed that primary fibroblasts stop dividing after serial passages in culture and named this phenomenon 'cellular senescence. Over the subsequent 60 years, several groups studied the mechanisms underlying senescence and identified the properties of senescent cells. More recently, several groups have noted that the elimination of senescent cells by targeted deletion in transgenic mouse models or with senolytics improves aged-related disease manifestations in animal models. SA- β- gal, senescence- associated β-galactosidase; SASP, senescence- associated secretory phenotype. Adapted from Mehdizadeh et al. The role of cellular senescence in cardiac disease: basic biology and clinical relevance. Nat Rev Cardiol. 2022 Apr;19(4):250-264. doi: 10.1038/s41569-021-00624-2. Epub 2021 Oct 19. PMID: 34667279.

1.4.2. Cellular senescence inducers and associated pathways

Senescent cells no longer divide.[85] Senescence growth arrest is mediated through two main pathways: p53/p21 and p16-retinoblastoma (RB) protein[109] (Figure 1.4). When these pathways become activated, they inhibit cyclin dependent kinase (CDK) 1, 2, 4 and 6, preventing RB-phosphorylation that is necessary for E2F transcription-factor activity needed to express genes involved in G1 to S-phase transition.[82] DNA damage stimulates the DNA damage response (DDR)

pathway leading to the activation of p53 and p21. Ageing and epigenetic derepression of the INK4a/ARF locus also leads to the activation of cell cycle inhibitors p16 and p14. [84,110]

There are other pathways that are also involved in cellular senescence, including those related to the activation of the TGFβ system, the mitogen-activated protein kinases (MAPKs) by the AKT signaling-pathway, ROS, all of which result in p15, p21 and p27 upregulation.[111,112] Cellular senescence is also induced by the prolonged activation of IGF-1 pathway in a p53-dependent manner.[112] Other triggers such as developmental cues and polyploidy activate the Ras-Raf-MEK-ERK pathway, protein kinase B (PKB, orAKT), mothers against decapentaplegic (SMAD) and p21 upregulation (Figure 1.4).[109] Both damage-induced senescence and developmental senescence pathways trigger cell cycle arrest. However, damage-induced senescence often leads to a more pro-inflammatory secretory phenotype, contributing to the local microenvironment's inflammatory response.[85,87]

There is a clear association between mitochondrial dysfunction and cellular senescence; however, whether senescence is a cause or consequence of mitochondrial dysfunction is under investigation.[113,114] Recent research suggests that dysfunctional mitochondria may have a wider role in senescence induction beyond just the production of ROS.[115,116] For example, the accumulation of damaged mitochondria due to impaired mitophagy is an important contributor to senescence, while, on the other hand, cellular senescence dysregulates mitophagy.[115] Several microRNAs (miRNA), endogenous small RNAs regulating gene-expression, are involved in senescence.[117-119] For example, the miR-17 family is associated with the suppression of p21 and TGFβ signaling.[120]

Importantly, senescent cells are remarkably resistant to apoptosis due to the overexpression of anti-apoptotic proteins, such as those in the B-cell lymphoma 2 (BCL2) family.[121] Resistance to apoptosis is an important characteristic of senescent cells because it explains why aging causes

the accumulation of senescent cells. It remains somewhat unclear why senescent cells do not enter apoptosis, since p53 activation is central to both processes.[110] One hypothesis is that senescent cells may only activate p53 partially, sufficient for senescence-associated genes activation but not enough to trigger apoptosis.[122] Consistent with this hypothesis, p53 levels are decreased by sequestration into DNA-damage foci adjacent to promyelocytic leukaemia (PML) bodies in senescent cells.[123] The administration of peptides such as forkhead box protein O4 (FOXO4)-DR that release p53 from these sites has been shown to rejuvenate old tissues in mice.[123]

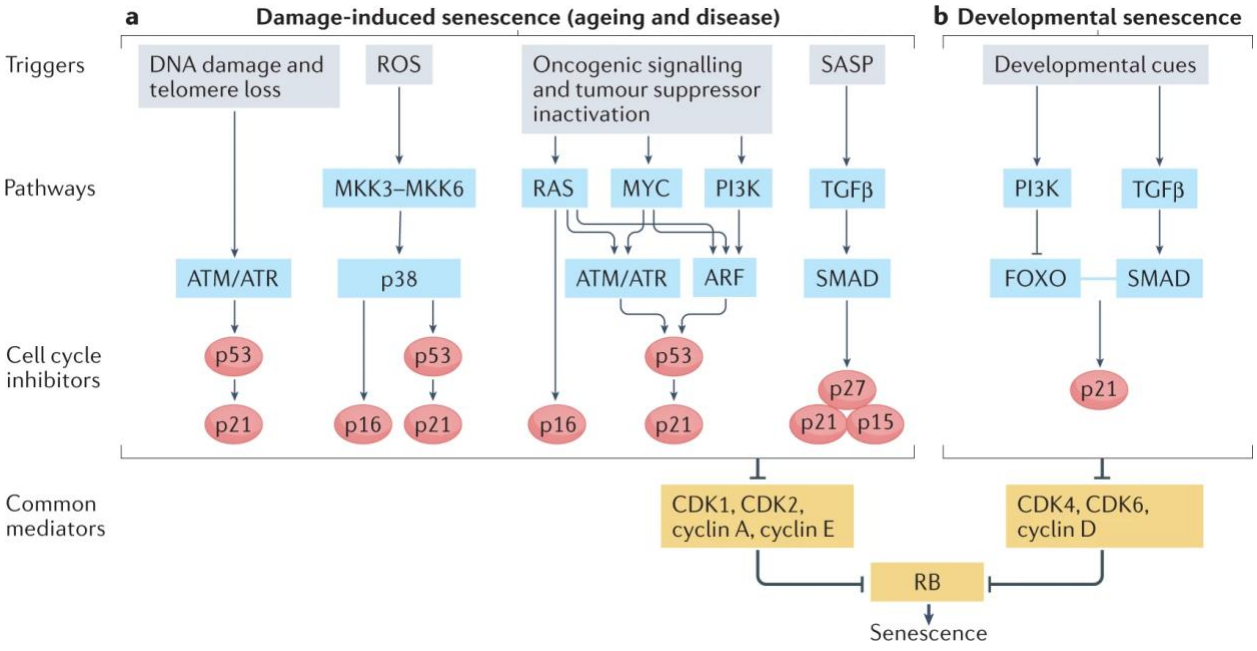

Figure 1.4. Overview of molecular mechanisms leading to cellular senescence.

The underlying pathways that lead to cellular senescence can vary depending on the triggers, context or cell type involved. a | In cell damage- induced senescence, which occurs with aging or disease, multiple pathways are involved that converge on the activation of cell cycle inhibitors, inhibition of retinoblastoma protein (RB) phosphorylation, and senescence. DNA damage and telomereloss activate a signaling cascade involving the DNA damage response via ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and RAD3- related protein (ATR) kinases, p53 upregulation, and increased p21. Reactive oxygen species (ROS) activate the mitogen- activated protein kinase kinase (MKK3 and MKK6) pathway and their downstream kinase effector p38, which leads to the upregulation of p16 (also

known as p16INK4A), p53 and p21. Oncogenic signaling or loss of tumour suppressors upregulates p16, p53 and p21 levels, mediated by RAS, MYC and phosphoinositide 3- kinase (PI3K) and their downstream effectors ATM, ATR and ARF. Lastly, transforming growth factor- β (TGFβ), a notable component ofcthe senescence- associated secretory phenotype (SASP) pathway, upregulates p15, p21 and p27 through SMAD complexes. b | Developmental cellular senescence changes occur via PI3K–forkhead box protein O (FOXO) signaling and TGFβ–SMAD signaling that lead to the upregulation of p21 but do not upregulate other cell cycle inhibitors such as p16 or p53. Adapted from Mehdizadeh et al. The role of cellular senescence in cardiac disease: basic biology and clinical relevance. Nat Rev Cardiol. 2022 Apr;19(4):250-264. doi:10.1038/s41569-021-00624-2. Epub 2021 Oct 19. PMID: 34667279.

1.4.3. The senescence-associated secretory phenotype

Senescent cells release high levels of pro-inflammatory cytokines, chemokines, lipids and proteases collectively referred to as the component of the SASP (Figure 1.5).[82] Campisi's group described the SASP for the first time in 2008.[124]The Peeper and Gil laboratories subsequently characterized the SASP.[125,126] In programmed senescence, the SASP restructures the extracellular matrix for remodeling and repair by attracting immune cells to eliminate the senescent cells, while attracting progenitors for repair.[127] However, in the context of aging, unprogrammed senescent cells accumulate in the tissue. Consequently, the SASP products are upregulated and affect healthy neighbouring cells through paracrine effects (Figure 1.5).[12,128,129]

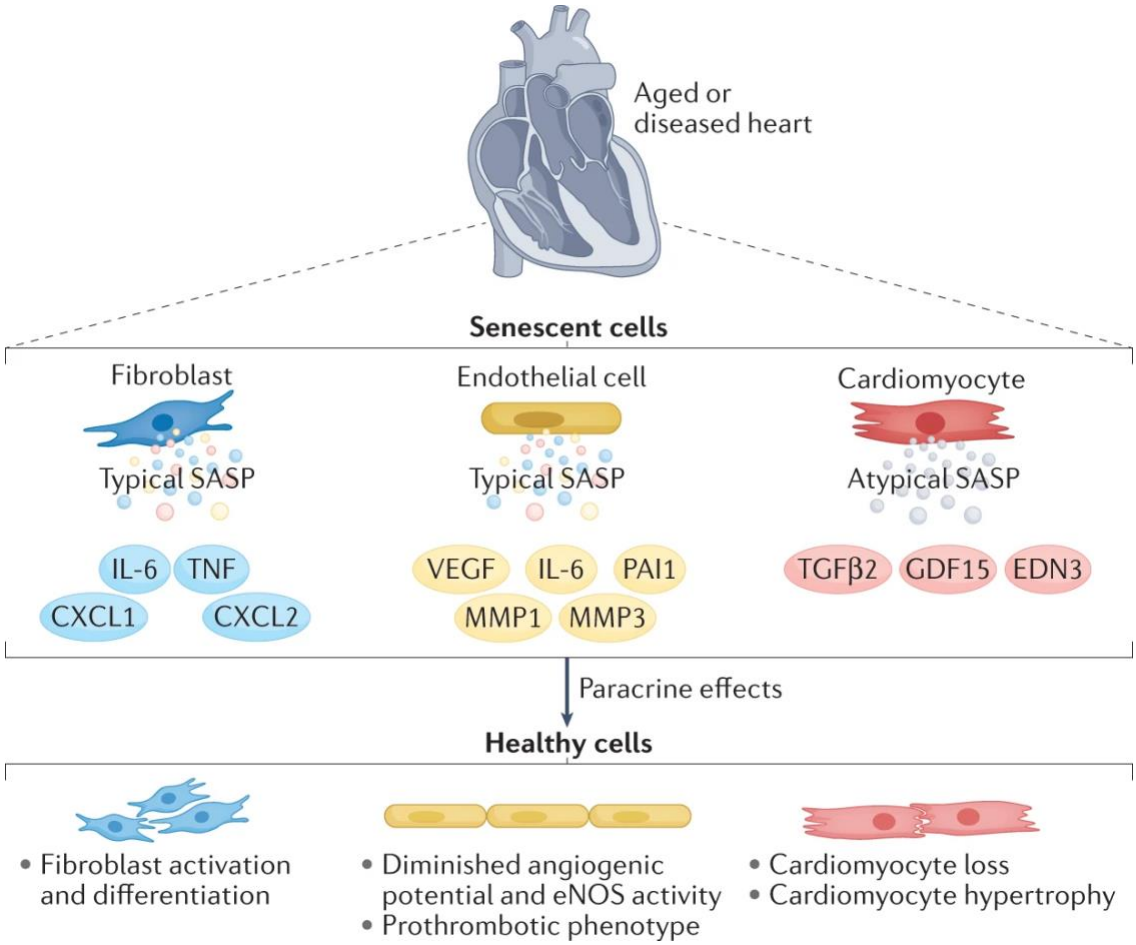

Figure 1.5. Intercellular paracrine communication between different cardiac cell types via SASP.

Senescent cells in the heart can adversely affect healthy neighbouring cells via senescence- associated secretory phenotype (SASP) products. Fibroblasts and endothelialcells produce typical SASP molecules. By contrast, senescent cardiomyocytes secrete atypical SASP products. The consequences of SASP paracrine effects on healthy neighbouring cells include fibroblast activation and differentiation; diminished angiogenic potential, decreased endothelial nitric oxide synthase (eNOS) activity and a prothrombotic phenotype in endothelial cells; and cardiomyocyte death and hypertrophy. Adapted from Mehdizadeh et al. The role of cellular senescence in cardiac disease: basic biology and clinical relevance. Nat Rev Cardiol. 2022 Apr;19(4):250-264. doi:10.1038/s41569-021-00624-2. Epub 2021 Oct 19. PMID: 34667279.

1.4.4. Experimental interventions targeting senescence

Senescent cells can have several detrimental effects on tissue.[130] For example, senescence causes cell-cycle arrest in progenitor cells resulting in the loss of tissue regenerative capacity.[130] Senescent cells further occupy important cellular niches and block the proliferation of healthy cells.[131] Finally, senescent cells' SASP components can contribute to tissue dysfunction *via* paracrine effects.[131] Figure 1.6 illustrates the approaches proposed to reduce the consequences of senescent-cell accumulation, including strategies such as preventing cellular senescence, modulating the deleterious effects of SASP components, and inducing apoptosis.

One effective method of apoptosis induction is the use of transgenic INK-ATTAC[132] and p16-3MR [133]mouse models to selectively clear $p16^{+}$ cells upon drug treatment. This method clarified the specific role of senescent cells in age-related pathologies and frailty. Another promising method has emerged through the discovery of senolytics, small-molecule drugs that induce apoptosis in senescent cells.[134] This new therapeutic class was developed through the testing of several drug compounds.[134]

1.4.4.1. Senolytics

The discovery of senolytic agents, drugs that can induce apoptosis of senescence cells, has supported the link between the burden of senescent cells and age-related diseases in animal models (Figure 1.7). [134] The first senolytics reported were the combination of dasatinib and quercetin which were shown to induce apoptosis in senescent cells by inhibiting the phosphoinositide 3-PI3K/AKT pathway and improves health and lifespan in aging mice.[135] FOXO4-DRI, a small peptide that disrupts FOXO4/p53 binding, neutralizes doxorubicin-induced chemotoxicity and restores renal function, fitness, and fur density in aged mice, as well as $Xpd^{TTD/TTD}$ mice with accelerated aging.[123] Intermittent treatment with navitoclax (ABT-263), a BCL-2 inhibitor, delays atherosclerosis progress in mice.[136] Furthermore, navitoclax prevents gliosis,

hyperphosphorylation of both soluble and insoluble tau proteins leading to neurofibrillary tangle deposition, and degeneration of cortical and hippocampal neurons, thus preserving cognitive function in a mouse model of tauopathy.[137] Fisetin, a flavonoid found in fruits, can selectively induce apoptosis in senescent cells by targeting different signaling pathways such as BCL-2 and PI3/AKT.[82] The potential clinical relevance of senolytics have been shown in tauopathy-associated cognitive decline[137] and atherosclerosis[136] among others; some clinical trials are already completed[138,139] and many are ongoing.[109]

1.4.4.2. SASP modulation

Another strategy to target senescent cells is SASP modulation. SASP inhibitors can attenuate the deleterious consequences of SASP-signaling and reduce senescence-induced inflammation without killing senescent cells. (Figure 1.7) [110] Specifically, SASP modulation can be achieved by inhibiting proliferation and proinflammatory -signaling pathways, such as the p38, MAPK, the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), and the mTOR.[140]

1.4.4.3. Prevention of senescence

Another potential approach to target of senescence is to prevent it. Caloric restriction, for instance, can reduce cellular senescence by inhibiting the activation of the mTOR signaling pathway through AMP-activated protein kinase (AMPK).[145] Various drugs targeting this pathway, such as the mTOR inhibitor rapamycin (sirolimus) and the AMPK activator metformin, have demonstrated their ability to prevent cellular senescence in animal models.[141,142] Intermittent fasting offers similar benefits to calorie restriction in controlling senescence by improving glucose control, lowering inflammation and cardiovascular risks. However, intermittent fasting can lead to the reduction in bone density and lean body mass.[145]

In addition, inhibiting the accumulation of ROS using ROS scavengers like N-acetyl-L-cysteine has shown promise in preventing senescence in human cells cultured *in vitro*.[143] Lastly, hypertension prevention can also be beneficial in mitigating senescence (Figure 1.7). [144]

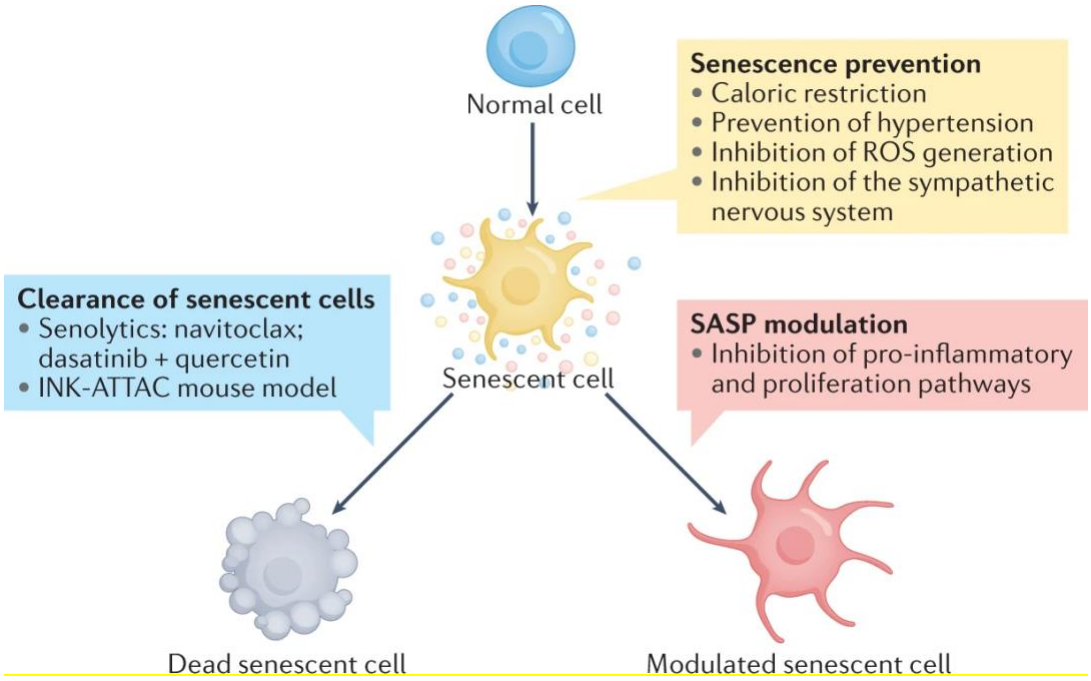

Figure 1.6. Therapeutic strategies for targeting cellular senescence in aging or diseased hearts.

Anti- senescence strategies include prevention of senescence, clearance of senescent cells and modulation of the senescence- associated secretory phenotype (SASP). Several interventions and drugs have been shown to prevent senescence in animal models, including caloric restriction and inhibition of reactive oxygen species (ROS) by suppressing ROS generation and/or increasing ROS scavenging. Senolytics, such as navitoclax and dasatinib plus quercetin, promote the clearance of senescent cells by interfering with the pro- survival signaling pathways that are active in senescent cells. In the INK- ATTAC mouse model, p16+ cells are selectively cleared from most tissues, including the heart, after drug treatment. SASP modulation is another approach to target senescent cells through the inhibition of pro- inflammatory and proliferation pathways. Adapted from Mehdizadeh et al.The role of cellular senescence in cardiac disease: basic biology and clinical relevance. Nat Rev Cardiol. 2022 Apr;19(4):250-264. doi:10.1038/s41569-021-00624-2. Epub 2021 Oct 19. PMID: 34667279.

1.4.5. Cardiac cell-type specificity of senescence

In targeting senescent cells, one major consideration is that senescence differentially affects cells depending on cell-type, tissue, and organ.[127] The heart contains a range of cell-types including cardiomyocytes, endothelial cells, fibroblasts, progenitor cells, immune cells, and epithelial cells. Several *in vitro* and *in vivo* studies have shown that all cardiac cell-types can undergo senescence with aging or cardiac disease.[10,12,145] Thus, the contributions of cellular senescence to aging-associated cardiac diseases can result from changes in a variety of cell-types. Although cellular senescence was initially thought to be a cell-cycle arrest response unique to dividing cells, recent research has proven that senescence is a more generalized response to cellular stress that also occurs in post-mitotic cells such as cardiomyocytes.[12,108,146] The occurrence and role of senescence in cardiomyocytes has been evaluated in only a limited number of studies and needs more investigation.[12,146]

Senescent cardiomyocytes are characterized by persistent DNA-damage at telomere-regions, independently of telomere length, which might be driven by mitochondrial dysfunction and oxidative stress. Senescent cardiomyocytes show upregulation of classical senescence markers such as p16, p21, and p53, along with atypical SASP components including growth/differentiation factor 15 (GDF15), TGFβ2, and endothelin-3 (EDN3).[12]

In contrast, senescent non-myocyte cardiac cells including endothelial cells, fibroblasts, immune cells, and epithelial cells are characterized by cell-cycle arrest, DNA-damage and the upregulation of p16, p21, and p53 as well as the production of SASP-components such as IL-6, chemokine (C-X-C motif) ligands 1 and 2 (CXCL1-2), TNFα, vascular endothelial growth factor (VEGF), MMP1-3, and plasminogen activator inhibitor-1 (PAI-1).[88] Importantly, the SASP-products from different cardiac cells adversely affect healthy neighbouring cells through paracrine effects, with consequences including fibroblast activation and differentiation, diminished

angiogenic potential and endothelial nitric oxide synthase 3 (eNOS) activity and prothrombotic properties of endothelial cells, and cardiomyocyte cell-death and hypertrophy (Figure 1.5). [12]

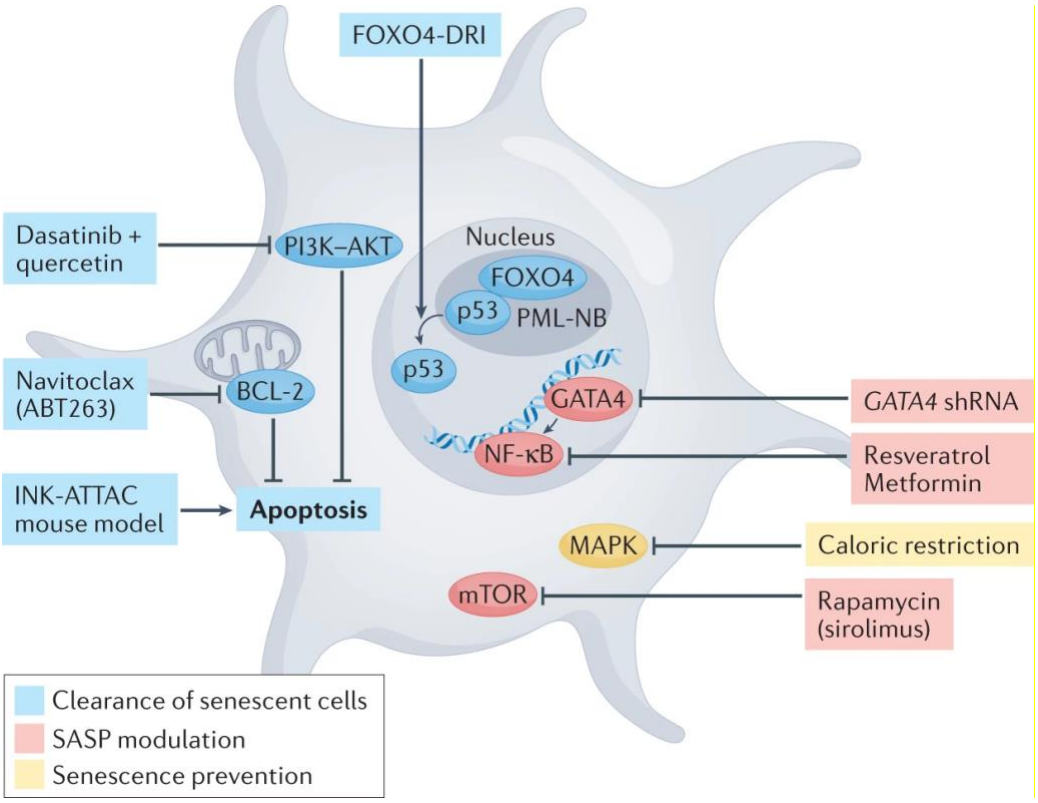

Figure 1.7. Molecular mechanisms underlying the therapeutic clearance of senescent cells and SASP modulation.

Strategies promoting the clearance of senescent cells are shown in blue. Dasatinib plus quercetin stimulate apoptosis in senescent cells by inhibiting the phosphoinositide 3- kinase (PI3K)–AKT pathway. Forkhead box protein O4(FOXO4)- DRI is a small peptide that disrupts the binding of FOXO4 to p53, causing p53to be released from the nucleus and activate apoptosis. Navitoclax (ABT263) inhibits antiapoptotic members of the BCL-2 family and consequently induces apoptosis in senescent cells. In the INK- ATTAC mouse model, senescent cells overexpress a 'suicide' gene (Casp8, encoding caspase 8) and undergo apoptosis after treatment with a polymerizing agent that activates the caspase. Senescence- associated secretory phenotype (SASP)- modulating strategies are shown in red. The mechanistic target of rapamycin (mTOR) pathway promotes SASP production and can be inhibited by the drug rapamycin (sirolimus). The transcription factor nuclear factor- κB (NF- κB) is essential for SASP production.

Resveratrol and metformin inhibit NF- κB activity and reduce the production of pro- inflammatory components of the SASP. The transcription factor GATA4 is an indirect regulator of the NF- κB pathway; therefore, inhibiting the translation of GATA4 by targeting with a short hairpin RNA (shRNA) can reduce SASP secretion. Approaches aimed at preventing cellular senescence are shown in yellow. Caloric restriction suppresses mitogen- activated protein kinase (MAPK) pathways, a necessary trigger for SASP production. PML- NB, promyelocytic leukaemia protein nuclear body. Adapted from Mehdizadeh et al. The role of cellular senescence in cardiac disease: basic biology and clinical relevance. Nat Rev Cardiol. 2022 Apr;19(4):250-264. doi: 10.1038/s41569-021-00624-2. Epub 2021 Oct 19. PMID: 34667279.

1.4.6. The role of senescence-associated pathways in the development of the heart

Numerous studies have explored the involvement of cellular senescence in cardiac embryonic development and tissue remodeling.[147,148] Senescence is a key process during embryogenesis, commonly known as developmental senescence, with the clearance of senescent cells being important for morphogenesis.[147] Lorda-Diez et al. noted an overlap between senescent and apoptotic cells in several areas of developing embryonic chick and mouse hearts, including the atrioventricular orifices, outflow tracts, and interventricular septum.[148] The presence of several typical components of the SASP, such as TGFβ-1, -2 and -3, MMP2 and MMP9, insulin-like growth factor-binding protein-5 (IGFBP5), and interleukin-1 β (IL-1β) in the outflow tract of developing chick hearts indicates an active involvement of cellular senescence in cardiac organogenesis.[148]

Additional evidence for this contribution comes from the observation that abnormalities in markers of senescence during development are associated with congenital heart defects.[149,150] For example, one abnormality is the mutation in sirtuin-1 (SIRT-1), an inhibitor of p53 transcriptional activity, which leads to congenital defects such as cardiac septal and valvular abnormalities as well as low perinatal survival.[149] This highlights the critical role of senescence-related signaling in the normal structural development of the heart. Furthermore, mice with knockout of paired box gene

8 (Pax8), a crucial regulator of murine heart development, develop cardiac dysplasia along with the upregulation of p21 and p53.[150] These studies suggest that senescence plays a role in the embryogenesis of the four-chambered mammalian heart, and dysregulated senescence signaling is associated with various congenital heart defects.

1.5. Cellular senescence in cardiac pathology

Mounting evidence suggests the significance of cellular senescence in the pathophysiology of age-related cardiac disease (Figure 1.8). Studies conducted over the past ten years confirm the role of aging as the primary risk factor for a wide range of cardiac pathologies, such as cardiac fibrosis, cardiac hypertrophy, heart failure, myocardial infarction, and cardiotoxicity.

1.5.1. Cardiac fibrosis

Studies in both animal models and in explanted human hearts have demonstrated the accumulation of senescent fibroblasts in fibrotic areas of the aged and diseased heart. [13,151,152] However, the precise mechanisms underlying the activation of senescence pathways in cardiac fibroblasts and their functional consequences are not well understood. The activation of profibrotic signaling pathways such as TGFβ and NLRP3 has been associated with cellular senescence in cultured fibroblasts.[129,153-156] Studies have identified several miRNAs, including miR-22, miR-17, miR-15, and miR-1468-3p, that are involved in fibroblast senescence.[117,118,157] These miRNAs have profibrotic properties and are involved in the pathogenesis of various cardiac conditions including AF and HF.[79,158]

The precise role of senescent fibroblasts in cardiac fibrosis is complex and research findings have reported conflicting results. On the one hand, some studies have reported an association between senescent fibroblast accumulation and fibrosis.[12,159,160] For example, dysfunctional fibroblasts derived from a knockout mouse model of MMP14, a collagenase essential for fibroblast

function, showed upregulation of p16 and SA-β-gal along with cardiac collagen accumulation.[159] Individuals with a mutation in the LEM domain containing protein 2 (LEMD2), an inner nuclear membrane protein, demonstrated cardiac fibrosis and the upregulation of senescence markers in fibroblasts.[160] However, it remains uncertain whether senescence plays a causal role in fibrogenesis or is a mere epiphenomenon in these studies. Notably, the global elimination of senescent cells in aged mice (using INK-ATTAC transgenesis or navitoclax treatment) reduces fibrosis, suggesting a potential causal role of senescent cells in cardiac fibrosis.[12] However, it remains unclear whether this effect is due to the elimination of senescent fibroblasts specifically or the elimination of senescent heart cells of other types that promote fibrosis.

On the other hand, several studies propose that cardiac fibroblast senescence can restrict fibrosis by attenuating fibroblast proliferation via cell-cycle arrest or anti-fibrotic SASP components.[105,151,161-163] For example, Sawaki et al. showed that visceral adipose tissue contributes to age-related cardiac fibrosis through the production of profibrotic factors such as osteopontin.[13] Osteopontin inhibition resulted in the upregulation of p16, p21 and SA-β-gal in fibrotic regions and a significant reduction in fibrosis, suggesting an anti-fibrotic role for cellular senescence.[13] Similarly, studies involving mice with myocardial infarction and left-ventricular hypertrophy showed that global p16 and/or p53 inactivation increases cardiac fibrosis, while the overexpression of cellular communication network factor 1 (CCN1), an inducer of cell senescence, showed anti-fibrotic effects in these conditions.[105,151,161] In another study, decreased fibroblast senescence in mice with haplodeficiency of ataxia telangiectasia mutated protein (ATM, an upstream signaling protein of p53) resulted in greater fibrosis and impaired left-ventricular ejection fraction.[162] Collectively, these findings indicate the complex role of cellular senescence in cardiac fibrosis. While the global clearance of senescent cells appears to reduce fibrosis, cell-cycle arrest due to senescence may reduce fibroblast proliferation and restrict fibrosis.

The role of senescence in fibrosis extends beyond cardiac fibrosis, as it has been studied in various other conditions such as lung, kidney and liver fibrosis. For example, Liu et al. showed that senescent fibroblasts accumulate in the lung tissue of bleomycin-injury idiopathic pulmonary fibrosis (IPF), a deadly condition characterized by lung tissue remodeling.[164] In this model, clearance of senescent cells with the combination of dasatinib and quercetin or INK-ATTAC transgene improved pulmonary function and physical activity, suggesting a detrimental effect of senescence in this condition.[165]

The role of senescence in kidney has been reported to be detrimental.[166] Chronic kidney cell senescence contributes to epithelial-to-mesenchymal transition (EMT) and triggers the SASP, which promotes inflammation and ultimately leads to fibrosis and chronic kidney disease (CKD).[167] Various factors such as inhibition of AMPK-mTOR signaling, activation of the Wnt-β-catenin pathway, or overexpression of Wnt9a can induce senescence in tubular epithelial cells, causing EMT and subsequent fibrosis.[167] In mouse model of ischemic acute kidney injury (AKI), treatment with nicotinamide mononucleotide reduced DNA damage and senescence in tubular cells, attenuating renal fibrosis.[168] Additionally, in mouse model of chronic renal ischemia and AKI progression to CKD, combination of dasatinib and quercerin alleviated kidney fibrosis.[169] This same combination therapy also halted the progression of AKI to CKD in mouse model of ischemia-reperfusion injury and cisplatin-induced injury.[170]

On the other hand, the role of senescence in the liver fibrosis was reported to be beneficial. Krizhanovsky et al. demonstrated that senescent hepatic stellate cells accumulate in mouse livers treated with a profibrotic agent, (C-C Motif Chemokine Ligand 4) CCL4.[171] Senescent stellate cells display gene expression patterns indicating cell-cycle arrest, reduced secretion of extracellular matrix components, increased secretion of enzymes that degrade the extracellular

matrix, suggesting that the senescence plays a beneficial role in limiting the fibrogenic response in acute liver damage.[171]

1.5.2. Cardiac hypertrophy

Cardiac hypertrophy is a physiological response to biomechanical stress leading to increased LV mass and decreased compliance.[172] While hypertrophy initially serves as an adaptive mechanism, uncontrolled hypertrophy can ultimately lead to HF and a higher susceptibility to sudden cardiac death.[172] At the cellular level, cardiomyocyte hypertrophy is characterized by the upregulation of contractile proteins.[172] Emerging evidence suggests that senescence actively contributes to the development of cardiac hypertrophy.[12,173,174]

The mechanisms underlying the senescence induction in cardiomyocytes, and their relationship to cardiac hypertrophy, are not fully understood. Several studies implicate ROS and mitochondrial dysfunction as significant contributor to the induction of cardiomyocyte age-related senescence.[12,175,176] The proposed mechanism involves increased ROS production and the downregulation of mitochondrial inner membrane proteins which leads to telomere-associated DNA damage, the upregulation of senescence markers, and SASP components. [12] Unlike other cell types, cardiomyocytes do not secrete the typical profile of SASP components.[11] The cardiomyocyte SASP (Figure 1.5) includes atypical components such as GDF15, EDN3, and TGFβ-2, all of which promote cardiomyocyte hypertrophy, fibroblast activation, and cardiac endothelial cell dysfunction. This suggests a potential paracrine effect of the cardiomyocyte SASP (Figure 1.5). [12]

Moreover, several studies showed the involvement of the IGF-1 signaling pathway in the SASP production of senescent cardiomyocytes, both *in vivo* and *in vitro.*[177-179] For example, aged cardiomyocyte-specific IGF-1 receptor knockout mice showed better cardiac function and less cardiac tissue expression of senescence markers and SASP compared to aged wild-type mice.[178]

In another study, the knockout of cathepsin K, a lysosomal protease, reduced hypertrophy while suppressing cardiac tissue expression of IGF-1, p21, and p16.[179] These findings suggest a potential link between IGF-1 signaling, cardiac hypertrophy, and senescence.

Some studies have investigated the impact of senescence suppression on cardiac hypertrophy.[12, 180-182] Anderson et al. used INK-ATTAC transgenic mice or navitoclax to globally remove senescent cells in aged mice, which led to reduced cardiomyocyte size and fibrosis without significant changes in left-ventricular mass or cardiac function. Based on these results, the authors concluded that cardiomyocyte senescence has a key role in age-related cardiac hypertrophy and that the elimination of senescent cardiomyocytes allows for cardiomyocyte regeneration, as indicated by the increased numbers of ethynyldeoxyuridine (EdU) and Ki-67-positive mononuclear cardiomyocytes.[12] Non-selective interventions, including calcium sensitizers, resveratrol, and calorie restriction were shown to ameliorate senescence in cardiac tissue and cardiac hypertrophy.[180-182] The mechanisms underlying these effects is still under investigation.

Together, the presence of senescence in cardiomyocytes is closely linked to cardiac hypertrophy, while the global clearance of senescent cells reduces cardiac hypertrophy. However, the mechanisms associated with senescence in cardiomyocytes and their pathophysiological significance need more investigation.

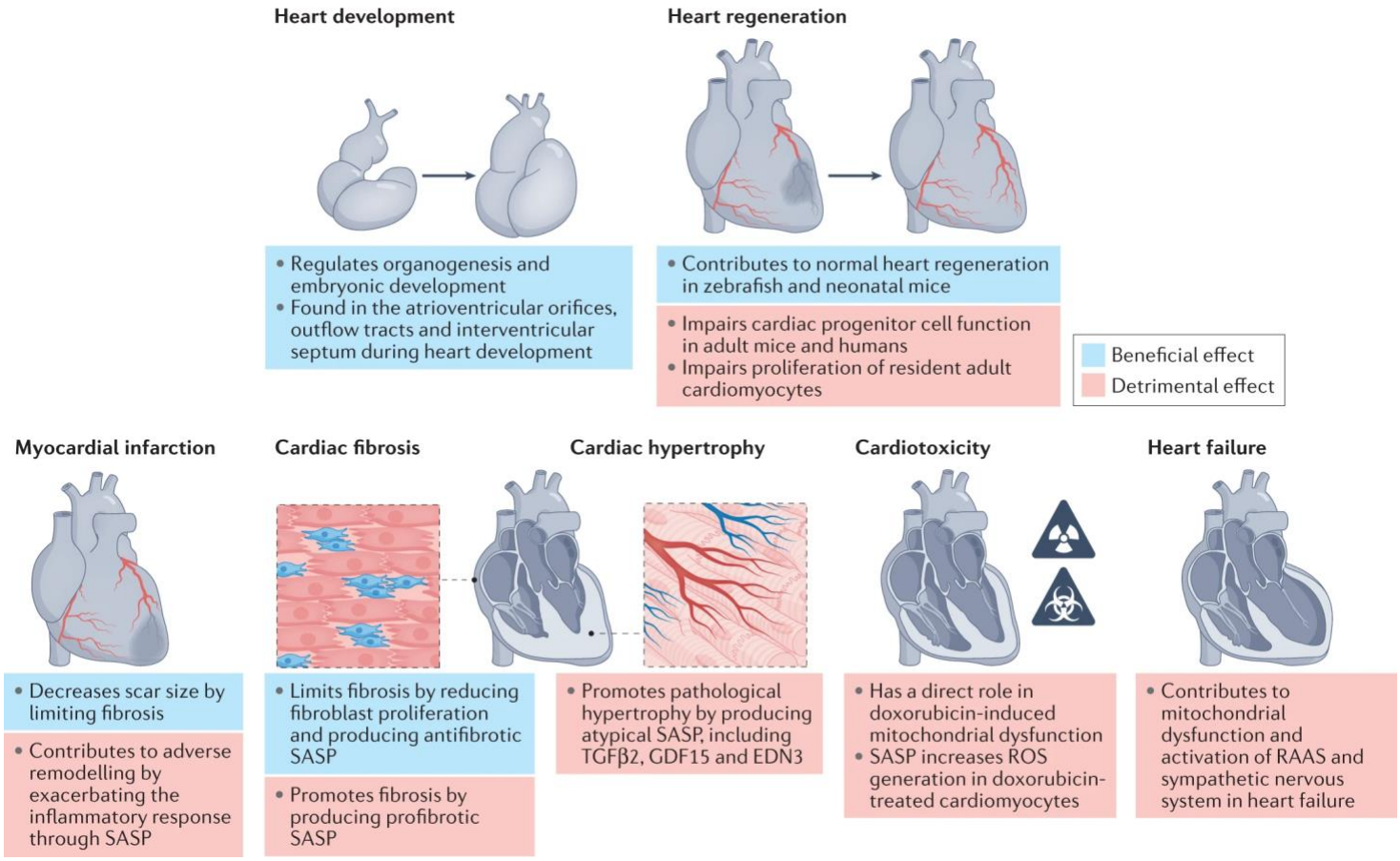

Figure 1.8. Involvement of cellular senescence in the pathogenesis of cardiac disease.

Senescent cells appear transiently in the heart and have physiological roles during heart regeneration (relevance shown in lower vertebrates) and heart development. However, when sustained, the presence of cellular senescence is believed to produce mainly deleterious effects in cardiac disease. Effects are colour coded in the figure: blue indicates beneficial effects and red indicates detrimental effects. The background in the box for each category reflects the balance between beneficial and detrimental effects for that category, according to the currently available literature. EDN3, endothelin 3; GDF15, growth differentiation factor 15; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species; SASP, senescence- associated secretory phenotype; TGFβ2, transforming growth factor β2. Adapted from Mehdizadeh et al.The role of cellular senescence in cardiac disease: basic biology and clinical relevance. Nat Rev Cardiol. 2022 Apr;19(4):250-264. doi: 10.1038/s41569-021-00624-2. Epub 2021 Oct 19. PMID: 34667279.

1.5.3. Heart failure

HF is a condition where the heart fails to adequately supply the peripheral tissues with sufficient blood and oxygen to meet their metabolic needs.[183] This leads to a decrease in cardiac output and can result in a pathological distribution of blood flow. Clinically, HF manifests as a syndrome characterized by symptoms including dyspnea or fatigue, elevated jugular venous pressure, tachycardia, and peripheral edema.[184] The primary cause of HF is typically an underlying disease such as ischemic heart disease, hypertension, and diabetes. Less common etiologies include endocardial or pericardial abnormalities, valvular disease, myocarditis, infections, systemic toxins, and cardiotoxic drugs.[183]

Among patients with diagnosed HF nearly half have preserved ejection fraction (≥45–50%; HFpEF), and the prevalence of HFpEF in comparison to HF with reduced ejection fraction (HFrEF) is increasing by 10% per decade.[185] The majority of HFpEF patients are diagnosed at the age of 60 or older, indicating that as life expectancy improves in western societies, the significant public health challenge posed by HFpEF will grow.[186] HFpEF is characterized by several key features including elevated arterial and myocardial stiffness, reduced relaxation of the LV, leading to elevated LV end-diastolic pressure and impaired LV filling.[187] HFrEF, on the other hand, is characterized by an impaired LV contractility accompanied with a reduced EF. The most common underlying causes of HFrEF are myocardial infarction, and heart valve diseases among others.[188]

1.5.3.1. Overview of heart failure pathophysiology

In HF, several underlying mechanisms play critical roles, with some of the most significant ones being activation of neurohumoral system, cardiomyocyte hypertrophy, apoptosis, necrosis, mitochondrial dysfunction with ROS production, fibrosis, and diastolic dysfunction (Figure 1.9).

