Senescent, dysfunctional human cardiac progenitor cells (CPCs) accumulate in the aged heart and elimination of senescent cells enhances CPC activation and cardiomyocyte proliferation in aged mice

Fiona C. Lewis-McDougall[1], Prashant J. Ruchaya[1], Eva Domenjo-Vila[1], Tze Shin Teoh[1], Larissa Prata[2], Beverley J. Cottle[1], James E. Clark[3], Prakash P. Punjabi[4], Wael Awad[5], Daniele Torella[6], Tamara Tchkonia[2], James L. Kirkland[2], Georgina M. Ellison-Hughes[1]

[1]School of Basic and Medical Biosciences, Faculty of Life Sciences & Medicine, Kings College London, London, UK; [2]Robert and Arlene Kogod Center on Aging, Mayo Clinic College of Medicine, Rochester, Minnesota, USA;[3]School of Cardiovascular Medicine & Sciences, Faculty of Life Sciences & Medicine, Kings College London, London, UK; [4]National Heart and Lung Institute, Imperial College London, London, UK; [5]Barts Health NHS Trust, London, UK; [6]Molecular and Cellular Cardiology, Department of Medical and Surgical Sciences, Magna Graecia University, Catanzaro, Italy.

Correspondence should be addressed to G.M.E-H (georgina.ellison@kcl.ac.uk)

Rationale: **Aging leads to increased cellular senescence and is associated with decreased potency of tissue-specific stem/progenitor cells.**

Objective: **To determine the impact of ageing and senescence on human cardiac stem/progenitor cell (CPC) biology and regenerative potential, and investigate whether elimination of senescent cells in aged mice enhances CPC activation and cardiomyocyte proliferation.**

Methods and Results: **CPCs were isolated from the right atrial appendage (~200mg) of human subjects with cardiovascular disease (n=119), aged 32-86 years, and assessed for expression of senescence-associated markers ($p16^{INK4A}$, SA-β-gal, DNA damage γH2AX, telomere length), Senescence-Associated Secretory Phenotype (SASP), cell growth, differentiation, and regenerative potential following transplantation into the infarcted mouse heart. Senescent cells were eliminated in aged mice (22 – 32 months) in vivo either genetically, using INK-ATTAC mice, which results in inducible elimination of $p16^{Ink4a}$-expressing senescent cells upon the administration of the drug AP20187, or pharmacologically using intermittent oral administration of combined senolytics, Dasatinib (D) and Quercetin (Q). In aged subjects (>74 years old) over half of CPCs are senescent, unable to replicate, differentiate, regenerate or restore cardiac function following transplantation into the infarcted heart. Aged-senescent CPCs secrete SASP factors, which renders otherwise healthy, cycling-competent CPCs to senescence. Elimination of senescent CPCs using senolytics abrogates the SASP and its debilitative effect *in vitro*. Elimination of senescent cells in aged mice (INK-ATTAC or wildtype mice treated with D+Q) *in vivo* activates resident CPCs (0.23±0.06% vs. 0.01±0.01% vehicle; $p<0.05$) and increased the number of small, proliferating Ki67-, EdU-positive cardiomyocytes (0.25±0.07% vs. 0.03±0.03% vehicle; $p<0.05$).**

Conclusions: **Human CPCs become senescent with age, negatively impacting their regenerative capacity. Therapeutic approaches that eliminate senescent cells may alleviate cardiac deterioration with aging and rejuvenate the regenerative capacity of the heart.**

Aging poses the largest risk factor for cardiovascular disease[1]. Although long-term exposure to known cardiovascular risk factors strongly drives the development of cardiovascular pathologies, intrinsic cardiac aging is considered to highly influence the pathogenesis of heart disease[2]. However, the fields of the biology of aging and cardiovascular disease have been studied separately, and only recently their intersection has begun to receive the appropriate attention.

Aging leads to increased cellular senescence in a number of tissues and work suggests senescent cell burden can be dramatically increased in various tissues and organs with chronological ageing or in models of progeria[3-5]. Cellular senescence is associated with increased expression of the senescence biomarker, $p16^{Ink4a}$ (also known as Cdkn2a), impaired proliferation and resistance to apoptosis[6-10]. Senescent cells disrupt tissue structure and function because of the components they secrete, which act on adjacent as well as distant cells, causing fibrosis, inflammation, and a possible carcinogenic response[10]. Indeed, senescent cells possess a senescence-associated secretory phenotype (SASP), consisting of proinflammatory cytokines, chemokines and ECM-degrading proteins, which have deleterious paracrine and systemic effects[10-13]. Remarkably, even a relatively low abundance of senescent cells (10-15% in aged primates) is sufficient to cause tissue dysfunction[14].

To test whether senescent cells are causally implicated in age-related dysfunction and whether their removal is beneficial, J. L. Kirkland, T. Tchkonia, D. Baker (Mayo), J. van Deursen (Mayo) and colleagues made use of the biomarker for senescence, $p16^{INK4a}$, and an inducible "suicide" gene designed by P. Scherer *et al*.[15] to develop a novel transgene, INK-ATTAC, to permit inducible elimination of $p16^{INK4a}$-positive senescent cells upon administration of a drug (AP20187)[9]. In these mice, eliminating a relatively small proportion (~30%) of senescent cells extends health span and prevents the development of multiple age-related morbidities in both progeroid and normal, chronologically aged mice[9,16-20]. Moreover, late life clearance attenuated the progression of already established age-related disorders[20]. In an effort to be applicable to humans, Kirkland and collaborators have identified a new class of drugs named senolytics. Through exploiting senescent cells' dependence on specific pro-survival pathways, senolytics specifically kill senescent cells without affecting proliferating or quiescent, differentiated cells[21-23]. Recent studies have documented the use of senolytic drugs for the selective clearance of senescent cells from 'aged' tissues[16-27]. Indeed, a combination of senolytics drugs (D, dasatinib, a FDA-approved tyrosine kinase inhibitor; and

Q, quercetin, a flavonoid present in many fruits and vegetables), administered to 20-month-old male C57BL/6 mice once monthly for 4 months, led to significantly lower senescent ($p16^{Ink4a}$- and SA-β-gal-expressing) cells in aorta, bone, adipose tissue, skeletal muscle, and kidney[11,16-20,24]. Whether eliminated genetically or through senolysis, it's been shown that removal of $p16^{Ink4a}$ senescent cells can delay the acquisition of age-related pathologies in adipose tissue, skeletal muscle, heart, blood vessels, lung, liver, bone, and eye[9,11,12,16-20,24-28]. Recently, the Kirkland lab has demonstrated that transplanting relatively small numbers of senescent preadipocyte cells into young (6 month old) mice causes persistent physical dysfunction, measured through maximal speed, hanging endurance and grip strength, 1 month after transplantation. Transplanting even fewer senescent cells into older (17 month old) recipients had the same effect and reduced survival, indicating the potency of senescent cells in shortening health- and lifespan. Intermittent oral administration of the senolytics, D and Q to senescent cell-transplanted young mice and naturally aged mice alleviated physical dysfunction and increased post-treatment survival by 36% while reducing mortality hazard to 65%[20]. Altogether these data indicate that cellular senescence is causally implicated in generating age-related phenotypes and that systemic removal of senescent cells can prevent or delay tissue dysfunction, physical dysfunction and extend health- and lifespan.

Mammalian aging is associated with gradual loss of the capacity of the tissue-specific stem/progenitor cells to maintain tissue homeostasis or to repair and regenerate tissues after injury or stress[29]. Indeed, in most tissues there is an overlap between aging and stem cell impairment[30-33]. Function of tissue-specific stem cells declines with age due to several factors including telomere shortening, increased senescence and elevated expression of $p16^{Ink4a}$ and other cyclin-dependent kinase inhibitors (CDKIs)[34], DNA damage and external influences affecting stem cell niche homeostasis[30,35-38].

The discovery that the adult mammalian heart possesses a pool of tissue-specific, resident cardiac stem/progenitor cells (CSCs or CPCs) was 15 years ago[39]. These cells express stem cell surface receptors, c-kit, Sca-1, which allows their isolation and purification from deep within the cardiac tissue[40]. They possess properties of stem cells, being clonogenic, self-renewing, and multipotent *in vitro* and *in vivo*[39-42]. Using a cardiac diffuse damage rodent model, which is in the presence of a patent coronary circulation, has a drop out of only ~10% cardiomyocytes, and recapitulates muscle wear-and-tear, we have demonstrated that the adult heart has intrinsic regenerative capacity[41]. Indeed, resident CSCs spontaneously restore

cardiac function by regenerating lost cardiomyocytes, and when CSCs and their expansion were ablated, no cardiac regeneration or functional recovery was apparent leading to overt heart failure. However, the regenerative process was completely restored by replacing the ablated CSCs with the progeny of one CSC. Conversely, selective suicide of these exogenous CSCs and their progeny abolished regeneration, severely impairing ventricular performance. Thus, we showed that when tested in the appropriate model, CSCs are necessary and sufficient for the regeneration and repair of the damaged heart[41].

Recently considerable controversy, confusion and debate has surrounded the role and significance of CSCs in cardiac homeostasis and repair. Most of this confusion stems from using a single marker - c-Kit - to genetically fate map c-kit-positive cells in mice. Such studies have indicated that these cells only minimally contribute cardiomyocytes during ageing and following injury[43,44]. However, these studies have not specifically tagged or lineage traced the CPCs, or isolated them and characterised their stem cell properties. Moreover, these studies test the regenerative potential of the heart using the non-physiological ischemic myocardial infarction model that has a drastic drop out of cardiomyocytes (~30% of LV). Furthermore, several limitations of the use of Kitcre-KnockIn strategies for CPC identification and cell-fate mapping have recently been revealed[45]. Indeed, the very low number of endogenous c-Kit^{pos} CPC-generated cardiomyocytes detected in the Kitcre mice simply reflects the failure to recombine the CPCs to track their progeny and the severe defect in CPC myogenesis produced by the Kitcre allele[45].

We have since re-assessed and refined the phenotype, characteristics, and regenerative potential of the CSCs, and showed that the majority (~90%) of the resident c-kit-positive cardiac cells are blood/endothelial lineage committed CD45-positive, CD31-positive cells. Among the cardiac c-kit-positive cell cohort, only a very small fraction (1-2%) has the phenotype and differentiation/regenerative potential of true multipotent CSCs; the rest are progenitor cells. We show that single CD45-, CD31-negative, c-kit-positive cell-derived clones, when stimulated with TGF-β/Wnt molecules, acquire full transcriptome and protein expression, sarcomere organisation, spontaneous contraction, and electrophysiological properties of differentiated cardiomyocytes *in vitro* and *in vivo*[42]. Therefore, the adult mammalian heart fits squarely with other organs and tissues as possessing a resident multipotent stem cell and progenitor population. These cells are rare but can regenerate

cardiomyocytes lost to wear and tear, but understandably not large, ischemic segmental loss of tissue, as occurs with a myocardial infarction.