1.5.3.1.1. Activation of Neurohumoral system

The decrease in cardiac output or vascular underfilling in the HF leads to baroreceptor-mediated sympathetic nervous activation.[189] This results in an elevation of heart rate, blood pressure, and vasoconstriction.[190] While this adaptation initially helps mitigate the acute drop in cardiac output, it eventually becomes maladaptive and leads to the downregulation of myocardial β-receptors, disrupting the normal coupling of contractility to stimuli.[191] In HF, there is an increased adrenergic tone along with pathological activation of the RAAS.[192] Excessive production of angiotensin II stimulates the adrenal glands to release more catecholamines, which further stimulates the kidney to release renin. Renin increases vascular tone and imposes pressure overload on a heart that is already susceptible to hemodynamic injury.[193] Furthermore, continuous neurohumoral activation induces transcriptional and posttranscriptional modifications in the genome, particularly affecting genes that regulate the structure and mechanical properties of cardiomyocytes.[194]

1.5.3.1.2. Cardiomyocyte hypertrophy, apoptosis, and necrosis

The interplay of pressure and volume overload, along with neurohormonal and cytokine signaling leads to cardiomyocyte hypertrophy, autophagy, apoptosis and necrosis.[183] These processes initiate with inflammatory, ischemic, or toxic injury, but are sustained by persistent oxidative stress and an increase in TNFα, norepinephrine, and angiotensin II.[195,196] Collectively, these processes result in decreased ventricular contractile function and progression of HF.[195,196]

1.5.3.1.3. Mitochondrial dysfunction and ROS production

An impaired coronary blood flow disrupts the balance between oxygen and energy demand and supply in the heart. This imbalance leads to an increased production of ROS, damaging mitochondrial DNA.[197] Due to the limited repair capacity of mitochondrial DNA, damaged DNA accumulates, further compromising energy homeostasis in the heart. This DNA damage and compromised energy homeostasis can further promote repetitive cycles of ROS generation results

in progressive cellular damage the heart.[198]Also, functional changes and structural abnormalities of mitochondria is detectable in HF; the number of mitochondria increases while the mitochondrial volume decreases.[199]

1.5.3.1.4. Fibrosis

HF is characterized by the progressive accumulation of interstitial collagen fibers, which impairs myocardial contractility and compliance, leading to ventricular dysfunction.[80] In HF, not only the quantity but also the quality of cardiac collagen changes, with a shift from insoluble to soluble collagen, resulting in reduced myocardial cross-linking and impaired ventricular contraction.[78,81]

1.5.3.1.5. Diastolic disfunction

Diastolic dysfunction, the heart's inability to adequately fill the ventricle with a sufficient preload volume at low pressures, is an important characteristic of HF, specifically HFpEF.[186,185] Diastolic function is conceptualized as the combination of two processes; active relaxation in early diastole, involving myofilament dissociation and calcium reuptake, and the passive stiffness.[186,185]

Several studies have shown that the isovolumic relaxation time is prolonged in HFpEF.[200-202] Regan et al. showed in a mouse model of HFpEF induced by a low dose of angiotensin II, the LV isovolumetric relaxation time increased along with the LV end-diastolic pressure without altering LV mass, dimensions, or ejection fraction.[203] Similarly, in patients diagnosed with HFpEF, the elevation of filling pressure was accompanied by prolonged relaxation time and LV stiffness.[204]

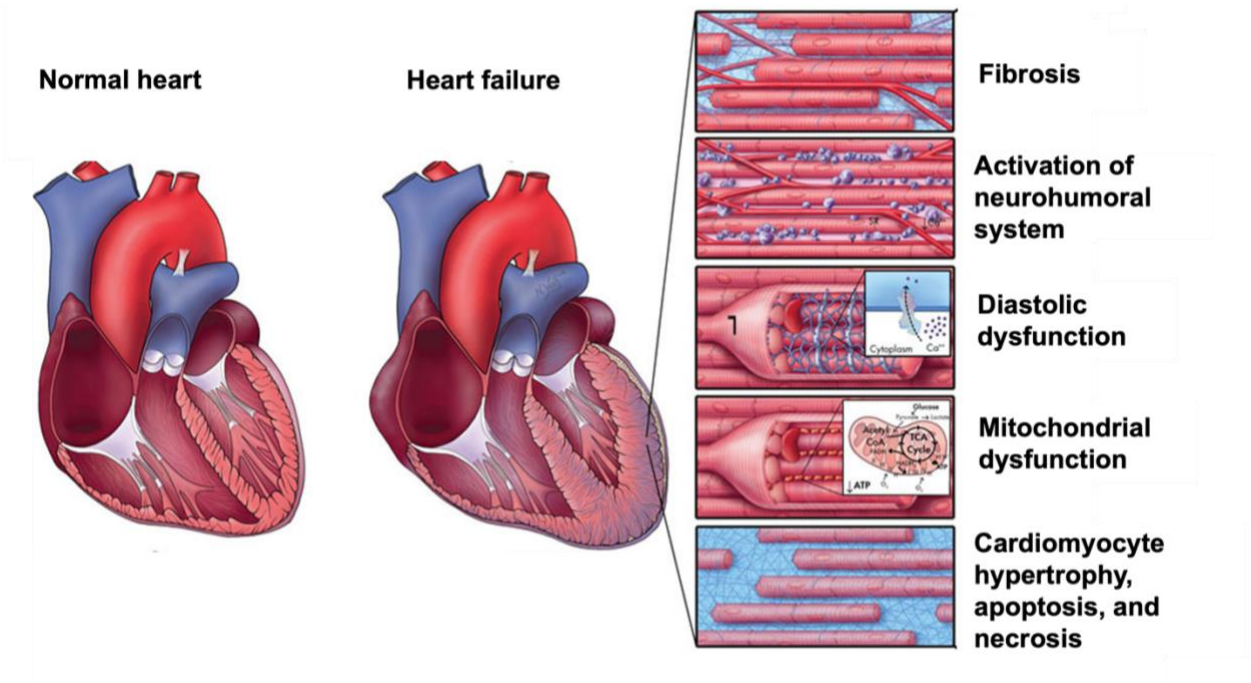

Figure 1.9. Schematic of processes and pathways contributing to heart failure.

Multiple processes likely contribute to heart failure, including fibrosis, inflammation, mechanical stiffening and diastolic dysfunction, mitochondrial dysfunction, and a growing imbalance between loss and birth of cardiomyocytes. Adapted from Li et al, Targeting Age-Related Pathways in Heart Failure. Circ Res. 2020 Feb 14;126(4):533-551. doi: 10.1161/CIRCRESAHA.119.315889. Epub 2020 Feb 13. PMID: 32078451; PMCID: PMC7041880.

1.5.3.2. Cellular senescence and heart failure

Several studies show a correlation between cellular senescence and HF pathophysiological pathways, suggesting that targeting senescence may hold therapeutic promise in prevention and/or treatment of HF.[12,209-213]

Mitochondrial dysfunction is frequently linked to cellular senescence.[12,205-207] Mice with global knockout of proto-oncogene serine/threonine-protein kinase-1 (PIM-1), a kinase with protective effects on telomere length and mitochondria, develop HF at 6 months of age and show high expression of SA-β-gal, p16 and p53 in cardiac tissue, accompanied by deterioration of mitochondrial structure and function.[205] Similarly, mice with diabetic cardiomyopathy show

overexpression of senescence markers in cardiac progenitor cells (CPCs) and cardiomyocytes, accompanied by mitochondrial dysfunction and augmented ROS production.[206]

Only few studies have assessed the association between RAAS activation and cardiac senescence, whereas the RAAS's role in vascular senescence has been studied extensively.[208,209] In a study using a diabetic mouse model, Kosugi et al. found that the administration of an angiotensin-II receptor blocker (candesartan) improves cardiac function while reducing cardiac-tissue expression of senescence markers.[210] Another study demonstrated that the global elimination of senescent cells with navitoclax improves cardiac electrophysiological and functional abnormalities in a mouse model of angiotensin II-induced HF.[211] The sympathetic nervous system also plays a crucial role in HF as a neurohormonal system in HF.[212] In the isoproterenol-induced HF model, Katsuumi et al. observed that mice with endothelial-specific p53 knock-out had better cardiac function, suggesting that p53-mediated endothelial cell-senescence pathways are involved in HF development.[144]

Autophagy, a process that involves bulk degradation and recycling of proteins and organelles, has an adaptive response with protective effects in HF.[213]Although the impact of cell-senescence on autophagy is not fully understood, a study by Inuzuka et al. showed that PI3K inhibition enhances autophagy and preserves cardiac function, while reducing the cardiac tissue expression of p16, p21, p53 and several SASP components in aged mice. This finding suggests an inverse relation between senescence and autophagy in HF.[214] Finally, clinical studies have found that the cardiac expression of SASP components including IGFBP7 and interleukin-6 (IL6) or reduction in SIRT1 correlate with the severity of HF disease.[215] These studies actively investigate molecules as potential biomarkers for the diagnosis and prognostication of HF patients.

Overall, these findings indicate that senescence may interact with several pathophysiological pathways involved in HF, such as mitochondrial dysfunction, autophagy, and neurohumoral-

system activation. However, further research is necessary to fully identify the role of senescence in HF, the mediating cardiac cell-types, and whether suppression of senescence has therapeutic potential for treating HF.

The development of novel medications for HFpEF is crucial due to limited treatment options.[185] Since aging is a predominant factor in HFpEF and considering the promising results of senolytics in various age-related diseases, they might be beneficial for HFpEF as well.[87] Senolytics have the potential to improve HFpEF by reducing senescent cells and the pro-inflammatory and profibrotic SASP productions, which can result in less hypertrophy and fibrosis, ultimately enhancing diastolic function. It is essential to investigate the effects of senolytic therapy on animal models of HFpEF, such as the model that combined aging, obesity and hypertension in mouse.[186]Assessing the efficacy of senolytics by measuring the reduction of SASP biomarkers in serum or evaluating cardiac diastolic function and fibrosis with imaging or echocardiography is crucial. Additionally, it is important to consider senolytic side effects, such as off-target effects that can affect healthy non-senescent cells. Moreover, certain senolytics, like dasatinib, may lead to the prolongation of the QT interval, which is significant to consider in any cardiac patients.[215]

1.5.4. Myocardial infarction (MI)

In MI, cardiac-tissue death occurs because of a critical imbalance between myocardial blood flow needs and supply, typically caused by the rupture of a vulnerable atherosclerotic plaque and subsequent thrombotic occlusion of a coronary artery.[216]

1.5.4.1. Overview of myocardial infarction pathophysiology

Cardiac repair following a MI involves a well-coordinated sequence of events.[217] It begins with an intense sterile inflammation and infiltration of immune cells, known as the inflammatory phase, which has the purpose of clearing damaged cells and extracellular matrix tissue.[218] This phase typically lasts around 3-4 days in mice. Subsequently, a reparative phase ensues, characterized by the resolution of inflammation, the proliferation of fibroblasts and myofibroblasts, scar formation, and neovascularization over the following days (Figure 1.10). [219]

The initial activation of inflammation is crucial for efficient clearance of apoptotic cardiomyocyte and the transition to the reparative and proliferative processes, however, if the inflammatory phase persists excessively, is too intense, or inadequately suppressed, it can cause sustained tissue damage, increased cell loss, cardiac rupture and impaired contractile function.[220]

The timely suppression and containment of the inflammatory response after MI rely on the release of secreted anti-inflammatory mediators, such as IL-10, members of the TGFβ family, and lipid-derived pro-resolving mediators. [221,222] These mediators inhibit the innate immune response and promote the resolution of inflammation.[221,222] Dysfunction in the molecular pathways responsible for suppressing and resolving the inflammatory response may contribute to adverse remodeling and HF following MI.[219]

During the subsequent proliferative phase of cardiac repair, cardiac fibroblasts proliferate and then differentiate into myofibroblasts in response to the presence of bioactive TGFβ family and changes in the surrounding extracellular matrix.[223] Furthermore, the extensive necrosis of cardiomyocytes in the healing infarct leads to the induction of chemokines, which can recruit and activate additional subsets of reparative fibroblasts, contributing to scar formation (Figure 1.10). [225]

After the proliferative phase, the maturation or late remodeling phase of cardiac repair begins, which is characterized by the formation of the scar.[223] The specific molecular signals that contribute to this phase are not yet fully understood. However, events such as the reduction in fibrogenic growth molecules, the activation of inhibitory signals that terminate TGFβ and angiotensin II signaling, and the clearance of matricellular proteins may play a role in suppressing myofibroblast proliferation and reducing their collagen-producing activity.[217] The secretion of antifibrotic mediators may also contribute to the termination of collagen synthesis.[224]

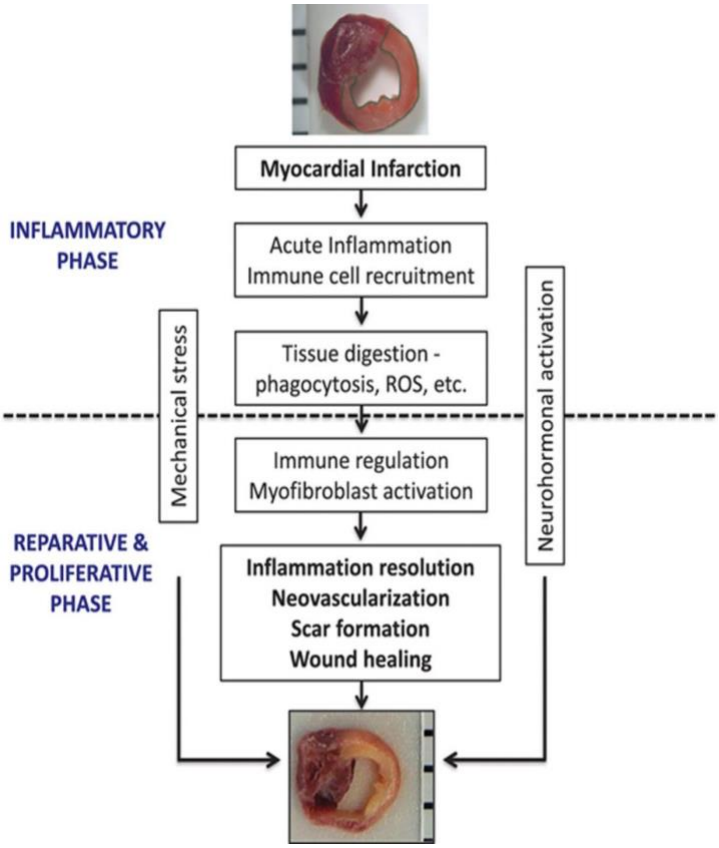

Figure 1.10. Biphasic nature of cardiac repair after myocardial infarction (MI).

Early after MI, tissue injury and necrosis initiate the inflammatory phase, consisting of intense sterile inflammation, and the dynamic recruitment of several immune cell subtypes including neutrophils, monocyte/macrophages, dendritic cells, and lymphocytes. After around 4 d in murine models, this transitions to a reparative and proliferative phase, with shift of immune cell polarity toward immunomodulation and resolution, myofibroblast proliferation, collagen

deposition and scar formation, and neovascularization, thereby resulting in wound healing. Neurohormonal activation and mechanical stress are other factors that influence this healing process. ROS indicates reactive oxygen species. Adapted from Prabhu SD, Frangogiannis NG. The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. Circ Res. 2016 Jun 24;119(1):91-112. doi: 10.1161/CIRCRESAHA.116.303577. PMID: 27340270; PMCID: PMC4922528.

1.5.4.2. Cellular senescence and myocardial infarction

Current evidence from research in animal models and human studies suggests a crucial role of cellular senescence in the infarcted heart.[108,161,225,226] Several studies have investigated the upstream signaling pathways that resulted in the activation of senescence following MI. In a mouse model of MI, the accumulation of senescence markers in the peri-infarct zone is linked to the upregulation of dynamin-related protein 1 (Drp-1), a key regulator of mitochondrial fission.[227] The type of cells involved was not identified. In an *in vitro* model of myocardial ischemia using H9c2 cells subjected to hypoxia/re-oxygenation, the inhibition of Drp-1 attenuates the expression of senescent markers, suggesting that ischemia-mediated mitochondrial dysfunction may trigger cellular senescence.[227] Additionally, the activation of the AKT/mTOR pathway in hypoxia-exposed H9c2 cells was found to with the activation of senescent markers, while the inactivation of this pathway is accompanied by reduced senescence markers.[228] These findings indicate that the discussed pathways may play an active role in the activation of senescence and could be subjected to therapeutic modulation in ischemic heart disease.

Despite this connection, the involvement of cellular senescence in the pathogenesis of MI is controversial, as both positive and negative effects have been reported.[146,226,229,230] The global clearance of senescent cells with navitoclax in aging mice prior to MI induction significantly improves their survival.[226] The navitoclax-treated mice also show better post-MI cardiac functional, indicating that senescent cells promote adverse remodeling.[226] Furthermore,

conventional drug therapies that enhance cardiac function post-MI were also shown to reduce cardiac-tissue senescence-marker expression.[182,231] In a diabetic rat MI model, the calcium sensitizer levosimendan improves cardiac function and reduces cardiac-tissue p16 expression.[230] Similarly, losartan, an angiotensin-II receptor blocker, partially reverses left-ventricular dysfunction and remodeling post-MI, while reducing p16 expression and oxidative stress marker and in cardiomyocytes.[231] On the other hand, several studies have proposed the beneficial role of cell senescence following MI.[108,162,232] For example, using an MI mouse model, Cui et al. showed that knocking down GATA binding protein-4 (GATA4), an upstream regulator of NF-κB, reduces SASP products in peri-infarct tissue.[210] This reduction in SASP production is associated with an increase in fibrosis and post-infarct systolic dysfunction[108], indicating that SASP components in the peri-infarct area have antifibrotic and cardioprotective effects post-MI. Other studies have also provided evidence suggesting that cell senescence reduces after MI fibrosis.[105,162,232]

In contrast to the many studies investigating the role of senescence in MI models, only few have assessed the impact of senescent cells in ischemia-reperfusion injury models.[233,234] In one such report, Dookun et al. showed that the global clearance of senescent cells with navitoclax following ischemia–reperfusion improves cardiac function, reduces the inflammatory response, and increases angiogenesis.[234]

Collectively, significant data suggests an association between cellular senescence and pathways involved in the pathogenesis of MI. However, much of the data show correlations rather than proving cause-and-effect relationships. The precise role of senescence in terms of mediating complications versus protecting against them, and the dependency of the effects on cardiac cell type as well as any phase within MI remain poorly understood.

1.5.5. Atrial fibrillation

AF, characterized by rapid and irregular atrial activation, is the most common cardiac arrhythmia in clinical practice.[235] The occurrence of AF increases drastically with age, with a progressive doubling with each decade after 65 years of age.[236] In addition to age, some clinical conditions such as MI, HF and hypertension are associated with AF.[237]

1.5.5.1. Overview of AF pathophysiology

There are four main mechanisms contributing to the pathophysiology of AF: (1) Electrical remodeling, (2) Calcium mishandling, (3) Structural remodeling, and (4) Autonomic nervous system changes.[238] These mechanisms, depicted as red boxes in Figure 1.11, contribute to the development of AF and are often associated with underlying cardiac conditions, which are represented by blue boxes in the diagram. Conversely, AF itself can further exacerbate AF-promoting abnormalities, as indicated by the red dashed arrows.[238] Focal ectopic firing, depicted as yellow boxes in Figure 1.11, refers to the generation of abnormal electrical impulses from specific sites other than SA node within the atria, such as the pulmonary veins. Focal ectopic firing is commonly due to delayed after depolarizations (DADs) that reach threshold and cause spontaneous AP generation. Focal ectopic firing can trigger reentry, a common mechanism in AF occurring when there is a circuit of electrical activity that perpetuates itself by continuously propagating through the atria (green boxes in Figure 1.11).[238]

Electrical remodeling

Electrical remodeling disrupts the expression and function of ion channels, creating conditions that favor the development of AF.[239] The principal components of electrical remodeling in AF are changes in multiple atrial ion channels; on one hand certain channels are upregulated, including the transient outward (Ito) current, the atrial-specific ultrarapid delayed-rectifier current (I_{Kur}), the sustained (I_{sus}) potassium, and the acetylcholine-induced potassium current (I_{KACh}).[38,238] On the

other hand, $I_{Ca,L}$ channel is downregulated in AF.[239] In addition to changes in ion channels, gap junction remodeling involving connexin 40 and connexin 43, which facilitate electrical coupling between cardiomyocytes, is another important aspect of electrical remodeling.[240] Importantly, electric remodeling contributes to various clinically significant phenomena, such as the early recurrence of AF after cardioversion, increasing resistance to drugs in longer-lasting AF cases, and the progression from paroxysmal AF to more persistent forms.[240]

Calcium mishandling

Calcium mishandling has direct pro-arrhythmic effects in the atria. Dysregulation of calcium handling can lead to the generation of spontaneous abnormal electrical activity.[241] The role of calcium mishandling in AF has been discussed in several animal models and human studies.[238,239,242,243,247]For example, Yan et al. showed an increase in c-Jun N-terminal kinase (JNK) activation in aged mice, which caused diastolic SR Ca^{2+} leaks and increased AF susceptibility. In this study, calcium/calmodulin-kinase type II (CaMKII), a key modulator of cell Ca^{2+} handling, was shown to be the downstream target of JNK.[244] AF patients' atrial samples have shown an elevation in spontaneous diastolic release of Ca^{2+} from the SR through leaky RyR2 channels. This is accompanied by phospholamban hyperphosphorylation, which enhances SR Ca^{2+} loading by disinhibiting the Ca^{2+} uptake protein, SERCA2a.[238,239,242,243]

Structural remodeling

Structural remodeling in AF is characterized by both atrial enlargement and the presence of fibrosis in the cardiac tissue.[249] Atrial dimension, which increases progressively with age in humans, plays an important role in the persistence of AF by promoting reentry mechanisms.[245] Atrial fibrosis is a significant pathophysiological factor contributing to AF recurrences, treatment resistance, and complications.[246-248] Furthermore, fibroblast-cardiomyocyte interactions can change the electrical properties of cardiomyocytes, potentially promoting reentry and/or ectopic activity.[249]

Additionally, AF itself promotes the development of atrial fibrosis, which can contribute to treatment resistance in patients with long-standing AF.[250]

Autonomic nervous system changes

Autonomic nervous system plays a significant role in the initiation and maintenance of AF.[240]On one hand, the activation of the adrenergic system leads to an increase in RyR2 leak, SR Ca^{2+} load, and the development of ectopic activity. [241,251] On the other hand, vagal stimulation enhances the I_{KACh}, leading to a reduction in APD and stabilization of reentries.[252]

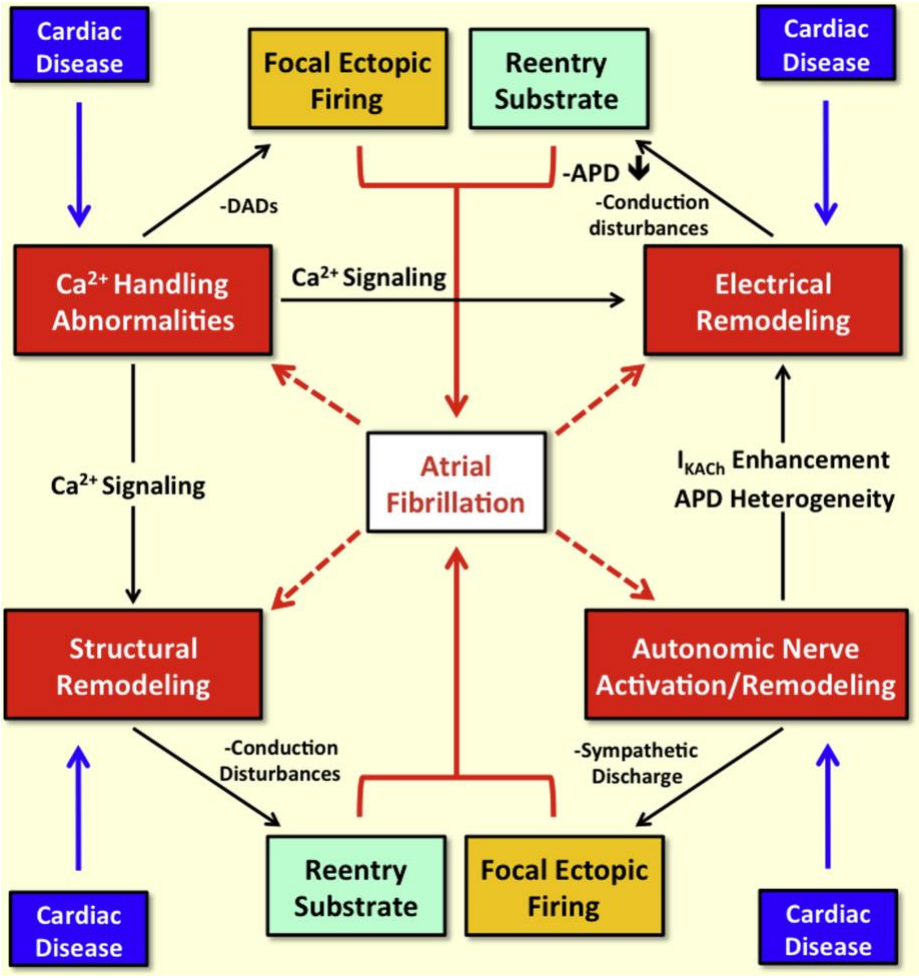

Figure 1.11. Major components of atrial Remodeling Underlying the Pathophysiology of AF.

The principal AF-promoting mechanisms resulting from AF-inducing remodeling are shown in red boxes. Focal ectopic firing is usually due to DADs that reach threshold and cause spontaneous AP generation. A susceptible reentry substrate requires abbreviated refractoriness (which depends primarily on APD) and/or conduction abnormalities.

These mechanisms can be the result of AF-promoting cardiac diseases but can also result from the consequences of AF itself. *AF*. atrial fibrillation; APD. action potential duration; DADs. delayed afterdepolarizations; I_{KACh} . acetylcholine-regulated K^+ current. Adapted from Stanley Nattel, Masahide Harada, Atrial Remodeling and Atrial Fibrillation: Recent Advances and Translational Perspectives, Journal of the American College of Cardiology, Volume 63, Issue 22,2014,Pages 2335-2345,mhttps://doi.org/10.1016/j.jacc.2014.02.555.

1.5.5.2. Cellular senescence and atrial fibrillation

Increased tissue expression of senescence markers in the heart is associated with greater AF progression and atrial fibrosis.[163,253] However, the mechanistic role of cellular senescence in AF pathophysiology and the potential consequences of removal of senescent cells for AF-substrate development have not been tested experimentally.

1.5.6. Senescence in other cardiac conditions

1.5.6.1. Cellular senescence and Cardiotoxicity

The administration of cancer chemotherapeutic drugs and radiation therapy are closely linked to cardiotoxicity and an increased risk of HF, coronary artery, valvular disease, and conduction system disease.[254] The mechanisms underlying the cardiotoxicity are complex and involve DNA damage, ROS generation, and the dysregulation of cardiac transcription factors.[255] Compelling evidence suggests that senescence plays a key role in mediating chemotherapy-/radiation-induced cardiomyopathy.[256,257]

However, there are distinct differences between senescent cells that accumulate due to aging and those induced by cytotoxic agents. Chemotherapy-/radiation triggers the formation of senescent cells in the heart through mechanisms such as DNA damage, oxidative stress, and activation of stress-response pathways.[88] This acute induction can result in the rapid onset of cellular senescence.[87] In contrast, age-related senescence occurs gradually over time due to accumulated

damage and stress from various sources, including metabolic dysfunction, telomere shortening, and immune system impairment. Both acute senescence and age-related senescence involve the upregulation of senescence markers such as p53, p21, and p16, but the components of the SASP can differ, although there is currently no study directly comparing these differences. In terms of morphology, chemotherapy-/radiation induced senescent cells exhibited a notably larger size compared to aged related senescent cells.[87,88] It has been suggested that aging is linked to increased irregularities or folds in the nuclear envelope.[85,89]

Human heart samples obtained from individuals treated with doxorubicin, an anthracycline chemotherapeutic agent, showed that the significant number of CPCs express p16 along with DNA damage, CPC migration impairment, and increased ROS production.[256]

Several animal models have been employed to study the mechanisms underlying cardiac senescence in induced by doxorubicin.[116,258,259] Sahin et al. showed that doxorubicin-treated mice have an increased cardiac expression of p53 and an enhanced binding of p53 to the promoter of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, a master regulator of mitochondrial function).[116] This process represses PGC-1α, but the repression is halted in p53 knockout mice, suggesting a direct role of cellular senescence in mitochondrial dysfunction subsequent to doxorubicin treatment.[116] Furthermore, an *in vitro* study showed that the inhibition of PAI-1, a SASP component, decreases ROS generation in doxorubicin-treated endothelial cells and cardiomyocytes.[258] Additionally, the activation of the PI3K/AKT pathway and its downstream target eNOS have potential senescence-suppressing effects in doxorubicin-induced cardiomyopathy.[111,259-261]

Doxorubicin-induced senescence is also associated with the altered expression of several non-coding RNAs.[262-265] The ectopic expression of circ-Foxo3 (a circular RNA) in mice exacerbates

doxorubicin-induced cardiomyopathy and induces senescence in cardiac tissue, whereas silencing endogenous circ-Foxo3 shows opposite effects.[264] Moreover, *in vitro* studies on CPCs from mice and rats show that doxorubicin increased the level of miR34a, inducing senescence through the p53-miR34-SIRT 1 axis, whereas the inhibition of miR34a suppresses senescence markers and upregulates SIRT-1.[262,263] Furthermore, Xie et al showed long non-coding RNA 21 (lncRNA-21) mediates doxorubicin-induced senescence in cardiac tissue, in part by increasing p53 binding to the p21 promoter.[266] Collectively, these findings implicate a significant role of non-coding RNAs in the induction of doxorubicin-induced senescence and point to the potential value of their modulation in preventing doxorubicin-induced cardiotoxicity.

While a considerable body of research investigates the involvement of senescence in doxorubicin-induced cardiotoxicity, the role of senescence in radiation-induced cardiac disease has not been studied in detail. Radiation therapy causes DNA damage, triggering senescence response.[267,268] Applying the INK-ATTAC model, Anderson et al. showed that thoracic irradiation triggers senescence and hypertrophy in the heart, these effects are mitigated by the global clearance of senescent cells.[12]

The cardiotoxic effects of cancer chemotherapeutic agents and/or radiation therapy limit cancer therapy; the prevention of cardiotoxic effects is an important but poorly addressed.[269] Clarifying the role of cellular senescence as a mediator and identifying the critical cell-types involved may lead to novel prophylactic and/or therapeutic modalities. Furthermore, cancer therapies can lead to both immediate and delayed cardiac damage. For example, injury to valves and the conduction system can occur several years after exposure[254]; it is plausible that cellular senescence plays a key role in the delayed effects, which might be amenable to senescence targeting.

1.5.6.2. Cellular senescence and heart regeneration

Unlike the hearts of lower vertebrates such as zebrafish, the adult mammalian heart lacks an effective regenerative response.[274] As a result, the loss of cardiomyocyte is generally considered an irreversible process leading to adverse remodeling, fibrosis and HF.[270-272] There has been great interest in developing regenerative therapies to enhance cardiac function in heart disease.[270-272] Since the degree of engraftment and differentiation of injected stem-cells into cardiomyocytes is very limited, other mechanisms, such as paracrine effects, may account for the beneficial effects reported from cell therapy.[273] Different types of cells have been studied, including CPCs, mesenchymal stem cells (MSCs), and cardiosphere-derived cells (CDCs), and were found to undergo senescence with stress or aging.[274,275,276] CPCs isolated from diseased or aged human hearts show upregulation of p16, p53 and shorter telomeres as well as impaired function. Senescent CPCs show reduced regenerative capacity and ability to reverse remodeling compared to healthy CPCs[277]. Conversely, suppression of senescence improves the function and therapeutic capacity of transplanted or resident stem cells.[145,277-287] For example, in an *in vitro* study, the knock down of p16 using short hairpin RNA (shRNA) reversed the senescent phenotype in human CPCs, while reducing ROS generation and increasing the levels of antioxidant proteins, including cytoglobin (CYGB), peroxiredoxin (PRDX1), and sulfiredoxin 1(SRXN1). Thus, modulating senescence might be a useful strategy to enhance the therapeutic benefits of cell therapy.[288]

Two approaches have been implemented to investigate whether the suppression of senescence can improve heart regeneration. Some studies focus on targeting senescence directly *via* the INK-ATTAC transgenesis, senolytics or knockdown of proteins involved in senescence.[12,277] For example, Lewis-McDougall et al. showed that the global elimination of senescent cells in aged mice (INK-ATTAC or wild type mice treated simultaneously with the senolytics dasatinib plus quercetin) leads to an increase in the population of resident CPCs.[277] In addition, there is evidence

suggesting that the depletion of senescent cells can result in the rejuvenation of stem cell populations and promote their proliferation.[12] For example, global clearance of senescent cells in the INK-ATTAC mouse model showed an increase in the numbers of Ki67 and EdU positive cardiomyocytes, indicating potential stimulation of cardiomyocyte proliferation.[12,277]

Alternatively, other studies have explored targeting molecular pathways linked to senescence, such as AMPK, AKT/mTOR and Wnt pathways. The activation of AMPK-pathway improves the reparative capacity of senescent MSCs and CPCs in a mouse MI model.[281] The inhibition of mTOR-pathway with rapamycin reduces senescence markers in human and mouse CPCs and improves their function.[289,290] In an *ex vivo* model, the activation of IGF-1 followed by the activation of AKT and the overexpression of SIRT-1 delays senescence and enhances proliferation of MSCs, while increasing their capacity to enhance heart function in a mouse MI model[282]. Finally, the overexpression of Wnt-pathway proteins such as Wnt3A increases the proliferative capacity of MSCs, while a Wnt antagonist, secreted frizzled related protein 1 (sFRP1), triggers senescence.[291]

Other studies suggested that resident cardiomyocytes may be stimulated to enter the cell cycle, proliferate and regenerate the injured heart; [273,292] however, the quantitative significance of such actions is controversial. Since most of the senescence-inducing pathways regulate cell-cycling, their manipulation might be able to trigger cardiomyocyte proliferation. For example, the silencing of senescence-inducing pathways in adult rat cardiomyocytes *in vitro* leads to the overexpression of proliferation-related genes like Ki67, Aurora-B and phosphor-histone H3 (PH3).[146]

Although most of the available data suggest that senescence decreases the regenerative capacity of stem cells, there are some cases, for example in zebrafish and neonatal mice, that cell-senescence contributes to heart regeneration after cryoinjury or apical resection[105,293]. Conversely, in these conditions clearing senescent cells with navitoclax or inactivation of senescence with p53

knockout impairs heart regeneration.[105,293] These conflicting observations may be explained by the differential role of senescence in actively regenerating contexts (such as zebrafish and neonatal mammals) versus adults with limited regenerative capacity.

Overall, senescence has the potential for both beneficial or detrimental roles in heart regeneration, and a better understanding of its role in this context is critical to exploiting the potential therapeutic.

1.6. Research goals and scope of thesis

This introduction highlights the critical role of age in cardiac pathologies. Among numerous mechanisms involved in cardiac aging, cellular senescence has gained considerable attention recently with regards to several age-related pathologies. Although current literature indicates that cellular senescence has an important contribution to different cardiac pathologies, there are still many crucial questions regarding the role of cellular senescence in cardiac disease that need to be addressed. While the role of senescence in some cardiac pathologies and processes has been extensively studied, such as cardiac regeneration, MI, HF or cytotoxicity induced by cancer chemotherapy or radiotherapy, its role in other important cardiac conditions (such as AF) has received limited attention. Furthermore, even in extensively studied cardiac pathologies, there are unanswered questions. In particular, the relative susceptibilities and consequences of senescence for the various types of cardiac cells, including fibroblasts, cardiomyocytes, endothelial cells, adipocytes, pericardial epithelial cells and neural cells, are poorly studied and understood. Importantly, it is still unknown which cardiac cell type(s) are most susceptible to senescence in aging-related diseases and which cell types drive cardiac disease processes.

For certain cardiac pathologies, such as cardiac fibrosis and MI, cellular senescence has been reported to have both detrimental and beneficial effects, but clarification is required to understand

these contradictory findings and determine the factors that influence the balance between these effects. Given the central importance of aging as a major risk factor for several cardiac diseases, a better understanding of the role of cellular senescence might be crucial for gaining deeper insights into the underlying pathophysiology and the development of new therapeutic opportunities.

This thesis aims to shed light on the role of cellular senescence in AF, an area that has not been extensively studied before. Additionally, it aims to provide further insights into the role of senescence, specifically different senescent cardiac cell types in other cardiac pathologies such as HF and MI, where the role of senescence requires clarification due to reported conflicting effects.

To achieve these objectives, the following projects were pursued:

Project1: Investigating the role of cellular senescence in the pathogenesis of atrial fibrillation.

Project 2: Investigating the role of cellular senescence in the age-related left ventricular remodeling.

Project 3: Investigating the role of cellular senescence in the early and late remodeling phases post-MI.

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2. Chapter 2. The Role of Cellular Senescence in Profibrillatory Atrial Remodeling Associated with Cardiac Pathology

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2.1. Abstract

Aims. Cellular senescence is a stress-related or aging response believed to contribute to many cardiac conditions; however, its role in atrial fibrillation (AF) is unknown. Age is the single most important determinant of the risk of AF. The present study was designed to: 1) Evaluate AF-susceptibility and senescence-marker expression in rat models of aging and myocardial infarction (MI); 2) Study the effect of reducing senescent-cell burden with senolytic therapy on the atrial substrate in MI-rats; 3) Assess senescence markers in human atrial tissue as a function of age and the presence of AF.

Methods and Results. AF-susceptibility was studied with programmed electrical stimulation. Gene and protein expression was evaluated by immunoblot or immunofluorescence (protein) and digital-PCR or RT-qPCR (mRNA). A previously-validated senolytic combination, dasatinib and quercetin (D+Q), (or corresponding vehicle) was administered from the time of sham or MI surgery through 28 days later. Experiments were performed blinded to treatment-assignment. Burst pacing-induced AF was seen in 100% of aged rats, 87.5% of young MI-rats and 10% of young-control rats ($P \leq 0.001$ vs. each). Conduction velocity was slower in aged (both left atrium, LA and right atrium, RA) and young-MI (LA) rats versus young-control rats ($P \leq 0.001$ vs. each). Atrial fibrosis was greater in aged (LA and RA) and young-MI (LA) versus young-control rats ($P < 0.05$ for each). Senolytic therapy reduced AF-inducibility in MI-rats (from 8/9 rats, 89% in MI-vehicle, to 0/9 rats, 0% in MI-D+Q, $P < 0.001$) and attenuated LA-fibrosis. Double staining suggested that D+Q acts by clearing senescent myofibroblasts and endothelial cells. In human atria, senescence-markers were upregulated in older (≥ 70 years) and longstanding-AF patients *versus* individuals ≤ 60 and sinus-rhythm controls respectively.

Conclusions. Our results point to a potentially significant role of cellular senescence in AF pathophysiology. Modulating cell senescence might provide a basis for novel therapeutic approaches to AF.

2.2. Introduction

Atrial fibrillation (AF) is the most common sustained arrhythmia observed in clinical practice.[1,2] Age is the single important AF risk factor: AF-incidence progressively doubles with each decade after 65 years of age.[3] A range of cardiovascular conditions, including left-ventricular (LV) dysfunction, valvular heart disease and myocardial infarction (MI), interact with age to cause left atrial (LA) remodeling and further increase AF-risk.[4,5] However, the mechanisms underlying the important age-dependence of AF-occurrence remain poorly understood.

Growing evidence indicates that acquired cellular senescence plays an important role in the pathophysiology of cardiac pathologies including cardiac hypertrophy, cardiotoxicity, MI and heart failure.[6-10] Cellular senescence is a stress-related and aging response that classically causes cell-cycle arrest in dividing cells, regulated through two main pathways: p16–retinoblastoma protein and p53–p21.[11,12] More recent work points to closely related pathways and changes in non-dividing cells like cardiomyocytes.[6] The main proteins in the senescence pathways, like p16, p21, and p53, are widely used as markers of senescence. Senescent cells are resistant to apoptosis and secrete proinflammatory cytokines, growth factors and matrix remodeling proteases, manifesting a "senescence-associated secretory phenotype" (SASP).[13,14]

Two studies have investigated the expression of senescence markers in the heart and their potential association with AF-promotion.[15,16] Xie *et al.* showed that greater expression-levels of senescence markers in atrial tissue are associated with more extensive atrial fibrosis and a greater AF-recurrence likelihood in patients undergoing combined valve replacement/MAZE surgery.[15] Jesel *et al.* showed stepwise increases in atrial p53 and p16 expression between sinus rhythm, paroxysmal AF and permanent AF groups.[16] While these studies suggest an association between senescence markers and AF-risk, they do not provide any direct evidence of causality.

Recent work has revealed that senolytic compounds, which selectively eliminate senescent cells by inducing apoptosis, may reduce the adverse cardiac consequences of ischemia-reperfusion and neurohormonal activation.[17-20] These studies used navitoclax, a compound that suppresses Bcl2-overexpression in senescent cells,[19,20] or a combination of the tyrosine-kinase inhibitor dasatinib (D) and the flavonoid quercetin (Q).[17]

Here, we address the potential roles of atrial cell-senescence in the pathophysiology of AF. Specific goals included: 1) To evaluate AF-susceptibility and senescence-marker expression in atrial tissue of rat models of aging and MI; 2) To study the effect of reducing atrial senescent-cell burden on AF-susceptibility and the atrial substrate of post-MI rats with senolytic therapy (a combination of dasatinib and quercetin (D+Q)); 3) To evaluate senescence-marker expression in atrial tissue from younger (≤ 60 years-old) versus older (≥70) human patients, and in patients with longstanding persistent (chronic) AF (cAF) versus sinus rhythm (SR).

2.3. Methods

Animal models

All experimental and animal-handling procedures were approved by the Animal Ethics Committee of the Montreal Heart Institute and were conducted in accordance with the Canadian Council on Animal Care (CCAC) and National Institute of Health (NIH**)** Guide for the Care and Use of Laboratory Animals. Male Sprague Dawley rats were obtained from Envigo (Indiana, USA) at 20 months (aged group), 3 months (young control) or 2 months (young MI). For the senolytic study, the male Sprague Dawley (2 months) were obtained from Charles River Laboratories (Montreal, Quebec). To induce MI, 2-month-old rats were injected subcutaneously (sc) with buprenorphine (0.05 mg/kg) and anesthetized with 2% isoflurane. The left anterior descending (LAD) coronary artery was ligated via left thoracotomy, sham-rats underwent the same procedure without LAD-

ligation. Buprenorphine (0.05 mg/kg sc) was given at 6 h and 24 h and ketoprofen (5 mg/kg sc) at 6 h post-operatively. For all MI and sham rats, all *in vivo*, ex vivo and tissue collection procedures were performed on day 28 post-operatively (at 3 months of age). Rats were euthanized by isoflurane overdose followed by cardiac excision.

Drugs and chemicals

Dasatinib (D-3307) was obtained from LC laboratories (Woburn, MA). Quercetin (Q4951) was obtained from Sigma-Aldrich (St. Louis, MI). Blebbistatin (B592490) was obtained from Toronto Research Chemical Inc (North York, ON) and di-4-ANEPPS (90134-00-2) was obtained from Cayman Chemical Company (Ann Arbor, MI). Taqman probes, including senescence markers, p16, p21, p53, galactosidase beta 1 (Glb1), and SASP markers, colony stimulating factor 2 (Csf2), chemokine (C-C motif) ligand 2 (*Ccl2*), insulin-like growth factor-binding protein 3(Igfbp3), insulin-like growth factor-binding protein 5(Igfbp5) and interleukin 6 (Il-6) were obtained from ThermoFisher Scientific (Waltham, MA). Primary antibody sources were: p16 antibody (for the rat study, ab54210), α-smooth muscle actin (αSMA) (ab5694), and troponin I (ab47003) from Abcam (Cambridge, UK); vimentin antibody (3932S) and p16 (for the human study, 80772) from Cell Signaling (Danvers, MA). The p21 antibody (05-655) was obtained from Sigma-Aldrich (St. Louis, MI) and the CD31 antibody (AF3628-SP) was obtained from R & D System (Minneapolis, MN); and GAPDH antibody (5G4-6C5) from Hytest Ltd (Turku, Finland). The 4′,6-diamidino-2-phenylindole (DAPI), wheat germ agglutinin (WGA)(W32466) and secondary antibodies, 555 donkey anti-mouse (A31570), 488 donkey anti-rabbit (A21206), 488 donkey anti-goat (A11055) were obtained from Invitrogen (Waltham, MA).

Senolytic study

After surgery, MI and sham rats were allocated (in a blinded fashion) to the following groups: Sham (vehicle); Sham (D+Q), MI (vehicle); and MI (D+Q). D+Q or vehicle treatment started 4

hours post-operatively. Rats received vehicle (20% PEG-400 in normal saline) or the senolytic combination (5 mg/kg dasatinib+50 mg/kg quercetin) by oral gavage once-daily for 3 consecutive days beginning the day of MI, with two courses of therapy separated by 2 weeks (Figure 2.4 A), a regimen chosen based on prior work.[17]

Echocardiography

Echocardiograms were recorded for aged and young rats (at end-study), and young MI-study rats (both sham and MI, at baseline and end-study) under 2% isoflurane anesthesia using a phased-array 10S probe (4.5 to 11.5 MHz) in a Vivid 7 Dimension system (GE Healthcare Ultrasound, Horten, Norway). M-mode echocardiogram was obtained in parasternal long-axis views to measure LA diameter at both end cardiac systole and diastole (LAD_s, LAD_d). M-mode echocardiogram was also obtained in parasternal short-axis view at the level of papillary muscles to measure LV diameter at both end cardiac systole (LVD_s) and diastole (LVD_d). LV-mass was calculated using the Reffelmann formula, and LV fractional shortening (FS) was calculated by $FS=(LVD_d-LVD_s)/LVD_d \times 100$. In apical 4-chamber view, right atrium minor (horizontal) diameter at end cardiac systole (RAD_s) was obtained by 2-dimensional echocardiogram, tricuspid annulus plane systolic excursion (TAPSE) by M-mode echocardiogram, and the right ventricular lateral systolic velocity (S_R) by tissue Doppler imaging. Pulsed-wave Doppler was used to record trans-mitral flow in apical 4-chamber view. Peak velocity of early-filling E-wave, atrial-filling A-wave, and E-wave deceleration time were measured.

Transesophageal electrophysiological study (EPS)

Transesophageal EPS was performed *in vivo* with a 4-F quadripolar catheter (2-mm interpolar distances. St. Jude Medical #401993, Saint Paul, MI). To assess atrial arrhythmia inducibility, 25-Hz burst pacing (pulse width 2 ms, 4x threshold voltage) was applied for 3 s, with 12 bursts separated by 2 s intervals; the cycle was repeated three times. AF was defined by a rapid (>800

bpm), irregular atrial rhythm ≥1 s. AF duration was the mean duration of all induced AF episodes. Surface ECG & catheter signals were recorded and analyzed using iox2 software (v.2.8.0.13, EMKA Technologies, Paris, France) and ECG auto (c3.5.5.25, EMKA Technologies, Paris, France). The experimenter was blinded to group identity throughout the experimental protocol and analysis.

***Ex vivo* optical mapping**

The heart was excised and perfused via the aorta with Krebs solution at 10 mL/min and 37°C. After 20 min for stabilization, a recirculating solution containing blebbistatin (15-μmol/L) was used to suppress mechanical contraction. Di-4-ANEPPS (10-μmol/L, 0.1 ml) was injected. Fluorescence signals were recorded at 2000 frames-per-second with a charge-coupled device camera (CardioCCD, Red Shirt Imaging, Decatur, GA) focused on a region up to 8-mm square in the LA free-wall. Conduction velocity and action potential duration to 80% repolarization (APD_{80}) was measured blinded to group assignment with a Matlab custom-written algorithm.[21]

Histology

Formalin-fixed, paraffin-embedded samples were cut at 6-μm thickness. To measure fibrosis, the samples were stained with Masson's Trichrome solution. Premier 9.3 Software (Media Cybernetics) was used to quantify fibrosis on Masson's Trichrome-stained images. Using immunofluorescence analysis, the protein expression of p16 in LA tissue was measured. The formalin-fixed, paraffin embedded LA sections were stained with p16 primary antibody (1/400) co-stained with either vimentin (1/200) or αSMA (1/100) or Troponin I (1/100) or CD31 (1/100) following DAPI (to stain the nuclei), WGA (to stain the membranes) and secondary antibodies (1/750). Entire LA-sections double-stained for p16 and cell-selective markers were scanned (Aperio VERSA Brightfield Scanner) and automatically analyzed with Visiomorph software (Visiopharm, Hoersholm, Denmark). All analyses were performed blinded to rat-group identity.