Over the years, accumulated evidence from human and mouse studies signified that cardiac aging and pathology affects the activity and potency of the resident cardiac stem/progenitor cells (abbreviated hereafter as CPCs)[46-49]. This translates into a diminished capacity of the aged and diseased myocardium to maintain homeostasis, and repair and regenerate following injury[50-55]. The aging *milieu* might therefore limit the success of cell transplantation therapies where the outcome is direct cardiogenic differentiation of transplanted cells and/or stimulation of endogenous regenerative mechanisms. As the majority of cardiovascular disease patients in need of regenerative therapies are of advanced age, regulation of CPC and cardiovascular aging/senescence is mission critical.

Here we provide new information about the existence and biology of CPCs and further show their importance and relevance in the aged and diseased human heart, which we hope will help resolve the controversy plaguing this important field. Indeed, we have carried out an extensive analysis of CPCs in the human failing heart with advanced age and show the accumulation of senescent-CPCs, which exhibit diminished self-renewal, differentiation, and regenerative potential *in vivo*. We show that Senescent-CPCs have a SASP that negatively affects healthy non-senescent, cycling-competent CPCs, rendering them senescent. Clearing the senescent-CPCs using combinations of senolytic drugs attenuates the SASP and its effect on promoting senescence *in vitro*. The effects of elimination of senescent cells on the heart and its regenerative capacity have not been elucidated. We report novel data that show systemic elimination of senescent cells *in vivo* in aged mice using senolytics (D+Q) or using the 'suicide' transgene, INK-ATTAC with administration of AP20187, results in CPC activation and increased number of small, immature, proliferating cardiomyocytes in the aged mouse heart. These findings are directly translational and transformative, which have great potential to inform the development and design of future clinical trials.

Methods

Expanded Methods are provided in the Online Data Supplement.

Myocardial samples (~200mg each) were obtained from the right atrial appendage (n=119) of human subjects with cardiovascular disease, aged 32-86 years. All subjects gave informed consent before taking part in the study (NREC #08/ H1306/91). Cardiac tissue was minced

then digested with collagenase II (0.3mg/ml; Worthington Laboratories) in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) at 37°C in a series of sequential digestions for 3 minutes each to release cardiac small cells. CPCs were purified from cardiac small cells by first depleting for $CD45^{pos}$ and $CD31^{pos}$ cells by immunolabelling with anti-human CD45 and CD31 magnetic immunobeads (Miltenyi), and then this CD45- CD31- depleted fraction was enriched for c-kit^{pos} cells through incubation with anti-human CD117 immunobeads (Miltenyi)[40-42]. CPCs were characterised by flow cytometry for other CPC markers, immunofluorescence for senescence-associated markers ($p16^{INK4A}$ expression, SA-β-gal, DNA damage γH2AX, telomere length by Q-FISH), cell growth and proliferation (BrdU incorporation assay, Roche), clonogenicity, sphereogenesis, differentiation, and SASP factors by qRT-PCR and Luminex® xMAP technology.

CPCs were labelled with PKH26 before transplantation into the infarcted myocardium. All surgical experimental procedures were conducted in accordance with the regulations for animal testing, directed by the Home Office and stipulated under the Animals (Scientific Procedures) Act 1986. Eight to ten week old non-obese diabetic (NOD), severe combined immunodeficiency (SCID) IL2Rgamma-c null mice [NSG; Charles River, UK] were randomly assigned to a treatment group (Sham (n=5), Cycling-CPCs (n=7), Sen-CPCs (n=5), c-kit^{neg} cells (n=6)). Myocardial infarction (MI) was induced through ligation of the left anterior descending (LAD) coronary artery performing a left-sided thoracotomy between the fourth and fifth rib to expose the heart. Immediately following MI, mice were injected intramyocardially with PKH26- labelled cycling-CPCs, Senescent-CPCs, or c-kit^{neg} cells (all $5x10^5$) delivered in a total volume of 15µl PBS, delivered across two sites at the border zone. Directly following MI+cell injection, mice were implanted with an osmotic pump (Alzet®, USA) loaded with a 0.2M solution of BrdU (MP Biomedicals), releasing the thymidine analogue for 14 days. Echocardiography measurements were taken at baseline (BL), 7 days, and 28 days post-MI, after which mice were sacrificed.

INK-ATTAC mice and senolytic drug treatment experimental procedures were conducted in accordance with Mayo Clinic Institutional Animal Care and Use Committee (IACUC) guidelines. Both stocks of INK-ATTAC and C57BL/6 wild type mice were bred and aged at Mayo Clinic. 24-32 month old mice were randomly assigned to treatment groups and injected intraperitoneally (i.p.) with either vehicle or AP20187 (B/B homodimerizer, Clontech, 10 mg AP20187/kg body mass) or administered by oral gavage vehicle (66% Phosal-50PG, 10% ethanol, and 30% PEG-400) or senolytic drugs (5mg/kg Dasatinib + 50mg/kg Quercetin diluted in vehicle) for 3 consecutive days every 2 weeks for 2 months. Another group of

INK-ATTAC mice aged 3 months (n=10) and 22 months (n=10) were randomly assigned to treatment groups and injected intraperitoneally (i.p.) with either vehicle or AP20187 for 2 consecutive days every 2 weeks for 2 months. These mice were injected (i.p) with EdU (123 $mgkg^{-1}$) 4 days and 2 hours prior to sacrifice.

Results

CPCs exhibit a senescent phenotype with increased age

Human CPCs were isolated from biopsies of right atria, obtained from subjects who had given informed consent before undergoing cardiac surgery (aortic disease, valve disease, coronary artery bypass graft (CABG), or multiple diseases), using sequential enzymatic digestion and dissociation, Optiprep density gradient to remove large debris, followed by magnetic activated cell sorting (MACS) (**Supplementary Figure 1a**). CPCs were magnetically enriched based upon a CD45-negative, CD31-negative, CD34-negative, and c-kit-positive sorting strategy[40] (**Supplementary Figure 1b**). Despite being recognised as a CPC marker, cells were not sorted for Sca-1 because its homology has not been confirmed in any species other than mouse. By flow cytometry analysis, CPCs showed expression of other recognised CPC markers such as CD90 (37±0.4%), CD166 (41±1%), CD105 (13±1%), and CD140α (5±0.4%) (**Supplementary Figure 1c**).

We isolated CPCs from 35 subjects of different genders, ages, and pathologies and found a linear increase (R^2=0.722) in the number of CPCs that expressed the senescence-associated marker, $p16^{INK4A}$, with age (**Figure 1a**). No differences were evident between males and females for $p16^{INK4A}$ expression or between aortic disease, valvular disease, coronary artery disease, and multiple other diseases for $p16^{INK4A}$ expression (**Supplementary Figure 2a,b**). On average, 22±9%, 31±4%, 48±9%, and 56±16% of CPCs expressed $p16^{Ink4a}$ isolated from 50-59, 60-69, 70-79, and 80-89 year old subjects, respectively. We also found an increase ($P<0.05$) in the number of senescence-associated β-galactosidase- (SA-β-gal; ~60%) and DNA damage marker, γH2AX-positive CPCs (~20%) isolated from old (71-79 years), compared to middle-aged (54-63 years) subjects (**Figure 1b,c**). Moreover, $p16^{INK4A}$-positive CPCs co-expressed γH2AX (**Figure 1c**). Further interrogation by Q-FISH revealed that, while the average telomere length of CPCs isolated from old and middle-aged subjects' hearts were comparable, CPCs isolated from old (78-84 years) subjects' hearts contained a 12% subpopulation with telomere length of <6Kb, which is regarded as being critically short (**Figure 1d**)[56]. Approximately 2% of the CPCs isolated from human hearts were Ki67-

positive, reflective of their mainly dormant, quiescent phenotype[41]. There were no differences between middle-aged and old subjects in number of Ki67-positive CPCs, and we did not see any Ki67-positive CPCs that were $p16^{INK4A}$-positive (**Supplementary Figure 2c**). These findings indicate that the aged human heart contains an increased proportion of aged senescent-CPCs, which could translate to their dysfunctionality.

CPCs from old subjects show impaired cell growth and differentiation

CPCs were isolated from 5 old (76-86 years) and 8 middle-aged (32-66 years) subjects, plated in growth medium, and propagated, where possible, to passage 11. CPCs isolated from 2 of the middle-aged (32 and 61 years) and 3 of the oldest (78, 80, and 86 years) subjects failed to grow and become established *in vitro*. Of the CPC cultures that did grow from all age groups (n=8), the CPCs, from P3 to P11, gradually lost their $p16^{INK4A}$–positive subpopulation (**Supplementary Figure 2d,e**), likely due to the cell culture activated, cycling-competent CPCs outgrowing their $p16^{INK4A}$–positive senescent counterparts. CPCs maintained the phenotype of c-kit-positive, CD31-negative over culture passage (**Supplementary Figure 2f**). To ensure that the effect of donor age could be effectively evaluated, all *in vitro* cell dynamic assays were performed between P2-P4.

CPCs isolated from old (77-86 years) subjects showed decreased ($P<0.05$) proliferation compared to CPCs isolated from middle-aged (34-62 years) subjects (**Figure 2a**). CPCs deposited as a single-cell in a 96-well plate generated a greater number ($P<0.05$) of clones if the single CPCs originated from middle-aged (34-62 years) subjects, compared to old (76-86 years) subjects (**Figure 2b**). Likewise, CPCs deposited at low dilution in bacteriological dishes for the generation of spheres in suspension were greater in number and size ($P<0.05$) for middle-aged (34-51 years) subject's CPCs, compared to old (76-77 years) subject's CPCs (**Figure 2c,d**). When CPCs were plated in cardiomyocyte differentiation medium they primed towards a cardiomyocyte-like precursor cell type that was Nkx2.5-positive, sarcomeric actin-positive, and Middle-aged (47-62 years) subject's CPCs had increased ($P<0.05$) differentiation potential, compared to CPCs from old (76-77 years) subjects (**Figure 2e-g**). Older subject's differentiated CPC-derived precursor cells showed disorganised sarcomeric structure (**Figure 2e**) and decreased ($P<0.05$) expression (**Figure 2g**), compared to differentiated CPCs from younger, middle-aged subjects.

Even though CPCs isolated from old hearts showed decreased proliferation, clonogenicity, and differentiation potential, only ~50% of CPCs are senescent in old myocardium (**Figure 1a**), therefore these data imply that a functionally cycling-competent CPC population still exists in old myocardium. Indeed, single CPC-derived clones from young, middle-aged, and old subjects were indistinguishable in terms of morphology, senescence, multipotency, self-renewing transcript profile, and differentiation (**Supplementary Figure 3**). These findings suggest that CPCs age and become senescent in a stochastic, non-autonomous manner. This resembles what was seen in rat preadipocytes[57].

Aged-senescent CPCs lose their regenerative capacity *in vivo*

To purify for a senescent population of CPCs we utilised the C_{12}-5-Dodecanoylaminofluorescein Di- β -D-Galactopyranoside (C12FDG) probe and pulled out SA-β-gal-positive CPCs through FACS (**Supplementary Figure 4a-d**). We also induced senescence in CPCs pharmacologically using Doxorubicin and Rosiglitazone (**Supplementary Figure 4e-g**), which we have used previously to render cells to senescence *in vitro*[11,20]. Senescent-CPCs, whether Doxorubicin-, or Rosiglitazone-induced or purified using the C12FDG probe, exhibited a senescent phenotype being $p16^{INK4A}$–positive, Ki67-negative, and with shorter telomeres (**Supplementary Figure 5**). Senescent SA-β-gal-positive CPCs were non-proliferative and did not form clonal colonies when deposited as single cells, or generate spheres *in vitro*, compared to SA-β-gal-negative, Ki67-positive cycling-competent CPCs (**Supplementary Figure 5**). FACS phenotyping revealed decreased surface expression of the progenitor markers, c-kit, CD90, CD105, and CD166 and increased expression of CD31 and CD34 in SA-β-gal-positive, senescent-CPCs compared to SA-β-gal-negative, cycling-competent CPCs (**Supplementary Figure 6**).

To determine whether the dysfunctional stem/progenitor cell properties of the senescent-CPCs translated *in vivo*, we tested the regenerative capacity of senescent SA-β-gal-positive CPCs and cycling-competent SA-β-gal-negative CPCs in the myocardial infarction-regeneration mouse model (**Figure 3a**). Male, immunodeficient NSG mice were subjected to permanent ligation of the left anterior descending (LAD) coronary artery. Immediately after ligation, 5 x 10^5 SA-β-gal-positive senescent or SA-β-gal-negative cycling-competent CPCs were injected intramyocardial in 15µl of PBS at 2 sites in the border zone. To serve as a cell control, a separate set of MI-mice were injected with 5 x 10^5 non-CPCs (c-kit^{neg} cardiac-

derived cells; containing 86±5% cardiac fibroblasts, 13±3% vascular smooth muscle, 1±1% endothelial cells[41]). Sham animals were treated the same way, except ligation of LAD coronary artery was not performed and they did not receive cells but were injected with the same volume of PBS. Mice were administered BrdU *via* osmotic mini pumps for 14 days after MI and cell injection to track new cell formation (**Figure 3a**). All cell populations were labelled prior to injection with PKH26 lipophilic membrane dye, which exhibited high labelling efficiency and label dye retention over population doublings in cycling-competent CPCs and c-kit^{neg} cardiac-derived cells *in vitro* (**Supplementary Figure 7**). We sacrificed a sub-set of MI-mice that had been injected with 5 x 10^5 SA-β-gal-negative cycling-competent CPCs at 4 days. There was high engraftment and survival of CPCs within the infarct/border zone at 4 days (**Figure 3b**) and by 28 days the engraftment was still ~10% of cycling-competent CPCs per total nuclei in the infarct/border zone (**Figure 3c**). The engraftment and survival of senescent-CPCs and c-kit^{neg} cells at 28 days was significantly ($P<0.05$) less (**Figure 3c**).

At 1 week after LAD ligation all groups had decreased ($P<0.05$) LV function, compared to baseline and sham controls, however the group that had been injected with cycling-competent CPCs had less of a decrease in LV function at 1 week, compared to the senescent-CPC and non-CPC c-kit^{neg} cell groups (**Figure 3d**). The degree of MI represented as the % Average Area at Risk (AAR) through Evans Blue staining immediately after MI was 33.0±1.6% (n=5), demonstrating the operator as being extremely consistent in inducing a similar size MI injury to each mouse. At 4 weeks after LAD ligation, MI hearts that had received cycling-competent CPCs showed an improvement ($P<0.05$) in LV ejection fraction (EF), fractional shortening (FS), LVEDD, and LVESD, which had almost returned to baseline values and to that of Sham controls (**Figure 3d**).

This extent of LV improvement was not apparent in MI-mice that were injected with SA-β-gal-positive senescent-CPCs, or the non-CPC c-kit^{neg} cell group that showed no recovery with worsened LV function and were all in heart failure at 4 weeks (**Figure 3d**). To accompany these functional changes, cycling-competent CPC injection resulted in a decreased ($P<0.05$) infarct size, whereas SA-β-gal-positive senescent-CPCs or non-CPC c-kit^{neg} cells did not change the extent of infarct size (**Figure 3e**). Immunohistochemical analysis of cross-sections revealed that at 4 weeks after MI, the transplanted PKH26-labelled cycling-competent CPCs had increased expression of sarcomeric proteins, α-actinin, as well as the endothelial lineage

marker vWF, evidencing their differentiation into cardiomyocyte-like precursor cells and endothelial cells, respectively (**Figure 3f**). Limited or no differentiation was evident in the infarcted/border zone of the hearts injected with PKH26-labelled senescent-CPCs and non-CPC c-kit^{neg} cells (**Figure 3f**). To determine whether the transplanted cells had participated in inducing a paracrine effect, the infarct/border zone of hearts that had received cells were analysed for formation of new cells that were BrdU-positive/PKH26-negative. Hearts that were injected with cycling-competent CPCs showed an increased number of BrdU-positive cells, compared to those injected with SA-β-gal-positive senescent-CPCs or non-CPC c-kit^{neg} cells (**Supplementary Figure 8**). Moreover, these BrdU-positive cells co-localised with vWF or α-sarcomeric actinin, indicating new endothelial (capillary) cell and cardiomyocyte formation, respectively. New cardiomyocyte and capillary formation was more evident ($P<0.05$) in the MI-cycling-competent CPC group (**Figure 3g**). These findings show the diminished regenerative and reparative capacity of senescent CPCs, compared to healthy, cycling-competent CPCs.

Aged-Senescent CPCs have a Senescence-Associated Secretory Phenotype (SASP)

Senescent cells exhibit a SASP[10]. Senescent SA-β-gal-positive CPCs showed increased expression of SASP factors, including MMP-3, PAI1, IL-6, IL-8, IL-1β, and GM-CSF, compared to non-senescent, SA-β-gal-negative, cycling-competent CPCs (**Figure 4a**). To determine whether the SASP factors were secreted from senescent-CPCs, we quantified the protein levels of seven of the highly expressed SASP factors in conditioned media from senescent-CPCs using Luminex technology. We found increased ($P<0.05$) quantities of all seven SASP factors in senescent-CPC conditioned medium, compared to conditioned medium of cycling-competent CPCs (**Figure 4b**). Next we treated cycling-competent CPCs with conditioned medium from senescent-CPCs and measured cell proliferation and senescence of the cycling-competent CPCs. Conditioned medium from senescent-CPCs resulted in decreased ($P<0.05$) proliferation (**Figure 4c**) and an increased ($P<0.05$) proportion of senescent p16^{INK4A}–positive, SA-β-gal-positive, and γH2AX-positive CPCs in the culture, compared to CPCs treated with conditioned medium from cycling-competent CPCs or unconditioned medium (**Figure 4d-f**). These findings show that the senescent-CPCs exhibit a SASP, which can negatively impact surrounding cells, rendering otherwise healthy, cycling-competent CPCs to lose proliferative capacity and switch to a senescent phenotype.

Elimination of senescent CPCs using senolytic drugs abrogates the SASP *in vitro*

Removal of p16^{Ink4a} senescent cells can delay the acquisition of age-related pathologies in adipose tissue, skeletal muscle, heart, blood vessels, lung, liver, bone, and eye[9,11,12,16-20,24,25,28]. Recent studies have documented the use of senolytic drugs for the selective clearance of senescent cells from 'aged' tissues[16-27,58]. We tested the potential of 4 senolytic drugs, Dasatinib (D; an FDA-approved tyrosine kinase inhibitor), Quercetin (Q; a flavonoid present in many fruits and vegetables), Fisetin (F; also a flavonoid), and Navitoclax (N; an inhibitor of several BCL-2 family proteins), alone and in combination to eliminate and clear senescent-CPCs *in vitro* (**Supplementary Figure 9a**). Measuring cell viability with crystal violet and number of SA-β-gal-positive CPCs, dose-response experiments on senescent- or cycling-competent CPCs from the same subjects showed D and N to effectively clear senescent-CPCs, whereas F and Q were less effective at clearing senescent-CPCs (**Supplementary Figure 9b,c**). However, D also decreased the viability of cycling-competent CPCs (**Supplementary Figure 9b**). A combination of D+Q, which has previously shown to yield effective senescent cell clearance[16-20,24,25] and which does not share the toxic anti-neutrophil and anti-platelet side effects of N[22,23] was tested, and at a dose of 0.5μM D with 20μM Q, cycling-competent CPC viability was preserved (**Figure 5a**), but senescent-CPCs were cleared and induced to selective apoptosis (**Supplementary Figure 9d**).

We next determined whether clearing senescent-CPCs using D+Q would abrogate the SASP and its paracrine impact on CPCs. Using transwell inserts, cycling-competent CPCs were seeded on the top chamber insert and co-cultured in the presence of senescent-CPCs seeded on the bottom chamber. Cultures were left for 7 days and then cycling-competent CPCs in the top chamber were analysed for proliferation and markers of senescence, p16^{INK4A}, SA-β-gal, and γH2AX, and conditioned medium analysed for SASP factors. The cultures were then treated with D+Q for 3 days to clear the senescent-CPCs on the bottom chamber, and then 7 days later cycling-competent CPCs in the top chamber were analysed for proliferation and the markers of senescence, p16^{INK4A}, SA-β-gal, and γH2AX, and conditioned medium analysed for SASP factors (total of 17 days; **Supplementary Figure 9a**). We found that cycling-competent CPCs co-cultured in the presence of senescent-CPCs for 7 days were decreased ($P<0.05$) in number and proliferation, and had increased ($P<0.05$) expression of p16^{INK4A}, SA-β-gal, and γH2AX (**Figure 5b-f**). Application of D+Q to co-cultures eliminated the senescent-CPCs (**Figure 5g**) and 7 days later, the cycling-competent CPCs had increased ($P<0.05$) in number (**Figure 5h**), proliferation (**Figure 5i**), and the number of p16^{INK4A} and SA-β-gal CPCs had decreased ($P<0.05$) compared to CPCs that had been in co-culture with

senescent-CPCs for 17 days (**Figure 5j,k**). Co-culture of cycling-competent CPCs with senescent-CPCs led to increased ($P<0.05$) secretion of SASP factors into the medium, but the level of SASP factors was reduced ($P<0.05$) with application of D+Q (**Figure 5l**). These findings document that senescent CPCs have a SASP, and clearance of senescent CPCs using a combination of D+Q senolytics abrogates the SASP and its detrimental senescence-inducing effect on healthy, cycling-competent CPCs.

Elimination of senescent cells *in vivo* activates resident CPCs and increases number of small, proliferating cardiomyocytes in the aged heart

Eliminating a relatively small proportion (~30%) of senescent cells using a 'suicide' transgene, INK-ATTAC, that permits inducible elimination of $p16^{Ink4a}$-expressing senescent cells upon the administration of a drug (AP20187) extends health span and prevents the development of multiple age-related morbidities in both progeroid and normal, chronologically aged mice[9,16-20,28]. Moreover, in 20-month-old male C57BL/6 mice either randomized to vehicle or D+Q treatment once monthly for 4 months, D+Q treatment led to significantly lower senescent ($p16^{Ink4a}$- and SA-β-gal-expressing) cells in bone, adipose tissue, skeletal muscle, and kidney[16-20,24]. Here 24-32 month INK-ATTAC transgenic or wild-type mice were randomised to either vehicle, AP20187, or D+Q treatment, administered in 4 cycles for 3 consecutive days/cycle, 12 days apart. Mice were sacrificed 4 days after the last dose of cycle 4 (**Figure 6a**). Tissues in which $p16^{Ink4a}$ expression and/or senescent cells are decreased by D+Q in wild type mice as well as by AP20187 in INK-ATTAC mice include the aorta, adipose tissue, cardiac and skeletal muscle, lung, liver, and bone[9,16-28]. We showed $p16^{Ink4a}$ mRNA expression was decreased ($P<0.05$) in the heart following D+Q or AP20187 treatment in aged INK-ATTAC or wildtype mice (**Figure 6b**).