WGA membrane staining was used to delineate cell borders, and cardiac cells were identified with positive troponin I (cardiomyocytes), vimentin (fibroblasts), αSMA (myofibroblasts) and CD31(endothelial cells) staining (cells with signal intensity that is 25% greater than background in green channel). Cells containing DAPI positive nuclei were considered to calculate % of $p16^{+}$ cells.

Human studies

Right atrial appendages (RAAs) were collected from patients (>18 years) undergoing open-heart surgery. Patients who were included in the study of aging and cell-senescence (young (≤ 60) and aged (≥ 70) patients) had no history of paroxysmal, persistent, or permanent AF. Patients who were included in the study of AF and cell-senescence had either a history of > 6-months continuous AF (the cAF group) or were in normal sinus rhythm (SR) at the time of surgery and had no prior AF-history (SR group) (Table S 2.1**,** Table S 2.2). We were not able to include RAAs from certain procedures, namely: off-pump and redo procedures, emergency surgery, or cases for which the surgeon judged it not appropriate to excise the RAA. All participants gave their written informed consent. The study was designed and conducted to conform to the principles of the declaration of Helsinki and was approved by the University Hospital Essen's ethical review boards (no. 12-5268-BO).

Immunoblot

Proteins were isolated from human atrial tissue homogenates, and protein levels were determined using Western blot according to standard protocols. Antibodies used in the study are listed below above in the Drugs and Chemicals section, at the following dilutions: p16 (1/1000), p21(1/1000), GAPDH (1/20000). We used the appropriate near-infrared fluorophore dyes (IRDye, all 1:20,000, LI-COR Biosciences, Lincoln, NE) as secondary antibodies and imaged with an Odyssey Infrared Imaging System (LI-COR Biosciences).

Digital PCR (human samples)

Total RNA was extracted human RAAs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently, 500 ng RNA was transcribed into cDNA using the reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. TaqMan probes were used for hydroxymethylbilane synthase (*HMBS*: Hs00609296_g1), beta-2-microglobulin (B2M: Hs99999907_m1), GATA binding protein 4 (*GATA4*: Hs00171403_m1), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*: Hs02758991_g1) as housekeeping genes, *CDKN1A*: Hs00355782_m1, *CDKN2A*: Hs00923894_m1, *TP53*: Hs99999147_m1, *GLB1*: Hs01035168_m1, growth differentiation factor 15 (*GDF15*): Hs00171132_m1, transforming growth factor beta-2 (*TGFB2*): Hs00234244_m1, *IGFBP5*: Hs00181213_m1, *IGFBP7*: Hs00266026_m1, *MMP2*: Hs01548727_m1. The reactions were run on a QIAcuity Digital PCR System (Qiagen, Hilden, Germany) using a 96-well Nanoplate with 8500 partitions and the QIAcuity master mix according to the manufacturer's instructions. The cycling program used for the reactions: 2 minutes at 50°C, followed by 10 minutes at 95°C, a total of 35 cycles (15 seconds at 95°C and 1 minute at 60°C), and 30 sec at 60°C. The relative mRNA levels to controls were calculated from the copies/µl values and normalized to the geometric mean of HMBS, B2M, GATA4 and GAPDH.

qPCR (animal tissues)

Tissue samples were freshly isolated on ice, snap-frozen in liquid nitrogen and homogenized in QIAzol Lysis Reagent. Extraction of RNAs was performed with RNeasy Mini Kit (QIAGEN, Hilden, Germany). RT-PCR was performed with Applied Biosystems Thermal Cycler Step One Plus (ThermoFisher Scientific, Watham, MA). Taqman probes were used for *p16* (*Cdkn2a*: Rn00580664_m1), *p21*(*Cdkn1a*: Rn00589996_m1), Galactosidase Beta 1 (*Glb1:* Rn01403379_m1), *B2m*: Rn00560865_m1, *Gapdh:* Rn01775763_g1, *Hprt1*: Rn01527840_m1),

Gdf15: Rn00570083_m1, *Igfbp3*: Rn00561416_m1, *Igfbp5*: Rn00563116_m1, *Csf2*: Rn01456850_m1, *Il-6*: Rn01410330_m1, *Serpine2*: Rn01400467_m1, *p53* (*Tp53*: Rn00755717_m1), Ccl2: Rn00580555_m1, Collagen1a1 (*Col1a1*: Rn01463848_m1), Collagen3a1 (*Col3a1:*Rn01437681_m1) and SYBR green primers for matrix metalloproteinase 9 (*Mmp9)* (forward: TCCAGTAGACAATCCTTGCAATGTG, reverse: CTCCGTGATTCGAGAACT TCCAATA), C-X-C Motif Chemokine Ligand 1 (*Cxcl1*) (forward: ACTCAAGAATGGTCGCGAGG, reverse: ACGCCATCGGTGC AATCTAT), Tumour Necrosis Factor-alpha (*TNFa*) (forward: GTGATCGGTCCCAACAAGGA, reverse: CTTGGTGG TTTGCTACGACG), insulin-like growth factor-binding protein 4 (*Igfbp4*) (forward: GGAGCTGTCGGAAATCGAAG, reverse: GAAGCTGTTGTTGGGATGCTC), chemokine (C-C motif) ligand 20 (*Ccl20)* (forward: TTCACAACACAGATGGCCGA, reverse: CAGCGCACACGGATCTTTTC), insulin-like growth factor-2 *(Igf2)* (forward: CGCTTCAGTTTGTCTGTTCGG, reverse: GGCCTGAGAGGTAGACACG) and transforming growth factor-2 *Tgfß2* (forward: CCATGACATGAACCGACCCT, reverse: TGCCG TACACAGCAGTTCTT). All mRNA values were normalized to the geometric mean of B2m, Hprt1, and Gapdh.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (San Diego, CA) and SAS release 9.4 (SAS Institute Inc., Cary, NC). For normally distributed data (assessed by Shapiro-Wilk test), we used Student's t-test (for 2-group only comparisons), 1-way ANOVA (followed by Dunnett's test), or 2-way ANOVA (followed by t-tests with Bonferroni's multiple comparison test). Homogeneity of variances among groups was verified with the Brown-Forsythe test. For non-normal data, Kruskal–Wallis or Mann–Whitney tests were used. Categorical variables (like AF inducibility)

were analyzed with Fisher's exact test. Results are expressed as mean±SEM and 2-tailed $P<0.05$ was considered statistically significant.

2.4. Results

Effects of aging and MI on AF vulnerability, electrophysiology and fibrosis

Transesophageal stimulation induced AF (Figure S2.1) in all aged rats (100%, $P<0.0001$ vs. control) and the majority of MI-rats (87.5%, $P=0.001$), but in only one rat in the young-control group (10%, Figure 2.1A*)*. Because of the rare inducibility of AF in young-control rats, AF-duration was not meaningfully quantifiable in this group (Figure S2.1. Representative ECGs and AF duration.D). Figure 2.1B shows representative activation maps for each group. LA conduction velocity was significantly slower in young-MI and aged rats. LA repolarization was significantly slower in aged rats but not significantly changed in young MI (Figure 2.1C). RA conduction velocity and RA repolarization were significantly slower in aged rats (Figure S2.2). ECG analysis revealed significant increases in P-wave duration and P-R interval in both MI and aged rats (Figure S 2.4A-B), consistent with atrial conduction slowing. QRS duration was not significantly different among groups (Figure S 2.4C), while QT intervals were significantly increased in aged rats (Figure S 2.4D). LA myocardial collagen content was significantly increased, indicating tissue fibrosis, in MI and aged rats compared to young control rats (Figure 2.1D). RA myocardial collagen content was significantly greater in aged rats compared to young control rats (Figure S 2.3A)

Cardiac function and structure in aged and MI rats

Echocardiography revealed a significant reduction in LA fractional shortening (LAFS) in both aged and MI rats (Figure S 2.5A), and increased LA dimensions in aged but not MI rats (Figure S 2.5A). E-wave deceleration time (EDT) and E/A wave ratio were not altered significantly (Figure S 2.5B), but LV fractional shortening (LV FS%) and LV ejection fraction (LV EF%) were reduced

and LV diastolic dimension (LVDd) was increased with both MI and aging (Figure S 2.5C). LV systolic dimension (LVDs) increased in aged but not MI rats (Figure S 2.5C). LV mass normalized to body weight was reduced in aged rats but not MI rats (Supplementary Material online, Figure S 2.5C). Tricuspid annular plane excursion (TAPSE) suggested right-ventricular dysfunction in MI, and RA dimension was increased in aged rats (Figure S 2.5D). Overall, the most consistent changes were reduced LA emptying function, impaired LV systolic function and LV diastolic dilation in both aged and MI animals.

Expression of senescence markers in MI and aged rats

The mRNA expression of senescence markers was measured by qPCR and was increased for p16 in LA of both MI and aged rats (Figure 2.2A), while no significant change was noted for p21, p53 or Glb1. Immunohistochemical analysis revealed increased protein expression of p16 in LA of MI and aged rats (Figure 2.2B). In LA, among typical SASP markers, upregulation was noted for Igfbp3, (in MI rats), Csf2 (in aged rats), Mmp9 (in both MI and aged rats) and Serpine2 (in aged rats; Figure 2.3B and Figure S 2.6). Mmp9 and Serpine2 are involved in remodeling of the extracellular matrix. In RA, the mRNA expression of the senescence marker p16, and SASP markers Csf2, Serpine2, and Mmp9 were upregulated in aged rats compared to the young control group, while there were no changes in the MI-rats (Figure 2.3A-C). Other SASP markers did not differ among groups (Figure S 2.7).

Effects of senolytic therapy on AF-substrate and cardiac function

The data described above suggest senescent-cell accumulation in the atria of both MI and aged rats. To test the role of cellular senescence in AF-occurrence, we administered a senolytic drug combination (D+Q) to MI-rats, beginning the day of MI (Figure 2.4A). D+Q significantly reduced AF inducibility 28 days post-MI (Figure 2.4B). Transoesophageal stimulation (performed and analyzed blinded to group identity) induced AF in 89% of MI-rats treated with vehicle, but none

of the MI-rats treated with D+Q or sham rats (P<0.0001; Figure 2.4B). The mean duration of AF in the MI-vehicle group was 25±11 s, but because of limited or no inducibility could not be quantified in Sham-vehicle, Sham-D+Q or MI-D+Q groups. D+Q treated MI-rats did not show significant changes in P-wave duration or PR-interval (Figure S 2.4E-H). On echocardiography, D+Q treatment had no effects on LA or LV function or structure (Figure S 2.8).

Figure 2.4C shows representative activation maps for each group. LA conduction velocity was significantly slower in MI groups versus sham. D+Q significantly attenuated the LA conduction velocity slowing caused by MI (Figure 2.4D), while strongly reducing changes in LA APD_{80}. Consistent with the changes in LA conduction, LA collagen content was significantly reduced in the MI group treated with D+Q compared to the MI vehicle group (D+Q: 2.4 ±0.2% vs. vehicle: 5.4±0.8%, P = 0.001, Figure 2.4E), as was RA fibrosis (Figure S 2.3B). Eighteen different collagen types have been identified, five of which are found in the extracellular matrix of the heart. Among those five, the interstitial collagens, types I and III are the most abundant components. In the normal condition, collagen plays a crucial role in providing structure and strength to the heart, however, in the pathological conditions, the amount and the ratio of the collagen subtypes can change and contribute to the pathogenesis of the disease.[25] For example, Xu et al. have illustrated that, in AF, the gradual increase in collagen type I, as opposed to type III, occurs from non-AF to permanent AF. Additionally, a selectively increased collagen type I/III ratio was associated with both the duration and recurrence frequency of AF. Conversely, in failing heart ventricular tissue, a predominant upregulation of collagen type III was observed, accompanied by a reduction in the collagen I/III ratio and a decrease in the abundance of collagen types I, IV, and VI.[47]

Effects of senolytic therapy on senescence markers

The expression of selected genes encoding proteins involved in senescence (Figure 2.5A), fibrosis (Figure 2.5B), and SASP (Figure 2.5C) were examined by qPCR.

The mRNA expression of p16 in LA was highly variable, and while it increased quantitatively in MI vehicle and not in MI D+Q, the differences were not significant after Bonferroni correction (Figure 2.5A). The expression of p21 and Serpine2, Igfbp4, Ccl20 mRNA was significantly reduced by D+Q treatment in MI-rats (Figure 2.5A-C). Other SASP markers with no changes in LA and RA are shown in Figure S 2.9 and Figure S 2.10. In the LV, p16 mRNA expression was upregulated in the peri-infarct and infarct zones of the MI-rats and significantly reduced by D+Q in the infarct zone (Figure S 2.11). The mRNA expression of Mmp2, a profibrotic SASP marker, was upregulated in MI-vehicle rats compared to sham-vehicle, and substantially reduced in the MI D+Q group (Figure 2.5B). The mRNA expression of Col1a1 was significantly reduced by D+Q treatment vs. vehicle (Figure 2.5B).

To quantify senescence in specific cardiac cell-types, we double-stained cross-sectioned samples with p16 along with cell-type selective markers in LA tissues of MI-rats (Figure 2.6). D+Q treatment did not alter the number of vimentin-positive cells expressing p16, but significantly reduced the percentage of p16-expressing cells in the CD31-expressing (endothelial cell) population. In sections not including blood vessels, αSMA-positive cells expressing p16 were significantly reduced by D+Q, while the number of troponin-I positive cells expressing p16 was unaffected. These results suggest that D+Q effects resulted in significant clearance of senescent myofibroblasts and endothelial cells in post-MI LA, but not quiescent fibroblasts or cardiomyocytes.

Senescence markers in human atrial tissues

To test the potential translational relevance of our findings, we analyzed senescence markers in human RAAs. RAA samples from older patients showed significantly higher protein expression

of p16 and p21 (Figure 2.7A). Human RAAs also showed significantly greater mRNA-expression of many senescence and SASP markers including p16, p21, GLB1, and GDF15 gene with aging (Figure 2.7B and Figure S 2.12). The RAAs of cAF-patients showed significantly greater gene-expression for TGFβ2 and protein expression for p21 than for SR-patients (Figure 2.7C-D). Original full-length immunoblot images are provided in Figure S 2.14, Figure S 2.15,Figure S 2.16.

2.5. Discussion

In this study, we examined the role of cellular senescence in the pathophysiology of AF using rat models of atrial remodeling associated with aging and MI, as well as human atrial tissue. We found evidence for the accumulation of senescent cells in the atria of both elderly rats and rats with LV-dysfunction due to recent MI, accompanying increased AF-susceptibility and atrial fibrosis. As a test of the pathophysiological role of cell-senescence, we used senolytic therapy (D+Q) to clear senescent cells and found that it prevented the atrial conduction and fibrotic changes caused by MI, along with the associated AF-vulnerability. We evaluated the potential relevance of cell-senescence to human AF by examining senescent-cell markers in human atrial tissue, noting enhanced expression of multiple markers in elderly patients and those with longstanding persistent AF.

The association of AF with aging and MI

Age is well-known to be the single most important determinant of the risk of AF[3] and recent work points to a primary role of biological age.[22] The available literature indicates that aging-related AF results from multiple structural, electrophysiologic, and molecular changes caused by aging.[3,4] Among these changes, the potential contribution of atrial fibrosis to aging-related AF has been

confirmed in several clinical and experimental studies.[23-25] Progressive atrial conduction abnormalities occur with aging in humans[26,27] and are believed to be important contributors to increasing susceptibility to AF with age.

While the accumulation of senescent cells is a hallmark of aging, accelerated cell-senescence is also believed to play a pathophysiological role in a range of cardiac disease conditions, including heart failure, MI, drug-induced cardiotoxicity and ischemia-reperfusion injury.[6] Almost all the work to date has been performed in ventricular tissues and cell lines. The present work provides the novel insight that MI-induced LV-dysfunction causes an accumulation of senescence markers in the atria, and that senolytic-cell clearance prevents adverse atrial remodeling and the development of an AF-promoting substrate.

Potential role of cellular senescence in increased AF susceptibility

While two clinical studies have shown that increased atrial expression of senescence markers accompanies AF progression and atrial fibrosis,[15,16] we are unaware of any studies directly evaluating the role of cellular senescence in experimental AF. Further work is needed to establish more clearly how cellular senescence affects AF. One potential avenue is clearly via the induction of tissue fibrosis, which plays a significant role in AF[25] and is well-documented to be associated with aging-related AF-susceptibility.[4] This notion is consistent with the prevention of atrial fibrosis that we noted with senolytic therapy in MI-rats, and with the associated clearance of senescent αSMA-positive cells. However, the role of senescent cells in cardiac fibrosis is complex and both beneficial and detrimental effects have been reported.[6] Whether cellular senescence can contribute to other recognized AF-promoting mechanisms associated with aging, like atrial connexin-dysregulation[28] Ca^{2+}-handling abnormalities[29] or ion-current abnormalities[4] remains to be established. Valvular heart disease, including mitral regurgitation, increases the risk of atrial fibrillation (AF). It has been suggested that mitral regurgitation may potentially promote atrial

senescence by decreasing silent information regulator 1 (SIRT1), an inhibitor of p53.[48] However, further research is necessary to fully identify the role of senescence in the pathogenesis of AF following valvular disease. The use of senolytics in AF patients with valve disease potentially can reduce atrial senescence, but senolytics probably can't improve the pathology of mitral regurgitation itself, as these patients are usually candidates for mitral valve replacement or repair.[4] Furthermore, it will be important to clarify to what extent the effects of cellular senescence are due to the cells directly affected, as opposed to paracrine actions on neighboring cells due to SASP mediators released by senescent cells.

Senolytic therapy

Senescent cells, similar to cancer cells, rely on anti-apoptotic pathways to survive following stress and damage. Zhu et al demonstrated that senescent cells are susceptible to selective clearance by targeting pro-survival mechanisms using a class of drugs called senolytics.[49] A variety of senolytic compounds have been documented experimentally to clear senescent cells; some of these are presently under clinical investigation.[6] Senolytics target anti-apoptotic pathways such as BCL-2 and phosphoinositide 3-kinase (PI3K)–AKT in senescent cells, thus inducing targeted senescent-cell death.[30] The global elimination of senescent cells with navitoclax, a senolytic compound, in aged mice reduces ventricular fibrosis and hypertrophy.[18] The administration of navitoclax prior to and following MI, improved adverse LV-remodeling and post-MI survival in elderly mice.[31] Dasatinib is a tyrosin kinase inhibitor that targets anti-apoptotic pathway PI3/Akt. Quercetin is a natural product, acts on anti-apoptotic hypoxia-inducible factor 1 (HIF-1), Bcl2 and insulin like factor (IGF-1) pathways.[17] Both dasatinib and quercetin can induce apoptosis in the senescent cells by inhibiting these anti-apoptotic pathways. In vitro studies indicate that these two drugs can impact distinct senescent cell types: dasatinib effectively eliminates senescent human fat cell progenitors, while quercetin demonstrates greater efficacy against senescent human endothelial

cells and mouse bone marrow-derived mesenchymal stem cells (BM-MSCs). The combination of dasatinib and quercetin proves effective in eliminating senescent mouse embryonic fibroblasts (MEFs). Consequently, in in vivo studies, aiming to target a diverse range of cell types, the combined treatment with dasatinib and quercetin has been consistently implemented across various studies in the literature.[49] We chose to use D+Q because of promising results with this combination in preclinical studies and ongoing clinical trials.[6] For example, the administration of D+Q in aged mice reduced LV fibrosis.[17] D+Q prevented mitochondrial-DNA induced inflammation and prolonged the survival of cardiac allografts.[32]

Potential role of senescent myofibroblasts and endothelial cells in AF

Double staining with p16 and cell-type selective markers (Figure 2.6) points to myofibroblasts and endothelial cells as senescent cell-types cleared by D+Q in post-MI LA. Atrial fibroblasts from patients with chronic AF show significant myofibroblast differentiation;[33] however, the role of senescent myofibroblasts in AF has not been studied. Meyer *et al.* showed that myofibroblasts are the predominant cardiac-cell population undergoing senescence in the LV of mice with transverse aortic constriction (TAC).[34] These researchers showed that genetic ablation of p53 or p16 results in reduced senescence, associated with increased LV fibrosis. In contrast, we found that clearance of senescent myofibroblasts with D+Q was associated with reduced LA fibrosis. The differences between studies may be due to differences between atrial and ventricular responses, or to technical differences like senolytic or genetic approach, models used (TAC vs MI) or species studied (mouse vs rat). The role of endothelial cells is unknown but might be related to the generation of fibrosis via endothelial-mesenchymal transition or via secreted profibrotic SASP products.[35,36]

Limitations

We studied the effects of senolytic therapy on atrial pathology underlying the AF substrate induced by LV-dysfunction resulting from acute MI. Our findings support the concept that pathologies

can cause cardiac abnormalities by inducing accelerated cellular senescence.[6] It would be of interest to determine whether senolytic interventions can also prevent senescent-cell accumulation and aging-related AF. The development of aging-related atrial fibrosis might be quite gradual and therefore months of senolytic therapy might be needed to prevent aging-related AF, as opposed to the rat MI-model in which atrial fibrosis and AF-susceptibility appear over the course of several weeks following the acute intervention.

We used a senolytic-drug approach for senescent-cell clearance. While D+Q is a well-established senolytic therapy,[37] there is always the risk of off-target drug effects, which can never be fully discounted. Senolytics might impact non-senescent cells, modifying cellular functions in healthy tissues, leading to undesirable side effects that could disrupt the normal physiological functions of these cells. Additionally, senolytics can affect the immune system by killing neutrophils, macrophages and possibly T cells.[13] Genetic approaches are available to clear senescent cells in transgenic mice.[38] It would be interesting to evaluate the effects of senescent-cell clearance on atrial remodeling in one of these systems.

The cell-types mediating the effects of cellular senescence on cardiac pathology is an important, but often ignored.[6] We used immunohistochemistry to address this question and found evidence for a possible role of myofibroblasts and endothelial cells (Figure 2.6). We recognize that our identification of the cell-types mediating the response to senolytics is not definitive. *In vitro* experiments could be used to investigate further the role of fibroblasts vs myofibroblasts, but our attempts to culture senescent fibroblasts were unsuccessful as we found that they failed to proliferate in culture (consistent with the cell-cycle arrest believed to result from senescence). The induction of *in vitro* senescence (e.g. with irradiation or doxorubicin treatment) could be used to compare the response of cultured non-senescent fibroblasts and myofibroblasts to senescence

induction, but the relationship of these artificially-induced forms of *in vitro* senescence to naturally-occurring *in vivo* senescence is unclear.

Our young-MI and corresponding control rats were studied at 3 months, whereas our aged rats were studied at 20 months. The issue of age equivalence between rats and humans is complex. It is difficult to extrapolate directly from rat age to human age. In a recent review on the topic,[39] the results of a survey completed by 611 labs was reported. The ages of rats studied as "adult" ranged from 8-16 weeks, with 8-12 weeks the most commonly used ages (considered equivalent to approximately 15 year old human).[39] We performed a Medline search with the term "aged rats atrial fibrillation" and identified 6 studies since 2011. The rats used for the aged groups ranged from 11 to 24 months, with a mean of 20.4,[40-45] so our aged rats were very close to the mean value in the literature. Most typically, 20-month-old rats are considered equivalent to approximately 60 year old humans; this must be considered in relating our findings in elderly rats to our human studies (Figure 2.7), which distinguished results in atria of humans younger than 60 with those over 70.

Our human studies were conducted in RAA tissues. Human LAA tissue is rarely available for experimental work from cardiac surgical procedures, whereas a piece of the RAA is routinely removed at the time of cannulation for extracorporeal circulation. We therefore have access to only a very limited number of human LAA samples, which does not allow meaningful analysis. For the rat work, we focused on the LA because coronary artery ligation causes primarily LV infarction and dysfunction and the LA is much more strongly affected than the RA.[46] Nevertheless, we present RA data for fibrosis, optical mapping and qPCR in the rat data (Figure 2.3, Figure S2.2, Figure S 2.3, Figure S 2.7, Figure S 2.10). RA changes for aged rats are qualitatively similar to those in aged humans. In MI-rats, RA fibrosis was significantly reduced with senolytic therapy but none of the senescence markers were significantly changed in MI-rats, which is not surprising,

given that the LA is predominantly affected by LV-dysfunction in the MI model, with much more limited changes in the RA.[46]

The specific molecular pathways involved in cell-senescence might differ in different contexts like LV dysfunction versus aging. Further work is needed to clarify the specific molecular pathways, signals and mediators involved and to evaluate the effects on these of senescent-cell clearance by D+Q, as well as by genetically-engineered approaches, in rodent models.

2.6. Conclusions

In the present study, we obtained evidence for the accumulation of senescent cells in the atria of aged rats and in the atria of rats with LV-dysfunction due to MI, in association with a pathological AF-substrate. Furthermore, we found that senolytic therapy was able to suppress markers of cellular senescence, prevent atrial fibrosis and obviate AF-substrate development in post-MI rats. The potential translational relevance of these findings is supported by the observation of overexpression of senescent-cell markers in RA-tissue from older humans and those with long-standing persistent AF. These findings have important potential implications for understanding the mechanisms linking aging and cardiac pathology to AF-occurrence and for the development of innovative therapeutic approaches.

2.7. References

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2.8. Figures

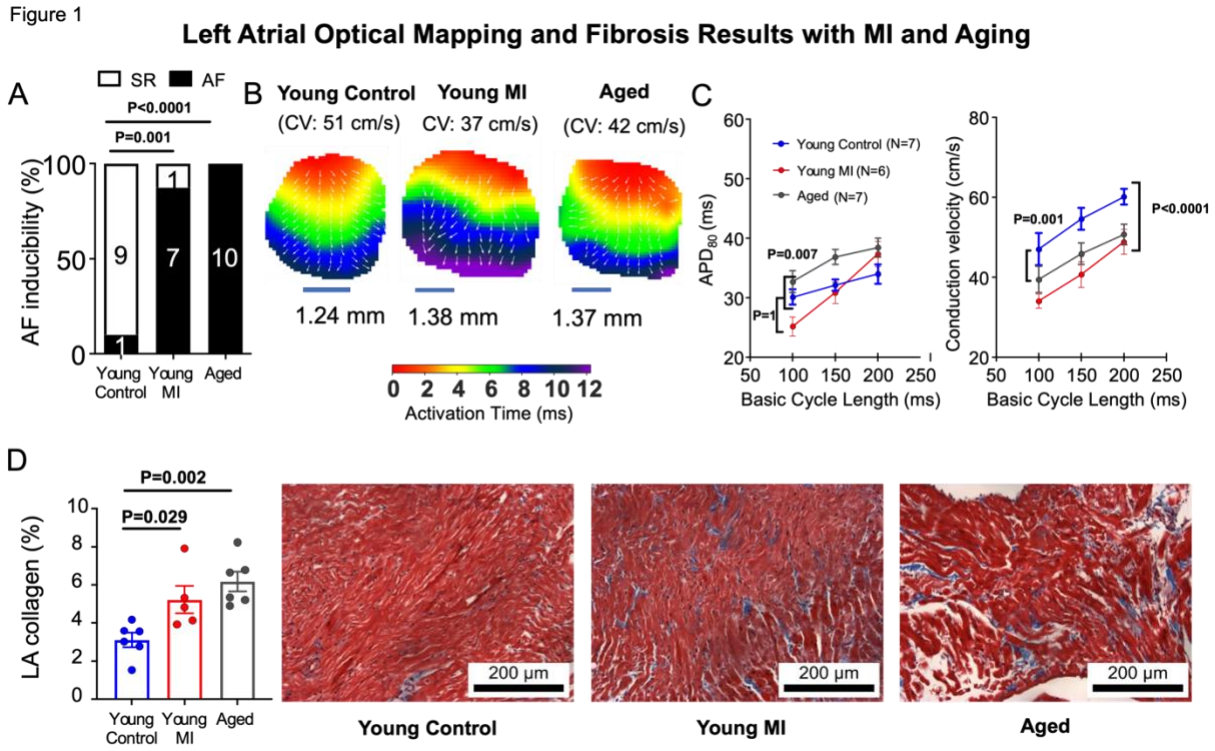

Figure 2.1. AF inducibility, optical mapping and fibrosis quantification in left atrium (LA) of young control (3 month), young MI (3 month) and aged (20 month) rats.

(*A*) Percentage of rats with inducible AF (*n*=8-10; Fisher's exact test). (*B*) LA activation-maps at BCL 100 ms from a control, young-MI, and elderly rat. (*C*) Mean±SEM LA conduction velocity and APD_{80} (*n*=6-7; two-way ANOVA followed by Bonferroni test; the p values indicate Bonferroni corrected differences between groups from data pooled from all BCLs). (*D*) Dot-plot graphs show mean±SEM percentage-fibrosis in LA. Points represents results from an individual animal (*n*=5-6; one-way ANOVA with Dunnett's test). AF, atrial fibrillation; APD_{80}, action potential duration to 80% repolarization; BCL, basic cycle length; MI, myocardial infarction; SR, sinus rhythm.

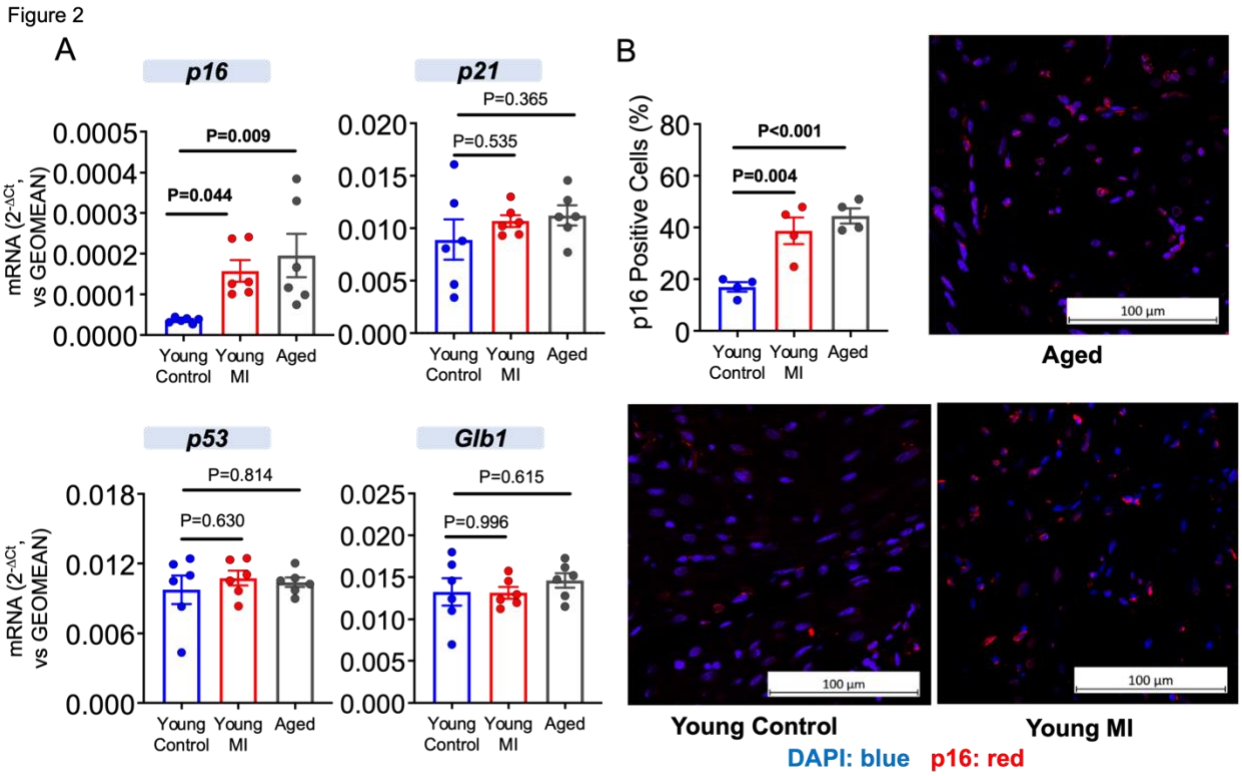

Figure 2.2. Senescence-marker expression in left atrium (LA).

(*A*) Messenger RNA expression (*n*=6; one-way ANOVA with Dunnett's test); (*B*) Immunofluorescence for p16 (red) and 4,6-diamino-2-phenylindole (DAPI, blue; *n*=4; one-way ANOVA with Dunnett's test). MI, myocardial infarction; qPCR, quantitative polymerase chain reaction.

Figure 3

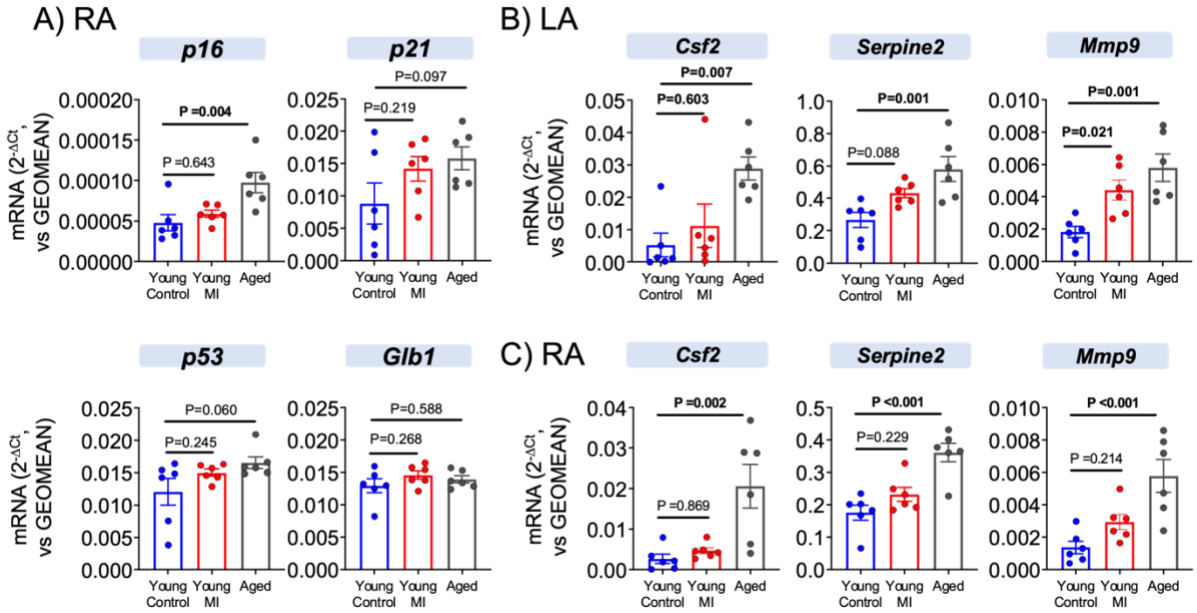

Figure 2.3. Senescence-marker expression in right atrium (RA) and components of senescence associated secretory phenotype (SASP) in LA and RA.

(*A*) RA senescence marker mRNA expression (*n*=6; one-way ANOVA with Dunnett's test); (*B*) LA SASP component mRNA expression (*n*=6; one-way ANOVA with Dunnett's test); *(C)* RA SASP component mRNA expression (*n*=6; one-way ANOVA with Dunnett's test).

Figure 4

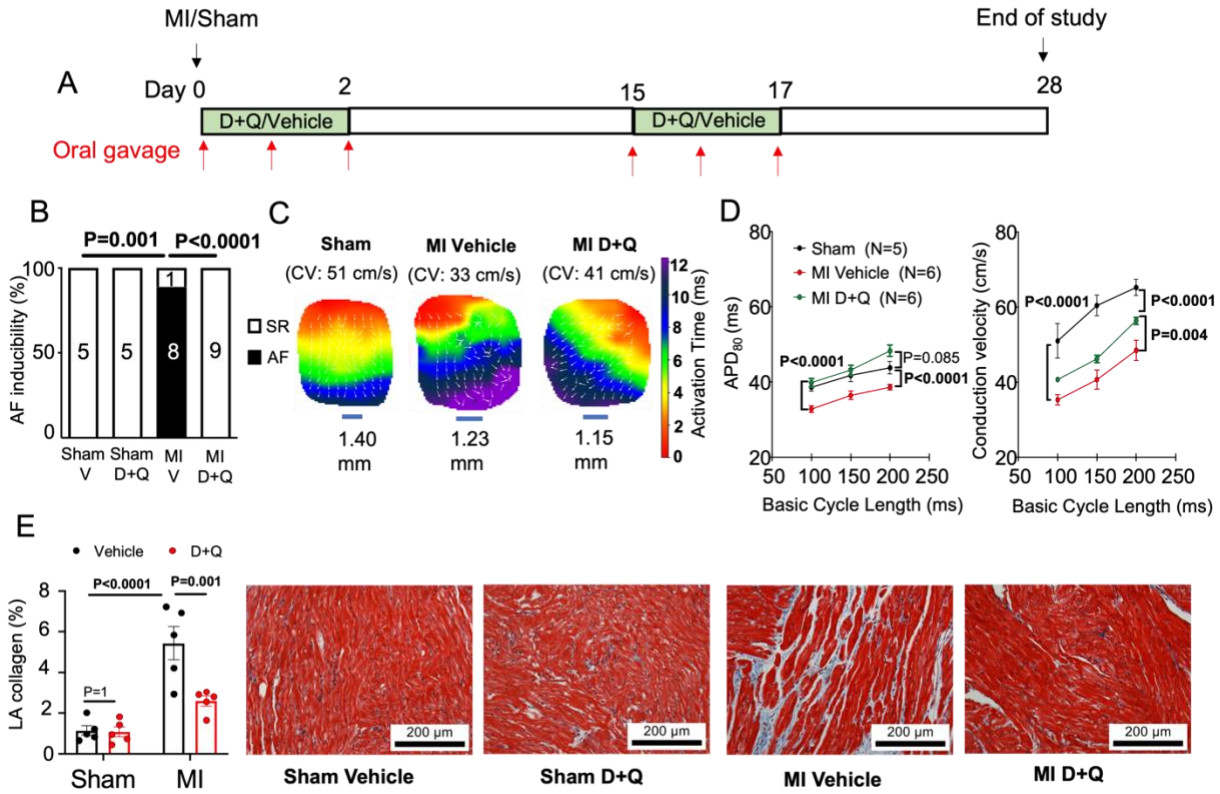

Figure 2.4. Study design, AF-inducibility changes, optical mapping and fibrosis quantification with Dasatinib+Quercetin (D+Q) senolytic therapy.

(*A*) Study design; (B) AF inducibility; (C) Left atrial (LA) activation maps at BCL 100 ms from a sham, an MI-Vehicle and a MI-D+Q rat; (*D*) Mean±SEM LA conduction velocity and APD_{80} (*n*=5-6; two-way ANOVA with Bonferroni test; the p values indicate Bonferroni corrected differences between groups from data pooled from all BCLs); (*E*) Fibrosis analysis. Dot-plot graphs show mean±SEM LA fibrous-tissue content (*n*=5; two-way ANOVA with Bonferroni test). Points represent results from individual animals. APD_{80}, action potential duration to 80% repolarization; BCL, basic cycle length; MI, myocardial infarction.

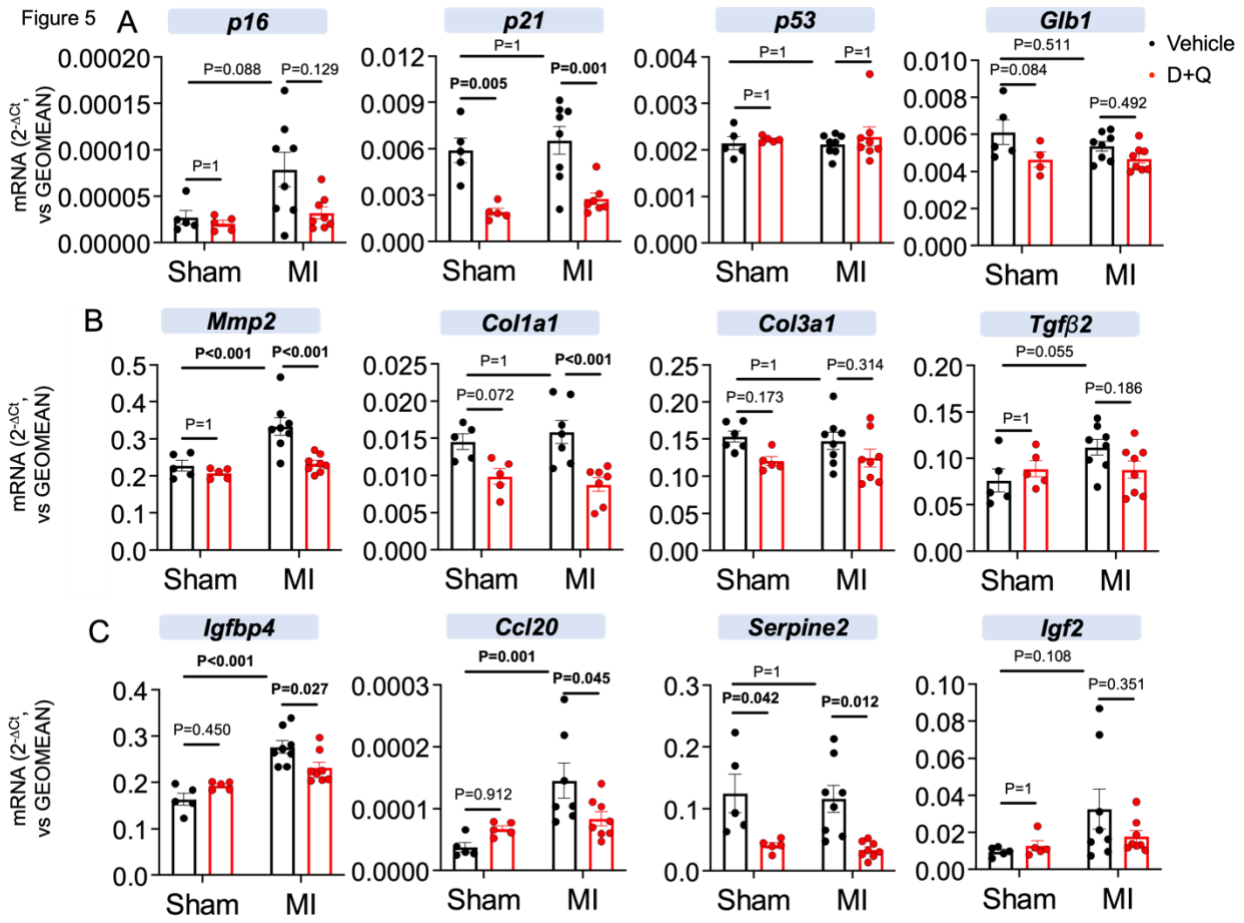

Figure 2.5. Left atrial (LA) fibrosis, cell-senescence and SASP marker gene expression without and with D+Q-Therapy.

Messenger-RNA expression for (*A*) Senescence markers *p16*, *p21*, *p53*, *Glb1* (N=5-8; two-way ANOVA with Bonferroni test). (*B*) Fibrosis markers *Mmp2*, *Col 1a1*, *Col 3a1*, and *Tgfß2 (*N=5-8; two-way ANOVA with Bonferroni test). (*C*) SASP components *Igfbp4*, *Ccl20*, *Serpine2* and *Igf2* (N=5-8; two-way ANOVA with Bonferroni test). *Mmp2*, matrix metalloproteinase 2; SASP, senescence associated secretory phenotype; *Tgfß2*, transforming growth factor 2; *Ccl20*, chemokine (C-C motif) ligand 20*; Igfbp4*, insulin-like growth factor binding protein-***4;*** *Igf2*, insulin-like growth factor 2.