Previously we have shown an improvement of heart function in old mice after D+Q treatment[24]. Analysis of cardiac cross-sections revealed significantly higher ($P<0.05$) CPC (Sca-1^+/c-kit^+/$CD45^-$/$CD31^-$/$CD34^-$)[42] numbers (**Figure 6c; Supplementary Figure 10a**) in AP20187-treated INK-ATTAC mice and D+Q-treated INK-ATTAC or wild-type mice, compared to vehicle-treated control. Interestingly, D+Q treatment showed increased ($P<0.05$) CPC number, compared to AP20187-treatment (**Figure 6c**). Roughly 10% of CPCs were activated and in the cell cycle (Ki67-positive) at the time of sacrifice, after AP20187 or D+Q treatment (**Supplementary Figure 10b**). Morphometric analysis of heart sections showed that AP20187-treated and D+Q-treated mice had increased number of smaller ventricular

myocytes (**Figure 6d**), suggesting these myocytes to be immature and newly formed, compared to vehicle-treated mice, which exhibited only rare small myocytes but a greater proportion of hypertrophied myocytes (**Figure 6d**). We found an increase ($P<0.05$) of small, proliferating Ki67-positive myocytes (~0.25%) in old hearts following AP20187- or D+Q-treatment, compared to vehicle-treated control (0.03±0.03%) (**Figure 6e,f**). To corroborate these data we injected EdU 4 days and 2 hours prior to sacrifice of old (22m) and young (3m) AP20187-treated INK-ATTAC mice (**Supplementary Figure 10c**). We found increased ($P<0.05$) number of small EdU-positive myocytes (0.25±0.06%; **Figure 6g,h**) in the hearts of old AP-treated mice, compared to old vehicle-treated (0.07±0.00%), young vehicle-treated (0.12±0.03%) and young AP-treated (0.11±0.04%) mice. The number of EdU-positive myocytes in the old AP-treated INK-ATTAC mice was the same in amount to Ki67-positive myocytes (~0.25%) in AP20187- or D+Q-treated mouse hearts (**Figure 6f**). Finally, we detected a decrease ($P<0.05$) in fibrosis in the LV following AP20187- and D+Q-treatment, compared to vehicle-treated control (**Figure 6i**). In contrast to the treatment of aged mice, treatment of young adult (2-3 Months) INK-ATTAC or wild-type mice with AP20187 or D+Q, respectively, did not alter EdU-positive myocyte number (**Figure 6h**), CPC numbers or myocyte diameter (*data not shown*). These findings show that clearance of senescent cells leads to stimulation of CPCs and cardiomyocyte proliferation and that this strategy is specific to the aged heart.

Discussion

In a large sample cohort our study shows that CPCs isolated from the failing human heart develop a senescent phenotype with age exhibited by increased expression of senescence-associated markers ($p16^{INK4A}$, SA-β-gal), DNA damage, shortened telomere length and a SASP. Aged human hearts with dilated cardiomyopathy showed greater numbers of $p16^{INK4A}$ –positive CPCs and cardiomyocytes with shorter telomeres than age-matched controls[47]. Similarly, CPCs isolated from failing, aged hearts show increased $p16^{INK4A}$ and inflammatory factor expression[48]. Reliably detecting senescent cells in vivo is an ongoing challenge, and it is important that a combination of senescent cell biomarkers are used for detection as any one marker used in isolation is prone to false positives. Our study used a combined panel of the senescence-associated biomarkers, $p16^{INK4A}$, γH2AX, telomere length, SA β-gal activity and SASP expression, to detect senescent CPCs. Our findings demonstrate that CPCs accumulate in the failing hearts of elderly subjects (>76 years) and are dysfunctional, showing impaired proliferation, clonogenicity, spherogenesis, and differentiation, compared to CPCs isolated

from the hearts of middle-aged (32-66 years) subjects. As the adult heart possesses very low numbers of cardiomyogenic CPCs[42], if by 80 years of age >50% of resident CPCs are senescent, this presents a bleak outcome for harvesting healthy, functional CPCs from patients who are candidates for regenerative therapies and their autologous use. Moreover, strategies to activate the regenerative capacity of the aged heart through delivery of growth factors or cell therapy will also be sub-optimal. Therefore, the success of cardiac regenerative therapeutic approaches thus far tested for treating patients with heart failure and disease could be of limited efficacy in promoting myocardial regeneration because of the increased number of senescent, dysfunctional CPCs and cardiomyocytes[47,48] and the resultant presence of a cardiac SASP in the aged and failing heart that impairs the function of the remaining non-senescent CPCs.

The present study found that CPCs age in a stochastic non-autonomous manner and it is possible to clonally select for a cycling-competent population of CPCs even from diseased or aged hearts. There are individual CPCs in older individuals that have replicative and functional capacities resembling those of CPCs in younger subjects. A similar scenario was found in the case of rat fat cell progenitors[57]. While the abundance of progenitors cloned from adipose tissue that had restricted capacities for replication and differentiation into adipocytes or that were non-replicative but viable (*i.e.*, senescent) increased progressively with aging in rat fat, there remained cells that had the capacities for replication and adipogenic differentiation characteristic of clones derived from young rats. Together, these findings indicate that: (1) it may be feasible to isolate CPCs even from older individuals that are functional, capable of supporting cardiac regeneration if removed from their toxic *milieu*, and that could be therapeutically relevant in treating patients, especially if they were autologously generated[59] and (2) that by clearing senescent CPCs with a toxic SASP from the aged heart, there remains a tissue-resident population of CPCs with capacity to regenerate damaged heart tissue.

When we purified for a homogenous SA-β-gal-positive, senescent CPC population, we showed that these cells had poor engraftment and survival, and were unable to contribute to cardiac regeneration, repair or restoration of cardiac function following transplantation into the infarcted myocardium. This is contrary to *in vitro*-selected, SA-β-gal-negative, proliferative cycling-competent ($Ki67^{pos}$) CPCs, which had high survival and engraftment in the infarct/border zone, restored cardiac function almost to baseline and sham control values

(LVEF 59±2% at 28 days vs. 66±2 at baseline), decreased infarct size, differentiated into endothelial and cardiomyocyte-like precursor cells as well as enhanced endogenous new cardiomyocyte and capillary formation. Although some of the transplanted CPCs expressed α-sarcomeric actin, these cells did not exhibit the typical cardiomyocyte phenotype as they were small and lacked a structured sarcomeric unit. Therefore, they could not be considered as new, immature myocytes which contributed physiologically to the substantially improved LV function. It is plausible that the reduced infarct size and increase in viable tissue relates to improved cardiomyocyte survival following cycling-competent CPC transplantation in the present study. Transplanted CPCs secrete pro-survival and regenerative factors that activate multiple mechanisms, including cardiomyocyte survival and protection, inflammation reduction, cell–cell communication, angiogenesis/vascularization and cardiomyogenesis[60]. These data emphasize the importance of taking into account the hostile infarcted environment, which does not favour engraftment, differentiation or maturation of newly formed cardiomyocytes derived from the injected cells. However, the presence of CPC-derived cardiomyocyte precursors expressing sarcomeric protein in the infarct/border zone is promising and further work should elucidate how to mature these cells into functionally competent contractile cells.

Outcomes of multiple clinical trials utilising bone marrow-derived cells (BMDCs) to treat myocardial infarction and heart failure patients show modest improvements in myocardial structure and function[61]. The major limitation of using BMDCs is their poor retention, survival and engraftment in the damaged myocardium. Injection of CPCs that show high survival and engraftment[62], and have the ability to generate new cardiomyocytes and vasculature[39-42] can facilitate physiologically significant repair and regeneration, with resultant improvements in cardiac function in both humans[63] and rodent models[39,41,42,64-67]. There is now a general consensus that the favourable effect of cell transplantation protocols is, at least in part, mediated by ‘paracrine’ effectors secreted by the transplanted cells, contributing to improved myocardial contractility and amelioration of ventricular remodelling (decreasing fibrosis, hibernation, and stunning), inhibition of the inflammatory response, increased cardiomyocyte survival and angiogenesis/neovascularisation[60]. Most importantly, the transplanted cells’ secretome activates the heart’s endogenous myocardial regenerative reserve, which then gives rise to new vasculature and cardiomyocytes, leading to endogenous (and autologous) myocardial regeneration[68,69]. Results from the SCIPIO clinical trial showed that CPCs injected into subjects with ischemic cardiomyopathy who had post-infarction

LVEF <40% had an increase of 12% LVEF at 1-year follow up compared to baseline. Moreover, there was a 30% decrease in infarct size measured by cMRI[63]. However, not all experimental studies have shown physiological regeneration of CPC-derived cardiac muscle following administration of CPCs[62,70]. This is most likely due to the heterogeneous nature of cardiac c-kit positive cells tested, with only a very small fraction (1-2%) being cardiomyogenic[42]. Bringing together the cardiomyogenic potential of CPCs, which can be amplified in number through *in vitro* clonal selection[41,42] and CPC-mediated cytokine release that regulates cardiac cells' behavior, advocate CPCs as an ideal cell candidate for cardiac regenerative therapies.

Senescent cells have emerged as bona fide drivers of aging and age-related CVD, which suggests strategies aimed at reducing or eliminating senescent cells could be a viable target to treat and prevent CVD[71]. Baker et al. (2016) showed that $p16^{INK4a}$-positive cells contribute to cardiac aging[28]. Hearts of 18 month old INK-ATTAC mice show increased SA-β-Gal activity at the atrial and ventricular surface and also smooth muscle cells in the aortic root wall. These senescent cells decreased following AP20187-treatment[28]. We show for the first time that the genetic and pharmacological approaches used here to reduce senescent cell burden leads to activation of the endogenous regenerative capacity in the aged heart. AP20187-treated INK-ATTAC and D+Q-treated INK-ATTAC and wild-type aged mice had an increased number of CPCs and smaller ventricular myocytes, which were Ki67-positive and EdU-positive, suggesting these myocytes to be immature and newly formed, compared to vehicle-treated mice, which exhibited very rare small myocytes but a greater proportion of hypertrophied myocytes. These findings are in line with those of Baker et al. (2016) who showed that AP-treated INK-ATTAC mice had smaller ventricular cardiomyocytes.

The frequency of resident cardiac stem/progenitor cells in the healthy myocardium of several mammalian species, including human, mouse, rat, and pig, is approximately one per every 1000–2000 myocytes, depending on age[72]. We detected a 16- and 23-fold increase in the number of CPCs following elimination of senescent cells by AP- or D+Q-treatment in the aged mouse heart, respectively. Moreover, ~10% of CPCs after elimination of senescent cells were activated, expressing Ki67. The number of Ki67-positive and EdU-positive cardiomyocytes increased 9- and 4-fold in the aged heart, respectively, following clearance of senescent cells by either D+Q- or AP-treatment. The number of proliferating cardiomyocytes present in the young (3 month old) mouse heart is 0.12±0.03% of total cardiomyocytes, and

the number of proliferating cardiomyocytes present in the old (22 month old) mouse heart is 0.07±0.00% of total cardiomyocytes. Elimination of senescent cells lead to double the amount of the proliferating cardiomyocytes found in a young heart, and triple the number found in an old heart. Therefore, the present data represent a significant and physiologically relevant increase and activation of the resident CPC compartment and cardiomyocyte proliferation following clearance of senescent cells.

Although clearing senescent cells using a genetic approach is not feasible in humans, the senolytic (D+Q) pharmacological approach described here is clearly translatable and can be used to target a fundamental aging mechanism present in most tissues, including the heart. Pharmacologically eliminating senescent cells or inhibiting the production of their SASP has been shown to improve cardiovascular function[16,24], physical function[20], enhance insulin sensitivity[11], prevent age-related bone loss[17], reduce frailty, and increase lifespan and health span[13,20]. Indeed, D+Q administration over 3 months decreased senescent cell markers (TAF+ cells) in the media layer of the aorta from aged (24 months) and hypercholesterolemic mice, which was met with improved vasomotor function[16]. The approaches used in the present study, and thus far in previous studies, target global elimination of all senescent cells. Therefore, whether cell-specific elimination or local delivery of senolytics results in a greater effect in eliminating senescent cells and rejuvenating tissue regenerative capacity is yet to be determined.

Previous work has shown that senescent human primary preadipocytes as well as human umbilical vein endothelial cells (HUVECs) develop a SASP with aging, and conditioned medium from senescent human preadipocytes induced inflammation in healthy adipose tissue and preadipocytes[13]. Recently, the translational potential of using D+Q senolytics on human tissue was reported. Freshly isolated human omental adipose tissue obtained from obese individuals (45.7±8.3 years) contained increased numbers of senescent cells and increased secretion of SASP cytokines. Treatment with D+Q (1μM + 20μM) or vehicle for 48 hours decreased the number of TAF-, $p16^{Ink4a-}$ and SA β-gal-positive cells, and decreased the secretion of key SASP components, PAI-1, GM-CSF, IL-6, IL-8 and MCP-1[20]. Clearance of senescent human CPCs using D+Q in the present study also abrogated the SASP, and the deleterious impact of the SASP on impairing proliferation and inducing senescence to healthy, cycling competent CPCs in a co-culture environment was abolished with D+Q treatment. Thus D+Q can kill human senescent cells, including tissue specific

stem/progenitor cells, and can attenuate the secretion of inflammatory cytokines associated with human age-related frailty[20].

The anthracycline doxorubicin (DOX) is an effective chemotherapeutic agent used to treat pediatric cancers, but is associated with progressive and dose-related cardiotoxicity that may not manifest itself until many years after treatment. In a juvenile mouse model of anthracycline-mediated late onset cardiotoxicity, Huang et al.[73] reported decreased number of CPCs, which showed impaired differentiation into the cardiomyocyte and endothelial lineage in the MI-border zone of animals that had been exposed to DOX as pups. DOX treatment also led to significantly less CPCs in the juvenile heart, and those that remained showed decreased proliferation and increased expression of $p16^{Ink4a}$ at 12 days of age. Moreover, 72 hours of DOX treatment (10nM or 100nM) on isolated CPCs in vitro led to attenuated proliferation, reduced telomerase activity and induced expression of $p16^{Ink4a56}$. The present study showed that DOX treatment (0.2µM) for 24 hours to CPCs induced $p16^{Ink4a}$, γH2AX and SA β-gal expression evidencing that CPCs had become senescent following DOX exposure. Future work should focus on the regenerative capacity of the heart following DOX exposure and whether senolytics could be used following DOX treatment and in later life to eliminate senescent cells and rejuvenate CPCs and the regenerative potential of the heart.

In conclusion, the present work demonstrates that in the aged and failing human heart a large majority of its resident CPCs are senescent and dysfunctional exhibiting impaired proliferation, clonogenicity and differentiation potential. Importantly, senescent CPCs have a SASP that can negatively affect cell behavior and render neighbouring cells into senescence. Elimination of senescent human CPCs *in vitro* attenuates the SASP and its deleterious effect. Senescent human CPCs are unable to contribute to repair and regeneration of the MI heart, in contrast to cycling competent, healthy human CPCs, which facilitate reparative processes in the damaged myocardium restoring LV function. The present work demonstrates approaches that eliminate senescent cells may be useful for treating age-related cardiac deterioration and rejuvenating the regenerative capacity of the aged heart. The next steps would be to determine whether senolytic approaches could be used in conjunction with cell therapy interventions to improve the environment (the ‘soil’) the cells (the ‘seeds’) are being transplanted into and the intrinsic reparative mechanistic processes that are compromised with age. Indeed, targeting senescent cells could also impact the potency of resident stem/progenitor populations in other aged organs. The present findings provide new insights

into therapies that target senescent cells to prevent an age-related loss of regenerative capacity.

References

1. North BJ and Sinclair DA. The Intersection Between Aging and Cardiovascular Disease. *Circ Res*. 2012;110:1097.
2. Dutta D, Calvani R, Bernabei R, Leeuwenburgh C and Marzetti E. Contribution of Impaired Mitochondrial Autophagy to Cardiac Aging. *Circ Res*. 2012;110:1125.
3. LeBrasseur NK, Tchkonia T, Kirkland JL. Cellular Senescence and the Biology of Aging, Disease, and Frailty. *Nestle Nutr Inst Workshop Ser*. 2015;83:11-8.
4. Varela I, Cadiñanos J, Pendás AM, Gutiérrez-Fernández A, Folgueras AR, Sánchez LM, Zhou Z, Rodríguez FJ, Stewart CL, Vega JA, Tryggvason K, Freije JMP and López-Otín C. Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation. *Nature*. 2005;437:564.
5. Zhu Y, Armstrong JL, Tchkonia T, Kirkland JL. Cellular senescence and the senescent secretory phenotype in age-related chronic diseases. *Curr Opin Clin Nutr Metab Care*. 2014;17:324-328.
6. Kuilman T and Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. *Nature Reviews Cancer*. 2009;9:81.
7. Kuilman T, Michaloglou C, Mooi WJ and Peeper DS. The essence of senescence. *Genes Dev*. 2010;24:2463-79.
8. Rodier F and Campisi J. Four faces of cellular senescence. *The Journal of Cell Biology*. 2011;192:547.
9. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL and van Deursen JM. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature*. 2011;479:232.
10. Tchkonia T, Zhu Y, van Deursen J, Campisi J and Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. *The Journal of Clinical Investigation*. 2013;123:966-972.
11. Xu M, Palmer AK, Ding H, Weivoda MM, Pirtskhalava T, White TA, Sepe A, Johnson KO, Stout MB, Giorgadze N, Jensen MD, LeBrasseur NK, Tchkonia T and Kirkland JL. Targeting senescent cells enhances adipogenesis and metabolic function in old age. *eLife*. 2015;4:e12997.
12. Jeon OH, Kim C, Laberge R-M, Demaria M, Rathod S, Vasserot AP, Chung JW, Kim DH, Poon Y, David N, Baker DJ, van Deursen JM, Campisi J and Elisseeff JH. Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nature Medicine*. 2017;23:775.
13. Xu M, Tchkonia T, Ding H, Ogrodnik M, Lubbers ER, Pirtskhalava T, White TA, Johnson KO, Stout MB, Mezera V, Giorgadze N, Jensen MD, LeBrasseur NK and Kirkland JL. JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age. *Proceedings of the National Academy of Sciences*. 2015;112:E6301-E6310.
14. Herbig U, Ferreira M, Condel L, Carey D and Sedivy JM. Cellular Senescence in Aging Primates. *Science*. 2006;311:1257.
15. Pajvani UB, Trujillo ME, Combs TP, Iyengar P, Jelicks L, Roth KA, Kitsis RN, Scherer PE. Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy. *Nat Med*. 2005;11:797-803.

16. Roos CM, Zhang B, Palmer AK et al. Chronic senolytic treatment alleviates established vasomotor dysfunction in aged or atherosclerotic mice. *Aging Cell*. 2016;15:973-977.
17. Farr JN, Xu M, Weivoda MM et al. Targeting cellular senescence prevents age-related bone loss in mice. *Nat Med*. 2017;23:1072-1079.
18. Ogrodnik M, Miwa S, Tchkonia T et al. Cellular senescence drives age-dependent hepatic steatosis. *Nature Communications*. 2017;8:15691.
19. Schafer MJ, White TA, Iijima K et al. Cellular senescence mediates fibrotic pulmonary disease. *Nature Communications*. 2017;8:14532.
20. Xu M, Pirtskhalava T, Farr, JN et al. Senolytics improve physical function and increase lifespan in old age. *Nature Med*. 2018;24:1246 -1256.
21. Kirkland JL and Tchkonia T. Clinical strategies and animal models for developing senolytic agents. *Experimental Gerontology*. 2015;68:19-25.
22. Kirkland JL, Tchkonia T, Zhu Y, Niedernhofer LJ and Robbins PD. The Clinical Potential of Senolytic Drugs. *Journal of the American Geriatrics Society*. 2017;65:2297-2301.
23. Kirkland JL and Tchkonia T. Cellular Senescence: A Translational Perspective. *EBioMedicine*. 2017;21:21-28.
24. Zhu Y, Tchkonia T, Pirtskhalava T et al. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging Cell*. 2015;14:644-658.
25. Lehmann M, Korfei M, Mutze K, Klee S, Skronska-Wasek W, Alsafadi HN, Ota C, Costa R, Schiller HB, Lindner M, Wagner DE, Günther A and Königshoff M. Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis. *European Respiratory Journal*. 2017;50: pii: 1602367.
26. Zhu Y, Tchkonia T, Fuhrmann-Stroissnigg H, Dai HM, Ling YY, Stout MB, Pirtskhalava T, Giorgadze N, Johnson KO, Giles CB, Wren JD, Niedernhofer LJ, Robbins PD and Kirkland JL. Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging Cell*. 2016;15:428-435.
27. Fuhrmann-Stroissnigg H, Ling YY, Zhao J et al. Identification of HSP90 inhibitors as a novel class of senolytics. *Nature Communications*. 2017;8:422.
28. Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, A. Saltness R, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaie K, Miller JD and van Deursen JM. Naturally occurring p16Ink4a-positive cells shorten healthy lifespan. *Nature.* 2016;530:184-189.
29. Jeyapalan JC and Sedivy JM. Cellular senescence and organismal aging. *Mechanisms of Ageing and Development.* 2008;129:467-474.
30. Sharpless NE and DePinho RA. How stem cells age and why this makes us grow old. *Nature Reviews Molecular Cell Biology*. 2007;8:703.
31. Rossi DJ, Jamieson CHM and Weissman IL. Stems Cells and the Pathways to Aging and Cancer. *Cell*. 2008;132:681-696.
32. Beltrami AP, Cesselli D and Beltrami CA. At the stem of youth and health. *Pharmacology & Therapeutics*. 2011;129:3-20.
33. Ellison-Hughes GM and Lewis FC. Progenitor Cells from the Adult Heart. In: M. Ieda and W.-H. Zimmermann, eds. *Cardiac Regeneration* Cham: Springer International Publishing; 2017:19-39.
34. Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L and Sharpless NE. Ink4a/Arf expression is a biomarker of aging. *The Journal of Clinical Investigation*. 2004;114:1299-1307.
35. Martin K, Kirkwood TBL and Potten CS. Age Changes in Stem Cells of Murine Small Intestinal Crypts. *Experimental Cell Research*. 1998;241:316-323.