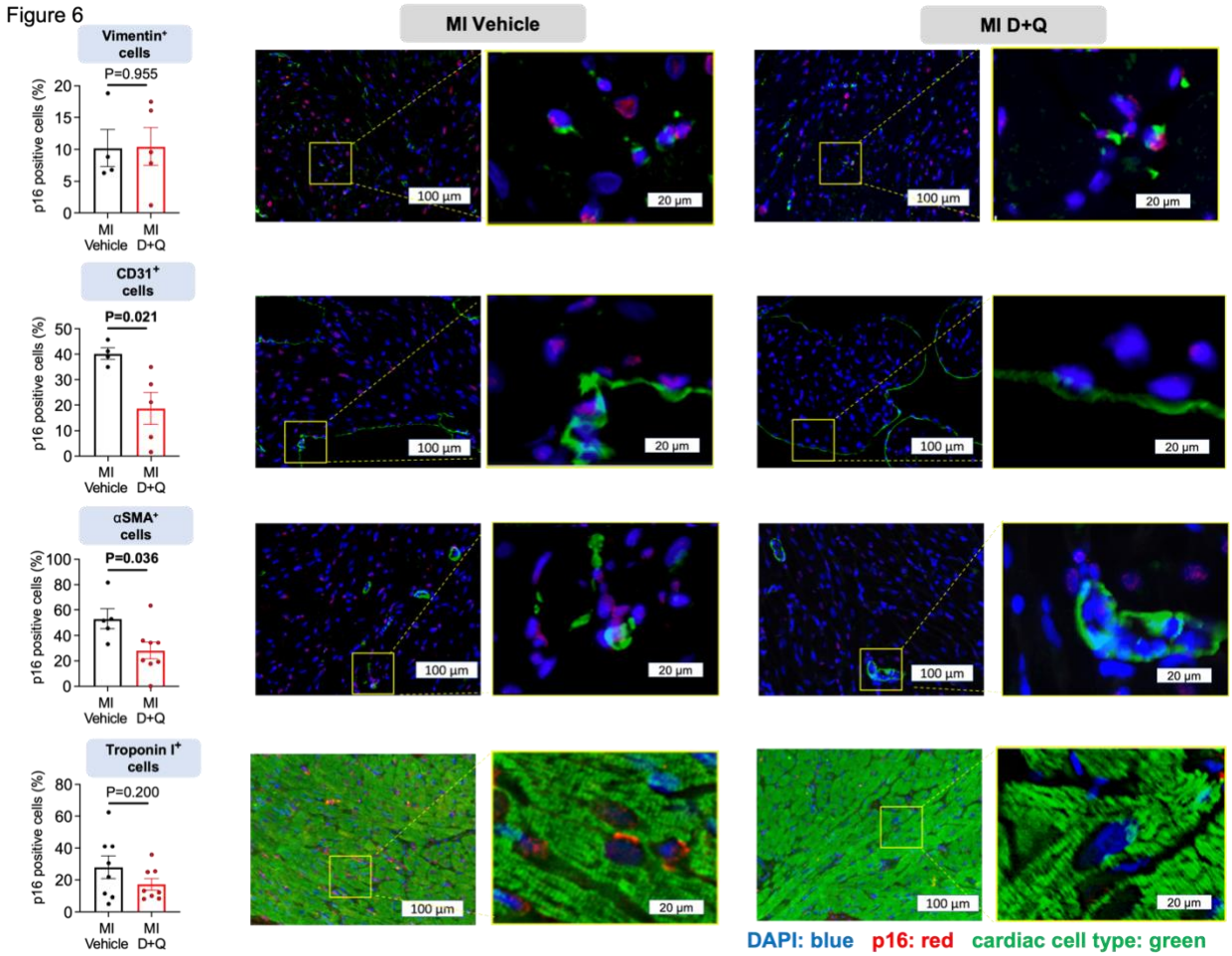

Figure 2.6. Double immunofluorescence for p16 and cardiac cell-type markers in left atrium (LA) of Sham and MI-rats treated with vehicle or Dasatinib+Quercetin (D+Q).

(*A*) Fibroblast-marker, vimentin (*n*=4-5; unpaired t-test). (*B*) Endothelial cell marker, CD 31 (*n*=4-5; unpaired t-test). (*C*) Myofibroblast marker, αSMA (*n*=5-8; unpaired t-test). (*D*) Cardiomyocyte marker, troponin I (*n*=8; unpaired t-test). All panels show cell-marker staining (green), staining for p16 (red) and 4,6-diamino-2-phenylindole (DAPI,blue). MI, myocardial infarction.

Figure 7

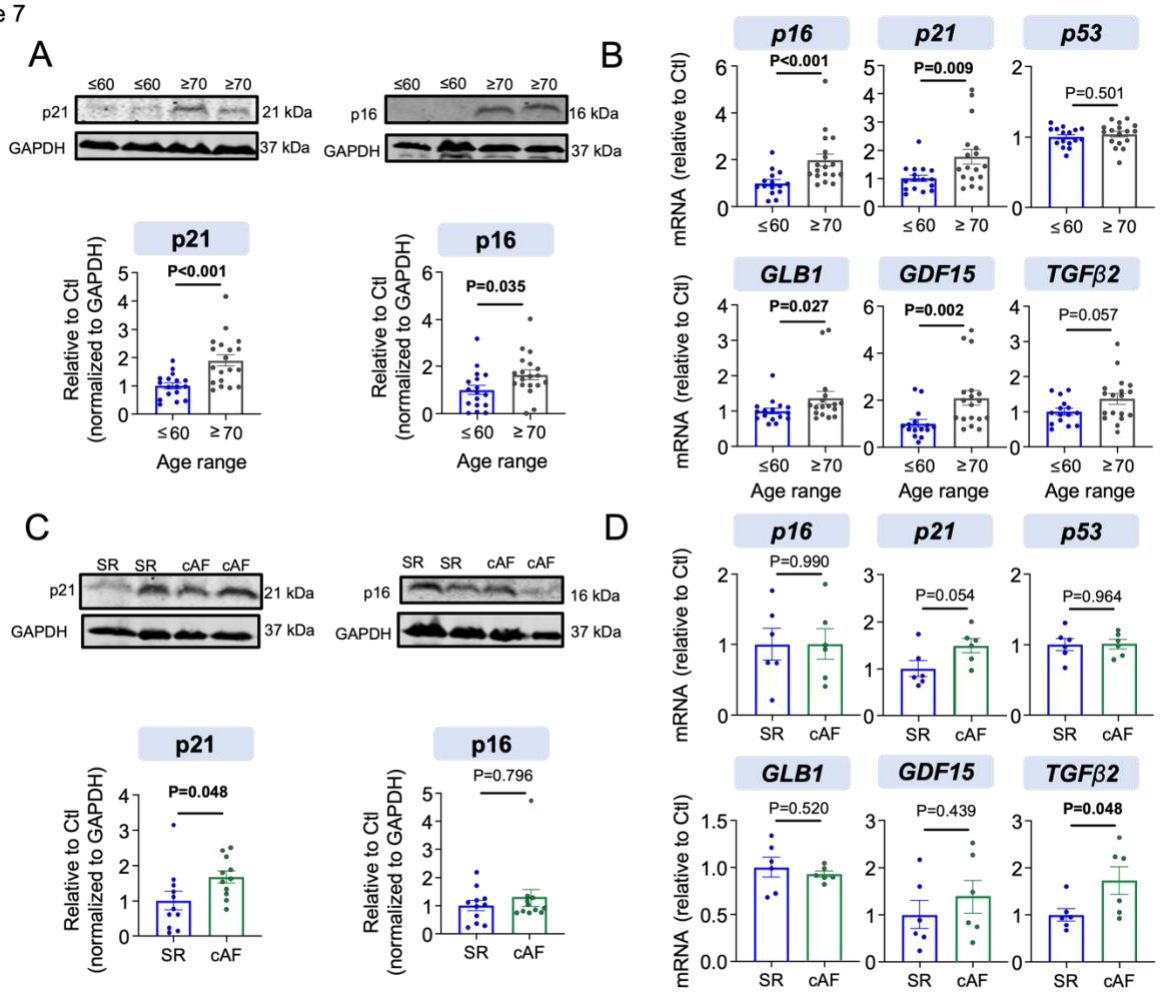

Figure 2.7. Senescence marker mRNA and protein expression in human atrial tissue.

(*A*) Immunoblot analysis of senescence markers, p16 and p21 in RAAs of young (≤ 60) and aged (≥ 70) patients (*n*=15-19; unpaired t-test for parametric or Mann-Whitney test for non-parametric variables). (*B*) Messenger RNA expression measured by dPCR in RAA of younger (≤ 60) and older (≥ 70) patients (*n*=15-19; unpaired t-test for parametric or Mann-Whitney test for non-parametric variables)**.** (*C*) Immunoblot analysis of senescence markers, p16, and p21 in RAA of SR and cAF patients (*n*=11; unpaired t-test for parametric or Mann-Whitney test for non-parametric variables). (*D*) Messenger RNA expression, as measured by dPCR in RAA of SR and cAF patients (*n*=6; unpaired t-test for parametric or Mann-Whitney test for non-parametric variables). cAF, chronic atrial fibrillation; dPCR, digital polymerase chain reaction; *GFD15*, growth differentiation factor 15; RAA, right atrial appendage; SR, sinus rhythm; *TGFβ2*, transforming growth factor 2.

Table S 2.1. Patient characteristics

	≤60	≥70	P-value
Patients, *n*	18	19	-
Sex, M/F	10/8	10/9	>0.999
Age, y[ǂ]	54.0±1.4	75.0±0.9	<0.001
Body mass index, kg/m^2[$]	30.4±1.5	27.6±1.2	0.115
CAD, *n* (%)	10 (56)	14 (74)	0.248
AVD/MVD, *n* (%)	8 (44)	5 (26)	0.248
CAD+AVD/MVD, *n* (%)	0 (0)	0 (0)	-
Hypertension, *n* (%)	15 (83)	18 (95)	0.264
Diabetes, *n* (%)	6 (33)	9 (47)	0.385
Hyperlipidemia, *n* (%)	11 (61)	13 (68)	0.642
LVEF, %	53.4±2.5	54.2±2.3	0.958
LA, mm[#]	40.9±1.6	39.9±1.8	0.589
Digitalis, *n* (%)	0 (0)	0 (0)	-
ACEi OR ARB, *n* (%)	14 (78)	15 (79)	>0.999
β-Blockers, *n* (%)	12 (67)	13 (68)	0.909
Dihydropyridines, *n* (%)	6 (33)	6 (32)	0.909
Diuretics, *n* (%)	8 (44)	7 (37)	0.637
Nitrates, *n* (%)	0 (0)	2 (11)	0.157
Lipid-lowering drugs, *n* (%)	11 (61)	14 (74)	0.414

≤60, sinus rhythm at age 60 and younger; ≥70, sinus rhythm at age 70 and older; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; AVD, aortic valve disease; CAD, coronary artery disease; LA, left atrial diameter; LVEF, left ventricular ejection fraction; MVD, mitral valve disease.

[ǂ]$P<0.05$ ≥70 vs. ≤60 from unpaired t test or Mann-Whitney test for continuous variables. [§]Data were not available for 1 ≤60. [#]Data were not available for 3 ≤60, and 3 ≥70. Continuous variables are presented as mean±SEM. Statistical

test: *P<0.05 versus CTL from Fisher's exact test for categorical variables. ǂP<0.05 versus CTL from unpaired t test or Mann-Whitney test for continuous variables.

Table S 2.2. Patient characteristics

	SR	cAF	P-value
Patients, *n*	11	11	-
Sex, M/F	6/5	6/5	>0.999
Age, y	70.0±2.5	73.4±1.5	0.267
Body mass index, kg/m^2	26.8±1.3	30.6±1.6	0.090
CAD, *n* (%)	6 (55)	2 (18)	0.076
AVD/MVD, *n* (%)	5 (45)	6 (55)	0.670
CAD+AVD/MVD, *n* (%)	0 (0)	3 (27)	0.062
Hypertension, *n* (%)	10 (91)	11 (100)	0.306
Diabetes, *n* (%)	4 (36)	3 (27)	0.647
Hyperlipidemia, *n* (%)	7 (64)	6 (55)	0.665
LVEF, %[§]	52.8±3.2	54.8±3.3	0.669
LA, mm[#]	44.6±2.5	49.4±2.0	0.142
Digitalis, *n* (%)	0 (0)	0 (0)	-
ACEi OR ARB, *n* (%)	8 (73)	9 (82)	>0.999
β-Blockers, *n* (%)	5 (45)	6 (55)	0.670
Dihydropyridines, *n* (%)	1 (9)	3 (27)	0.269
Diuretics, *n* (%)	4 (44)	5 (45)	0.665
Nitrates, *n* (%)	0 (0)	0 (0)	-
Lipid-lowering drugs, *n* (%)	8 (73)	5 (45)	0.193

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; AVD, aortic valve disease; CAD, coronary artery disease; cAF, chronic atrial fibrillation; LA, left atrial diameter; LVEF, left ventricular ejection fraction; MVD, mitral valve disease; SR, sinus rhythm as control.

[§]Data were not available for one cAF patients. [#]Data were not available for 2 SR, and 2 cAF patients. Continuous variables are presented as mean±SEM. Statistical test: *P<0.05 versus CTL from Fisher's exact test for categorical variables. ǂP<0.05 versus CTL from unpaired t test or Mann-Whitney test for continuous variables.

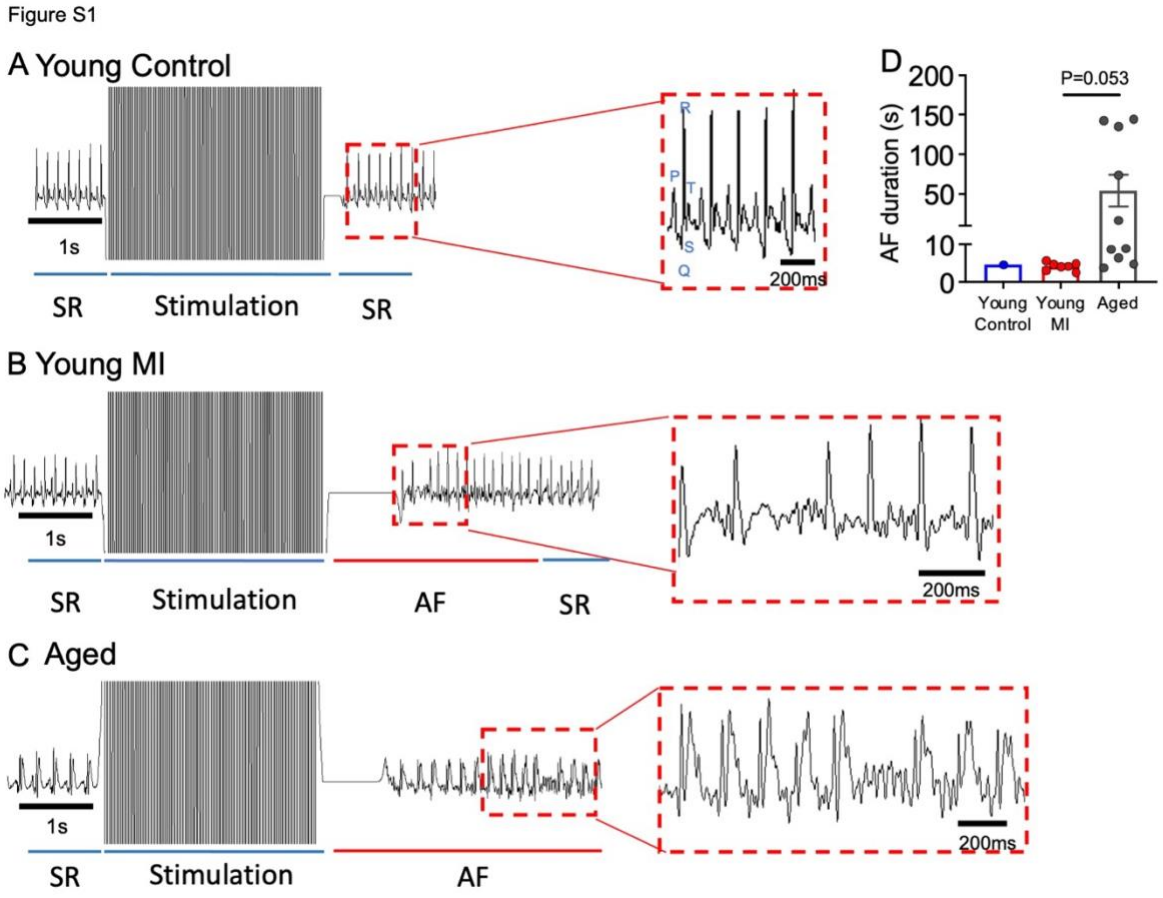

Figure S2.1. Representative ECGs and AF duration.

Showing sinus rhythm (SR), 3s burst stimulation followed by an induced AF episode in a young MI rat (*B*) and an aged rat (*C*), and SR in a young control (*A*). (*D*) Dot-plot graphs show Mean ± SEM of AF duration in each group (statistical test: unpaired t-test). SR: sinus rhythm. AF: atrial fibrillation. MI: myocardial infarction.

Figure S2

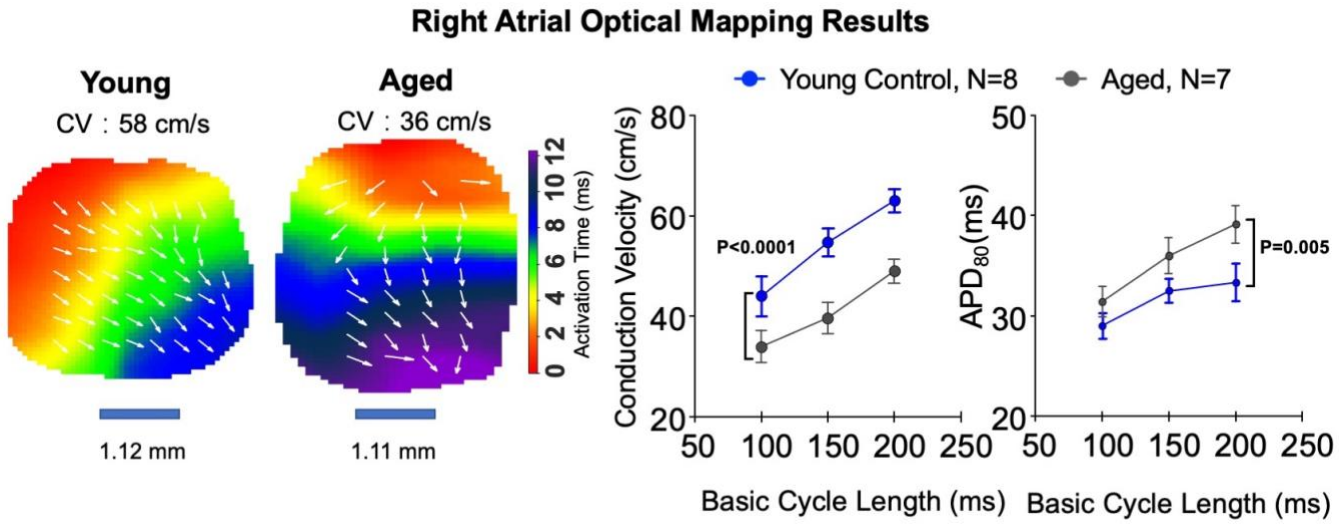

Figure S2.2. Optical mapping in right atrium (RA) of young control (3 months), young MI and aged (20 months) rats.

(A) RA activation-maps at BCL 100 ms from a control, young-MI, and elderly rat. (*B*) Mean±SEM RA APD_{80} and conduction velocity (*n*=7-8; two-way ANOVA followed by Bonferroni test; the p values indicate Bonferroni corrected differences between groups from data pooled from all BCLs).

Figure S3

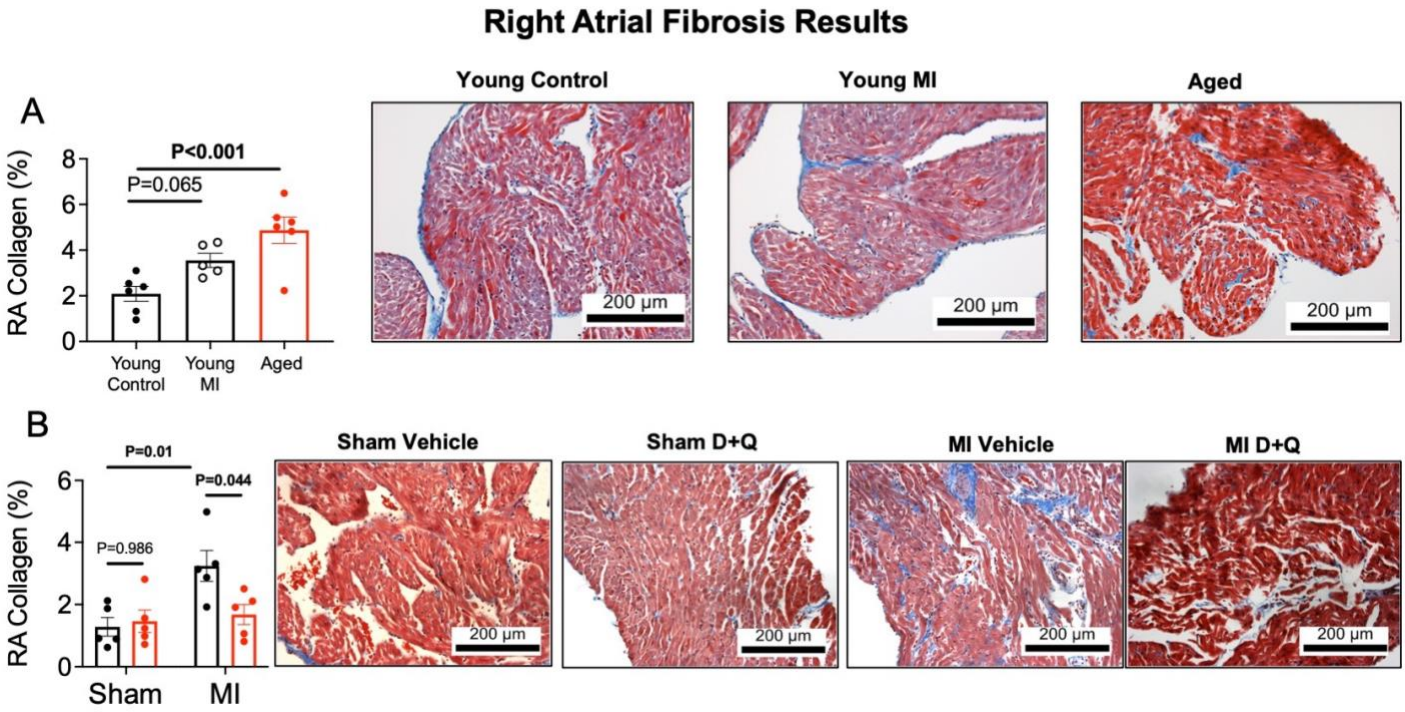

Figure S 2.3. Fibrosis quantification in right atrium (RA).

A) Dot-plot graphs show mean±SEM percentage of collagen content in RA in young control (3 month), young MI and aged (20 month) rats (*n*=5-6; one-way ANOVA with Dunnett's test). B) Percentage of collagen content in RA with and without Dasatinib+Quercetin (D+Q) senolytic therapy. (*n*=5; two-way ANOVA with Bonferroni test).

Figure S4

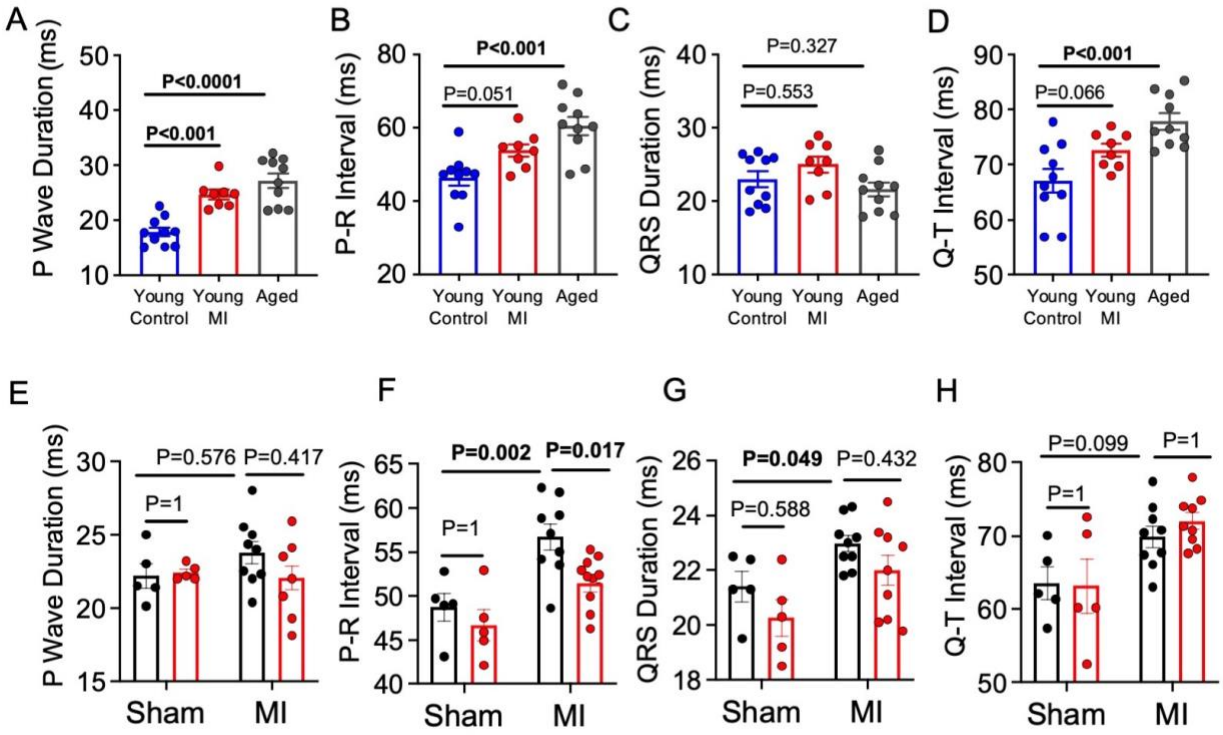

Figure S 2.4. ECG parameters including (A) P-wave duration, (B) P-R interval, (C) QRS duration, and (D) QT interval (in milliseconds) of young control (3 months), young MI and aged (20 months) rats.

In A, B, C and D panels, each point represents an individual animal (*n*=8-10; statistical analysis: one-way ANOVA followed by Dunnett's test, significance level *P*<0.05). (*E*) P-wave duration, (*F*) P-R interval, (*G*) QRS duration, and (*H*) QT interval of sham and MI rats treated with vehicle or dasatinib and quercetin (D+Q). In E, F, G and H panels, each point represents an individual animal (statistical analysis: two-way ANOVA for the group effect followed by Bonferroni's test, significance level *P*<0.05). AF: atrial fibrillation. ECG, electrocardiogram; LA, left atrium; MI, myocardial infarction.

Figure S5

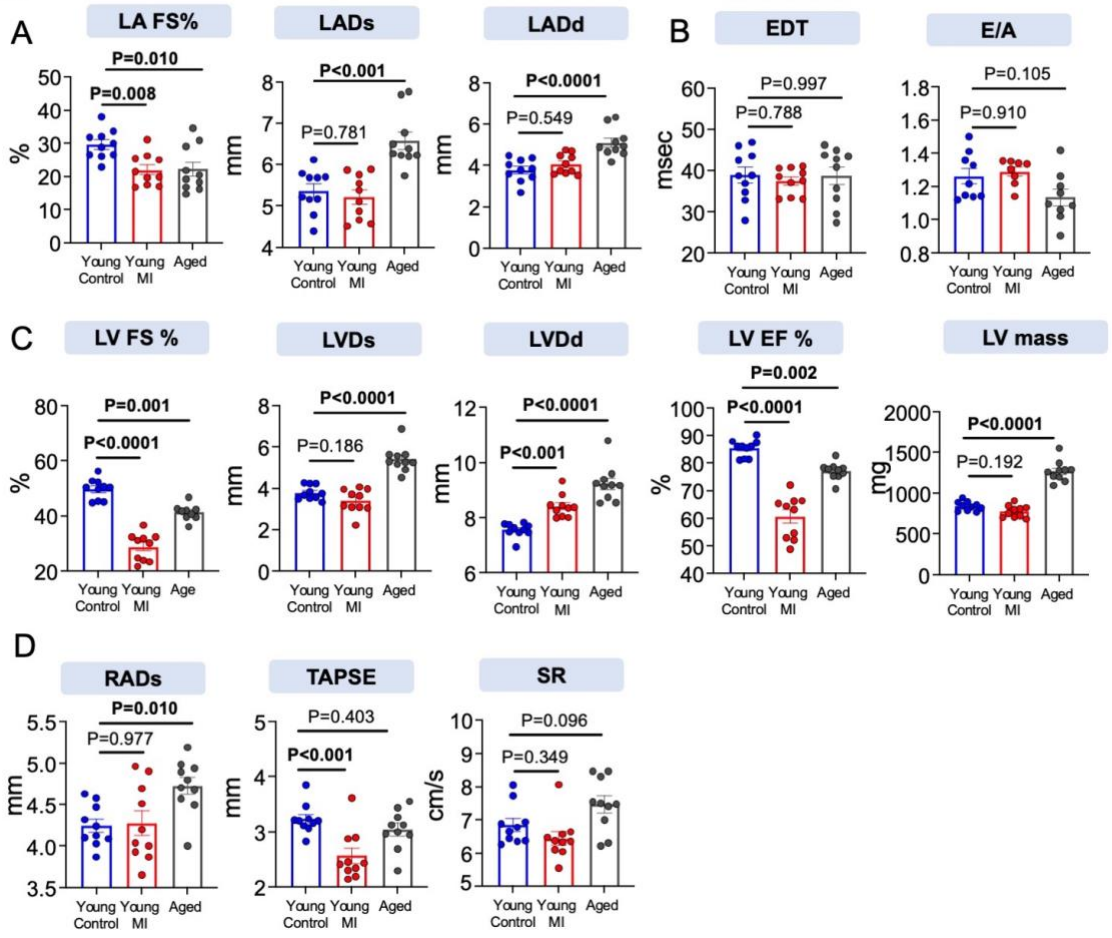

Figure S 2.5. Echocardiographic data of young control (3 months), young MI and aged (20 months) rats.

(*A*) Left atrial (LA) parameters: LA FS%: fractional shortening of LA; LADs: LA dimension at end systole; LADd: LA dimension at end diastole. (*B*) LV diastolic function parameters: EDT: early filling deceleration time; A: trans mitral flow atrial filling; E: trans mitral flow early filling. (*C*) left ventricular (LV) systolic function and structure parameters: LV FS%: LV fractional shortening; LVDs: LV dimension in systole; LVDd: LV dimension in diastole; LV EF%: LV ejection fraction; LV mass (*D*) right atrial (RA) structure and ventricular systolic function: RADs: RA dimension at end systole; TAPSE: tricuspid annulus plane systolic excursion; S_R: right ventricular lateral wall systolic contractility (n=10). For all panels, the statistical analysis was one-way ANOVA followed by Dunnett's test, significance level P<0.05.

Figure S6

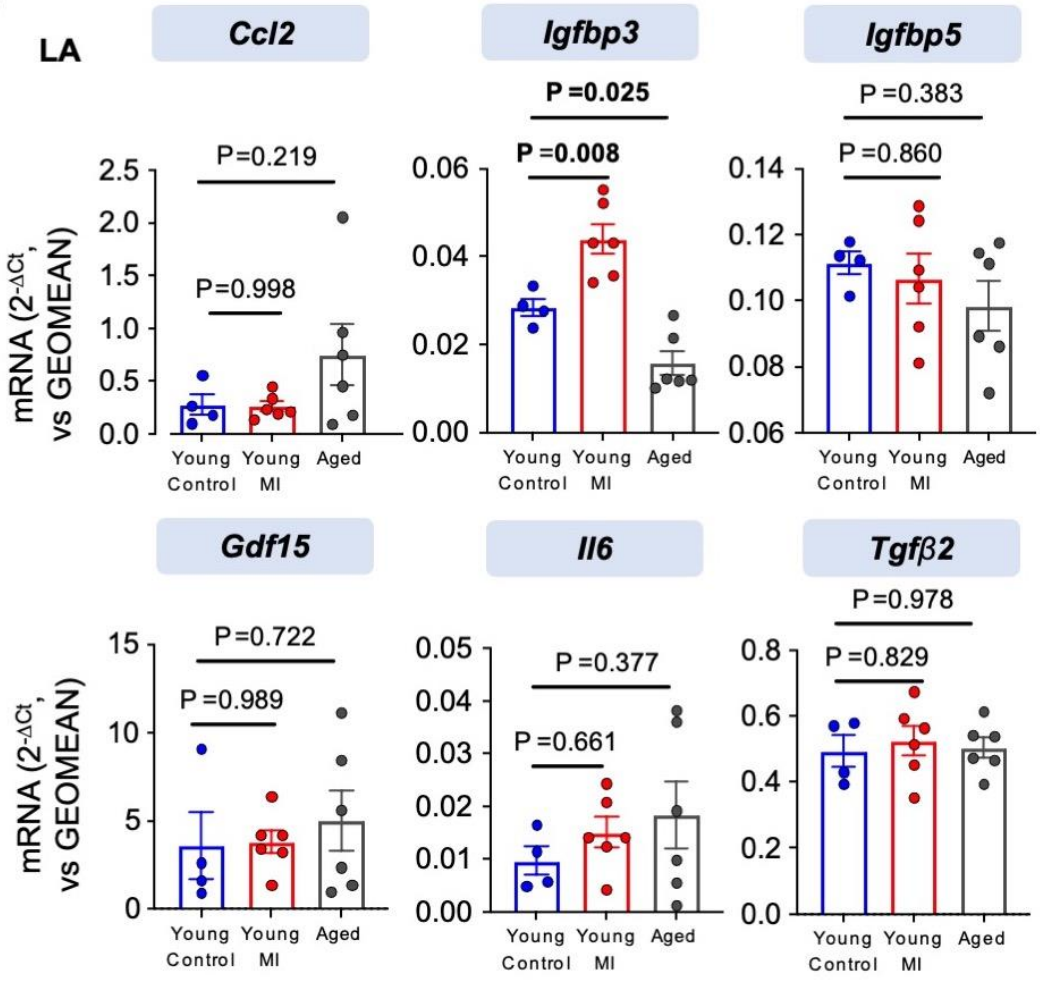

Figure S 2.6. SASP marker gene expression in left atrium of young control (3 months), young MI and aged (20 months) rats.

Messenger RNA expression, measured by qPCR, relative quantities ($2^{-\Delta Ct}$) calculated with the geometric mean of 3 reference genes (hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta-2-microglobulin (*B2m*) as internal standards (n=4-6; statistical analysis: one-way ANOVA followed by Dunnett's test, significance level P<0.05). *Ccl2*, C-C Motif Chemokine Ligand 2; *Gdf15*, growth differentiation factor 15; *Igfbp 3,5*, Insulin-like growth factor-binding protein 3 and 5; *Il6*, interleukin 6; *Tgfβ2*, transforming growth factor 2; MI, myocardial infarction; qPCR, quantitative polymerase chain reaction.

Figure S7

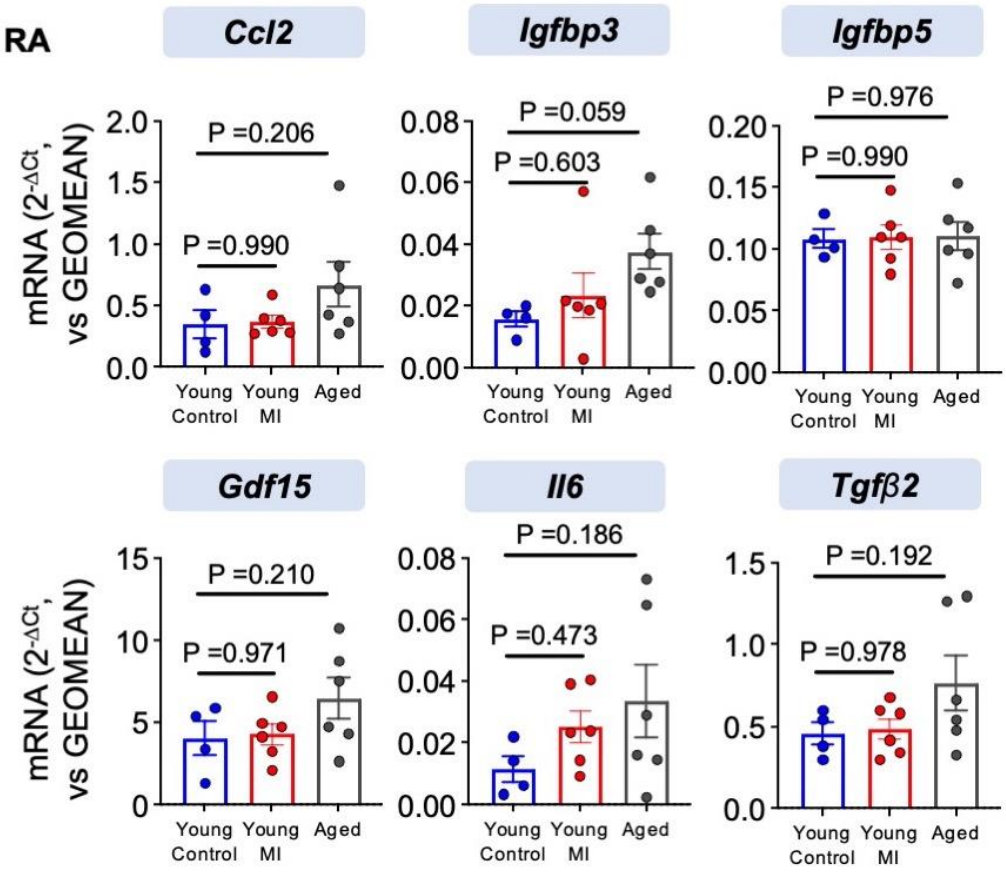

Figure S 2.7. Senescence and SASP marker gene expression in right atrium (RA) of young control (3 months), young MI and aged (20 months) rats.

Messenger RNA expression, measured by qPCR, relative quantities ($2^{-\Delta Ct}$) calculated with the geometric mean of 3 reference genes (hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta-2-microglobulin (*B2m*) as internal standards (*n*=4-6; statistical analysis: one-way ANOVA followed by Dunnett's test, significance level $P<0.05$). *Ccl2*, C-C Motif Chemokine Ligand 2; *Gdf15*, growth differentiation factor 15; *Igfbp 3,5*, Insulin-like growth factor-binding protein 3 and 5; *Il6*, interleukin 6; *Tgfβ2*, transforming growth factor 2; MI, myocardial infarction; qPCR, quantitative polymerase chain reaction.

FigureS8

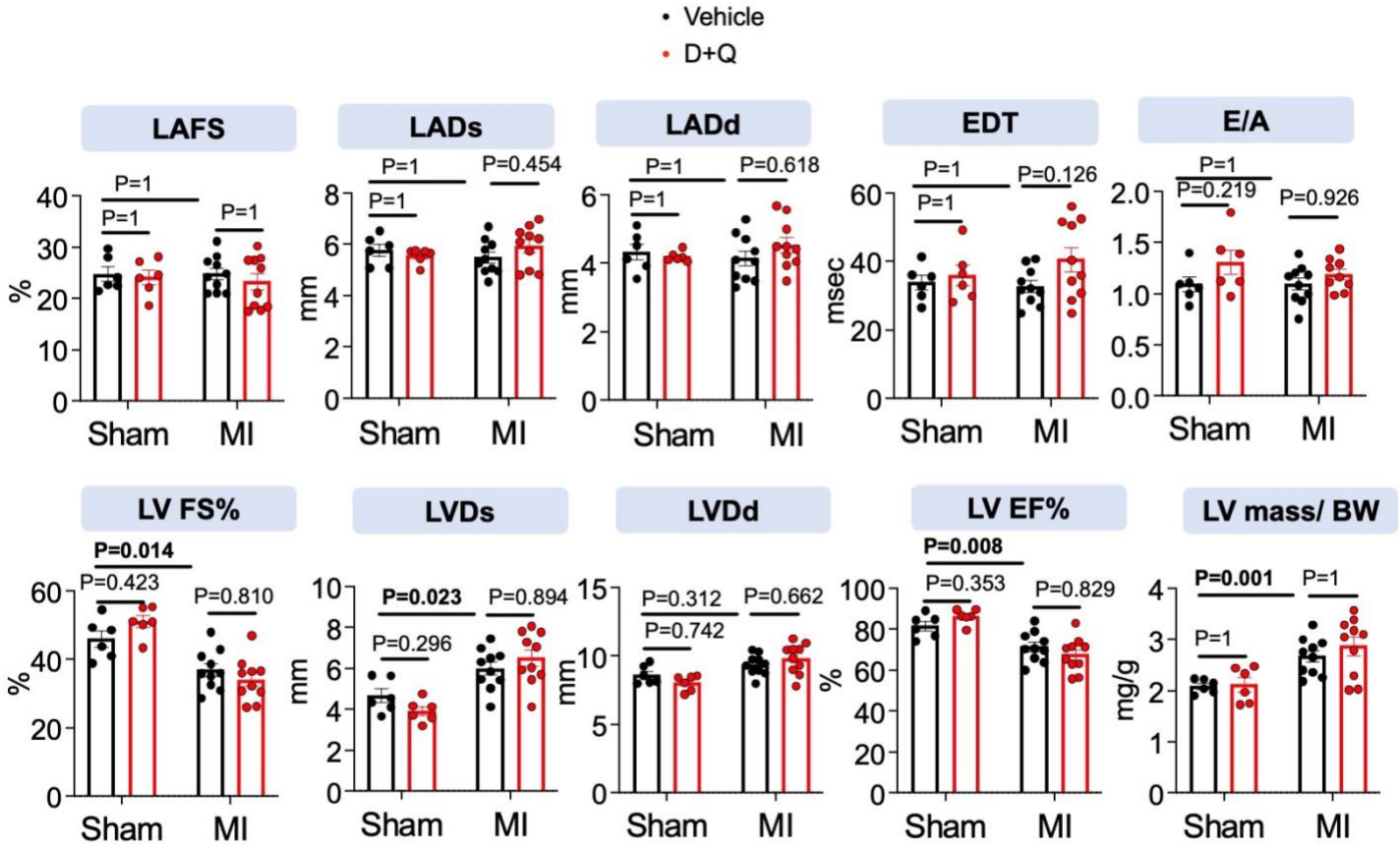

Figure S 2.8. Echocardiographic data of Sham and MI rats treated with vehicle or Dasatinib + Quercetin (D+Q).

(*A*) Left atrial (LA) parameters: LA FS%: fractional shortening of LA; LADs: LA dimension at end systole; LADd: LA dimension at end diastole. (*B*) LV diastolic function parameters: A: transmitral flow atrial filling wave; E: transmitral flow early filling wave; EDT: early-filling deceleration time. (*C*) left ventricular (LV) systolic function and structure parameters: LV FS%: LV fractional shortening; LVDs: LV dimension in systole; LVDd: LV dimension in diastole; LV EF%: LV ejection fraction; LV mass/BW: LV mass normalized to body weight (n=6-10; statistical analysis: two-way ANOVA for the group effect followed by Bonferroni's test, significance level P<0.05).

Figure S9

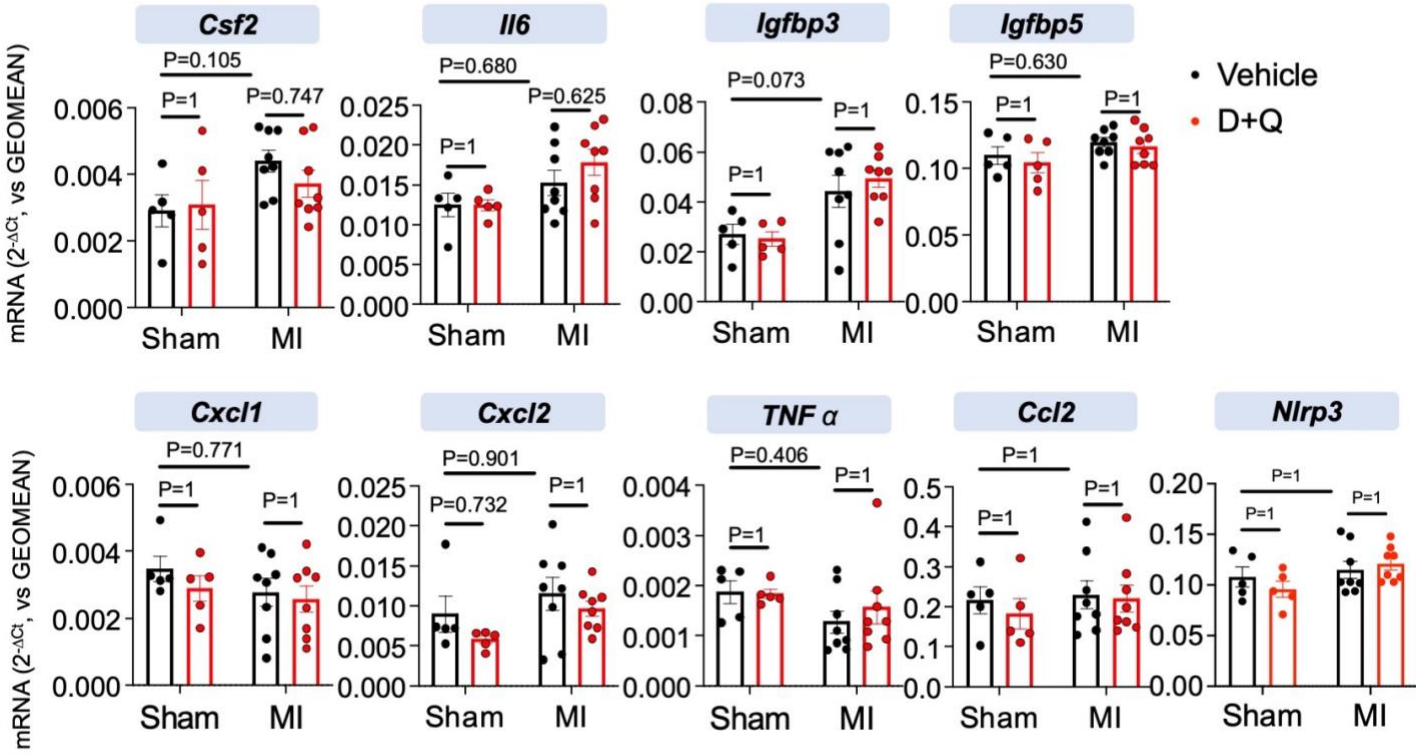

Figure S 2.9. SASP marker gene expression in left atrium (LA) of Sham and MI rats treated with vehicle or Dasatinib + Quercetin (D+Q).

Messenger RNA expression, measured by qPCR, relative quantities ($2^{-\Delta Ct}$) calculated with the geometric mean of 3 reference genes (hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta-2-microglobulin (*B2m*) as internal standards (*n*=5-8). Statistical analysis: two-way ANOVA for the group effect followed by Bonferroni's test, significance level $P<0.05$. *Ccl2*, C-C Motif Chemokine Ligand 2; *Csf2*, Granulocyte-macrophage colony-stimulating factor; *Cxcl1,2*, C-X-C motif chemokine ligand 1 and 2; *Igfbp 3,5*, Insulin-like growth factor-binding protein 3 and 5; *Il6*, interleukin 6; qPCR, quantitative polymerase chain reaction; SASP, senescence associated secretory phenotype; *TNFα,* Tumour Necrosis Factor alpha.; *Nlrp3*, nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3.

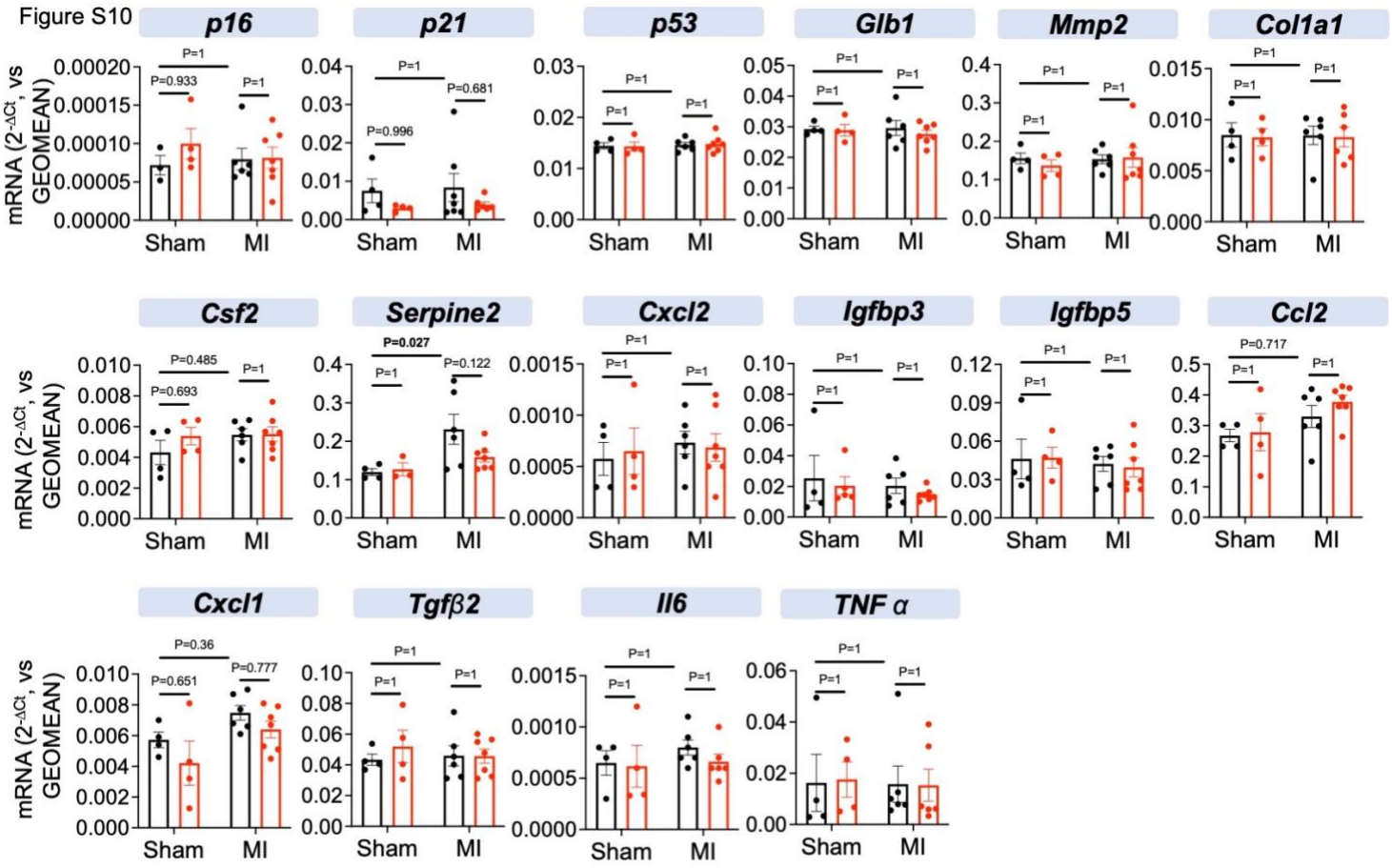

Figure S 2.10. Senescence and SASP marker gene expression in right atrium (RA) of Sham and MI rats treated with vehicle or Dasatinib + Quercetin (D+Q).

Messenger RNA expression, measured by qPCR, relative quantities ($2^{-\Delta Ct}$) calculated with the geometric mean of 3 reference genes (hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta-2-microglobulin (*B2m*) as internal standards (*n*=4-7). Statistical analysis: two-way ANOVA for the group effect followed by Bonferroni's test, significance level $P<0.05$. *Ccl2*, C-C Motif Chemokine Ligand 2; *Csf2*, Granulocyte-macrophage colony-stimulating factor; *Cxcl1*,*2*, C-X-C motif chemokine ligand 1 and 2; *Igfbp 3*,*5*, Insulin-like growth factor-binding protein 3 and 5; *Il6*, interleukin 6; qPCR, quantitative polymerase chain reaction; SASP, senescence associated secretory phenotype; *TNFα,* Tumour Necrosis Factor alpha.

Figure S11

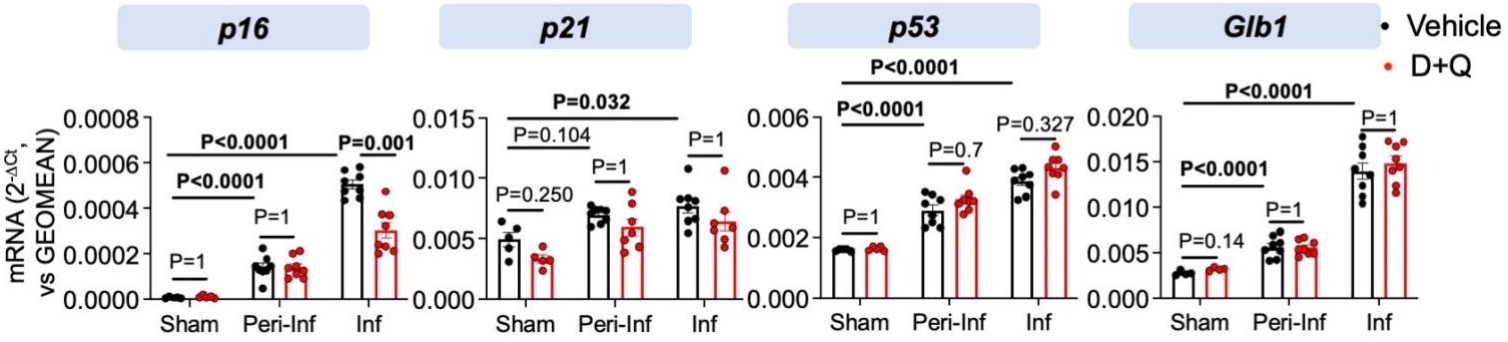

Figure S 2.11. Senescence marker gene expression in ventricular tissues of MI rats treated with vehicle or Dasatinib + Quercetin (D+Q).

Messenger RNA expression, measured by qPCR, relative quantities ($2^{-\Delta Ct}$) calculated with the geometric mean of 3 reference genes (hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta-2-microglobulin (*B2m*) as internal standards for senescence markers, p16, p21, p53, Glb1 in sham an and peri infarct (peri-inf) and infarct (inf) regions of MI rats (n=5-8). Statistical analysis: two-way ANOVA for the group effect followed by Bonferroni's test, significance level P<0.05. qPCR, quantitative polymerase chain reaction.

Figure S12

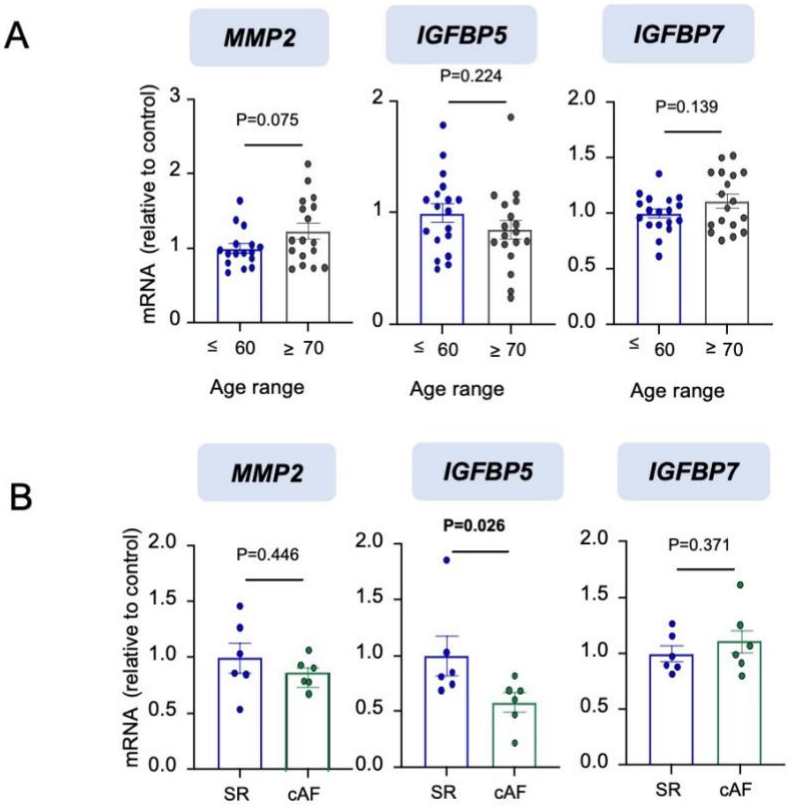

Figure S 2.12. SASP marker gene expression in human atrial tissue.

Messenger RNA expression, as measured by dPCR, expressed in relative to control. Relative quantities ($2^{-\Delta\Delta Ct}$) calculated with the geometric mean of 4 reference genes, hydroxymethylbilane synthase (HMBS), beta-2-microglobulin (*B2M*), GATA binding protein 4 (*GATA4*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal standards for SASP markers, *MMP2, IGFBP5, IGFBP7* in RAAs of (*A*) young (≤ 60) and aged (≥ 70) patients (*n*=15-19). (*B*) SR and cAF patients (*n*=6). For all panels, statistical analysis was unpaired t-test for parametric or Mann-Whitney test for non-parametric values, significance level $P < 0.05$. Each point represents an individual animal. cAF, chronic atrial fibrillation; dPCR, digital polymerase chain reaction; *IGFBP5,7*, Insulin-like growth factor-binding protein 5 and 7; *MMP2*, matrix metalloproteinase 2; SASP, senescence-associated secretory phenotype; SR, sinus rhythm.

Figure S13

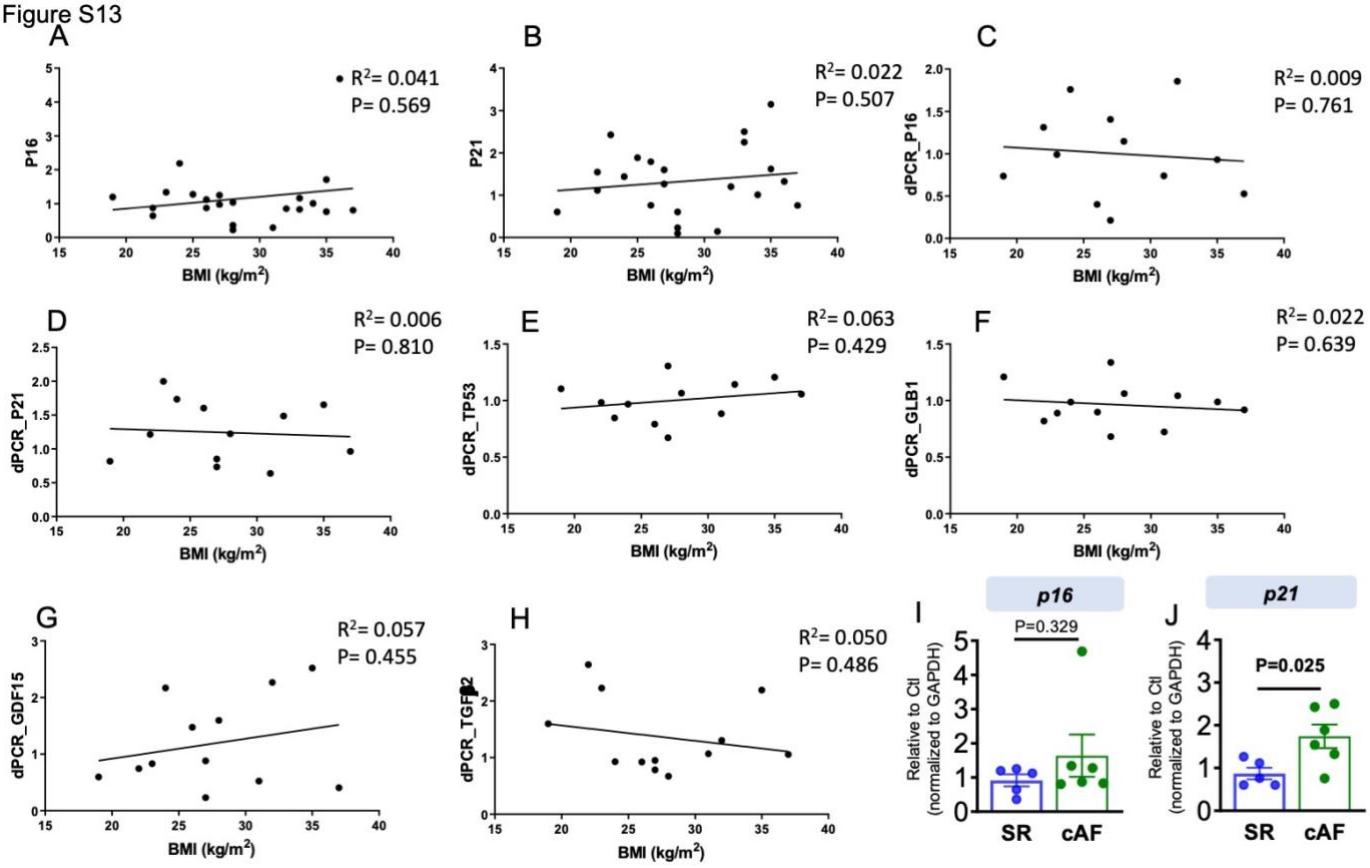

Figure S 2.13. Association of BMI (body mass index) and CAD (coronary artery disease) diagnosis with senescence markers.

A-H: Association of BMI with A) WB- p16 B) WB- p21 C) dPCR-p16 D) dPCR-p21 E) dPCR-TP53 F) dPCR-GLB1 G) dPCR-GDF15 H) dPCR-TGFB2. I-J: dPCR level of senescence markers in patients with CAD: I) p16 J) p21.

Figure S14

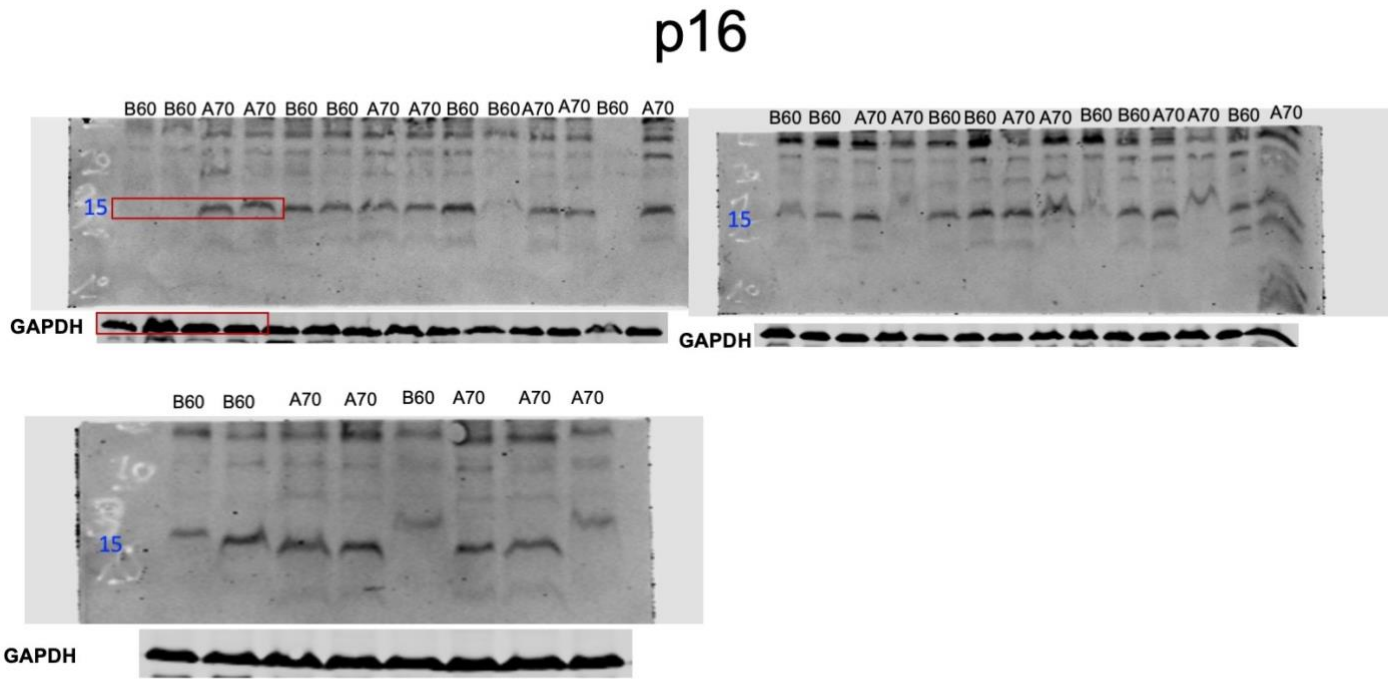

Figure S 2.14. Original full-length immunoblot.

Immunoblot of p16 in RAAs of young (≤ 60) and aged (≥ 70) patients.

Figure S15

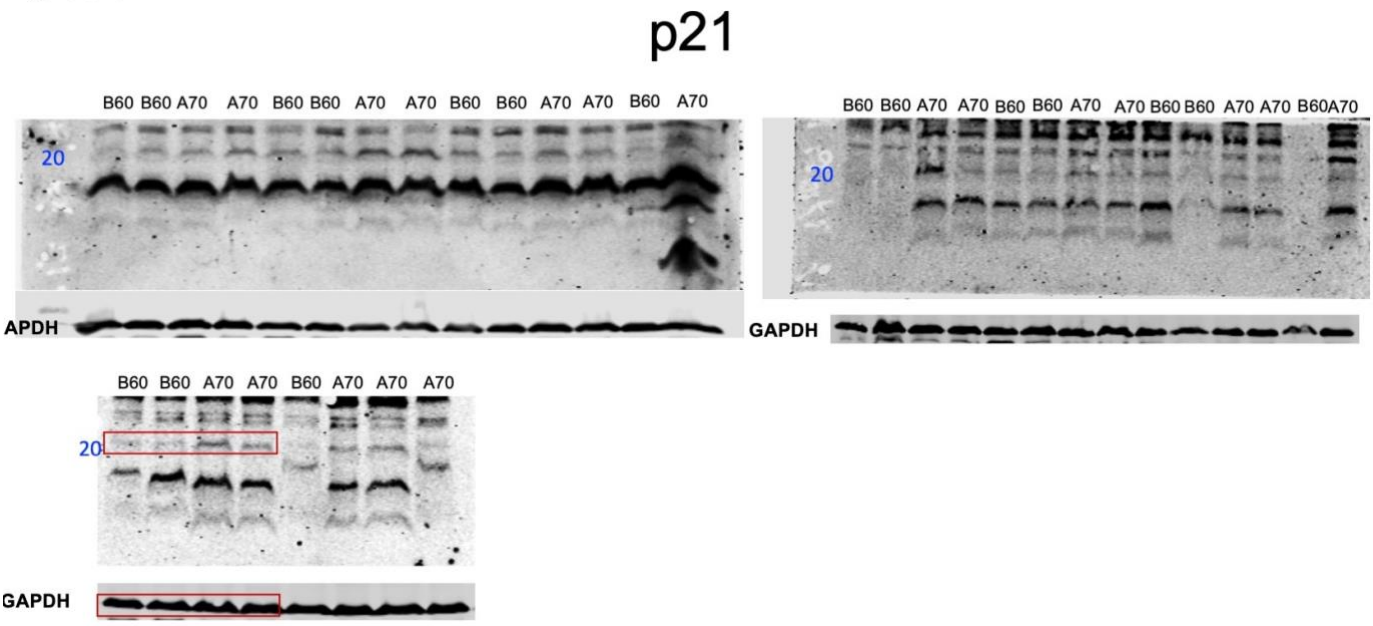

Figure S 2.15. Original full-length immunoblot.

Immunoblot of p21 in RAAs of young (≤ 60) and aged (≥ 70) patients.

Figure S16

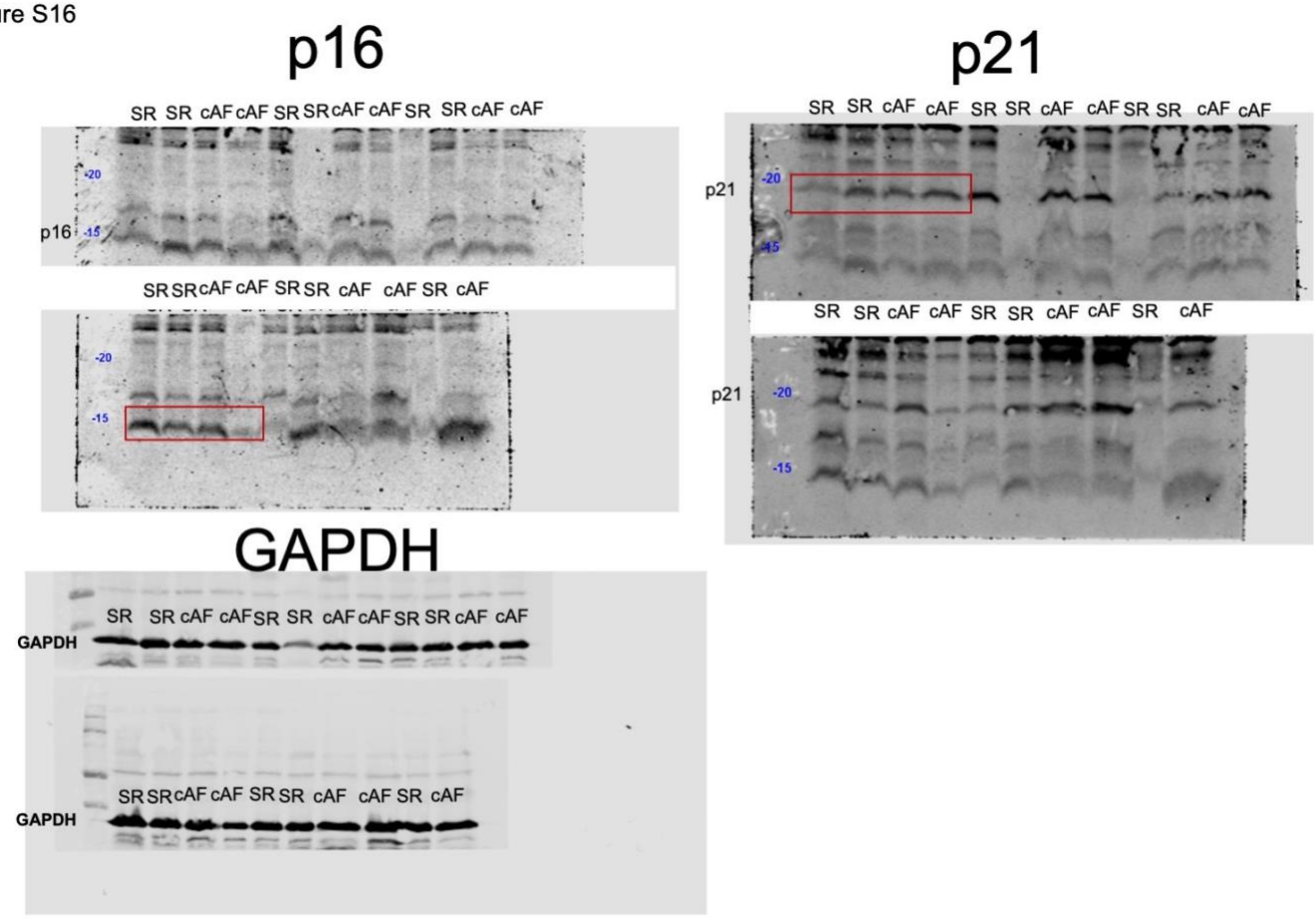

Figure S 2.16. Original full-length immunoblot.

Immunoblot of p16 and p21 in RAAs of SR and cAF patients. cAF, chronic atrial fibrillation; SR, sinus rhythm.

Connecting statement: Chapter 2 and 3

In chapter 2, we showed promising results of senolytic therapy in AF prevention. Due to potential off-target effects of senolytics, we further investigated the role of senescence using the INK-ATTAC transgenic mouse model, in which senescent cells express undergo apoptosis following the treatment of mice with a dimerizing agent AP, that activates caspase 8. In INK-ATTAC mice, p16-positive senescent cells undergo apoptosis, however, dasatinib and quercetin affect the pro-survival pathways such as PI3/Akt and Bcl2 in senescent cells and may have an impact on various types of senescent cells, not limited solely to p16-positive cells. Baker et al., the researchers who developed the INK ATTAC mouse model at Mayo Clinic, investigated whether the administration of AP caused any off-target effects by conducting a healthspan study on wild-type mice lacking ATTAC. In contrast to INK ATTAC mice, which exhibited significant healthspan improvement, wild-type mice did not show any enhancements in healthspan when treated with AP. This suggests that AP demonstrates selectivity for INK ATTAC mice.[10] Aged INK-ATTAC mice did not show inducible AF, whether treated with AP or control-vehicle; however, we noticed a significant improvement in LV diastolic function upon the clearance of senescent cells with the AP treatment in aged INK-ATTAC. This finding prompted us to change our focus to study the effects of senescent cell clearance on LV remodeling, a process known to render the heart more susceptible to HF.

3. Chapter 3. Clearance of Cardiac Senescent Cells Improves Age-related Cardiac Remodeling in Mice

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Manuscript in preparation for submission.

3.1. Abstract

Background: Age is a major risk factor for heart failure (HF); however, the underlying mechanisms are poorly understood. Cellular senescence is an aging and stress related response; Senescent cells secrete profibrotic and proinflammatory factors as parts of a senescence associated secretory phenotype (SASP). The role of senescence in age-related cardiac remodeling leading to HF is unknown.

Objectives: To study the effect of clearance of senescent cells on cardiac function, structure and left ventricular (LV) pressure in a mouse model of aging. To evaluate the role of different senescent cardiac cell-types in age-related cardiac remodeling.

Methods: We studied INK-ATTAC mice, in which senescent cells undergo targeted clearance following treatment with a dimerizing agent, AP20187 (AP). Mice were treated with AP or vehicle from 12 months to 18 months of age. Cardiac function and structure were studied with echocardiography. Hemodynamic parameters were measured with a Millar catheter. The percentage of senescent cells in different cardiac-cell populations was analyzed with the use of Fluorescence-Activated Cell Sorting (FACS) and immunofluorescence imaging. The potential paracrine effects of senescent fibroblast SASP components were assessed *in vitro* by co-culturing senescent fibroblasts with healthy cardiomyocytes.

Results: Echocardiography revealed a significant reduction LV mass/ LV diameter at end diastole (LVDd) and LV anterior wall thickness at end diastole (LVAWd) in the Aged-AP vs Aged-Vehicle group. Prolonged isovolumic relaxation time indicated diastolic dysfunction in vehicle mice and improved with AP. Measuring GFP positive cells, indicating senescent cells with FACS, suggests senescent fibroblasts reduced with AP, whereas immune and endothelial

cells were not affected. Double staining of p16 (marker of senescence) and cardiac cell type markers showed the percentage of senescent cardiomyocytes reduced after AP treatment. These observations suggest that positive effects of senescent cell clearance on age-related cardiac remodeling might be mediated through the clearance of senescent fibroblasts and cardiomyocytes. Co-culturing senescent fibroblasts with healthy cardiomyocytes resulted in the upregulation of hypertrophy markers, suggesting a potential paracrine effects of senescent fibroblast SASP components on cardiomyocytes.

Conclusions: Our study points to a significant role of senescent cells, likely senescent fibroblasts and cardiomyocytes, in the age-related cardiac remodeling. Modulating senescence might provide the basis for novel therapeutic approaches for age-related cardiac disease, particularly HF.

3.2. Introduction

Age is the leading risk factor for cardiac disease.[1-3] The prevalence of heart failure (HF) rises from <2% in adults younger than 60 years of age to 14.7% of men and 12.8% of women over 80.[4,5] The underlying changes include impaired left ventricle (LV) relaxation, diastolic dysfunction, LV hypertrophy and fibrosis.[1,4] The mechanisms underlying how aging makes the heart more susceptible to HF remain poorly understood.

Molecular mechanisms that underly cardiac aging are multifactorial and interactive.[6] Cellular senescence is a stress- and age-related response that causes cell-cycle arrest in dividing cells and is regulated through two key pathways: (i) p16–retinoblastoma protein and (ii) p53–p2.[7,8] Senescent cells are resistant to apoptosis and secrete proinflammatory cytokines, growth factors, and matrix remodeling proteases, manifesting a "senescence-associated secretory phenotype" (SASP). Although cellular senescence was initially considered unique to dividing cells, more recent research points to the occurrence of senescence in non-dividing cells like cardiomyocytes.[9]

Recent work suggests the possible therapeutic potential of targeting senescence in the prevention and/or treatment of age-related cardiac disease. Baker et al. showed that the clearance of senescent cells in aged mice reduces cardiomyocyte size.[10] Similarly, Anderson et al. showed that the clearance of senescent cells with the senolytic drug navitoclax or in the INK-ATTAC transgenic mouse model results in reduced cardiomyocyte size and fibrosis.[9] To our knowledge, no data are available in the literature regarding the effects of senescent-cell clearance on LV hemodynamic parameters, systolic and diastolic function, and electrophysiological properties. Also, the cell-types mediating the effects of cellular senescence on age-related cardiac remodeling is often ignored.

Here, we tested the hypothesis that cellular senescence contributes to age-related cardiac remodeling. To test our hypothesis, we studied aged INK-ATTAC mice treated with vehicle or a dimerizing agent AP20817 (AP) that induces apoptotic death in senescent cells expressing p16. Our specific goals were: (1) to evaluate the effect of senescent-cell clearance on cardiac hypertrophy and fibrosis as well as LV systolic and diastolic function in aged mice; (2) to study the effect of senescent-cell clearance on hemodynamic and conduction indices; (3) to evaluate the role of different senescent cardiac cell-types in age-related cardiac remodeling; (4) to evaluate potential paracrine effects of senescent fibroblasts.

3.3. Methods

Animal Model

INK-ATTAC mice were developed by the Kirkland and J. van Deursen laboratories at Mayo Clinic. The INK-ATTAC mouse model aims to eliminate senescent cells by leveraging a genetic approach based on the dimerization of specific protein, a membrane-bound myristoylated FK506-binding-protein–caspase 8 (FKBP–Casp8) fusion protein. This dimerization process is induced by the synthetic drug AP20187. The dimerization specifically occurs within senescent cells due to the expression of this protein fusion via a modified p16 gene promoter, and p16 is mostly expressed in senescent cells. After the fusion proteins dimerize, Caspase 8 becomes activated, initiating an apoptosis response in senescent cells. Consequently, senescent cells undergo apoptosis and are subsequently cleared. [10] The mice were bred at the Montreal Heart Institute. All experimental and animal-handling procedures were approved by the Animal Ethics Committee of the Montreal Heart Institute and were conducted in accordance with the Canadian Council on Animal Care and National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Animal Treatment

INK-ATTAC mice were randomly allocated (in a blind fashion) to the following groups: Young (4 months, without treatment), Aged-AP (18 months, with AP treatment), and Aged-Vehicle (18 months, with vehicle treatment). AP (2μg/g) or vehicle (4%Ethanol, 10% PEG400, 86% Tween 2% in water) treatment for aged groups began when the mice were 12 months old (Figure 3.1A). The mice received these treatments by intraperitoneal injection two times a week for six months, a regimen chosen based on prior work.[10]

Drugs and Chemicals

AP20187 (635069) was obtained from Takara Bio (Shiga, Japan), taurine (TAU303) from Bioshop Canada (Burlington, ON), blebbistatin (B592490-10) from Toronto Research Chemical (North York, ON) and RH-237 (S1109) from Invitrogen (Waltham, MA). Conjugated antibodies for the FACS experiment, Alexa Fluor 647 Rat Anti-Mouse CD31(563608) and BV421 Rat Anti-Mouse CD45 (563890) were obtained from BD Bioscience (Mississauga, ON). Primary antibody sources for immunofluorescence were: p16 (ab54210), α-smooth muscle actin (αSMA) (ab5694), and troponin I (ab47003) from Abcam (Cambridge, UK); vimentin antibody (3932S) from Cell Signaling (Danvers, MA); the CD31 antibody (AF3628-SP) from R & D System (Minneapolis, MN). The 4′,6-diamidino-2-phenylindole (DAPI), wheat germ agglutinin (WGA)(W32466) and secondary antibodies, 555 donkey anti-mouse (A31570), 488 donkey anti-rabbit (A21206), 488 donkey anti-goat (A11055) were obtained from Invitrogen (Waltham, MA). FcR blocker (purified anti-mouse CD16/32) (101302) from Biolegend (San Diego, CA). Fixable Viability Stain 780 (FVS) (565388) from BD Bioscience (Mississauga, ON). The 12 mm Transwell® with 0.4 μm

Pore Polycarbonate Membrane Insert (29442-086) was obtained from Coring Inc (Coring, NY). The hydroxyproline assay (MAK008-1KT) was obtained from Sigma-Aldrich (Missouri, USA).

Echocardiography

Echocardiographic studies were performed as described previously.[11] Briefly, mice were anesthetized with 2% isoflurane and kept on a heated platform to maintain the body temperature around 37°C. The cardiac function and structure were assessed by a transthoracic echocardiography using an i13L probe (10-14 MHz) and Vivid 7 Dimension ultrasound system (GE Healthcare Ultrasound, Horten, Norway). LV anterior thickness at the end of diastole (LVAWd), LV dimension at end cardiac diastole (LVDd) and systole (LVDs) were measured by M-mode echocardiography (M-mode). LV fractional shortening and ejection fraction were obtained by formula available within the Vivid 7 system. LV trans mitral filling velocities in early (E) and atrial (A) filling were measured with pulsed wave Doppler (PW). LV mass was calculated using a previously described formula.[12] Left atrium dimension at end cardiac systole and diastole (LADs, LADd) were measured by M-mode. The average of 3 consecutive cardiac cycles was used for each measurement. Throughout the experiments, the operator was blinded to group/treatment assignment.

Histology

Formalin-fixed, paraffin-embedded samples were cut at 6-µm thickness. Image Pro Premier 9.3 Software (Media Cybernetics) was used to quantify fibrosis on Masson's Trichrome-stained images. Entire left ventricle sections, double-stained for p16 and cell-selective markers were scanned (Aperio VERSA Brightfield Scanner) and automatically analyzed with Visiomorph software (Visiopharm, Hoersholm, Denmark). All analyses were performed blind to mouse-group identity.

Hydroxyproline Assay

Total collagen content in the LV was determined by hydroxyproline assay as described previously.[13] The results were expressed as μg hydroxyproline/mg dry tissue.

Cardiac Cells Isolation: Langendorff Perfusion

To isolate cardiomyocytes and non-myocyte cardiac cells (including fibroblasts, myofibroblasts, immune cells, and endothelial cells), we performed the Langendorff perfusion of cannulated mouse hearts with the previously described method.[14] The mice were injected with 10,000 UI heparin (0.2 mL/mouse) for anticoagulation, anesthetized with 2% isoflurane and euthanized by cardiac excision. The whole heart was cannulated and perfused with perfusion buffer to wash out the remaining blood in the heart, followed by a 16-minute perfusion with the same solution containing collagenase II (2.4 mg/mL) (first 6-minute without calcium and the next 10-minute with 40 μM calcium). Tissues were minced and cellular dissociation was completed by gentle trituration. The cell suspension was first passed through a 500 μm filter and centrifuged at 60 g to obtain cardiomyocytes (the pellet). Then, the supernatant was passed through a 20 μm filter and centrifuged at 500 g to obtain non-myocyte cardiac cells.

Fluorescence Activated Cell Sorting (FACS)

The isolated non-myocyte cardiac cells were resuspended in the sorting buffer (PBS (Ca^{2+}/Mg^{2+} free), 20 mM HEPES, pH 7.4, 5mM EDTA, 0.5% BSA) and stained with FVS (to distinguish dead cells), then they were blocked for Fc receptors using Fc blockers. Following, the cell surface markers of endothelial cells (Alexa Fluor 647 Rat Anti-Mouse CD31) and immune cells (BV421 Rat Anti-Mouse CD45); as there are no unique or common surface markers for fibroblasts, the double negative population was considered fibroblasts. As INK-ATTAC transgene contains green fluorescent protein (GFP) sequence, senescent cells were identified as GFP positive cells. Cells

were washed, resuspended in the sorting buffer, and sorted with a FACS Aria Fusion (BD Biosciences) cell sorter into four different populations: double negative GFP^+, Double negative GFP^-, $CD31^+$ and $CD45^+$. Cells were then used for qPCR experiments to quantify specific genes of interest. The percentage of senescent cells in all cell populations were determined based on the GFP signal. The data was analyzed with FlowJo V10.7.1 (BD Biosciences). Prior to sorting, we performed a compensation to set the gates using beads for each antibody.

Hemodynamic Study

The mice were anesthetized with 2% isoflurane on a temperature-regulated operating table to maintain the body temperature around 37°C. Through the cervical incision, the right carotid was isolated. With a surgical microscope (Leitz Wild M650, Wild Surgical Microscopes, Heerbrugg, Switzerland) a Millar catheter (SPR 671, TX, USA) was inserted and advanced into the ascending aorta, subsequently into the LV. At each step during insertion and advancement into the aorta and the LV, transmitted pressure and surface ECG were recorded and analyzed with iox2 software (v.2.8.0.13, EMKA Technologies, Paris, France).

Ex Vivo Optical Mapping

The heart was excised and perfused via the aorta with Krebs solution at 4 mL/min and 37°C, bubbled with 95% O2/5% CO2 to maintain a pH of 7.4. After 20 min of stabilization, a Krebs solution containing blebbistatin (10 μM) was used to suppress mechanical contraction and prevent motion artifacts. The voltage-sensitive dye RH-237 was injected as following: a 750 μL bolus injection of 100 uM in ~200 μL increments over 5 minutes directly into the perfusion line. Experiments were performed in sinus rhythm or paced (8Hz). RH-237-loaded whole hearts were

illuminated with light from the X-Cite Xylis Broad Spectrum LED Illumination System (model No: XT720L, Excelitas Technologies, ON, Canada) and filtered with a 520/35 nm excitation filter. Emitted fluorescence was separated by a dichroic mirror (560 nm cut-off) and filtered by a 695 long pass emissions filter. Recordings were captured using a high-speed CMOS camera (MiCAM03-N256, SciMedia). We mapped conduction and measured APD50 and APD70 in the LV. Data was captured at a frame rate of 1818 or 2914 frames/s using BrainVision software (BrainVision Inc.). The spatial resolution was 0.02643 mm/px. Magnification was constant (1.6X) in all experiments and no pixel binning was used. All recordings were analyzed with custom MatLab software created by Alexander Quinn, Dalhousie University.

qPCR

Total RNA was extracted using the RNeasy Mini Kit. RT-PCR was performed with Applied Biosystems Thermal Cycler Step One Plus (ThermoFisher Scientific, Watham, MA). SYBR green primers used for p16 (Fwd: CCCAACGCCCCGAACT, Rev: GCAGAAGAGCTGCTACGTGAA) p21 (Fwd: GGCAGACCAGCCTGACAGAT, Rev: TTCAGGGTTTTCTCTTGCAGAAG), myosin heavy chain 6 (*Myh6*) (Fwd: CCAACACCAACCTGTCCAAGT, Rev: AGAGGTTATTCCTCGTCGTGCAT) and *Myh7* (Fwd: CTCAAGCTGCTCAGCAATCTATTT, Rev: GGAGCGCAAGTTTGTCATAAGT), Interleukin-6 (Il-6) (Fwd: TCCGGAGAGGAGACTTCACA, Rev: TGCAAGTGCATCATCGTTGT), transforming growth factor beta-2 (Tgfβ2) (Fwd: TGCCTTCGCCCTCTTTACATT, Rev: AGCGGAAGCTTCGGGATTTA). All mRNA values were normalized to the geometric mean of *B2m*, *Hprt1*, and *Gapdh*. Throughout the experiments, the operator was blinded to group/treatment assignment.

Induction of Senescence in Cardiac Fibroblasts with H_2O_2

Primary mouse fibroblasts were isolated with the Langendorff perfusion as mentioned above. Fibroblasts were collected from the cell suspension by spinning at 500 g for 5min. Fibroblasts were plated in culture using maintenance medium (Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 1 %penicillin and streptomycin and 0.1% amphotericin B). The cells were then washed, and the medium was changed after 2 h and 24 h to remove unattached cells, leaving a highly pure population of fibroblasts. The fibroblasts were cultured for 4 days to reach confluency; they were then trypsinized (passage1), counted, and seeded (30,000-40,000 cells) on each well of 12 well plates. The day after passage 1, the cells almost reached 60-70% confluency and were treated with different concentrations of H_2O_2 including 800 μM, 400 μM, 200 μM, 100 μM, 50 μM and 25 μM for two hours, repeated 3 times in two days. The 200 μM concentration was selected because of its high efficiency in senescence induction. Next, the senescent fibroblasts were cocultured with freshly isolated cardiomyocytes using Coring Transwell inserts.

Coculture of Senescent Fibroblasts and Cardiomyocytes

Freshly isolated cardiomyocytes were seeded on laminin coated Coring Transwell inserts after calcium reintroduction described previously.[14] The cardiomyocytes were left to attach for 1h and then washed. The inserts containing cardiomyocytes were then suspended over the senescent (treated with H_2O_2) or control fibroblasts in the 12 well plate, so that the base of the inserts was within the culture medium on the fibroblasts but did not touch the bottom of the plate. Following this, the co-cultures were incubated at 37°C and 5% CO_2 for 48 hours before analysis.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 (San Diego, CA). For normally distributed data (assessed by Shapiro-Wilk test), we used Student's t-test (for 2-group only comparisons), 1-way ANOVA (followed by Tukey). For non-normal data, Kruskal–Wallis or

Mann–Whitney tests were used. Categorical variables (like AF inducibility) were analyzed with Fisher's exact test. Results are expressed as mean±SEM and 2-tailed $P<0.05$ was considered statistically significant.

3.4. Results

Effect of the Clearance of Senescent Cells on Cardiac Function and Structure

The echocardiographic data are reported as changes from 12 to 18 months in each group (Aged-Vehicle, Aged-AP) (Figure 3.1B-D, Figure S 3.1). Echocardiography revealed a significant reduction LV mass/LVDd and LVAWT in the Aged-AP vs Aged-Vehicle group (Figure 3.1B). The LV mass, LVDs, LVDd and LV mass/BW changes were not significantly different between the two groups (Figure 3.1D, Figure S 3.1A). Left-ventricular systolic function (LVFS% and LVEF%) was also not statistically different between the groups (Figure S 3.1B).

The A wave amplitude was significantly lower in the AP treated group (Figure S1C) whereas the E wave amplitude was not different between the groups (Figure S 3.1C). The mean E/A change was negative for the vehicle treated group, while it was positive in the AP treated group without reaching statistical significance (Figure S 3.1C). Overall, the most apparent echocardiographic differences between groups were a reduction in hypertrophy parameters (LV mass/ LVDd and LVAWT).

The mice were euthanized, and the hearts were weighted. The mouse heart weight normalized to body weight (HW/BW) was significantly higher in the Aged-Vehicle group compared to the young group while it was normalized in the AP group (Figure 3.1E).

Effect of the Clearance of Senescent Cells on Arterial Pressure, LV Pressure and Other Hemodynamic Parameters

Hemodynamic studies showed a prolongation in LV isovolumic relaxation time in Aged-Vehicle vs Young which was significantly improved in the Aged-AP (Figure 3.2A), compatible with an improvement in diastolic function. There were no significant changes in other hemodynamic parameters of diastolic function such as the LV end-diastolic pressure and the maximum rate of decrease in pressure during isovolumetric relaxation (min -dP/dt) among the groups (Figure 2B-C). The mean of arterial pressure (Figure 3.2D) and several LV systolic function parameters were assessed and found to be not statistically different among the groups (Figure S 3.2).