36. Flores I, Cayuela ML and Blasco MA. Effects of Telomerase and Telomere Length on Epidermal Stem Cell Behavior. *Science*. 2005;309:1253.
37. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE and Scadden DT. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature*. 2006;443:421.
38. Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE and Morrison SJ. Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature*. 2006;443:448.
39. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B and Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*. 2003;114:763-76.
40. Smith AJ, Lewis FC, Aquila I, Waring CD, Nocera A, Agosti V, Nadal-Ginard B, Torella D, Ellison GM. Isolation and characterization of resident endogenous c-Kit+ cardiac stem cells from the adult mouse and rat heart. *Nat Protoc*. 2014;9:1662-81.
41. Ellison G M, Vicinanza C, Smith Andrew J, Aquila I, Leone A, Waring Cheryl D, Henning Beverley J, Stirparo Giuliano G, Papait R, Scarfò M, Agosti V, Viglietto G, Condorelli G, Indolfi C, Ottolenghi S, Torella D and Nadal-Ginard B. Adult c-kitpos Cardiac Stem Cells Are Necessary and Sufficient for Functional Cardiac Regeneration and Repair. *Cell*. 2013;154:827-842.
42. Vicinanza C, Aquila I, Scalise M, Cristiano F, Marino F, Cianflone E, Mancuso T, Marotta P, Sacco W, Lewis FC, Couch L, Shone V, Gritti G, Torella A, Smith AJ, Terracciano CMN, Britti D, Veltri P, Indolfi C, Nadal-Ginard B, Ellison-Hughes GM and Torella D. Adult Cardiac Stem Cells are Multipotent and Robustly Myogenic: C-kit Expression is Necessary but not Sufficient for their Identification. *Cell Death And Differentiation*. 2017;24:2101.
43. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marbán E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature*. 2014;509:337-41.
44. Sultana N, Zhang L, Yan J, Chen J, Cai W, Razzaque S, Jeong D, Sheng W, Bu L, Xu M, Huang GY, Hajjar RJ, Zhou B, Moon A, Cai CL. Resident c-kit(+) cells in the heart are not cardiac stem cells. *Nat Commun*. 2015;6:8701.
45. Vicinanza C, Aquila I, Cianflone E, Scalise M, Marino F, Mancuso T, Fumagalli F, Giovannone ED, Cristiano F, Iaccino E, Marotta P, Torella A, Latini R, Agosti V, Veltri P, Urbanek K, Isidori AM, Saur D, Indolfi C, Nadal-Ginard B, Torella D. Kitcre knock-in mice fail to fate-map cardiac stem cells. *Nature*. 2018;555:E1-E5.
46. Hariharan N and Sussman MA. Cardiac Aging — Getting to the Stem of the Problem. *Journal of Molecular and Cellular Cardiology*. 2015;83:32-36.
47. Chimenti C, Kajstura J, Torella D, Urbanek K, Heleniak H, Colussi C, Di Meglio F, Nadal-Ginard B, Frustaci A, Leri A, Maseri A and Anversa P. Senescence and Death of Primitive Cells and Myocytes Lead to Premature Cardiac Aging and Heart Failure. *Circulation Research*. 2003;93:604.
48. Cesselli D, Beltrami AP, D'Aurizio F, Marcon P, Bergamin N, Toffoletto B, Pandolfi M, Puppato E, Marino L, Signore S, Livi U, Verardo R, Piazza S, Marchionni L, Fiorini C, Schneider C, Hosoda T, Rota M, Kajstura J, Anversa P, Beltrami CA and Leri A. Effects of Age and Heart Failure on Human Cardiac Stem Cell Function. *The American Journal of Pathology*. 2011;179:349-366.
49. Castaldi A, Dodia RM, Orogo AM, Zambrano CM, Najor RH, Gustafsson ÅB, Heller Brown J, Purcell NH. Decline in cellular function of aged mouse c-kit+ cardiac progenitor cells. *J Physiol*. 2017;595:6249-6262.

50. Torella D, Rota M, Nurzynska D, Musso E, Monsen A, Shiraishi I, Zias E, Walsh K, Rosenzweig A, Sussman MA, Urbanek K, Nadal-Ginard B, Kajstura J, Anversa P and Leri A. Cardiac Stem Cell and Myocyte Aging, Heart Failure, and Insulin-Like Growth Factor-1 Overexpression. *Circulation Research*. 2004;94:514.
51. Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini F, Bolli R, Leri A, Kajstura J and Anversa P. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. *PNAS*. 2005;102:8692-8697.
52. Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME, Esposito G, Vitale S, Urbanek K, Casarsa C, Giorgio M, Lüscher TF, Pelicci PG, Anversa P, Leri A and Kajstura J. Diabetes Promotes Cardiac Stem Cell Aging and Heart Failure, Which Are Prevented by Deletion of the p66 Gene. *Circulation Research*. 2006;99:42.
53. Gonzalez A, Rota M, Nurzynska D, Misao Y, Tillmanns J, Ojaimi C, Padin-Iruegas ME, Müller P, Esposito G, Bearzi C, Vitale S, Dawn B, Sanganalmath SK, Baker M, Hintze TH, Bolli R, Urbanek K, Hosoda T, Anversa P, Kajstura J and Leri A. Activation of Cardiac Progenitor Cells Reverses the Failing Heart Senescent Phenotype and Prolongs Lifespan. *Circulation Research*. 2008;102:597.
54. Kajstura J, Gurusamy N, Ogórek B, Goichberg P, Clavo-Rondon C, Hosoda T, Amario D, Bardelli S, Beltrami AP, Cesselli D, Bussani R, del Monte F, Quaini F, Rota M, Beltrami CA, Buchholz BA, Leri A and Anversa P. Myocyte Turnover in the Aging Human Heart. *Circulation Research*. 2010;107:1374.
55. Hariharan N, Quijada P, Mohsin S, Joyo A, Samse K, Monsanto M, De La Torre A, Avitabile D, Ormachea L, McGregor MJ, Tsai EJ and Sussman MA. Nucleostemin Rejuvenates Cardiac Progenitor Cells and Antagonizes Myocardial Aging. *Journal of the American College of Cardiology*. 2015;65:133-147.
56. Canela A, Vera E, Klatt P and Blasco MA. High-throughput telomere length quantification by FISH and its application to human population studies. *PNAS*. 2007;104:5300-5305.
57. Kirkland, J.L., Hollenberg, C.H., Gillon, W.S.: Age, anatomic site, and the replication and differentiation of adipocyte precursors. *Am. J. Physiol*. 1990;258:C206-C210.
58. Zhu Y, Doornebal EJ, Pirtskhalava T, Giorgadze N, Wentworth M, Fuhrmann-Stroissnigg H, Niedernhofer LJ, Robbins PD, Tchkonia T and Kirkland JL. New agents that target senescent cells: the flavone, fisetin, and the BCL-XL inhibitors, A1331852 and A1155463. *Aging*. 2017;9:955-963.
59. Kanisicak O, Vagnozzi RJ and Molkentin JD. Identity Crisis for Regenerative Cardiac cKit+ Cells. *Circulation Research*. 2017;121:1130.
60. Broughton KM, Wang BJ, Firouzi F, Khalafalla F, Dimmeler S, Fernandez-Aviles F, Sussman MA. Mechanisms of Cardiac Repair and Regeneration. *Circ Res*. 2018;122:1151-1163.
61. Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM. Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. *Eur Heart J*. 2008;29:1807-18.
62. Tang XL, Li Q, Rokosh G, Sanganalmath SK, Chen N, Ou Q, Stowers H, Hunt G and Bolli R. Long-Term Outcome of Administration of c-kit(POS) Cardiac Progenitor Cells After Acute Myocardial Infarction: Transplanted Cells Do not Become Cardiomyocytes, but Structural and Functional Improvement and Proliferation of Endogenous Cells Persist for at Least One Year. *Circ Res*. 2016;118:1091-105.

63. Bolli R, Chugh AR, D'Amario D et al. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. *Lancet*. 2011;378:1847-57.
64. Bearzi C, Rota M, Hosoda T et al. Human cardiac stem cells. *PNAS*. 2007;104:14068-73.
65. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res*. 2004;95:911-21.
66. Williams AR, Hatzistergos KE, Addicott B, McCall F, Carvalho D, Suncion V, Morales AR, Da Silva J, Sussman MA, Heldman AW, Hare JM. Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial infarction. *Circulation*. 2013;127:213-23.
67. Avolio E, Meloni M, Spencer HL et al. Combined intramyocardial delivery of human pericytes and cardiac stem cells additively improves the healing of mouse infarcted hearts through stimulation of vascular and muscular repair. *Circ Res*. 2015;116:e81-94.
68. Loffredo FS, Steinhauser ML, Gannon J, Lee RT. Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. *Cell Stem Cell*. 2011;8:389-98.
69. Ellison GM, Nadal-Ginard B, Torella D. Optimizing cardiac repair and regeneration through activation of the endogenous cardiac stem cell compartment. *J Cardiovasc Transl Res*. 2012;5:667-77.
70. Li TS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, Matsushita N, Blusztajn A, Terrovitis J, Kusuoka H, et al. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. *J Am Coll Cardiol*. 2012;59:942–953.
71. Childs BG, Li H and van Deursen JM. Senescent cells: a therapeutic target for cardiovascular disease. *The Journal of Clinical Investigation*. 2018;128:1217-1228.
72. Torella D, Ellison GM, Karakikes I, Nadal-Ginard B. Resident cardiac stem cells. *Cell Mol Life Sci*. 2007;64:661-73.
73. Huang C, Zhang X, Ramil JM, Rikka S, Kim L, Lee Y, Gude NA, Thistlethwaite PA, Sussman MA, Gottlieb RA and Gustafsson ÅB. Juvenile Exposure to Anthracyclines Impairs Cardiac Progenitor Cell Function and Vascularization Resulting in Greater Susceptibility to Stress-Induced Myocardial Injury in Adult Mice. *Circulation*. 2010;121:675.

ACKNOWLEDGEMENTS

We acknowledge Thomas Theologou and Mark Field from Liverpool Heart & Chest Hospital, Institute for Cardiovascular Medicine and Science (ICMS), Liverpool, UK for giving us some myocardial samples. We thank Carl Hobbs (King's College London) for tissue processing, histology, and microscopic analysis assistance. Confocal microscopy was carried out in the Nikon Imaging Centre, King's College London.

FUNDING

This work was supported by British Heart Foundation project grant PG/14/11/30657 (GME-H), NIH grant AG13925 (JLK), the Connor Group (JLK), Robert J. and Theresa W. Ryan (JLK), Robert and Arlene Kogod (JLK), the Noaber Foundation (JLK), and a Glenn/American Federation for Aging Research (AFAR) BIG Award (J.L.K.).