Clearance of Senescent Cells with AP in Different Non-Myocyte Cardiac Cells

The percentage of senescent cells in different non-myocyte cardiac cells (fibroblasts, endothelial cells, and immune cells) was determined by FACS. As INK-ATTAC transgene contains a GFP sequence, senescent cells were identified as GFP positive cells. Young INK-ATTAC mice were used to determine the GFP background signal. FACS data suggests that in all cell populations, the number of senescent cells was increased in aged animals, most significantly in immune cells and fibroblasts (Figure 3.3B-C). We identified two distinct populations in the $CD45^{+}$ cells with different granularity (with high side scatter (SSC) or low SSC value). Comparison of the GFP signal in each population suggests that AP treatment was effective in reducing senescent cells only in the fibroblast population (Figure 3.3D). The FACS experiments were validated with qPCR experiments on fibroblast (discoidin domain receptor tyrosine kinase2 (Ddr2), transcription factor 21(Tcf21)), immune cells (CD45) and endothelial cells (CD31) gene markers in the sorted cells (Figure S 3.4).

The FACS data was further validated by double immunofluorescence staining of p16 and cardiac cell-type selective markers (Figure 3.4). AP treatment significantly reduced the percentage of p16-expressing cells in the vimentin-expressing population (fibroblasts), while the number of

αSMA-positive cells (myofibroblasts) or CD31-positive cells (endothelial cells) expressing p16 were unaffected (Figure 3.4D).

Effect of the Clearance of Senescent Cells on Cardiomyocytes

Due to the large size of the cardiomyocytes, we were unable to measure the GFP signal in that population with FACS. Instead, we double stained p16 and troponin I (TNI, a marker for cardiomyocyte) to determine senescent cardiomyocytes. The percentage of $p16^+$ cardiomyocytes were significantly higher in the Aged-Vehicle group vs the Young group while it was normalized with the AP treatment (Figure 3.5A-B). We also used the cardiomyocytes obtained with the Langendorff perfusion for qPCR of genes of interest. The mRNA level of myosin heavy chain 7 (*Myh7*), a marker of hypertrophy, was significantly upregulated in the Aged-Vehicle group compared to the Young group, and significantly reduced in the aged AP group (Figure 3.5C). Several ion channel mRNA expressions or other markers of hypertrophy were assessed, but no significant changes were identified among the groups (Figure S 3.3).

Effect of the Clearance of Senescent Cells on LV Fibrosis and Conduction Velocity

Our hydroxyproline assay and Masson's Trichrome staining suggest that the total content of collagen increased in the aged mice and was normalized with the AP treatment (Figure 3.6A-B). Profibrotic markers gene expression such as Il-6 and Tgfβ2 were upregulated in the aged mice and was normalized with the AP treatment (Figure 3.6C). LV conduction velocity and action potential duration were not different among different groups (Figure 3.6D-E)

Paracrine Effects of Senescent Cardiac Fibroblasts on Healthy Cardiomyocytes

To induce senescence in primary fibroblasts in our *in vitro* model, we used 200 μM concentration of H_2O_2 (Figure 3.7A). To validate the senescence phenotype in the treated fibroblasts, we

measured gene expression of p16 and SASP markers such as Il-6 and Tgfβ2. Il-6 and p16 gene expression were significantly upregulated in the fibroblasts treated with 200 μM H_2O_2, indicating a senescence phenotype in our fibroblast culture (Figure 3.7B). Next, we cocultured senescent or control fibroblasts with freshly isolated cardiomyocytes using Transwell inserts. After 48 hours of coculture, we found that the gene expression of hypertrophy markers such as Myh6 and Myh7 were significantly upregulated in the cardiomyocytes cocultured with senescent fibroblasts compared to those cocultured with control fibroblasts (Figure 3.7C).

3.5. Discussion

In this study, we investigated the role of cellular senescence in aged-related cardiac remodeling using a mouse model of aging INK-ATTAC. We found evidence for the accumulation of senescent cells in the LV of aged mice accompanied by increased hypertrophy, fibrosis, and prolongation of LV relaxation time. In the INK-ATTAC mouse model, reduction of senescent fibroblasts and cardiomyocytes with AP treatment decreased fibrosis and hypertrophy, as well as normalizing prolonged isovolumic relaxation time. These results point to a direct role of senescent fibroblasts and cardiomyocytes in the age-related cardiac remodeling. Furthermore, the co-culture of senescent fibroblasts with healthy cardiomyocytes resulted in upregulation of hypertrophic markers' gene expression in cardiomyocytes, suggesting potential paracrine effects of senescent fibroblast SASP components on cardiomyocytes.

Cellular Senescence and Age-related Cardiac Remodeling

The risk of cardiac disease such as HF increases with age.[15] An aged heart undergoes several functional, structural, cellular, and molecular changes that may promote HF. Underlying mechanisms of cardiac aging are multifactorial and include, but are not limited to, oxidative stress,

systematic inflammation, cardiomyocyte hypertrophy, telomere shortening and damage, epigenetic alterations, and cellular senescence.[6]

Cellular senescence is described as a hallmark of aging; however, relatively little is known about the role of senescence in age-related cardiac remodeling. Our study provides the novel insight about the accumulation of different senescent cardiac cell types in the LV of aged mice, clearance of which improves hypertrophy, fibrosis, and diastolic dysfunction. Our study provides the novel insight about the accumulation of different senescent cardiac cell types in the LV of aged mice, clearance of which improves hypertrophy, fibrosis, and diastolic dysfunction. These factors contribute to increasing the heart's vulnerability to HF.

Potential Role of Cellular Senescence in Age-related Cardiac Remodeling: Cardiac Hypertrophy, Fibrosis and Diastolic Dysfunction

Cardiac hypertrophy

One potential role of senescence in age-related cardiac remodeling is to induce hypertrophy, which is a significant pathophysiological feature of cardiac aging.[7,16] Anderson et al. showed that ROS and mitochondrial dysfunction in cardiomyocytes lead to telomere-associated DNA damage followed by the induction of senescence and hypertrophy.[9] In the same study, senescent cell clearance in aged mice with navitoclax or in the INK-ATTAC mouse model reduced the size of cardiomyocytes with no changes in LV mass or cardiac function. However, the investigators have not determined which senescent cell types were affected by the treatment in their mouse model.[9] Our study shows that the clearance of senescent cells in INK-ATTAC mice with AP prevents hypertrophy, evident in the reduction of LV mass, LVAWT, and Myh7 gene expression (a hypertrophy marker). Our immunofluorescence experiment showed a significant reduction of senescent cardiomyocytes with AP treatment. The reduction of senescent cardiomyocytes in the

AP group may have a role in the reduction of hypertrophy in that group. On the other hand, among nonmyocyte cardiac cells, in our FACS experiment, we noted that the beneficial effect of AP treatment is uniquely associated with clearance of senescent fibroblasts, suggesting a possible role of senescent fibroblasts in LV hypertrophy which was tested in our *in vitro* model.

Cardiac fibrosis

Another potential role of senescence in cardiac aging might be to induce fibrosis.[7,17,18] This notion is consistent with the reduction in LV fibrosis that we noted in aged INK-ATTAC mice treated with AP and with the associated clearance of senescent fibroblasts. Studies in animal models and in human hearts have confirmed the accumulation of senescent fibroblasts in fibrotic areas of the heart during aging and in association with cardiac disease.[19] However, in these studies, whether senescent fibroblasts have a causal role in the state of cardiac disease is unclear. To our knowledge, ours is the first study describing the causal role of senescent fibroblasts in cardiac fibrosis by showing clearance of senescent fibroblasts reduces fibrosis.

Diastolic dysfunction

Diastolic dysfunction refers to abnormal mechanical properties of the myocardium which represents a combination of characteristics such as impaired LV relaxation and filling.[20]

We investigated the effects of senescent cell clearance in diastolic dysfunction. Our results show that clearance of senescent fibroblasts normalizes prolonged isovolumic relaxation time which is consistent with an improvement in diastolic function. Other hemodynamic and echocardiographic parameters of diastolic function were not significantly different among groups.

Cardiac Cardiomyocyte-Fibroblast Paracrine Communications in Cardiac Hypertrophy

Evidence suggests that different cardiac cell types such as myocytes and nonmyocyte cells, including fibroblasts, endothelial cells, and immune cells, communicate with each other via a variety of autocrine or paracrine mediators and actively participate in the development of cardiac hypertrophy.[21-23]

Cardiac fibroblasts are one of the most prevalent nonmyocyte cells in the heart, accounting for between one fifth and two thirds of cardiac cells.[21] Recent research has pointed to the critical role of fibroblasts in inducing cardiomyocyte hypertrophy through different paracrine secretion factors such as fibroblast growth factor 2 (Fgf-2) , transforming growth factor beta 1(Tgfβ1), and insulin like growth factor 1 (IGF-1), which was demonstrated by several *in vitro* and *in vivo* studies.[22,23] In our study, we show for the first time that the coculture of senescent fibroblasts with healthy cardiomyocytes increases the gene expression of hypertrophic markers in cardiomyocytes, suggesting a direct role of senescent fibroblasts SASP components in cardiomyocyte hypertrophy.

Potential Limitations

The INK-ATTAC mouse model has several considerable advantages: the model is specific, it has minimal off-target effects, and it has been used in several senescence studies.[7,24] However, the model has the weakness of only inducing apoptosis in $p16^{+}$ cells. While p16 is among the most important senescence markers, not all senescent cells express p16. Thus, not all senescent cells are cleared using this system. Moreover, in our FACS experiments, we considered double-negative population fibroblasts, as there is no unique common surface marker for fibroblasts. At the same time, this population might contain smooth muscle cells. Nonetheless, since the quantity of smooth muscle cells in the heart is significantly less than fibroblasts, and qPCR of fibroblasts markers (*Ddr2* and *Tcf21*) showed significantly higher expression in the double-negative population compared to the other populations, we have associated the phenotype with fibroblasts. Finally,

while H_2O_2 is a well-established way of inducing senescence *in vitro*, it does not fully mimic the senescence caused by aging.

3.6. Conclusion

In the present study, we showed the accumulation of different senescent cardiac cells in the LV of aged mice in association with age-related cardiac remodeling. Furthermore, we showed that a the clearance of senescent fibroblasts and cardiomyocytes in aged mice reduces cardiac hypertrophy, fibrosis, and improved the LV isovolumic relaxation time. These findings have important potential implications for understanding the underlying mechanism of cardiac hypertrophy, fibrosis and diastolic dysfunction in the aged heart and development of innovative therapies for age related cardiac disease, particularly HF.

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3.8. Figures

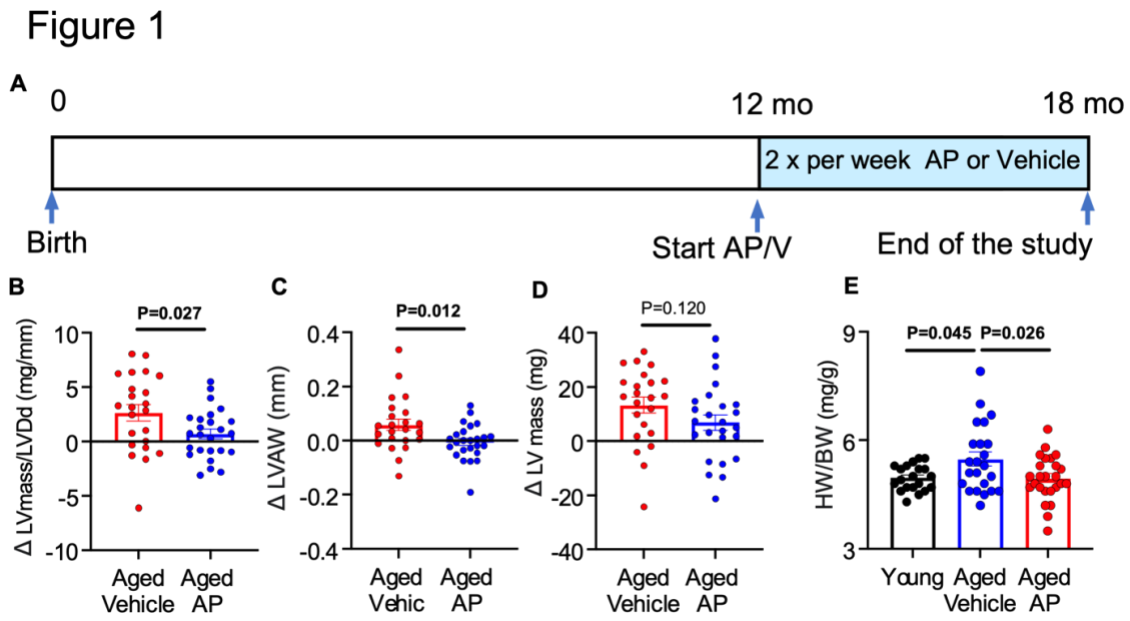

Figure 3.1. Study design, echocardiographic data and normalized heart weight of aged INK-ATTAC mice treated with vehicle of AP.

A) Study design: INK-ATTAC mice were treated from 12 months until 18 months, 2 times a week. The echocardiographic data are reported as changes from 12 to 18 months in each group (Aged-Vehicle, Aged-AP). B) changes of left ventricle (LV) mass/LV end diastole dimension (LVDd). C) changes of LV anterior wall thickness (LVAWT). D) changes of LV mass. E) The excised heart weight (HW) normalized to body weight (BW). Statistical analysis for B, C, D: unpaired t-test, for E: one-way ANOVA followed by Tukey's test, significance level $P<0.05$.

Figure 2

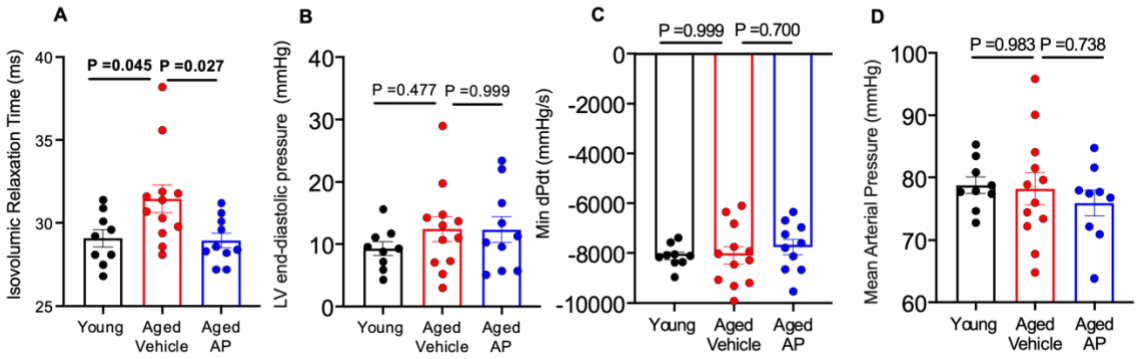

Figure 3.2. Hemodynamic parameters in Young, Aged-Vehicle and Aged-AP INK-ATTAC.

A) Isovolumic relaxation time. B) End diastolic left ventricle pressure (Ped). C) Maximum rate of decrease in pressure during relaxation (Min dpdt). D) Mean arterial pressure. Statistical analysis for all panels: one-way ANOVA followed by Tukey's test, significance level $P<0.05$.

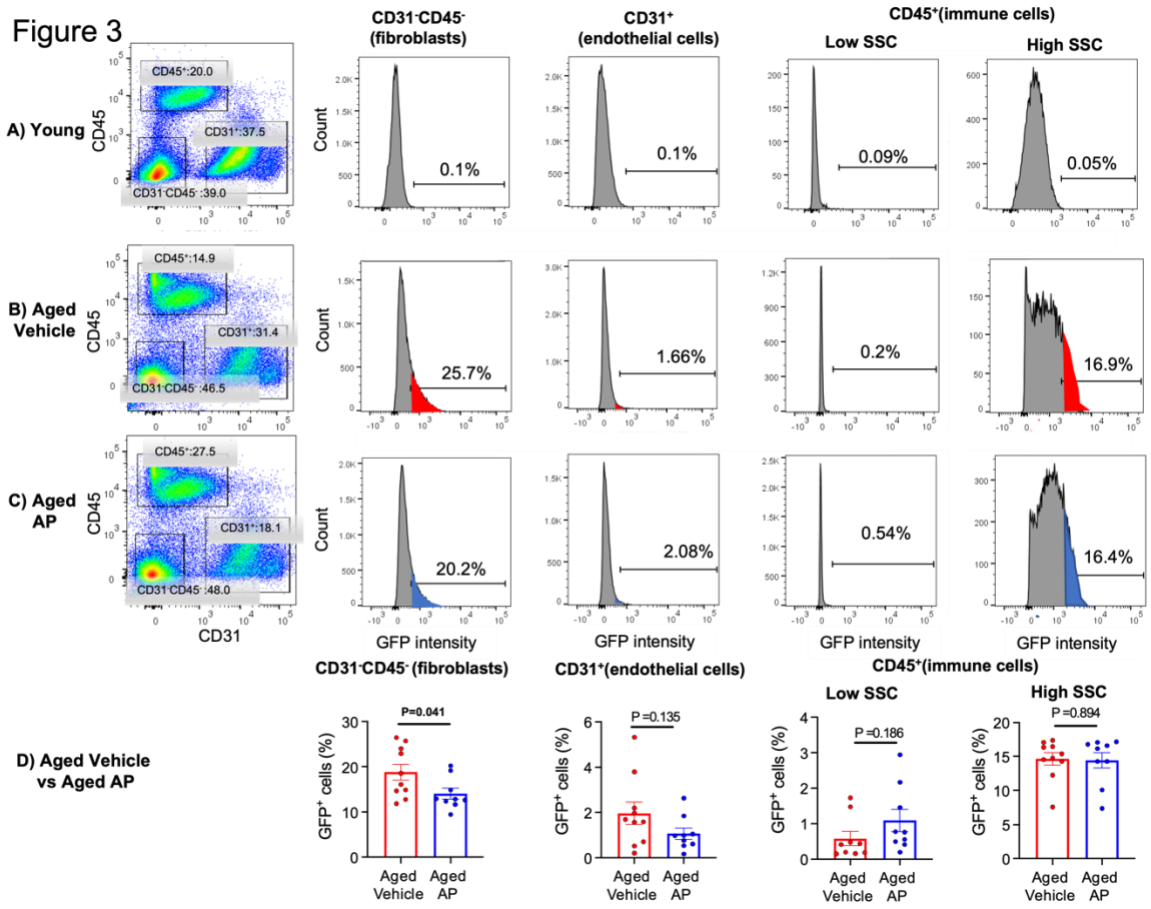

Figure 3.3. Fluorescence-activated cell sorting (FACS) for non-cardiomyocyte cardiac cells in Young, Aged-Vehicle and Aged-AP groups.

A) Young group: left panel diagram showing the distribution $CD31^-$ $CD45^-$, $CD31^+$, $CD45^+$ populations, second, third and fourth panels showing the GFP signal intensity in $CD31^-$ $CD45^-$, CD 31^+, $CD45^+$ population. B) Aged-Vehicle group: left panel diagram showing the distribution $CD31^-$ $CD45^-$, $CD31^+$, $CD45^+$ populations, second, third and fourth panels showing the GFP signal intensity in $CD31^-$ $CD45^-$, $CD31^+$, $CD45^+$ population. C) Aged-AP group: left panel diagram showing the distribution $CD31^-$ $CD45^-$, $CD31^+$, $CD45^+$ populations, second, third and fourth panels showing the GFP signal intensity in $CD31^-$ $CD45^-$, $CD31^+$, $CD45^+$ population. D) Percentage of GFP positive cells (senescent cells) in each cell population of Aged-Vehicle and Aged-AP. Statistical analysis for all panels: unpaired t-test significance level $P<0.05$. SSC: side scatter, indicating granularity of the cells.

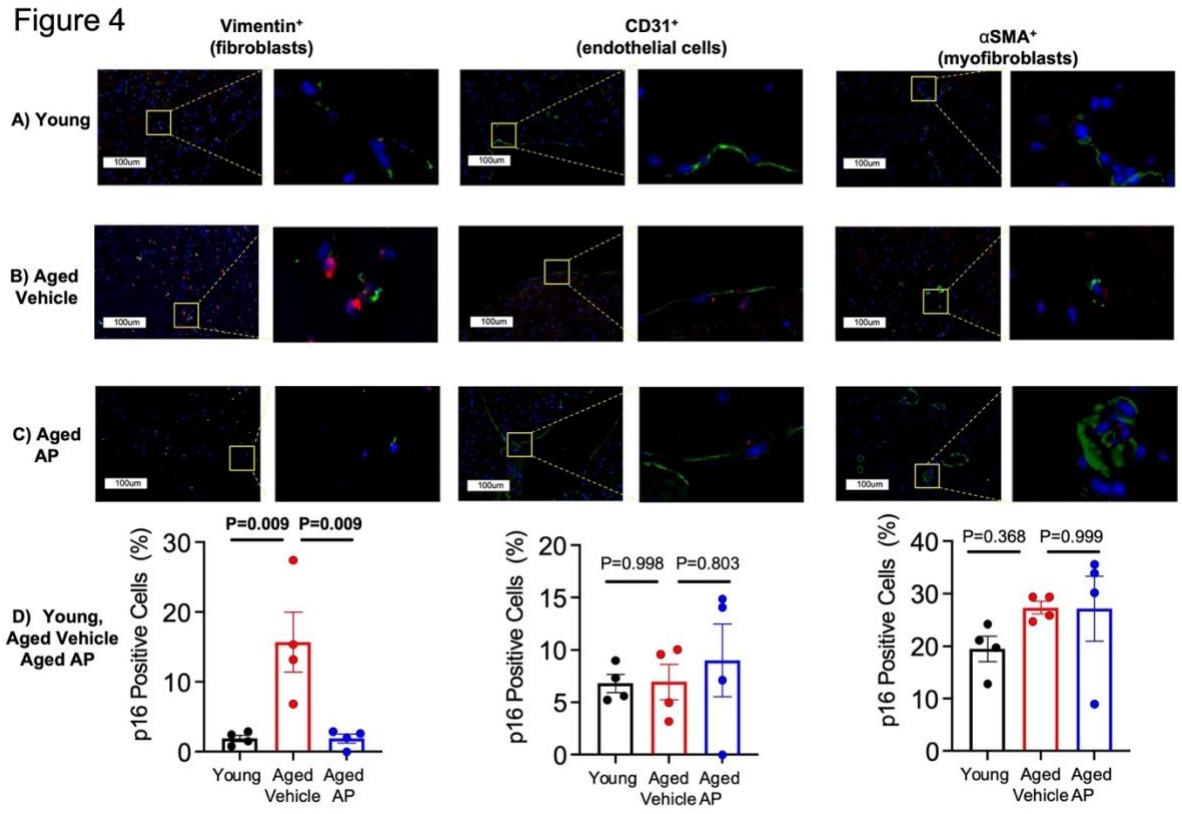

Figure 3.4. Double immunofluorescence for p16 and cardiac cell type markers in left ventricle of Young, Aged Vehicle and Aged AP groups.

A) Young B) Aged-Vehicle C) Aged-AP. In all groups, left panel: double staining of p16 and Vimentin (marker of fibroblasts), middle panel: double staining of p16 and CD31 (marker of endothelial cells), right panel: double staining of p16 and aSMA (marker of myofibroblasts). All panels show cell-marker staining (green), staining for p16 (red) and 4,6-diamino-2-phenylindole (DAPI,blue). Statistical analysis for all panels: one-way ANOVA followed by Tukey's test, significance level $P<0.05$.

Figure 5

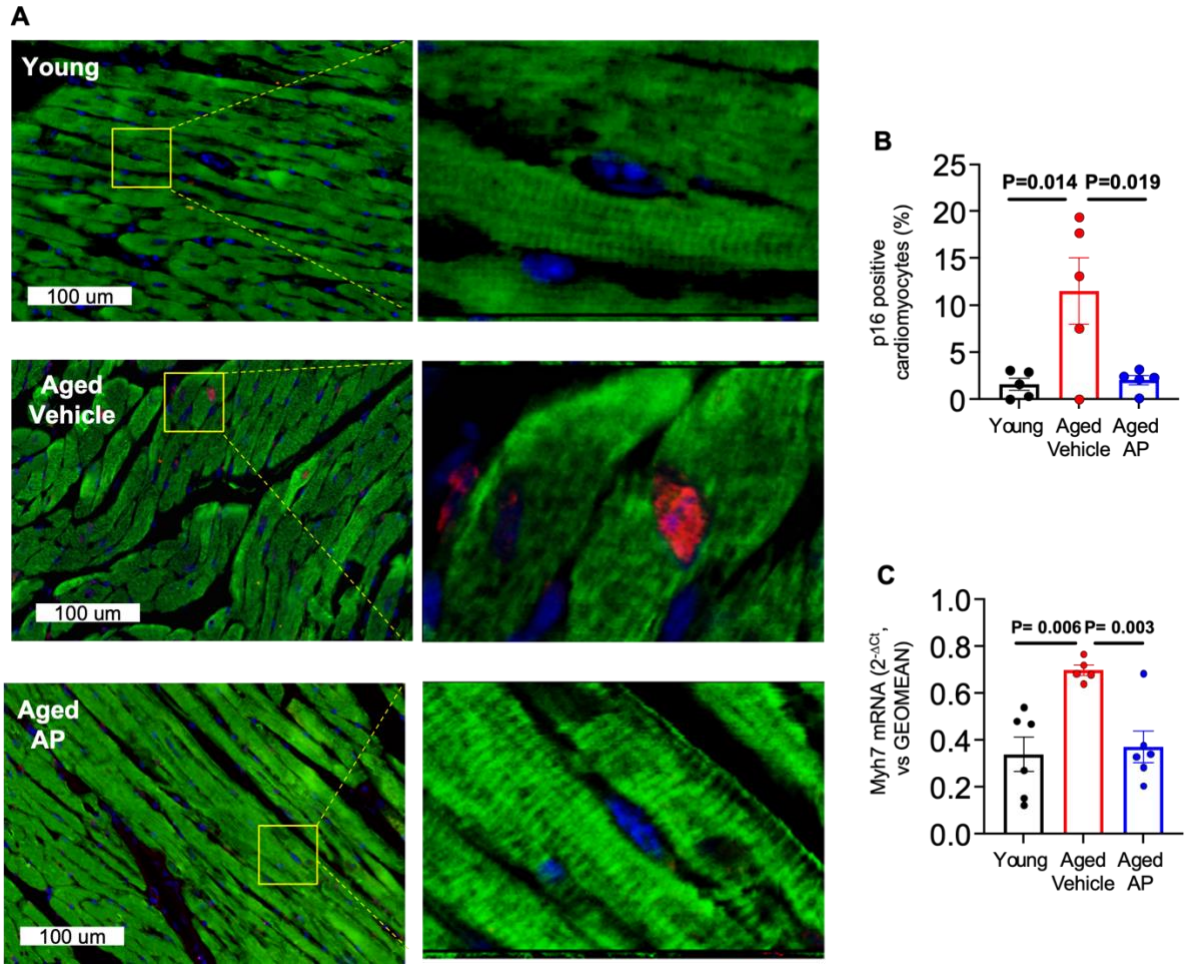

Figure 3.5. Double immunofluorescence for p16 and cardiomyocyte marker, senescence and hypertrophy markers gene expression in Young, Aged-Vehicle and Aged-AP INK-ATTAC groups.

A) p16 (red) and 4,6-diamino-2-phenylindole (DAPI; blue), cardiomyocyte marker, troponin I (green). B) Percentage of p16 positive cardiomyocytes. C) Gene expression of myosin heavy chain 7 (Myh7). Statistical analysis for all panels: one-way ANOVA followed by Tukey's test, significance level $P<0.05$.

Figure 6

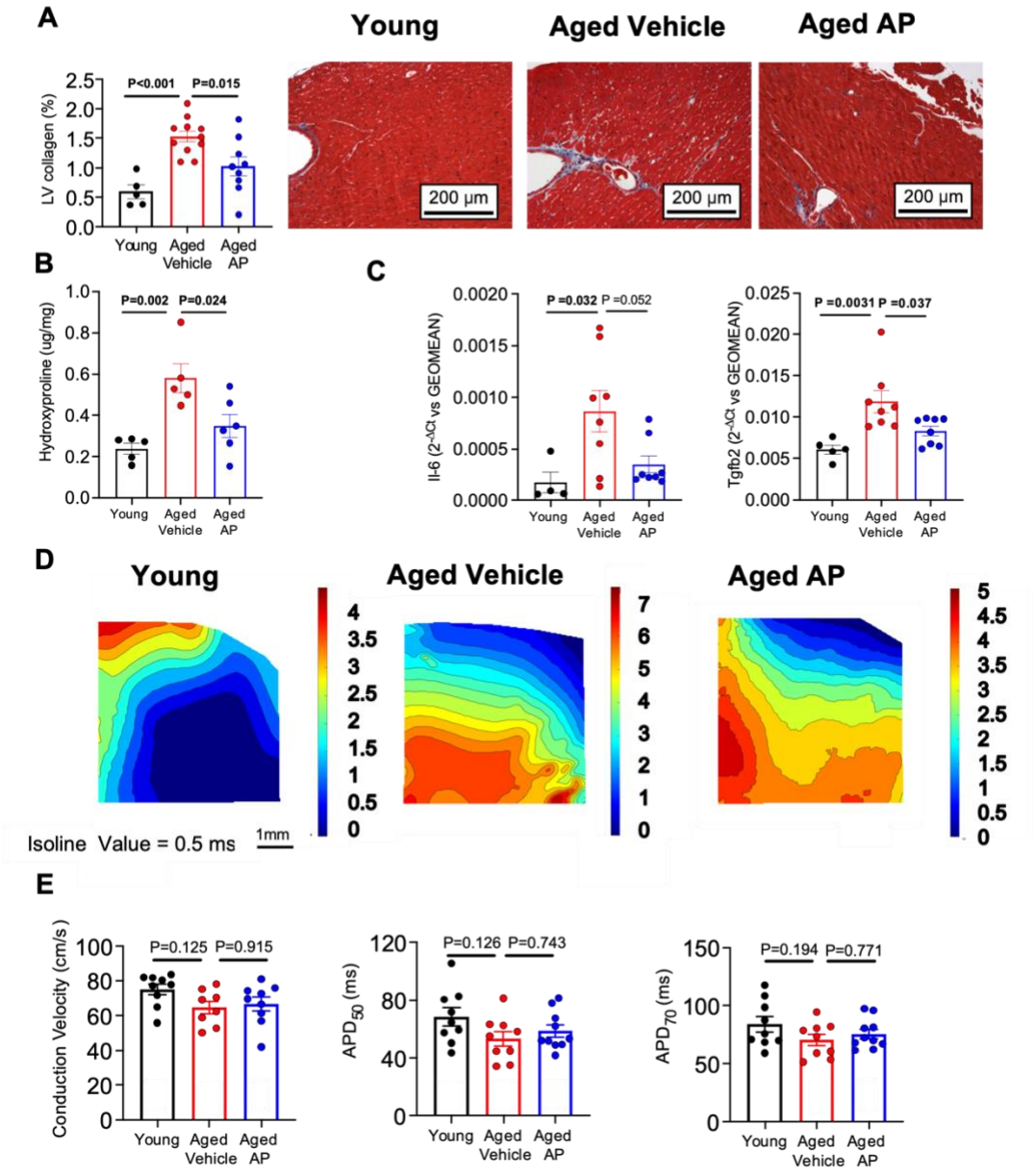

Figure 3.6. Fibrosis quantification, profibrotic markers gene expression and Optical mapping of left ventricle in Young, Aged-Vehicle and Aged-AP groups.

A) Collagen content analyzed with Masson's Trichrome immunostaining. B) Hydroxyproline quantification. C) Profibrotic markers gene expression. D) Activation map. E) Conduction velocity, APD 70, APD 50. Statistical analysis for all panels: one-way ANOVA followed by Tukey's test, significance level $P<0.05$.

Figure 7

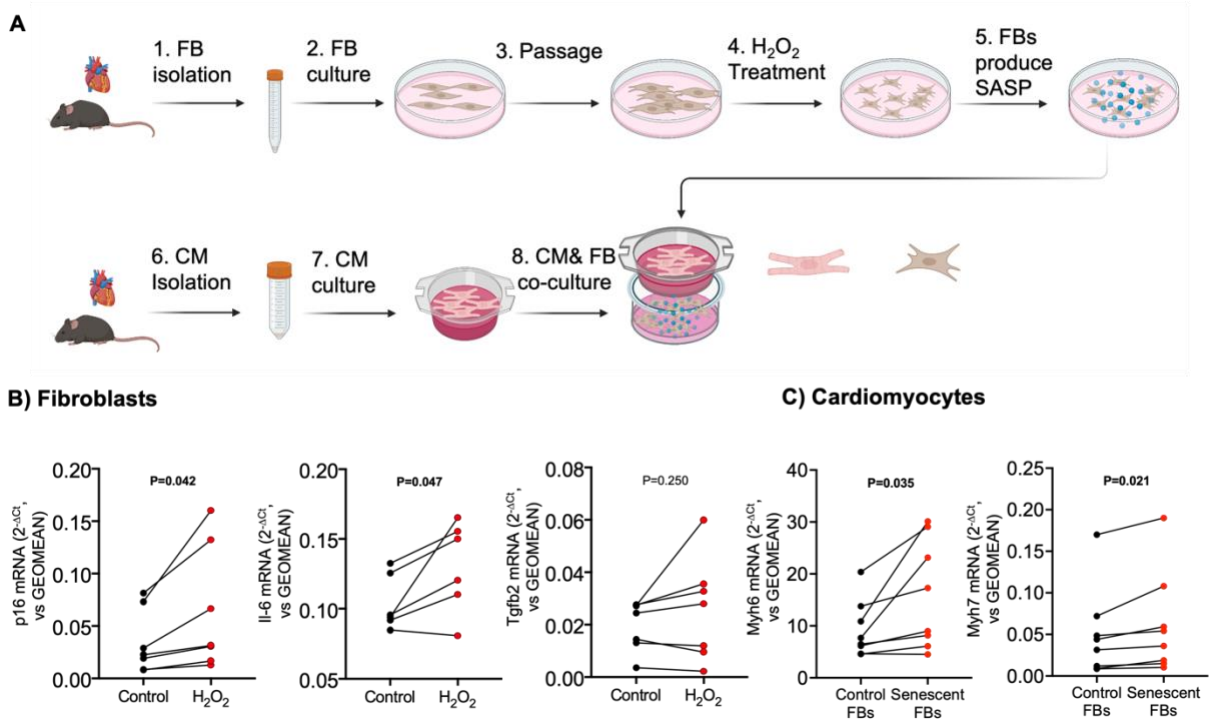

Figure 3.7. In vitro model of cardiac senescent fibroblasts and their co-culture with healthy cardiomyocytes.

A) Study design B) Senescence and SASP markers gene expression in control fibroblasts and those treated with H_2O_2. C) Hypertrophy markers' gene expression in cardiomyocytes co-cultured with control and senescent fibroblasts. Statistical analysis for all panels: unpaired t-test significance level $P<0.05$.

Figure S1

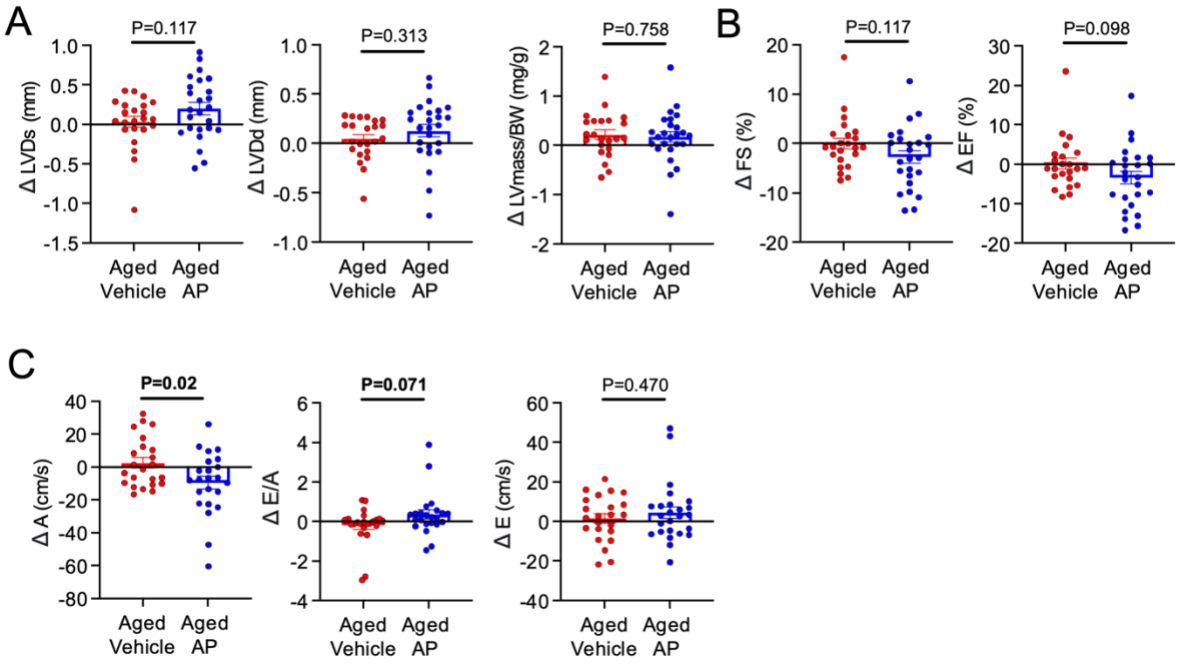

Figure S 3.1. Echocardiographic data.

The echocardiographic data are reported as changes (Δ) from 12 to 18 months in each group (Aged-vehicle, Aged-AP). **A)** Left ventricle (LV) structural parameters: LV dimension at end systole (LVDs); LV dimension at end diastole (LVDd); LV mass/body weight (BW). B) systolic function parameters: fractional shortening % (FS%), ejection fraction % (EF%), cardiac output (CO) **C)** LV diastolic function parameters: trans mitral flow atrial filling (A), trans mitral flow early filling (E) Statistics: Unpaired t-test for parametric or Mann-Whitney test for non-parametric variables.

Figure S2

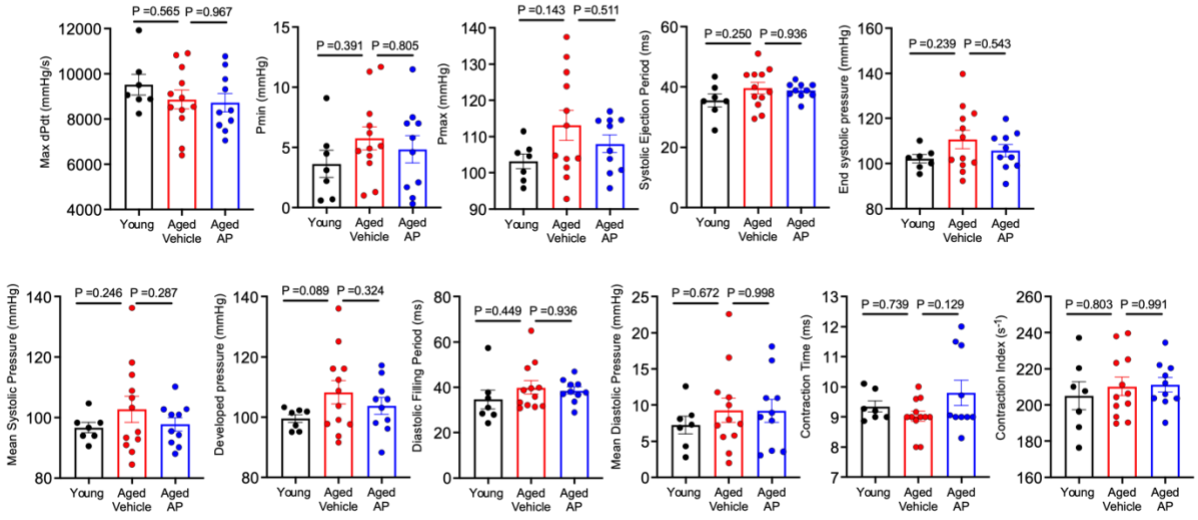

Figure S 3.2. Hemodynamic parameters.

Statistical analysis for all panels: one-way ANOVA followed by Tukey's test, significance level $P<0.05$.

Figure S3

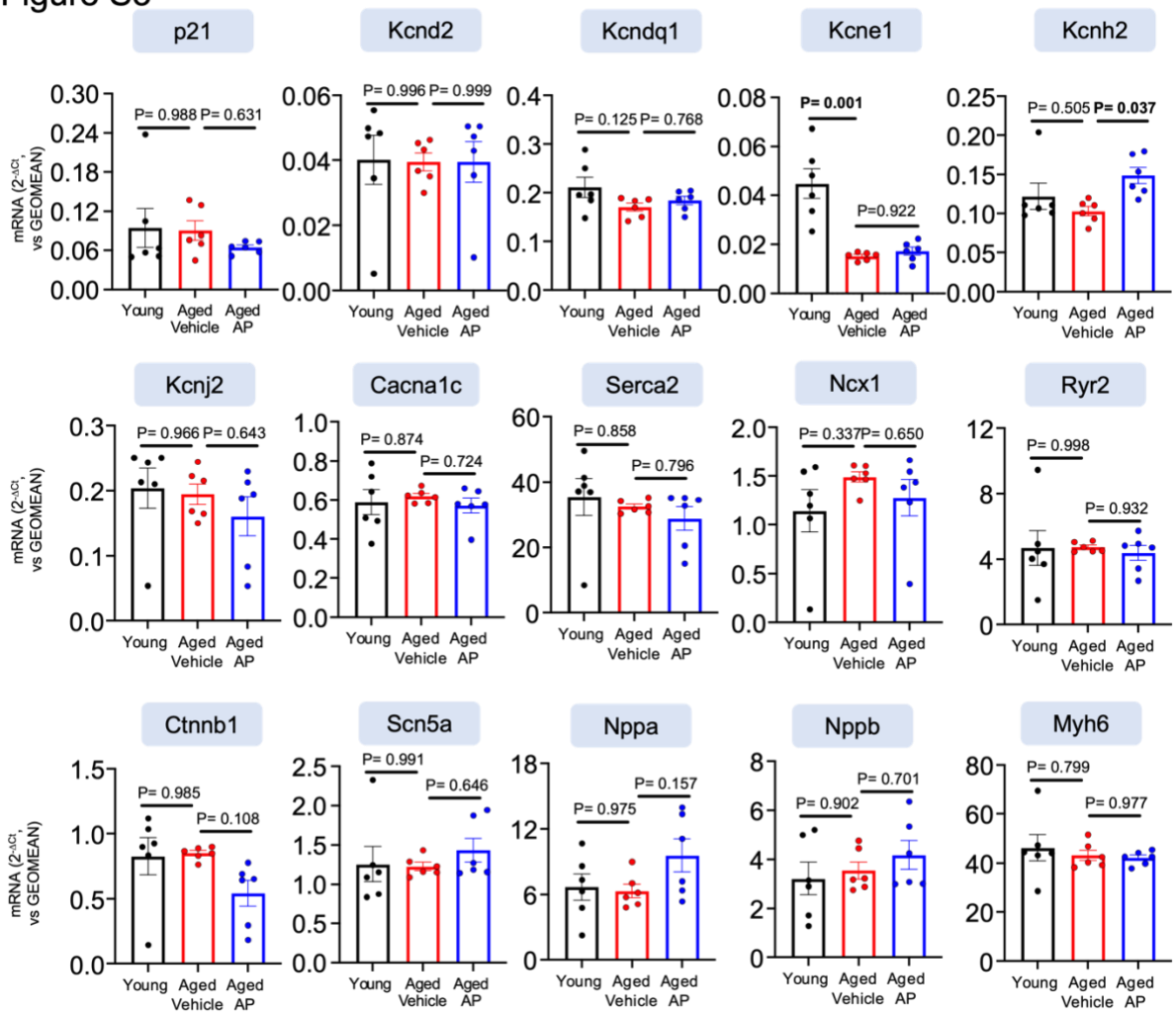

Figure S 3.3.Hypertrophy markers, ion channels and calcium handling proteins gene expression.

Statistical analysis for all panels: one-way ANOVA followed by Tukey's test, significance level P<0.05.

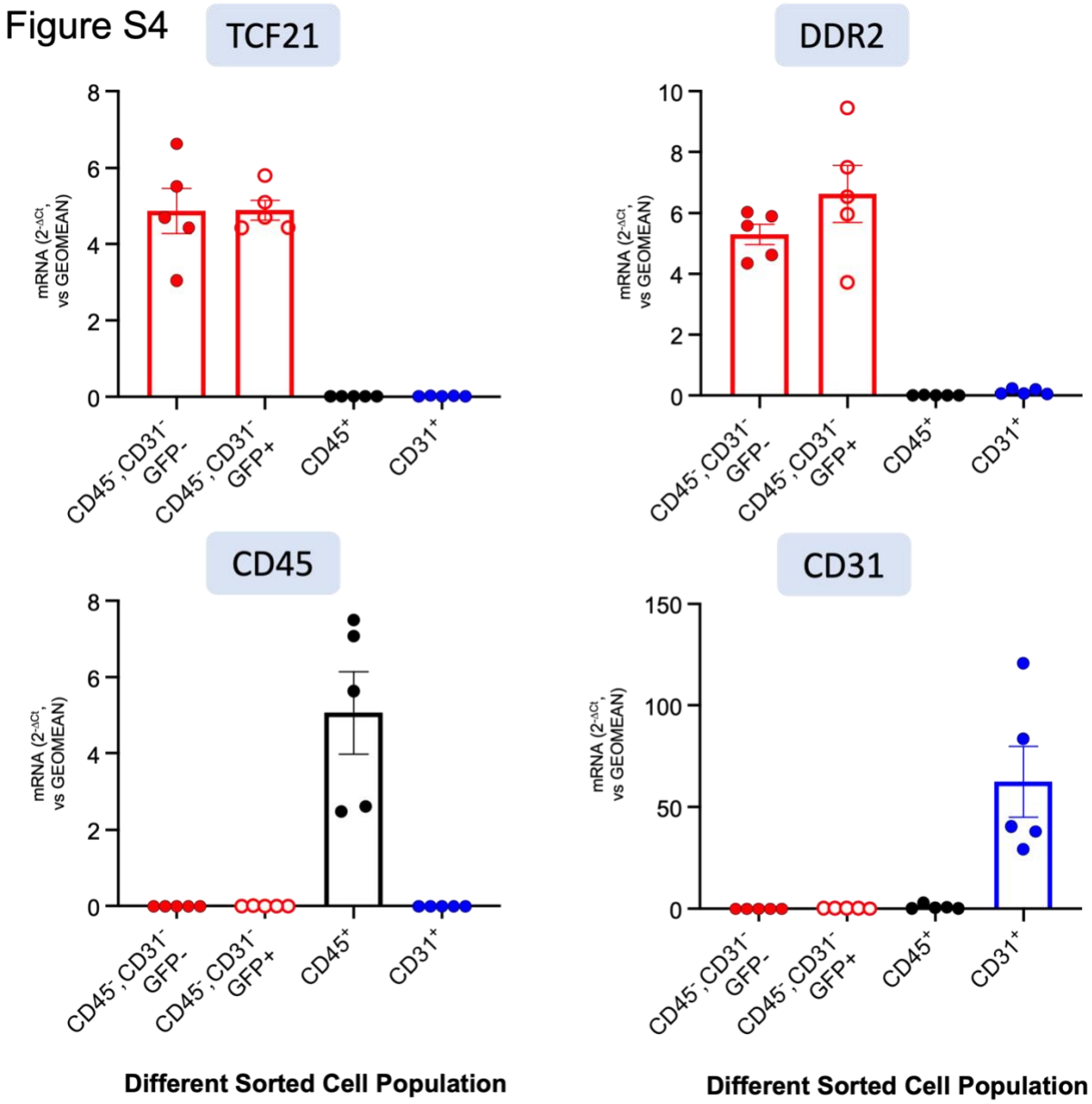

Figure S 3.4. Validation of sorted cell population post FACS with qPCR.

A) marker of fibroblasts (Tcf21 and Ddr2). B) marker of immune cells (CD45). C) marker of endothelial cells (CD31). Ddr2: discoidin domain receptor tyrosine kinase2; Tcf21: transcription factor 21.

Connecting statement: Chapter 3 and 4

In chapter 3, we showed a significant role of cellular senescence in age-related LV remodeling which render the heart more susceptible to HF. This finding motivated us to investigate the role of senescence in another LV disease, MI. The role of cellular senescence in MI is controversial, with conflicting reports of both positive and negative effects, primarily based on indirect evidence. The use of INK-ATTAC mice allowed us to evaluate the direct effects of senescent-cell clearance on LV remodeling post-MI, providing direct insights into causality.

4. Chapter 4. Clearing Senescent Cells Prevents Cardiac Dysfunction and Death in Mice with Acute Myocardial Infarction

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Manuscript in preparation for submission.

4.1. Abstract

Background: Cellular senescence, a stress and age-related response, is often characterized by p16 expression in p16 positive ($p16^{+}$) senescent cells. Senescent cells secrete profibrotic and proinflammatory factors as parts of a "senescence associated secretory phenotype". The role of cellular senescence in cardiac remodeling post myocardial infarction (MI) is unclear.

Objectives: To study the effect of senescent cell clearance on surviva andcardiac remodeling post-MI. To identify potential pathways affected by MI and senescent-cell clearance in MI-tissue using single nucleus RNA-sequencing (snRNA-seq) technology.

Methods: We used INK ATTAC transgenic mice, in which $p16^{+}$ cells undergo targeted clearance upon exposure to a dimerizer, AP20187 (AP). MI mice were treated with AP or vehicle (v), twice a week for 1 month, beginning 3-4 hours post-MI. Cardiac function and structure were evaluated with echocardiography, hemodynamic parameters with a Millar catheter, infarct area with Masson's Trichrome, gene expression with qPCR and single-nucleus RNA-sequencing (snRNAseq).

Results: Survival post MI was dramatically increased in MI-AP vs MI-V mice ($P<0.001$), primarily via prevention of cardiac rupture. Left ventricular (LV) ejection fraction (LVEF) and LV anterior wall thickness at end diastole (LVAWd) decreased in MI-V vs Sham and improved in MI-AP mice, indicating preservation of cardiac function and structure with $p16^{+}$ cell clearance. Hemodynamic indices of LV function (max and min dP/dt) improved with AP. Fibrosis-related gene expression (*Col1a1, Col3a1 and Lox*) increased with MI and improved with AP, paralleling changes in infarct size. Our snRNA-seq data show the clearance of $p16^{+}$-cells affects the

inflammatory response by reducing the number of myeloid cells and point to the significant gene expression differences between the vehicle and AP group in a specific subset of macrophages, (arginase-1) Arg1_macrophages.

Conclusion: These findings indicate a substantial involvement of senescent cardiac cells in the cardiac remodeling post MI, likely through the modulation of inflammatory signaling. This highlights the significance of targeting cellular senescence as a potential avenue for treatment and prevention of MI.

4.2. Introduction

Myocardial infarction (MI) is one of the most common causes of heart failure (HF), hospitalization, and death worldwide.[1,2] Current strategies for acute management of MI are mainly based on reperfusion therapy and hemodynamic support in case of advanced acute HF.[3] Reperfusion is not fully effective for all patients- some present too late for reperfusion; others do not present in the acute phase and reperfusion is not attempted.[1] Post-MI outcomes are strongly influenced by the cardiac remodeling processes.[4,5] Post-MI cardiac repair occurs through a complex process.[5,6]

Recent research indicates that acquired cellular senescence plays an important role in the pathophysiology of several cardiovascular pathologies including atherosclerosis, cardiac hypertrophy, anticancer induced cardiotoxicity, and heart failure.[6] Cellular senescence is an age and stress related response regulated through two main pathways: $p16^{INK4a}$ (p16) –retinoblastoma protein and p53–p21.[7] Furthermore, the main proteins in the senescence pathways, like p16, p21, and p53, are commonly used as markers of senescence.[8] Senescent cells are often resistant to apoptosis and secrete proinflammatory cytokines, growth factors and matrix remodeling proteases, manifesting a "senescence-associated secretory phenotype" (SASP).[7] SASP components were shown to be involved in several biological processes associated with maladaptive cardiac remodeling such as induction of cardiac fibrosis, hypertrophy and inflammation as well as attenuated regeneration.[9,10]

Although a potential role of cellular senescence in MI-related remodeling has been suggested by previous studies [6,10-13], the precise impact of senescence at the cardiac cellular level as well as its involvement in pathways associated with MI remains unestablished. In the present study, we hypothesize that cellular senescence has a direct role during MI-related cardiac remodeling. More

specifically, we assessed the impact of senescent-cell clearance using the INK-ATTAC (p16Ink4a apoptosis through targeted activation of caspase) mouse model, in which $p16^+$ cells undergo apoptosis following the administration of a dimerizing agent, AP20817.[8] Our specific goals were: (1) To evaluate the effect of the clearance of $p16^+$ cells on survival, hemodynamic parameters, cardiac function and structure of MI-mice at 3 and 28 days post-MI, (2) To study the effect of $p16^+$-cell clearance on SASP markers, senescence-indicators; and (3) To identify potential pathways affected by MI and senescent-cell clearance in MI-tissue using single nucleus RNA-sequencing (snRNA-seq) technology.

The results point to an important role of acquired cellular senescence in MI healing and outcome, potentially mediated *via* a specific macrophage subpopulation.

4.3. Methods

Animal Models

All experimental and animal-handling procedures were approved by the Animal Ethics Committee of the Montreal Heart Institute and were conducted in accordance with the Canadian Council on Animal Care and National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. INK-ATTAC mice were bred at the Montreal Heart Institute. To induce MI, 3-month-old INK-ATTAC mice were injected subcutaneously (sc) with buprenorphine (0.05 mg/kg) and anesthetized with 2% isoflurane. The left anterior descending (LAD) coronary artery was ligated *via* left thoracotomy, sham-mice underwent the same procedure without LAD-ligation. Both in MI and sham mice, buprenorphine (0.5 mg/kg, sc) was given at 6h and 24 h and ketoprofen (20 mg/kg, sc) at 6h post-operatively.

Animal Treatment

INK-ATTAC mice were randomly allocated (in a blind fashion) to the following groups: (A) To study the late remodeling phase post-MI (end of the study: 28 days post-MI): 1) Sham-AP (with AP treatment), 2) Sham-Vehicle (with vehicle treatment), 3) MI-AP, and 4) MI-Vehicle. (B) To study early inflammatory phase post-MI (end of the study: 3 days post-MI): 1) Sham-AP, 2) Sham-Vehicle, 3) MI-AP, and 4) MI-Vehicle. AP or vehicle treatment for all groups began 3-4 hours post-MI/Sham surgery. The mice received these treatments by intraperitoneal injection two times a week for until 28- or 3-days post-MI (Figure 4.1A and Figure 4.4A).

Drugs and Chemicals

AP20187 (635069) was obtained from Takara Bio (Shiga, Japan). Chromium Next GEM Chip G Single Cell Kit, 16 rxns PN-1000127 and Chromium Next GEM Single Cell 3′ Kit v3.1, 4 rxns PN-1000269 from 10x Genomics (California, USA). Nuclei Extraction Buffer (130-128-024) from Miltenyi Biotec (North Rhine-Westphalia, Germany). Protector RNase Inhibitor (3335402001) from Sigma Aldrich (Missouri, USA).

Echocardiography

Echocardiographic studies were performed as described previously.[14] Mice were anesthetized with 2% isoflurane and kept on a heated platform to maintain the body temperature around 37°C. The cardiac function and structure were assessed by a transthoracic echocardiography using an i13L probe (10-14 MHz) and Vivid 7 Dimension ultrasound system (GE Healthcare Ultrasound, Horten, Norway). Left ventricle (LV) anterior thickness at the end of diastole (LVAWd), LV dimension at end cardiac diastole (LVDd) and systole (LVDs) were measured by M-mode echocardiography (M-mode). LV fractional shortening (FS%) and ejection fraction (EF%) were obtained by formula available within the Vivid 7 system. LV trans mitral filling velocities in early (E) and atrial (A)

filling were measured with pulsed wave Doppler (PW). LV mass was calculated using a previously described formula.[15] The average of 3 consecutive cardiac cycles was used for each measurement. Throughout the experiments, the operator was blinded to group/treatment assignment.

Histology

Formalin-fixed, paraffin-embedded samples were cut at 6-µm thickness. Image Pro Premier 9.3 Software (Media Cybernetics) was used to quantify fibrosis on Masson's Trichrome-stained images. All analyses were performed blind to mouse-group identity.

Hemodynamic Study

The mice were anesthetized with 2% isoflurane on a temperature-regulated operating table to maintain the body temperature around 37°C. Through the cervical incision, the right carotid was isolated. With a surgical microscope (Leitz Wild M650, Wild Surgical Microscopes, Heerbrugg, Switzerland) a Millar catheter (SPR 671, TX, USA) was inserted and advanced into the ascending aorta, subsequently into the LV. At each step during insertion and advancement into the aorta and the LV, transmitted pressure and surface ECG were recorded and analyzed with iox2 software (v.2.8.0.13, EMKA Technologies, Paris, France). Throughout the experiments, the operator was blinded to group/treatment assignment.

qPCR

Total RNA was extracted using the RNeasy Mini Kit. RT-PCR was performed with Applied Biosystems Thermal Cycler Step One Plus (ThermoFisher Scientific, Watham, MA). SYBR green primers were used for p16 (Fwd: CCCAACGCCCCGAACT, Rev: GCAGAAGAGCTGCTACGTGAA) p21 (Fwd: GGCAGACCAGCCTGACAGAT, Interleukin-6 (Il-6) (Fwd: TCCGGAGAGGAGACTTCACA, Rev: TGCAAGTGCATCATCGTTGT), transforming growth factor beta-2 (Tgfβ2) (Fwd: TGCCTTCGCCCTCTTTACATT, Rev:

AGCGGAAGCTTCGGGATTTA), collagen 1a1 (*Col1a1*) (Fwd: TGGATACGCGGACTCTGTTG, Rev: GGCCCTTTCGTACTGATCCC), collagen 3a1 (*Col3a1*) (Fwd: AGCTTTGTGCAAAGTGGAACC, Rev: GGTGGCTGCATCCCAATTCA) , α-smooth muscle actin (*αSMA*) (Fwd: AAGACAGCTATGTGGGGGATG, Rev: GGCCACACGAAGCTCGTTA) , tumour necrosis factor alpha (*Tnfα*)(Fwd: ATAGCAAATCGGCTGACGGT, Rev: AGCCGATGGGTTGTACCTTG), matrix metalloproteinase 9 (Mmp9) (Fwd: CCCTGGAACTCACACGACAT, Rev: TGGTTCACCTCATGGTCCAC) , Mmp13 (Fwd: TTGGCCACTCCCTAGGTCTG, Rev: GTTGGGGTCTTCATCGCCTG) lysyl oxidase (Lox) (Fwd: GCTGCGGAAGAAAACTGCCTGGC, Rev: TCCGATGTCCCTTGGTTCTTCACTCTT), interleukin 1-β (Il-1β) (Fwd: GCTGAAAGCTCTCCACCTCAA, Rev: GGGTGTGCCGTCTTTCATTAC) . All mRNA values were normalized to the geometric mean of Beta-2-Microglobulin (*B2m)*, hypoxanthine phosphoribosyltransferase 1 (Hprt1), and Glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

Single nucleus RNA sequencing

Since AP treated mice and non-treated mice presented a marked difference in survival rate at 3 days post-MI, we conducted snRNA-seq using tissue samples collected from the peri-infarct and infarct areas at this timepoint. Our experimental groups included in 3 mice treated with AP, 3 with vehicle and 2 shams.

Sample preparation and sequencing

To isolate nuclei, we used a modified version of the gentleMACS™ nuclei extraction protocol (https://www.miltenyibiotec.com/upload/assets/IM0027287.PDF). All steps were performed on ice or maintained at 4°C. For each sample, we loaded approximately 25mg of tissue with 1.5ml of nuclei lysis

buffer (0.5U/μL Protector RNase inhibitor) in the gentleMACS™ C tubes and lunched the 4C_nuclei_1 program on the gentleMACS™ Octo Dissociator. Once the run was completed, we rinsed the tubes with an additional 1ml of lysis buffer, passed the solution in a 40 μm filter (Miltenyi catalogue no. 130-101-812) sequentially, and performed a first round of centrifugation at 500g and 4°C for 5 min in a swing bucket centrifuge. We then removed the supernatant, added 1ml of suspension buffer (PBS, 2% BSA, 0.5U/μL Protector RNase inhibitor), waited 5 min for buffer exchange, re-suspended the nuclei with an additional 1mL of suspension buffer, passed the solution through a 20μm filter and performed a second centrifugation at 500g and 4°C for 5 min. Finally, we re-suspended the nuclei in 200μl suspension buffer, quantified them on a Countess® II FL, adjusted concentrations in an optimal range for 10,000 cells yield per sample and proceeded to loading the chip on the Chromium controller and downstream steps from the manufacturer protocol. We sequenced libraries at genome Quebec on a Novaseq 6000 S4 PE100 with a targeted 62,500 paired-reads per cells.

Alignment and pre-processing

We aligned FASTQ to Cellranger's X reference (https://support.10xgenomics.com/single-cell-multiome-atac-gex/software/downloads/latest) using the **count** function of cellranger-X-X.0.0 for each sample. For downstream analyses, we used Seurat v4.[16] We removed low quality cells using the following thresholds: > 500 counts and <10% mitochondrial reads. We used SCTransform for normalization, regressing out the percentage of mitochondrial gene counts and integrated the data using Harmony[17] on 30 PCs. We then annotated cells by scoring them with the AddModuleScore function using the Azymuth heart detailed cell-type marker genes list *celltype.l2*.

We removed doublets using both scDblFinder[18] scores and manual sub-clustering curation. We first calculated a doublet score using scDblFinder and removed cells labelled as doublets by the algorithm. We then sub-clustered each cell-type and removed sub-clusters that showed an

enrichment of the scDblFinder scores and the top marker gene of another cell-type (labeled sub-cell-type doublets). Finally, we re-clustered the filtered dataset as described above. To infer cell-cycle stages, we used the tricycle package.[19]

Pseudobulk differential expression

We first aggregated gene counts by sample using Seurat's function AggregateExpression(). Then, we used DESeq2[20] Wald test to contrast the AP vs Vehicle MI groups and log2 fold change shrinkage 'apeglm' method. We categorized differentially expressed genes (DEG) with a fasle discovery rate (FDR) < 0.05 and an absolute log2 fold change > 0.5. For all pre-ranked gene set enrichment analyses (GSEA), we used the fgsea package.[21] Gene sets were downloaded from https://maayanlab.cloud/Enrichr/ to the exception of the curated library. We assessed gene sets enrichments in MI-AP vs MI-Vehicle using the signed log10(FDR) of their pseudobulk DEG, signed based on the value of the log2 fold change with a positive value associated with a higher expression in the MI-AP condition. When multiple gene sets had an FDR < 0.1, we report the top 5 gene sets with the lowest FDR per library and condition.

Cell-type and cell-state proportion tests

To test for composition changes between groups, we used the sccomp package (https://github.com/stemangiola/sccomp) sccomp_glm() function with an FDR of 0.05.

Cell-type and cell-state DEG attribution

To attribute DEG to a specific cell-type or cell-state, we calculated gene expression specificity using the presto package (https://github.com/immunogenomics/presto). We then attributed DEGs to a cell-type or cell-state based on the strongest area under the curve (AUC) after removing results with AUC < 0.55.

Sub-clustering analyses

To remove unwanted sources of variations, we removed ribosomal sub-unit genes, mitochondrial encoded genes and long non-coding RNAs. For cardiomyocytes, we sub-clustered cells as stated above using 10 PCs without regression on mitochondrial gene percentage. For immune cells, we sub-clustered cells as stated above using 20 PCs. To investigate the different cell-states and infer their function, we conducted GSEA on sub-cluster marker genes with an FDR < 0.01, ranked based on AUC-0.5.

MI snRNA-seq time course dataset integration

We downloaded the Calcagno et al. dataset GSE214611 from GEO.[22] We kept nuclei present in the zenodo metadata file (https://zenodo.org/record/7055957/files/Nikatag/Single-Cell-Spatial-Transcriptomics-for-Border-zone-BZ_Cell_Mapping.zip?download=1) without further threshold filtering. We used the same clustering steps used for our data. We removed doublets called by scDblFinder. Then, we integrated the filtered nuclei with our MI INK-ATTAC dataset using the same clustering parameters. We used this integrated map for the immune (Myeloid, T-cells and B-cells) sub-clustering analysis.

For the integrated pseudobulk PCA, we first aggregated gene counts per samples and transformed counts using variance stabilizing transformation from DESeq2. Then, we used the top 500 most variable genes in the GSE214611 dataset to compute PCs on all samples from both dataset.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 (San Diego, CA) and R v 4.2.2. For normally distributed data (assessed by Shapiro-Wilk test), we used Student's t-test (for 2-group only comparisons), 1-way ANOVA (followed by Tukey test), 2-way ANOVA (followed by post-hoc analysis using a Bonferroni-corrected t-test). For non-normal data, Kruskal–Wallis or Mann–

Whitney tests were used. Categorical variables (like AF inducibility) were analyzed with Fisher's exact test. Results are expressed as mean±SEM and 2-tailed $P<0.05$ was considered statistically significant.

4.4. Results

Expression of senescence-related markers following MI and the impact of $p16^+$ cell clearance on survival rate, cardiac function, and structure 28 days post-MI

At 28 days, all selected senescence markers (p16, p21, and p53) were upregulated in the infarct-zone of MI-Vehicle vs Sham groups (Figure 4.1B). We noted a statistically significant decrease in infarct-zone p16 in the AP group, indicating that AP effectively cleared $p16^+$ cells in the Infarct-zone (Figure 4.1B); p21 and p53 were not significantly altered in the AP group. Survival rate post-MI was significantly improved in the AP group compared to the vehicle group (Figure 4.1C). Death appeared to be due to cardiac rupture in many cases, observed in 40% of dead mice in the AP group and 68% in the vehicle group.

Consistently, echocardiography revealed an attenuation of LVAWT thinning in the MI-AP group (Figure 4.2A), and infarct-size was reduced (Figure 4.3A). The LVDs and LVDd were significantly greater in the MI vs Sham groups, but no statistically significant changes were observed between MI-Vehicle and MI-AP (Figure 4.2B-C). LVFS% and LVEF% were significantly reduced in MI-vehicle vs Sham while AP-treatment significantly improved both vs MI-Vehicle (Figure 4.2D-E).

Hemodynamic study showed significant impairment in a wide range of indices post-MI, including max dPdt, contraction index, contraction time, and min dPdt with significant

improvements in max dPdt, contraction index, and min dPdt in AP-treated mice vs Vehicle (Figure 4.2F-I). The mean arterial pressure was not statistically different among the groups (Figure 4.2J). *Col1a1, Col3a1, Lox, αSMA,* and *Tgfβ2* mRNA-expression in infarct-zone were strongly upregulated in MI vs Sham (Figure 4.3B). AP treatment significantly reduced the expression of *Col1a1, Col3a1,* and *Lox* vs Vehicle, indicating that $p16^{+}$-cell clearance altered the myocardial fibrotic process following MI (Figure 4.3B). These data suggest a significant improvement in MI-size, LV systolic function, fibrosis and outcome resulting from $p16^{+}$-cell clearance after MI.

Effect of $p16^{+}$-cell clearance on cardiac function and SASP markers 3 days post-MI

We noted a marked improvement in post-MI survival with AP-treatment, with the survival curves diverging after 3 days and 2 doses of AP. We therefore focused on the 3-day time point with the goal of obtaining insights into the underlying mechanisms (Figure 4.4A). We noted that the senescence markers p16 and p53 were significantly upregulated in MI-Vehicle vs Sham in the infarct area, whereas p21 was not significantly changed (Figure 4.4B-D); AP-exposure did not attenuate these changes. Echocardiography revealed a larger LVAWT in the MI-AP vs MI-Vehicle group (Figure 4.4E), consistent with thicker infarct wall in the AP group (Figure 4.5A). The LVDs and LVDd were significantly higher in the MI vs Sham groups, however, no significant changes were observed between MI-Vehicle and MI-AP (Figure 4.4F-G). Systolic function parameters such as LVFS% and LVEF% did not significantly differ among groups (Figure 4.4H-I). Similarly, hemodynamic parameters did not differ among groups (Figure 4 S.1). These data suggest that the difference in survival rate observed in the early inflammatory phase was not related to early hemodynamic improvement.

We then proceeded to analyze proinflammatory SASP markers: *Il-6, Tnfα* and *Il-1β* were strongly upregulated in MI group vs Sham in both infarct and remote zones. AP treatment

significantly reduced *Il-6, Tnfα* and *Il-1β* gene expression in remote-zone, as well as Il-6 in the infarct-zone (Figure 4.5B-C). Profibrotic SASP markers, *Col 1a1, Col3a1, Mmp9, Mmp13,* and *Lox* were strongly upregulated in MI group vs Sham in both infarct and remote zones (Figure 4.6A-B). AP treatment significantly reduced the expression of *Col1a1, Col3a1* and *Lox* genes in both infarct and remote zones, as well as *Mmp9* and *Mmp13* in the remote zone (Figure 4.6A-B). Thus, senescent cell clearance induced early changes in MI-related inflammation and fibrotic process.

SnRNA-seq on infarct-zone 3 days post-MI

Given the significant improvement in survival rate after MI in the early inflammatory phase through the clearance of $p16^{+}$-cells and considering that early changes in cell-subsets can influence myocardial remodeling, we sought to analyze the properties of specific cellular populations in the infarct-zone affected by removing $p16^{+}$-cells using snRNA-seq.

Principal component analysis (PCA) of the aggregated counts (pseudobulk) per sample, showed distinct clustering among groups, with the most prominent separation occurring between the sham and MI groups on PC1, (~90% of the variance explained) (Figure 4.7A). Figure 4.7B shows proportions of cell-types for each sample.

We then sought to quantify contrast gene expression between AP and vehicle treated animals. We found a total of 1027 differentially expressed genes (DEG), with 766 upregulated (FDR < 0.05 and log2 fold change > 0.5) and 261 downregulated (FDR < 0.05 and log2 fold change < -0.5) with AP treatment (Figure 4.7C). Upregulated genes in the MI-AP group were enriched for cardiomyocytes (CMs) and fatty acid metabolism-related pathways whereas the most DEG in the MI-Vehicle group were enriched for macrophages, neutrophils, and inflammatory response pathways (Figure 4.7D). Furthermore, the most upregulated genes in the MI-AP group were specific to CMs, while the most downregulated genes were specific to myeloid cells (Figure 4.7E-

F). MI-AP mice had a cell-type profile closer to the sham animals (Figure 4.7A, B, E, Figure 4S.2A).

We then sought to characterize the cell states that distinguished the AP treated animals. CM subsets were first characterized based on a sub-cluster analysis. Four CM subsets were identified. Most sham CMs were found in sub-cluster 0, reflecting healthy-CMs (Figure 4.8A). Subclusters 1 and 2 were reported within the infarct border zone[22] (Figure 4.8A). In addition to a larger amount of CMs (Figure S2B), AP-treated mice had a greater proportion of healthy-CMs (Figure 4.8B). Finally, we explored whether DEG were enriched in these CM states. We found that the vast majority of DEG were enriched in the healthy-CM state (Figure 4.8C). These results suggest that $p16^{+}$-cell clearance improves the survival of healthy-CMs in the infarct-zone.

Since pseudobulk analysis revealed enrichment in inflammation-related genes in Vehicle-MI mice, we sought to characterize immune-cell states following MI. We therefore performed sub-clustering analysis of the immune-cell cluster (Figure 4.8D). By far the largest number of DEG for AP vs vehicle were expressed in the (arginase-1) Arg1_macrophage sub-cluster (Figure 4.8E). Figure 4.8F and Figure 4.8G show a comparison between the percentage of Arg1-positive macrophages and Arg1 expression level in our study vs a time-course analysis based on previously-published snRNAseq data.[22] In the published data-base, both the percentage of Arg1_ macrophages and Arg1 expression-level in the Infarct-zone increased post-MI, with the largest number of Arg1_macrophages at day-1. In our day-3 data, the Infarct-zone Arg1 expression-level of Arg1_macrophages in MI-Vehicle mice was comparable to the published level at days 1 and 3, while the number of Arg1_macrophages was more comparable to day-1. The percentage of Arg1_macrophages decreased in the AP group; however, this shift did not reach statistical

significance after correction (FDR=0.059). We noted significant enrichment in the hypoxia and cardiac rupture pathways in Arg1_macrophages (Figure 4.8H).

Finally, we performed an analysis focused on quantification of $p16^+$ cells across samples. We found relatively low proportions (~1%) of $p16^+$ cells in all samples with no detectable difference between AP or vehicle treated MI groups (Figure 4 S.2 C-D).

4.5. Discussion

In this study, we examined the role of cellular senescence in the cardiac remodeling post-MI, using the INK-ATTAC mouse model. We found that MI induces a marked increase in senescence markers in the infarct-zone, accompanying by high mortality, cardiac disfunction and large infarct area. The clearance of $p16^+$-cells, using the INK-ATTAC model, improves survival rate and cardiac function, reduces infarct size, and alters inflammatory response post-MI. Our snRNA-seq data from the infarct-zone 3 days post-MI suggests that $p16^+$-cell clearance reduces the inflammatory response post-MI. Our data point to a significant role of cellular senescence in the cardiac remodeling post-MI and cardiac rupture by altering the composition of myeloid cells, specifically Arg1_ macrophages.

Cellular senescence and MI

MI is associated with DNA damage and over-production of ROS in the in the infarct-zone.[23,24] The DNA damage response and ROS products potentially cause cell senescence with the upregulation of senescence markers such as p16.[7] Some evidence from animal models suggests that cellular senescence plays an important role in the infarcted heart.[6] Aged mice showed a significant upregulation of senescence markers post-MI within the infarct area.[25,26] Clearance of senescent cells using senolytic agent navitoclax or combination of dasatinib and quercetin (senolytic agents) improved cardiac function post-MI.[25,26] These observations are consistent with

the cardiac function improvement following p16^{+}-cell clearance that we noted in our INK-ATTAC model. We have, for the first time to our knowledge, shown that the removal of senescent cells leads to an improvement in hemodynamic parameters and post-MI survival rate. Some research has reported a positive role of cell senescence in MI through inducing cell cycle arrest in fibroblasts or the secretion of certain anti-fibrotic factors.[27,28] However, these studies showed that the prevention of senescence led to detrimental effects, while in our INK-ATTAC model, apoptosis was induced in senescent cells once senescence is achieved. While senescence may initially reduce fibrosis in the short term after MI by reducing the fibroblast proliferation, the accumulation of senescent cells may contribute to chronic inflammation, leading to cardiac fibrosis [6].

Potential role of cellular senescence in cardiac remodeling post-MI

The process of cardiac repair following MI is initiated by an inflammation response and the infiltration of immune cells.[5,29] Multiple studies have demonstrated that excessive inflammatory responses can result in cardiac rupture, impaired healing, increased cardiomyocyte loss, systolic dysfunction, and ultimately raise the risk of HF.[5,30,31] Senescent cells can potentially contribute to this inflammation response by producing proinflammatory SASP components.[9,32] This potential contribution is supported by our qPCR data showing the clearance of p16^{+}-cells reduces proinflammatory SASP markers such as *Il-6, Tnfα*, and *Il-1β* in the infarct area. Our pseudo-bulk analysis from the snRNA-seq dataset shows the clearance of p16^{+}-cells results in a reduction in several proinflammatory SASP markers such as chemokine (C-X-C motif) ligand 2 (*Cxcl2*) and C-C motif chemokine ligand 24 (*Ccl24*). This clearance is accompanied by a decrease in genes involved in inflammatory response, cardiac rupture and senescence pathways.

Current literature shows the cardiac myeloid cell composition changes in response to MI.[33,34] Among myeloid cells, macrophages are known to be the primary cell type involved in regulation of healing post-MI.[33] Classically, macrophages were classified as inflammatory (M1) and alternatively activated (M2) macrophage types based on results of in vitro experiments. M1 macrophages are distinguished by their production of elevated pro-inflammatory cytokines. On the other hand, M2 macrophages are identified by their participation in tissue remodeling, immune regulation, and effective phagocytosis. However, recent research indicates that the composition of macrophages in vivo is more complex and cannot be simplified by the traditional M1 and M2 classifications.[33] Considering the significance of macrophages in the pathology of myocardial infarction (MI), it is crucial to study them in more detail. With snRNA-seq, we identified 4 subclusters of macrophages. Our data show the clearance of $p16^{+}$-cells reduces the number of myeloid cells and point to the significant gene expression differences between the vehicle and AP group in a specific subset of macrophages, Arg1_macrophages. Our findings align with recent insights into the complexity of macrophage subtypes, emphasizing the necessity for additional research to better comprehend the phenotype of Arg-1 macrophages in MI. Additionally, investigating how senescent cell clearance impacts the various subpopulations of macrophages and understanding the contributions of these effects on different subclasses of macrophages to the resulting cardioprotection is essential for a comprehensive understanding of the role of senescence in MI pathogenesis.

Reduction of proinflammatory response has been suggested as a therapeutic target to reduce extension of cardiomyocyte injury flowing ischemia.[35] The analysis on CMs population in our snRNA-seq dataset suggests the clearance of $p16^{+}$ cells leads to a greater quantity of healthy -

CMs. This observation could be linked to the changes we noted in the proinflammatory response following the $p16^{+}$-cell clearance.

Senescence may also contribute to scar formation through profibrotic SASP components [6]; this potential role is consistent with the reduction of infarct size and profibrotic factors that we noted in the AP treatment group 28 days post-MI. However, the role of senescence in cardiac fibrosis is complex and both beneficial and detrimental effects were reported.[6]

Limitations

Although the INK-ATTAC mouse model offers significant advantages including specificity in clearance of senescent cells and the absence of observed off-target effects as seen with senolytics, the model has the limitation of inducing apoptosis solely in $p16^{+}$ cells. While p16 is a crucial marker for senescence, it is not expressed by all senescent cells. Consequently, this system fails to clear all senescent cells present. It would be important to study the senescence in other mouse models such as those that $p21^{+}$ cells are cleared [36] to fully understand the role of senescence in MI.

In our snRNA-seq experiment, we detected only few numbers of $p16^{+}$ cells mostly in endothelial cells and fibroblasts. Also, we did not observe any significant differences in $p16^{+}$ cells between MI-Vehicle vs MI-AP. One possible explanation for this finding is that some $p16^{+}$ cells may have been lost during the nuclei isolation process. Another plausible explanation is based on previous reports indicating that the majority of p16 mRNA is localized in the cytoplasm of brain cells, which suggests a similar pattern could exist in cardiac cells.[37] snRNAseq does not capture cytoplasmic RNA, potentially missing certain cytoplasmic transcripts that may be important. We chose snRNA-seq over single-cell RNA-seq because it enables us to capture data from CMs, which are large in size, making them unsuitable for single-cell RNA-seq methods. Moreover, the isolation of individual nuclei doesn't allow for detailed information regarding their precise location within

tissues, limiting the effectiveness of snRNA-seq in interpreting gene expression within specific physiological or pathological microenvironments. Addressing this challenge, in 2016, Stahl et al. introduced spatial transcriptomics technologies. These methods capture mRNA while retaining location data within tissues, utilizing unique location barcodes.[38]

Although findings from snRNA-seq study highlight the relative importance of immune cells shift resulting from $p16^{+}$ cells clearance, the underlying mechanism remains unclear; since INK-ATTAC model is not cardiac-selective, the effect might be due to extracardiac $p16^{+}$-cell clearance.

4.6. Conclusion

In the present study, we obtained evidence for the accumulation of senescent cells in the infarct-zone of MI mice. Furthermore, we found that the clearance of $p16^{+}$ cells improves survival rate, cardiac function, and structure potentially due to altered inflammatory response. These findings have important potential implications for understanding the pathophysiology of MI and for the development of innovative therapeutic approaches.

4.7. References

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4.8. Figures

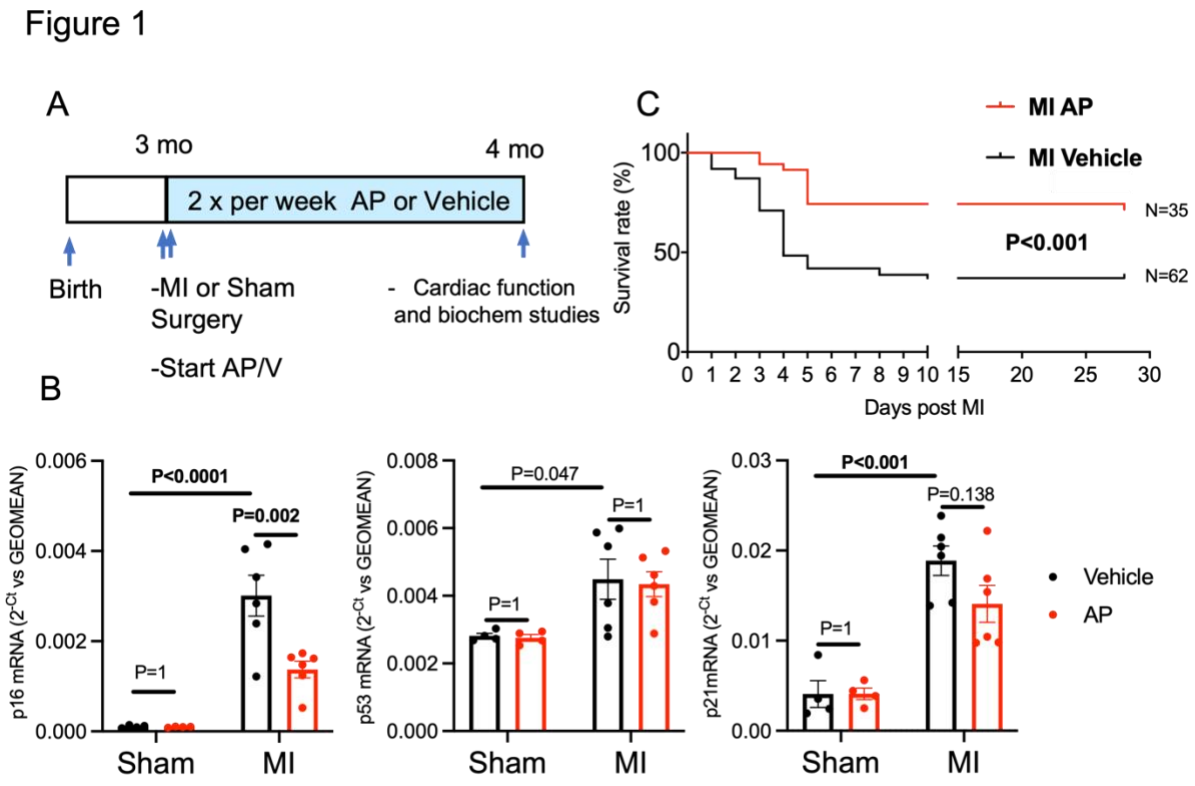

Figure 4.1. Study design, gene expression of senescence markers and survival rate 28 days post-MI.

A) Study design: 3 months old INK-ATTAC mice were treated 2 times a week with AP or Vehicle for 28 days. B) Senescence markers, p16, p21, p53 gene expression. Statistical analysis: Two-way ANOVA followed by Bonferroni, significance level $P<0.05$. C) Survival rate reported as days post-MI. Statistical analysis: Log-rank (Mantel-Cox) test.

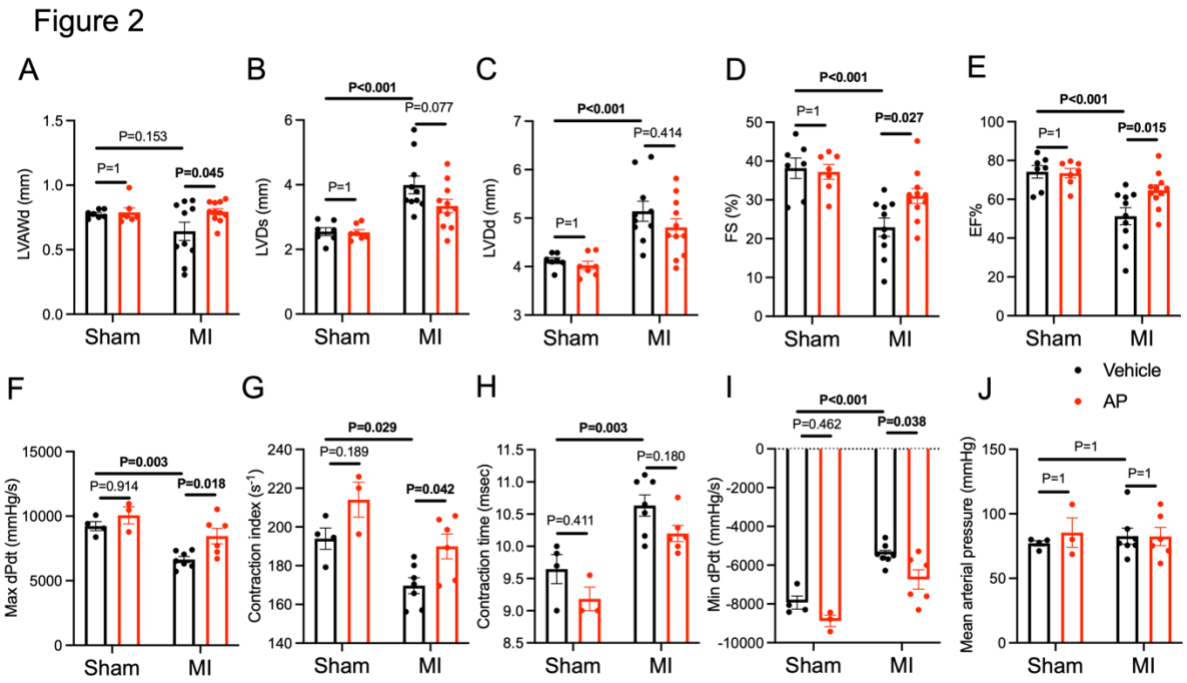

Figure 4.2. Echocardiographic parameters and hemodynamic parameters 28 days post-MI.

A) Left ventricle anterior wall thickness at end diastole (LVAWd).B) Left ventricle dimension in systole (LVDs).C) Left ventricle dimension in diastole (LVDd).D) Fractional shortening (FS). E) Ejection fraction (EF).F) Maximum rate of increase in pressure during contraction (Max dpdt). G) Maximum rate of decrease in pressure during relaxation (Min dpdt). H) Contraction index. I) Contraction time. J) Mean arterial pressure. Statistical analysis: Two-way ANOVA followed by Bonferroni, significance level $P<0.05$.

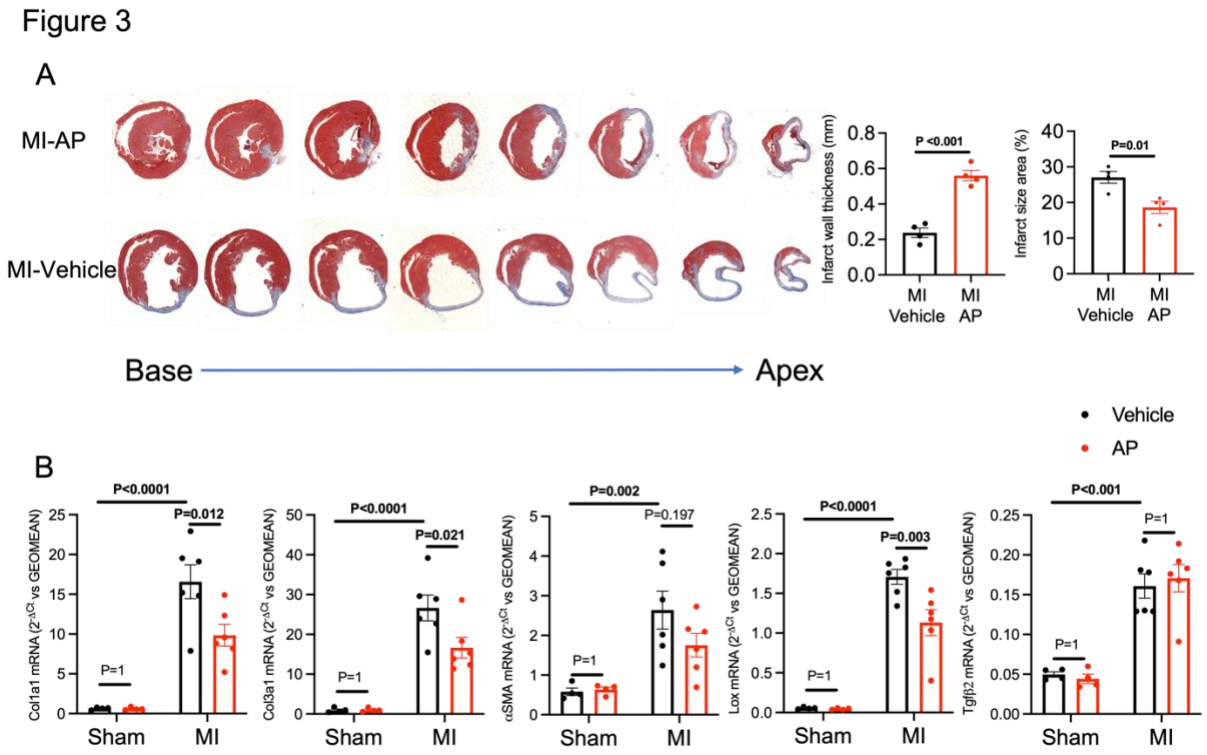

Figure 4.3. Infarct size and fibrosis related gene expression in the infarct-zone 28 days post-MI.

A) Serial section from ligature to apex, Masson's Trichrome staining. B) Quantification of infarct size and thickness. Statistical analysis: Unpaired t-test, significance level $P<0.05$. C) Fibrosis related gene expression in the infarct-zone. Statistical analysis for all panels: Two-way ANOVA followed by Bonferroni, significance level $P<0.05$.