AUTHOR CONTRIBUTIONS

F.C.L performed the isolation and characterisation of CPCs from subjects' myocardial samples, functional CPC assays, expression and secretion of SASP factors, senolytic clearance of senescent cells *in vitro*, immunohistochemical and confocal microscopy analysis of mouse cardiac sections. P.J.R performed the myocardial infarction-regeneration mouse surgery model and echocardiography. E.D-V performed the characterisation of senescent-CPCs for SASP factors and the effect of SASP factors on CPC fate. T.S.T performed isolation and characterisation of CPCs for $p16^{Ink4a}$ expression. B.J.C analysed mouse cardiac sections using immunohistochemical and confocal microscopy. J.E.C oversaw and assisted with the myocardial infarction-regeneration mouse surgery model and echocardiography. P.P.P and W.A provided myocardial samples and contributed to experimental design. D.T contributed to experimental design. T.T and J.L.K contributed the senolytic drugs, performed the genetic and pharmacological elimination of senescent cells in mice, contributed to experimental design, and directed the elimination of senescent cells aspect of the study. L.P carried out the EdU INK-ATTAC experiments. G.M.E-H directed and supervised all aspects of the study, and wrote the manuscript with input from F.C.L, P.J.R, D.T, T.T., and J.L.K. All authors reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

Conflict of Interest: J.L.K., T.T., and Mayo Clinic have a financial interest related to this research. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic conflict of interest policies.

List of Supplementary Materials

Materials and Methods
Tables S1 – S3
Figures S1 – S10
References

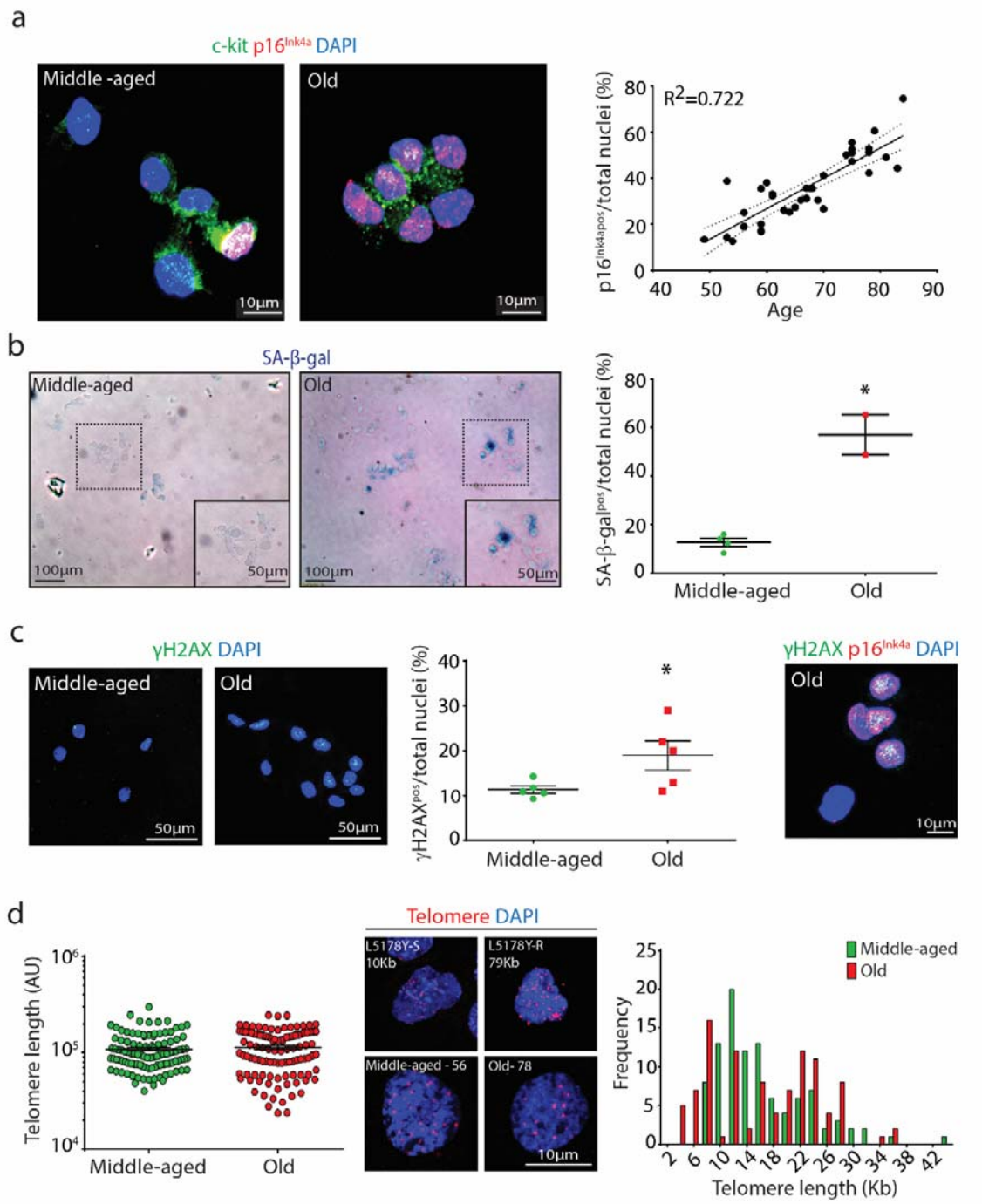

Figure. 1. Over half of CPCs in the aged human heart are senescent. (**a**) Representative immunofluorescence images and quantification of freshly isolated c-kit^{pos} CPCs (green), p16^{INK4A}-positive (red) expression, each data point represents the mean number of p16^{INK4A} CPCs per total nuclei for each individual donor (R^2=0.722, n=32). (**b**) Representative immunofluorescence images and quantification of freshly isolated c-kit^{pos} CPCs stained for SA-β-gal (blue), each data point represents the mean number of SA-β-gal^{pos} cells per total nuclei for each individual donor (**P*=0.0014, n=4 middle-aged, n=2 old). (**c**) Representative immunofluorescence images and quantification of freshly isolated c-kit^{pos} CPCs stained for γ-

H2AX (green), each data point represents the mean percentage of γ-H2AX^{pos} cells per total nuclei for each individual donor (**P*=0.0264, n=5). Nuclei stained in blue by DAPI. (**d**) Representative immunofluorescence images and quantification of freshly isolated c-kit^{pos} CPC Q-FISH telomere staining, each data point represents the telomere signal intensity (red) per total nuclei (DAPI, blue) calculated from 20 cells for each individual donor (n=5). Mouse cell lines with known telomere length, L5178Y-S (10 Kb) and L5178Y-R (79 Kb), were used to determine telomere length histograms (n=5). In each scatter graph, data points represent individual measurements and error bars represent SEM. Statistical analysis by Student's t-test.

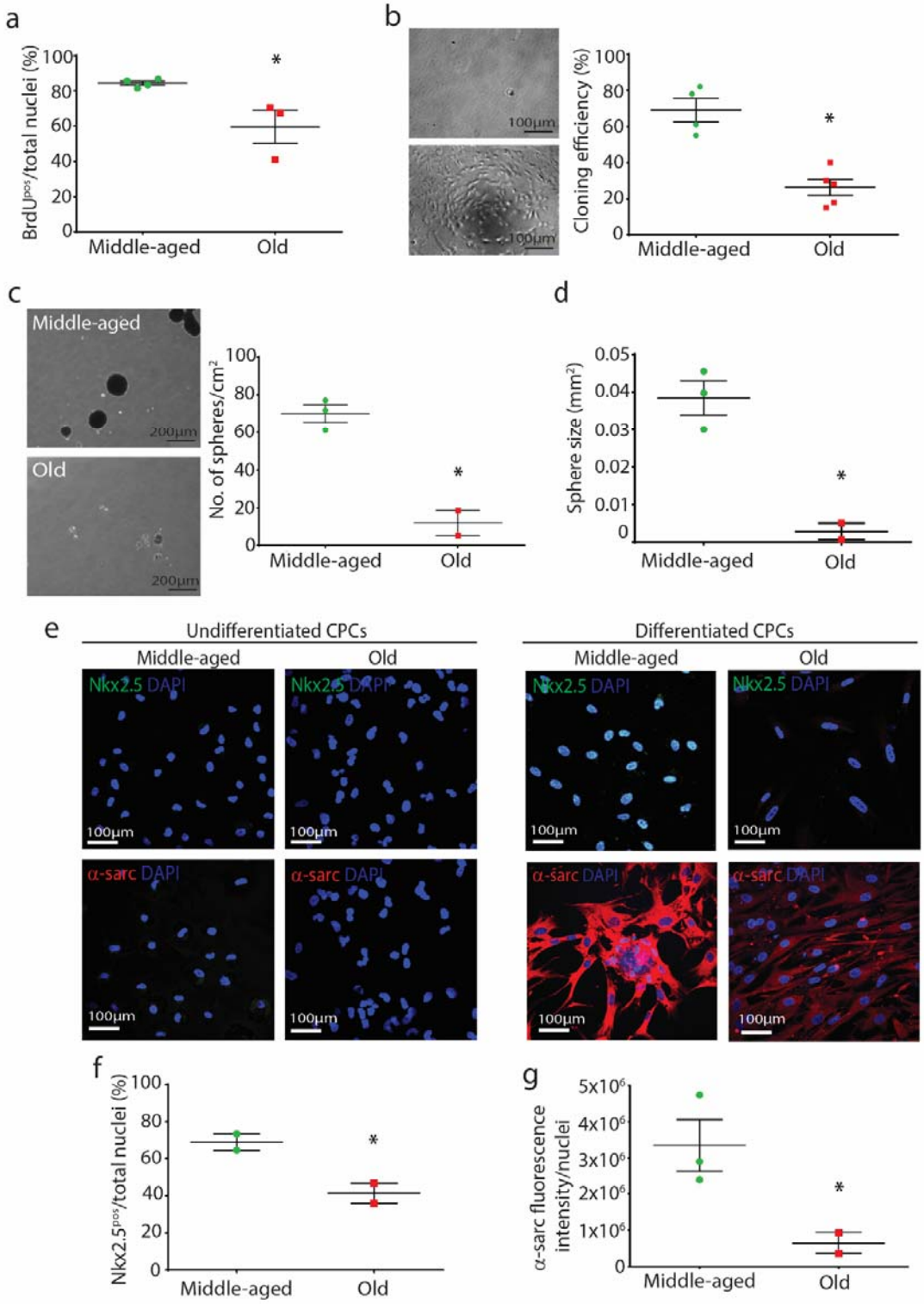

Figure. 2. CPCs isolated from aged hearts exhibit diminished proliferation, clonogenicity, and cardiomyocyte differentiation potential (**a**) CPC proliferation was determined using a BrdU incorporation assay, each data point represents the mean number of $BrdU^{pos}$ cells per total nuclei for each individual donor (**P*=0.0266, n=4 middle-aged, n=3 old). (**b**) Representative micrographs and quantification of a single CPC (top) and CPC-

derived clone (bottom), each data point represents the mean cloning efficiency for each individual donor (**P*=0.0008, n=4 middle-aged, n=5 old). (**c**) Representative micrograph and quantification of CPC-derived spheres from middle-aged (top) and old CPCs (bottom), each data point represents the mean number of spheres for each individual donor (**P*=0.0051). (**d**) Quantification of the mean sphere size per donor (**P*=0.01, n=3 middle-aged, n=2 old). (**e**) Representative immunofluorescence images of old and middle-aged donors' CPCs stained α-sarcomeric actin (red), Nkx2.5 (green) after 14 days of differentiation. Nuclei stained in blue by DAPI. (**f**) Quantification of Nkx2.5^{pos} cells per total nuclei for each individual donor (**P*=0.0295, n=2). (**g**) Quantification of α-sarcomeric actin fluorescence intensity per nuclei for each individual donor (**P*=0.023, n=3 middle-aged, n=2 old). In each scatter graph, data points represent individual measurements and error bars represent SEM. Statistical analysis by Student's t-test.