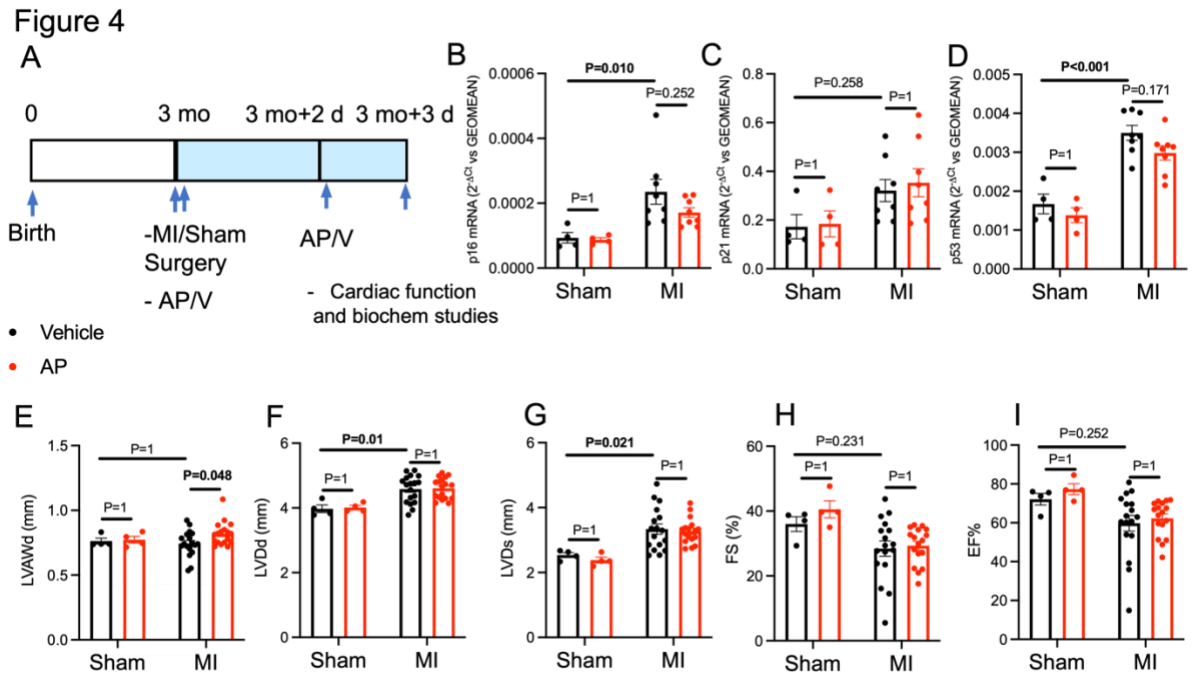

Figure 4.4. Study design, gene expression of senescence markers and echocardiographic parameters 3 days post-MI.

A) Study design: 3 months old INK-ATTAC mice were treated 2 times with AP or Vehicle, end of study was 3 days after surgery. Gene expression data: B) p16 C) p21D) p53. Echocardiographic data: E) left ventricle anterior wall thickness in diastole (LVAWd).F) Left ventricle dimension in diastole (LVDd). G) Left ventricle dimension in systole (LVDs). H) Fractional shortening (FS). I) Ejection fraction (EF). Statistical analysis: Two-way ANOVA followed by Bonferroni, significance level $P<0.05$.

Figure 5

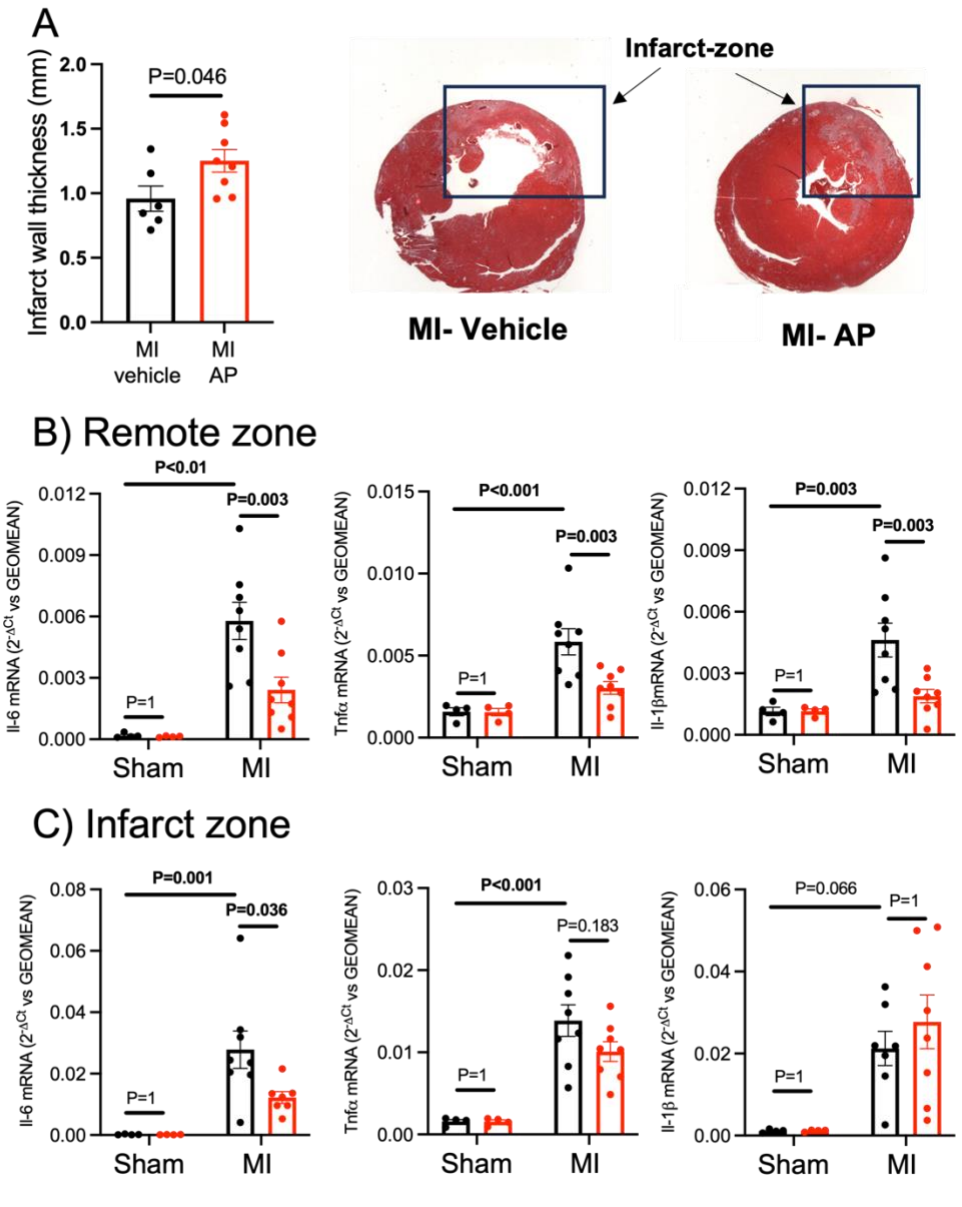

Figure 4.5. Infarct size and gene expression of inflammatory markers 3days post-MI. A) Masson's Trichrome staining and quantification of infarct thickness.

Gene expression in B)Remote zone. C) Infarct-zone. Statistical analysis: Two-way ANOVA followed by Bonferroni, significance level P<0.05.

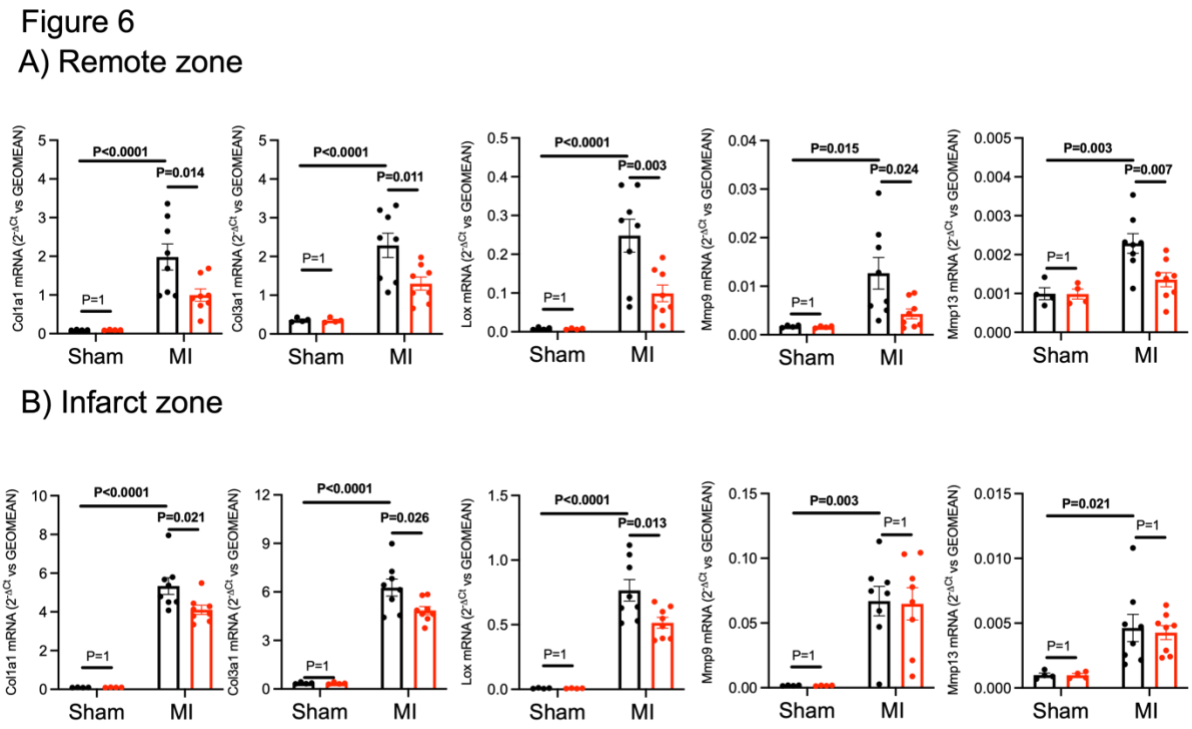

Figure 4.6. Gene expression of fibrosis markers 3 days post-MI.

A) Remote zone. B) Infarct-zone. Statistical analysis: Two-way ANOVA followed by Bonferroni, significance level $P<0.05$.

Figure 7

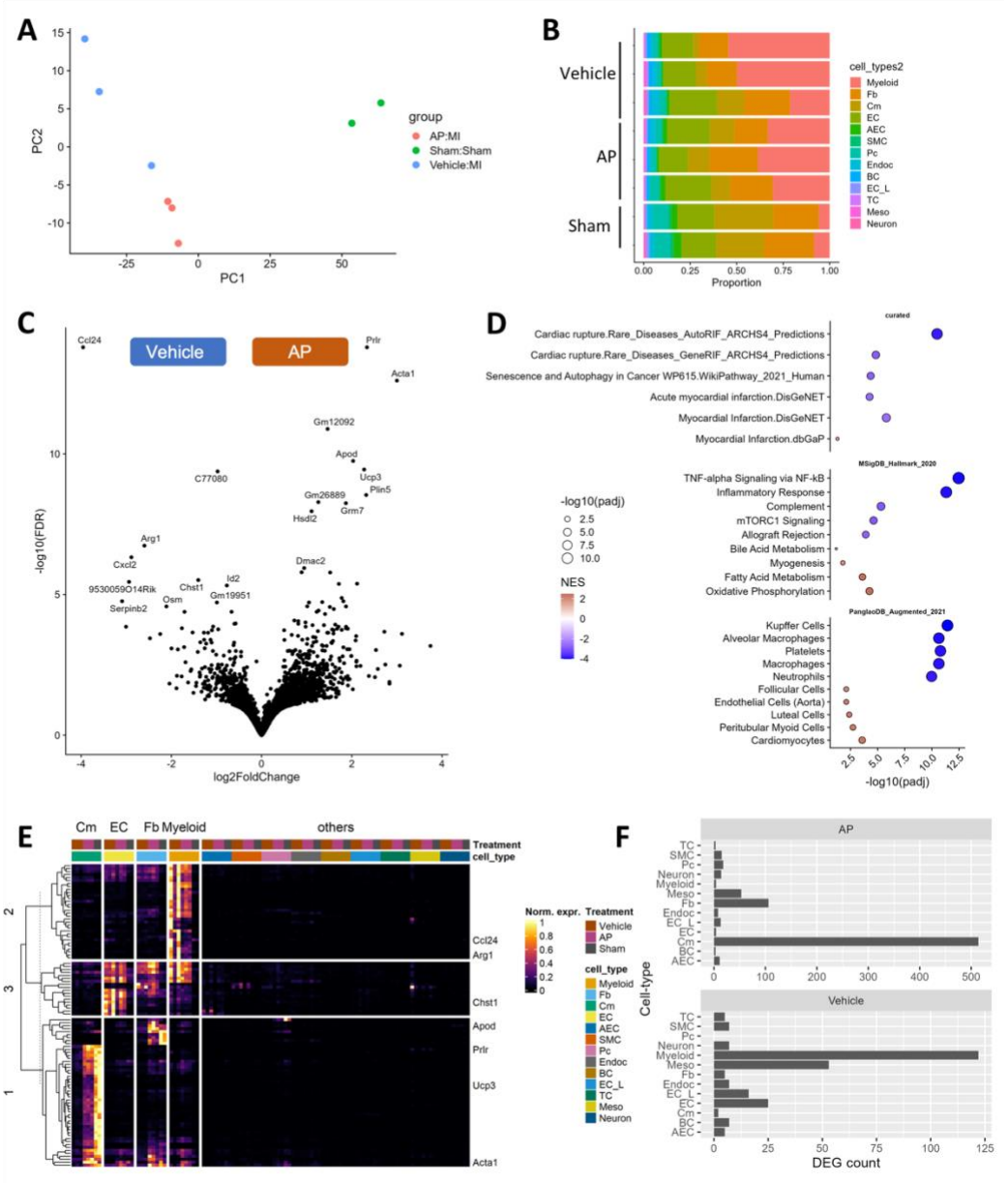

Figure 4.7. Single nucleus RNA sequencing data.

A) Principal component analysis of pseudobulk samples (aggreging cells counts by sample, methods) using the 500 genes with the highest variance. B) Bar plot showing the proportions of cell-types for each samples. C) Volcano plot of the pseudobulk differential expression analysis between MI-Vehicle vs MI-AP showing genes DESeq2 false discovery rates (FDR) and log2 fold change. Positive log2 fold change show increased expression of the gene in the MI-AP group. D) Pre-ranked gene set enrichment analysis of the results in C). Genes were ranked based on signed

log10(FDR) using the sign of the log2 fold change. A positive normalized enrichment score (NES) indicates an enrichment in the MI-AP group. E) Heatmap of pseudobulk sample-cell-types (aggreging cells counts by sample and cell-type, methods) for the top 50 upregulated and top 50 downregulated genes with cell-type specificity (methods). F) Bar plot of the number of differentially expressed genes (FDR< 0.05 and |log2 fold change| > 0.5) upregulated in MI-AP or MI-Vehicle classified by cell-type based on specificity of expression.

Figure 8

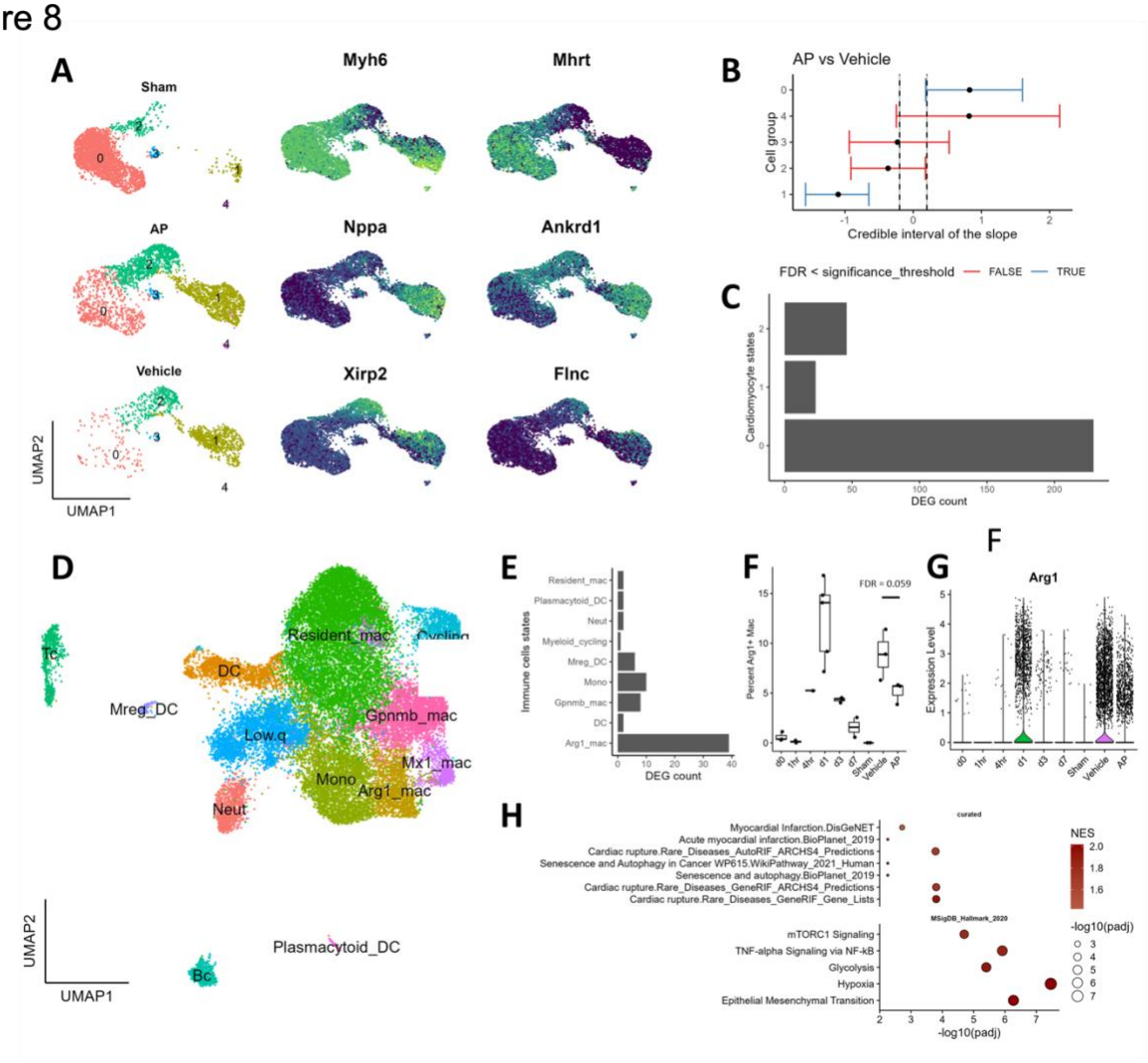

Figure 4.8. Single nucleus RNA sequencing data.

A) Sub-clustering uniform manifold approximation and projection of cardiomyocytes. The 3 lefts panels show the distribution of cells by samples across the sub-clusters. The 6 middle and right panels show the expression of MI zones markers i.e., remote zone (Myh6, Mhrt), boarder zone 1 (Nppa, Ankrd1), boarder zone 2 (Xirp2, Flnc). B) Generalized linear model sub-cluster proportion test between MI-AP and MI-Vehicle. Blue intervals show significant differences between the groups (false discovery rate; FDR < 0.05). The means of each group are represented by the black dot in the center of the interval and a mean above 0 indicates higher proportion in the AP group. C) Bar plot of number of differentially expressed genes (FDR < 0.05 and |log2 fold change| > 0.5) upregulated in MI-AP or MI-Vehicle classified by cardiomyocyte sub-cluster based on specificity of expression

(methods). D) Sub-clustering uniform manifold approximation and projection of the integrated immune MI cells (methods). E) Bar plot of differentially expressed genes (FDR < 0.05 and |log2 fold change| > 0.5) upregulated in MI-AP or MI-Vehicle classified by cell-type based on specificity of expression. F) Boxplot of the Arg1_mac immune sub-cell-type percentage across groups in the integrated immune MI cells (methods). G) Violin plot of Arg1 expression in immune cells divided by groups. H) Pre-ranked gene set enrichment analysis of the marker genes in the Arg1_mac sub-cell-type. Genes were ranked based on their area under the curve statistic obtained with the preso package. A positive normalized enrichment score (NES) indicates an enrichment in the Arg1_mac sub-cell-type.

Figure S1

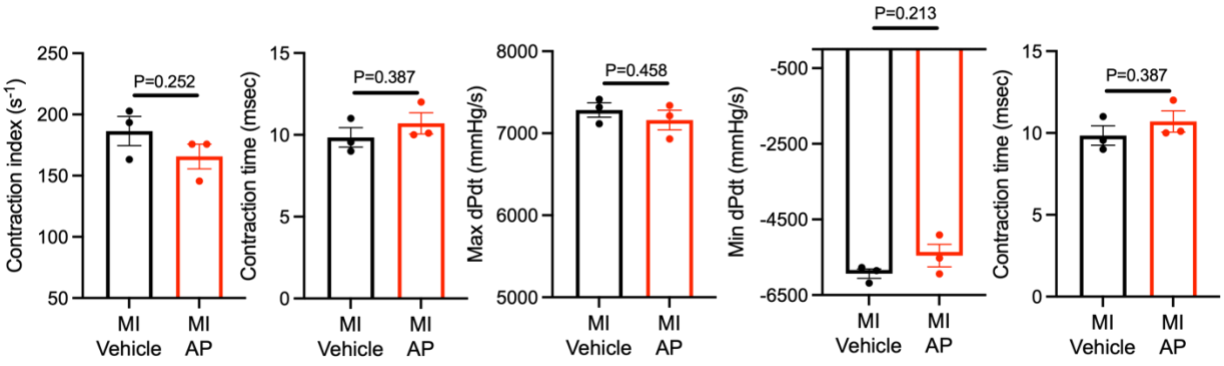

Figure 4 S.1. Hemodynamic parameters 3 days post-MI.

Max dpdt : Maximum rate of increase in pressure during contraction. Min dpdt: Maximum rate of decrease in pressure during relaxation.

Figure S2

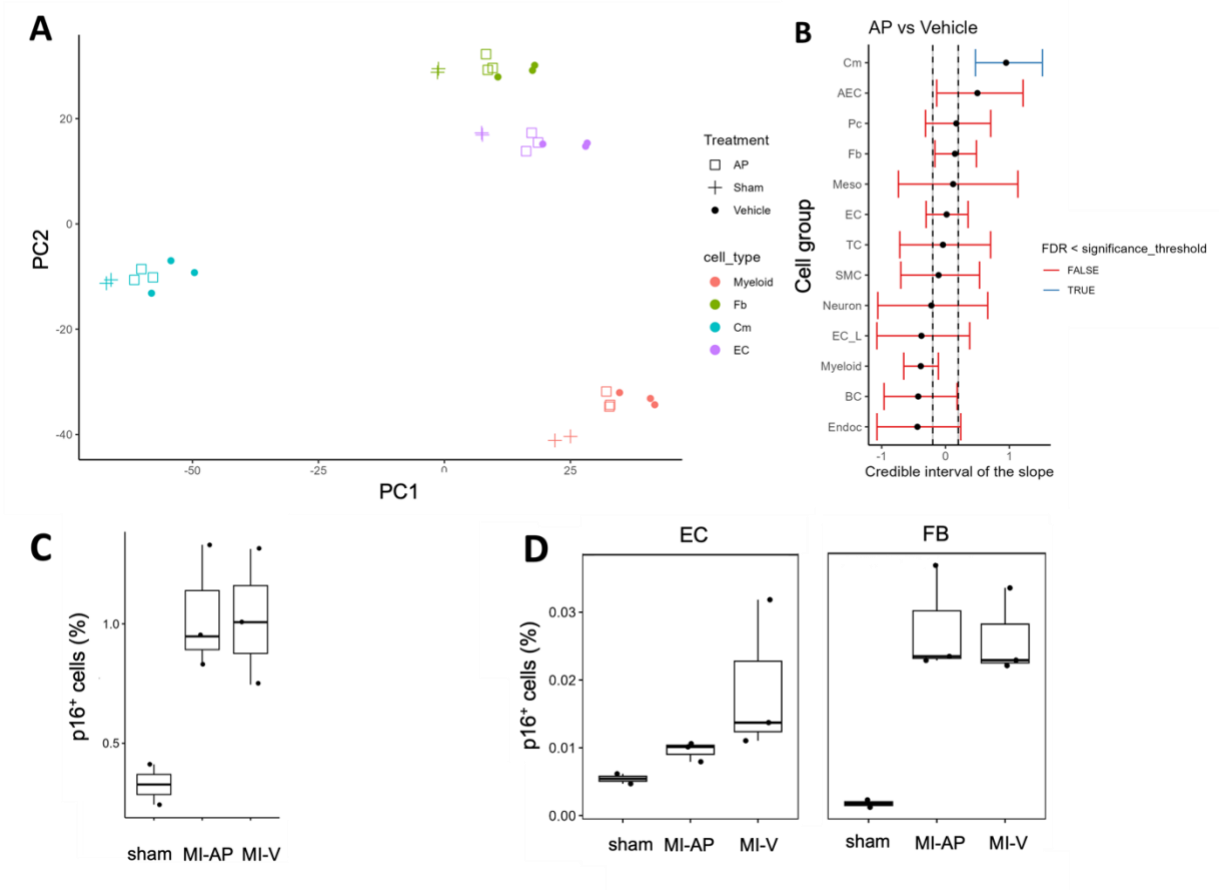

Figure 4 S. 2. Single nucleus RNA sequencing data.

A) Principal component analysis of pseudobulk sample-cell-types (aggreging cells counts by sample and cell-type, methods) using the 500 genes with the highest variance. Only the most prevalent cells-types are shown. **B)** Generalized linear model sub-cluster proportion test between MI-AP and MI-Vehicle. Blue intervals show significant differences between the groups (false discovery rate; FDR < 0.05). The means of each group are represented by the black dot in the center of the interval and a mean above 0 indicates higher proportion in the AP group. **C)** Boxplot of proportion of cells with p16 expression > 0 by group. **D)** Boxplot of proportion of cells with p16 expression in FB and EC.

EC; endothelial cells, AED; arterial endothelial cells, EC_L; endothelial cells lymphatic, Endoc; endocardial cells, TC; T-cells, BC; B-cells, Cm; cardiomyocytes, Fb; fibroblasts, Meso; mesothelial cells, Pc; pericytes, SMC; smooth muscle cells.

5. Chapter 5. General discussion

5.1. Novel findings

Evidence from both clinical and experimental studies points to the significant role of cellular senescence in cardiac pathology.[1] However, this area of research is still in its infancy and the precise role of senescent cells in cardiac pathology is poorly understood. One significant limitation of the existing literature on cellular senescence in the heart is its focus on tissue-level properties, rather than defining the specific role of senescence in different cardiac cell types. Therefore, the susceptibility of various cardiac cell types to senescence during cardiac disease and the specific cell types that drive cardiac disease remain largely unknown. This thesis provides novel findings regarding the role of senescence in three different cardiac conditions: AF, MI and age-related LV remodeling. Focusing on the identification of the role of different senescent cardiac cell types in these conditions, this thesis has shed light on important aspects of senescence in cardiac pathology that were previously unknown.

5.1.1. The role of cellular senescence in AF

Aging is widely recognized as the most significant determinant of AF risk.[2] Various structural, electrophysiological, and molecular changes associated with aging contribute to AF.[2,3] Among these changes, atrial fibrosis has been confirmed as a substantial factor in age-related AF through clinical and experimental studies.[4] Several lines of evidence point to a potential role for the accumulation of senescent cells with age or pathology in AF.[5,6,7] The expression of senescence markers in fibroblasts is associated with more extensive atrial fibrosis and greater AF-recurrence in patients with valve replacement surgery.[5] AF progression is associated with the p16 and p53 expression in the atrial tissue of AF patients.[6] A transcriptomic analysis of atria of AF patients showed significant upregulation of angiopeptin-like 2 (ANGPTL-2), which is proinflammatory

SASP component.[7] Inflammasome-activation is one of the crucial regulators of cellular senescence[8]; It has been reported that cardiomyocyte NLRP3 inflammasome activation is an important AF-promoting system in AF-patients and animal models.[9] Therefore, senescent cell accumulation is a potential candidate to contribute to AF-susceptibility. Despite this clinical evidence, there was a lack of direct investigation into the role of cellular senescence in AF.

Experiments in chapter 2 provide novel insights by demonstrating that aging and MI-induced LV dysfunction lead to the accumulation of senescence markers in the atria, which accompany increased AF susceptibility, slower conduction velocity and atrial fibrosis. Importantly, the clearance of senescent cells through senolytic therapy in the MI model prevents adverse atrial remodeling and the development of an AF-promoting substrate. The prevention of atrial fibrosis with senolytic therapy in MI rats is accompanied by the clearance of senescent myofibroblasts. Myofibroblasts potentially play a crucial role in AF, since studies have shown atrial fibroblasts from patients with chronic AF significantly differentiate to activated, collagen-producing myofibroblasts.[10] In addition to myofibroblasts, senolytic therapy clears senescent atrial endothelial cells. The role of endothelial cells in the pathogenesis of AF is largely unknown but might be related to the induction of fibrosis via endothelial-mesenchymal transition or via secreted profibrotic SASP products.[11,12]

5.1.2. The role of cellular senescence in age-related LV remodeling

The risk of HF increases drastically with age.[13] Mechanisms implicated in cardiac aging leading to HF are diverse and include oxidative stress, systemic inflammation, cardiomyocyte hypertrophy, telomere shortening and damage, epigenetic alterations, and cellular senescence.[14] The available literature suggests that senescence potentially interacts with a number of pathways involved in the HF pathophysiology such as neurohumoral system activation, mitochondrial

dysfunction and autophagy.[14,15] However, more research is needed to understand the role of senescence in HF, to identify which cardiac cell types mediate HF, and whether the suppression of senescence has therapeutic potential for HF treatment. In Chapter 3, we show the accumulation of different types of cardiac senescent cells, including cardiomyocytes, fibroblasts, endothelial cells, and immune cells in the LV of aged INK-ATTAC mice. Clearing senescent cells with the dimerizing agent AP leads to significant improvements in cardiac hypertrophy, fibrosis, and diastolic dysfunction, which potentially contribute to aging-related HF. Cellular senescent-marker analysis pointed to significant clearance of senescent fibroblasts and cardiomyocytes in mediating AP effects.

Our findings, along with evidence from other studies in the literature, suggest that senescence may play a vital role in age-related cardiac remodeling by inducing fibrosis and hypertrophy. The mechanisms underlying the role of senescence in cardiac hypertrophy are complex, and some studies indicate the involvement of senescent cardiomyocytes in this process.[16,17] For instance, Anderson et al. demonstrated that ROS and mitochondrial dysfunction in cardiomyocytes lead to telomere-associated DNA damage, triggering senescence and the secretion of SASP factors, which promote hypertrophy.[18] In their study, the clearance of senescent cells in aged mice using navitoclax or an INK-ATTAC mouse model reduced cardiomyocyte size without affecting LV mass or cardiac function. However, they did not identify which specific senescent cell types were affected by the treatment.[18] In our study, we show that the clearance of senescent cells in INK-ATTAC mice with AP treatment prevents hypertrophy, as evidenced by a reduction in LV mass, left ventricular wall thickness, and decreased expression of *Myh7* gene (a marker of hypertrophy). Our immunofluorescence experiments reveal a significant decrease in the number of senescent cardiomyocytes with AP treatment which may contribute to the observed decrease in hypertrophy.

Furthermore, our FACS experiment demonstrate that among nonmyocyte cardiac cells, the beneficial effects of AP treatment are specifically associated with the clearance of senescent fibroblasts. These findings suggest a potential role of senescent fibroblasts in LV hypertrophy, which we further investigate using an *in vitro* model; co-culturing senescent fibroblasts with healthy cardiomyocytes results in an upregulation of hypertrophic marker gene expression in the cardiomyocytes, suggesting potential paracrine effects of senescent fibroblast's SASP components on cardiomyocytes.

Another potential role of senescence in cardiac aging is its involvement in inducing fibrosis. This idea aligns with our observation of reduced LV fibrosis in aged INK-ATTAC mice following AP treatment, which is correlated with the clearance of senescent fibroblasts. Studies conducted on animal models and human hearts have confirmed the accumulation of senescent fibroblasts in fibrotic areas of the heart during aging and in association with cardiac diseases.[19-22] However, the current literature lacks definitive evidence to establish the direct causal role of senescent fibroblasts in cardiac disease. To our knowledge, our study is the first to provide direct evidence for a causal role of senescent fibroblasts in cardiac fibrosis by showing that their clearance, confirmed with FACS and immunofluorescence, leads to a reduction in fibrosis. Furthermore, we show that the clearance of senescent fibroblasts and cardiomyocytes normalizes prolonged isovolumic relaxation time in the aged mice, indicating an improvement in diastolic function due to reduced fibrosis and hypertrophy.

5.1.3. The role of cellular senescence in MI

MI is one of the most common causes of HF, hospitalization and death worldwide.[23] One of the characteristics of MI is DNA damage and excessive production of ROS in the infarct zone, which can lead to cell senescence with upregulation of senescence markers like p16.[24,25] Some animal

studies have provided evidence for the involvement of cellular senescence in the infarcted heart.[15,26,27] For example, in aging mice, the administration of navitoclax led to a significant improvement in survival rate post-MI.[26] The navitoclax-treated mice also showed reduced functional deterioration post-MI, indicating that senescent cells may contribute to adverse cardiac remodeling .[26] Furthermore, conventional drug therapies known to improve cardiac function after MI such as levosimendan and losartan have shown to reduce the expression of senescence markers in cardiac tissue.[27,28] Although these studies suggest a contribution of senescence to MI pathogenesis, the mechanistic link between senescence and remodeling post-MI, specifically at the cellular level, remains unestablished.

In Chapter 4, we show that the clearance of $p16^{+}$ cells in the INK-ATTAC model results in substantial improvements in cardiac remodeling and mortality rate post-MI, highlighting the detrimental impact of senescent cells following MI. Consistent with our findings, previous studies demonstrated that global clearance of senescent cells using senolytic agents improved survival and cardiac function post-MI in aging mice.[26,29] However, none of these studies explored the underlying mechanisms through which senescence causes cardiac remodeling post-MI. In contrast to the previous studies which focused on the tissue level, we explore cellular-level contributions of senescence using snRNA-seq.

In the early phase post-MI, timely resolution of the inflammatory response is crucial for proper cardiac healing, as excessive inflammation can lead to adverse outcomes such as cardiac rupture, impaired healing, cell loss, systolic dysfunction, and an increased risk of HF[30]. Senescent cells can potentially contribute to the early post-MI phase by releasing proinflammatory SASP components, which can alter the inflammatory response. Our qPCR and snRNA-seq data indicate that the clearance of $p16^{+}$ cells in the early inflammatory phase of MI reduces proinflammatory SASP

markers gene expression such as Cxcl2 and Ccl24 and genes involved in cardiac rupture. Furthermore, mice treated with AP exhibit changes in immune cell subsets, particularly Arg1_macrophages. The percentage of Arg1_macrophages decreased with senescent cell clearance; however, this shift did not reach statistical significance. We noted significant enrichment in the hypoxia and cardiac rupture pathways in Arg1_macrophages, indicating an important role of this cells in the MI pathophysiology.

The subsequent repair phase after the inflammatory response involves fibroblast proliferation, differentiation into myofibroblasts, and scar formation, characterized by upregulated collagen expression and enzymes responsible for cross-linking collagens.[23] These changes contribute to ventricular remodeling, including systolic and diastolic dysfunction and an increased risk of arrhythmias.[23] Cellular senescence may play a role in driving fibrosis and scar formation through the release of profibrotic SASP components.[25] This potential role aligns with our observations of reduced infarct size and profibrotic factors in the AP treatment group, as well as improvements in cardiac function, LVEF%, and hemodynamic parameters. These findings suggest an active role of senescence in the late remodeling phase following MI. There is also a possibility that the improvement in later remodeling is due to the improvement in the early inflammatory phase.

5.2. The importance and implications of findings

The novel findings of this thesis point to a significant contribution of cellular senescence to cardiac pathologies. This thesis provides important insights into the mechanistic role of senescence in cardiac pathologies like AF, MI and HF, potentially leading to novel treatment targets and biomarkers. Given the emergence of clinically feasible strategies to target senescence, exploration in this area offers intriguing possibilities for novel clinical interventions.

5.3. Unanswered questions, limitations and future direction

5.3.1. Characterization of cardiac senescent cells

Understanding the cell types responsible for the effects of cellular senescence on cardiac pathology is a crucial and has been frequently overlooked. To address this problem, in this thesis we utilize several techniques such as immunofluorescence, FACS and snRNA-seq to distinguish different cardiac senescent cells. However, we acknowledge that our identification of the specific senescent cell types with these tools are not definitive and needs improvement.

In chapters 2 and 3, we performed immunofluorescence experiments using the p16 antibody to identify senescent cells. Nevertheless, it is essential to investigate the development of alternative assays for the identification of senescent cells. Characterizing cells as senescent is complex, and relying solely on a single specific biomarker can lead to limitations in identification.[31] Although p16 is considered an important marker of senescence, diversifying the assays by incorporating other markers such as SA- β- gal, p21, p53, and γH2AX will enhance the reliability of senescent cell identification.[32]

In chapter 4, we used snRNA-seq to study senescence at the cellular level. However, snRNA-seq presents challenges in identifying senescent cells due to various reasons. Firstly, the technique is complex and technically demanding, involving multiple steps such as nuclei isolation and library preparation. Senescent cells are fragile and may be prone to destruction during these procedures, potentially leading to the loss or misrepresentation of their gene expression profiles. Therefore, advancing snRNA-seq protocols is essential to ensure the preservation of senescent cell integrity and minimize any potential biases.

Previous reports indicate that the majority of p16 mRNA is localized in the cytoplasm of brain cells and not in the nuclei, suggesting a similar pattern could exist in cardiac cells.[33] This is in line with our findings, as we detected very few $p16^{+}$ nuclei. Hence, approaches to enhance the

sensitivity of snRNA-seq in detecting low-abundance transcripts in senescent cell nuclei are needed. We chose to do single nucleus analysis rather than analysis of single-cell contents because it enables us to capture data from cardiomyocytes, which are large in size, making them unsuitable for single-cell RNA-seq methods bu,\t further studies with single-cell analysis from intact cells rather than single nuclei might be needed in the future.

5.3.2. Senolytics and animal models

In chapter 3, we utilize the combination of dasatinib and quercetin as senolytics. Although this combination is a well-established senolytic therapy, it is important to acknowledge the potential risk of off-target effects of these compounds, which cannot be entirely ruled out.[32] Therefore, it is crucial to adopt multiple approaches to optimize and identify novel senolytics. Some of these approaches are summarized as follow[34]:

1) Utilizing bioinformatics to identify novel molecular targets for senolytics in different types of senescent cells.
2) Applying medicinal chemistry to existing senolytics to enhance their senolytic activity.
3) Developing prodrugs that are selectively activated in senescent cells, for example, through hydrolysis by SA- β- gal.
4) Investigating the dynamics of senescent cells *in vivo* to optimize dosing with senolytics, thus ensuring their safety and effectiveness.

These diverse strategies will improve the safety and efficacy of senolytics and bring us closer to effective interventions for age-related conditions.

In chapter 3 and 4, we took advantage of the INK-ATTAC mouse model developed in the Mayo Clinic.[16] INK-ATTAC is a transgenic mouse model in which $p16^{+}$ cells undergo apoptosis following the treatment of mice with a dimerizing agent (AP20187). While the INK-ATTAC

mouse model is quite specific to the cells targeted, it is essential to recognize the limitation of inducing apoptosis solely in $p16^+$ cells. Not all senescent cells express p16, which means that this system cannot clear all senescent cells.[35] To gain a comprehensive understanding of the role of senescence, it would be useful to explore the role of cardiac senescence in other mouse models, such as those in which $p21^+$ cells are cleared.[36]

The INK-ATTAC model is also not cardiac-selective; it is possible that the observed effects are influenced by the clearance of non-cardiac $p16^+$ cells, including those found in the vascular or autonomic nervous systems. To gain a deeper understanding of the impact of senescence in cardiac pathology, it would be valuable to develop animal models that specifically target senescent cells in the heart.

5.3.3. *In vitro* model of senescence

In chapter 3, our attempts to culture senescent fibroblasts collected with FACS were unsuccessful as senescent fibroblasts do not proliferate in culture, consistent with the cell-cycle arrest associated with senescence. Therefore, we established an *in vitro* model of senescence in primary cardiac fibroblasts using H_2O_2. Our *in vitro* study shows promising insights in the role of SASP components and their paracrine effects through specific markers. While *in vitro* models are valuable tools for studying specific aspects of senescence, they fail to fully recapitulate the complexity and heterogeneity of cellular senescence observed *in vivo*.[37] Therefore, strategies to enhance the relevance of *in vitro* models including organ on a chip technology or 3D culture systems are required. These strategies mimic the structure, function, and dynamic microenvironment of specific organs or tissues integrating living cells, typically derived from human sources, within a controlled environment.[38] These strategies have demonstrated their ability

to provide a more realistic environment, in contrast to the conventional 2D plating-type structures.[38,39]

5.3.4. Cellular senescence in cardiomyocytes

Initially cellular senescence was thought to be a cell-cycle arrest response unique to dividing cells, it is now clear that senescence is a more generalized response to cellular stress that also occurs in postmitotic cells such as cardiomyocytes[18].

In our INK-ATTAC aging model, we show senescent cardiomyocyte clearance with AP treatment, which is associated with less hypertrophy and diastolic dysfunction. This observation highlights the potential role of senescent cardiomyocytes in cardiac aging. Nevertheless, to fully understand their impact, a more comprehensive characterization of senescent cardiomyocytes is required. Specifically, how do they differ from non-senescent cardiomyocytes in terms of phenotype and function?

It's important to recognize that our presumption of senescence playing a role in cardiomyocytes might be associated with the effects of other cardiac senescent cells via SASPs. Delivering INK-ATTAC transgene to cardiomyocytes using adeno-associated virus serotype 9 (AAV9) is a potential way to clarify the role of senescence in cardiomyocytes. However, the feasibility of establishing such a model remains to be determined.

5.3.5. Cellular senescence and AF

In this thesis, we investigate the impact of senolytic therapy on atrial pathology in the context of AF substrate induced by left ventricular dysfunction resulting from acute MI. Our findings provide support that certain pathologies like MI can trigger cardiac abnormalities by accelerating cellular senescence. It would be intriguing to explore whether senolytic interventions can also prevent the accumulation of senescent cells and age-related AF. The development of age-related atrial fibrosis

may occur gradually, requiring months of senolytic therapy to potentially prevent age-related AF. This time-course contrasts with the rat MI-model, where atrial fibrosis and AF-susceptibility manifest over several weeks following the acute MI. Several challenges associated with testing senolytics in aged rats include, but are not limited to age-related mortality, uncertainties regarding the optimal dosage and duration of senolytic therapy, and the substantial costs involved.

In this thesis, we show the role of senescence in atrial fibrosis, however, whether cellular senescence can contribute to other recognized AF-promoting mechanisms associated with aging, like atrial connexin-dysregulation Ca^{2+}-handling abnormalities or ion-current abnormalities remains to be established.[40]

5.3.6. Clinical relevance of senolytics in cardiac pathology

The promising results obtained from senolytics in preclinical models offer significant therapeutic and preventive opportunities for age-related diseases.[32,35,41] These encouraging findings have led to proof-of-concept clinical trials that are currently investigating the use of senolytics for age-related diseases.[32,35]

Clinical trials in non-cardiac diseases have already shown promising results and demonstrated a favorable safety profile.[42,43] For example, the combination of dasatinib and quercetin improved physical performance in patients with idiopathic pulmonary fibrosis[42]. The same senolytic therapy reduced the number of senescent cells in adipose tissue in patients with diabetic kidney disease.[43]

Certain senolytic compounds, including dasatinib and navitoclax, can lead to adverse effects. For instance, dasatinib may cause myelosuppression, hemorrhage, fluid retention, including pleural effusion, as well as adverse cardiac events (e.g., QT prolongation). Navitoclax can result in dose-limiting thrombocytopenia due to the clearance of mature platelets.[44]

Since senescent cells do not proliferate, adopting intermittent administration and lower dosages of these compounds may reduce their toxicity. In our rat AF study, we utilized a regimen involving dasatinib (5 mg/kg) and quercetin (50 mg/kg), administered intermittently (once-daily for 3 consecutive days beginning the day of MI, with two courses of therapy separated by 2 weeks). Clinical trials in noncardiac diseases utilizing dasatinib and quercetin reported dosages of dasatinib: 100 mg/day and quercetin: 1000-1250 mg/day, three-days/week over three-weeks.[42,43] Neither our animal study nor clinical trials reported any severe side effects, specifically any heart-related side effects such as QT interval prolongation. This observation suggests that the regimen utilized in these trials may be suitable and relatively safe to be used in the cardiac clinical studies. However, conducting more controlled clinical trials is essential to better determine the benefits and potential risks of senolytics and adjusting the dosages and therapy duration based on the type of disease.

In the context of cardiac pathology, only one clinical trial exploring the effects of senolytic (quercetin) has been initiated in patients undergoing coronary artery bypass graft surgery, though results are yet to be reported.[32] Thus, the feasibility of senolytic therapy for acute cardiac conditions such as MI or more chronic conditions such as AF and HF, remains to be determined.

5.4. Summary and Concluding remarks

The findings presented in this thesis highlight the role of cellular senescence in cardiac pathology. We show the contribution of cellular senescence in three different cardiac conditions including AF, MI and age-related LV remodeling.

In chapter 2, we show evidence for the accumulation of senescent cells in the atria of aged and MI rats which is associated with AF-substrate. Additionally, we demonstrate that senolytic

therapy suppresses cellular senescence in atria, prevent atrial fibrosis, and reduce AF susceptibility in MI rats.

In chapters 3, we show evidence for the accumulation of senescent cells in the LV of aged INK-ATTAC mice. Furthermore, we demonstrate that the clearance of senescent fibroblasts and cardiomyocytes improves cardiac diastolic function, hypertrophy and fibrosis in the LV of aged mice.

In chapter 4, we show senescent cells accumulate in the infarct area of MI INK-ATTAC mice post-MI. Clearance of senescent cells improve survival rate, cardiac function and structure potentially due to reduced inflammatory response.

Overall, these findings have important potential implications for understanding the mechanisms linking cellular senescence to cardiac pathology. Furthermore, given the development of clinically applicable senolytic therapies, work in this area presents interesting possibilities for breakthroughs in cardiac therapeutics.

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