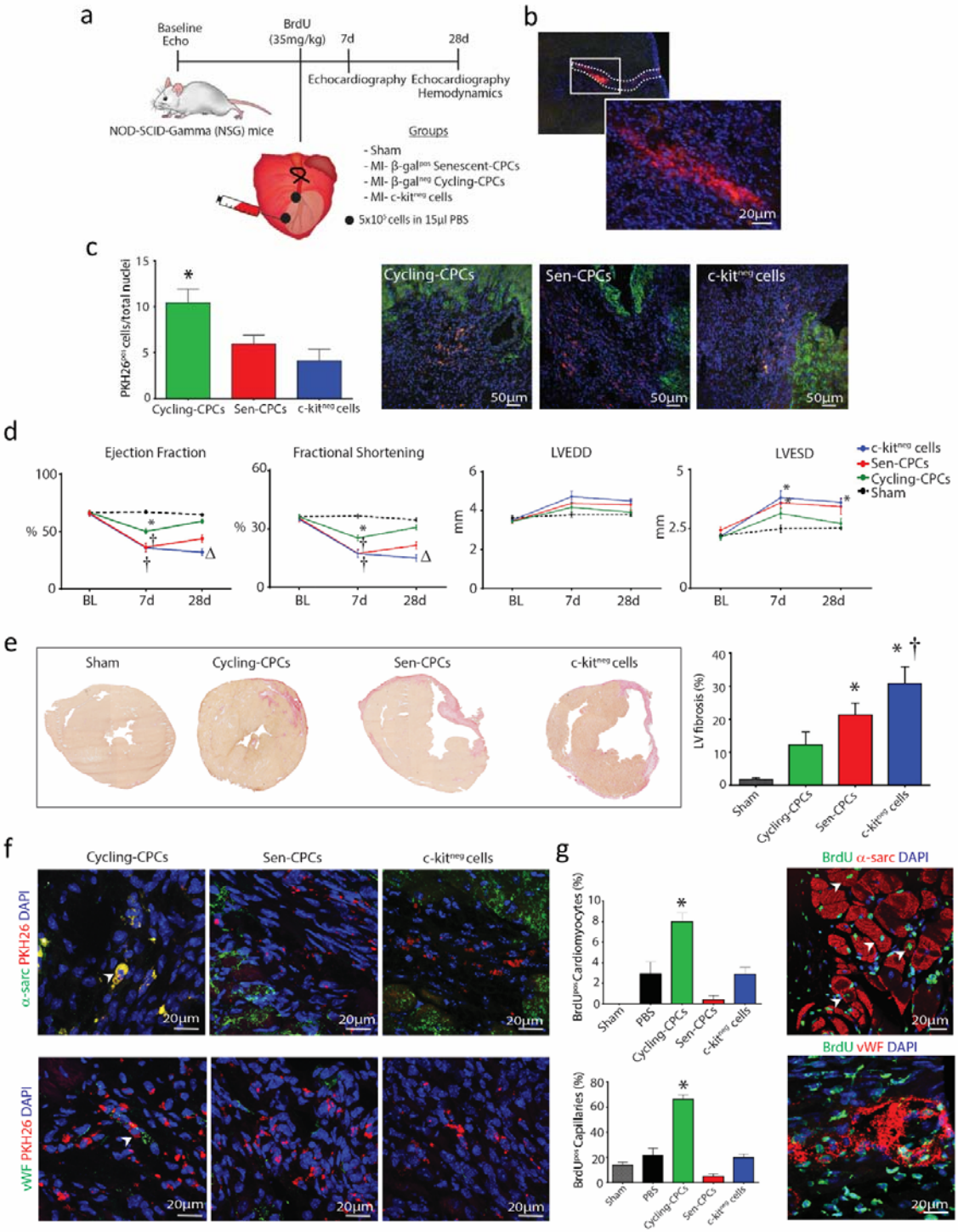

Figure. 3. Aged-senescent CPCs show decreased reparative potential (**a**) Experimental design for Nod-Scid-Gamma (NSG) mice left anterior descending (LAD) coronary artery ligation and treatment regime. (**b**) Representative immunohistological images showing PKH26 labelled CPCs in the myocardium 4 days post-MI. (**c**) Representative immunohistological images and quantification of engraftment rate of PKH26^{pos} cells (red) within the myocardium (α-sarcomeric actin; green) 28 days post-MI (**P*=0.015 *vs.* c-kit^{neg} cells, n=4-5). (**d**) Echocardiography measurements of NSG mice at 7 and 28 days after MI

and cell injection, compared to baseline (BL) showing LV ejection fraction (EF), fractional shortening (FS), left ventricular end-diastolic diameter (LVEDD), and left ventricular end-systolic diameter (LVESD) for Sham (black), MI-Cycling-competent CPCs (green), MI-Sen CPCs (red) and MI-c-kit^{neg} cells (blue) (**P*<0.05 *vs*. Sham, †*P*<0.05 *vs*. MI-cycling-competent CPCs, Δ*P*<0.05 *vs*. MI-Sen CPCs, n=5-7). (**e**) Representative micrographs and quantification of HVG fibrosis staining in treated hearts (**P*=0.0105 Sen CPCs *vs*. Sham, (**P*=0.0003 c-kit^{neg} cells *vs*. Sham, †*P*=0.0112 *vs*. MI-cycling-competent CPCs, n=5-6). (**f**) Representative immunohistological images showing PKH26^{pos} cycling-competent CPCs co-expression of α-sarcomeric actinin (green) and vWF (green) (arrowheads) 28 days post-MI. (**g**) Representative immunohistological images and quantification of newly formed BrdU^{pos} (green) α-sarcomeric actinin^{pos} cardiomyocytes (red) and BrdU^{pos} (green) vWF^{pos} capillaries (red) in treated hearts (**P*<0.05 *vs*. all treatment groups, n=5-7). Nuclei stained in blue by DAPI. All data are means ± SEM. Statistical analysis by one-way ANOVA with Tukey's multiple comparison test.

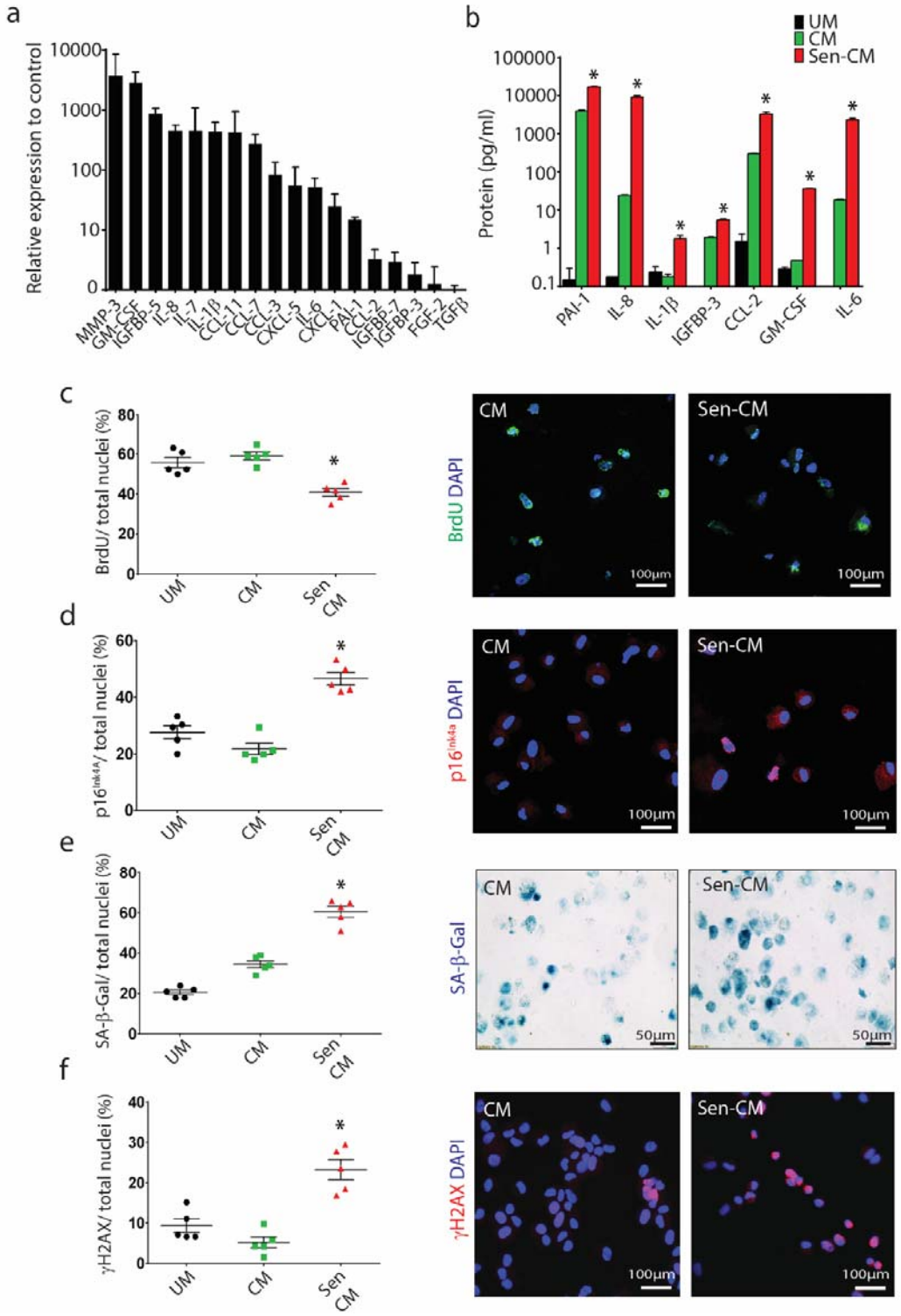

Figure. 4. Aged-senescent CPCs have a SASP (**a**) Gene expression of Senescent-CPC SASP factors relative to cycling-competent CPCs (control). (**b**) SASP factor protein levels quantified by Luminex analysis of unconditioned media (UM), Cycling-competent CPC (CM) and Senescent-CPC (Sen CM) conditioned media, PAI-1 (**P*=0.0006), IL-8 (**P*=0.003), IL-1ß (**P*=0.0283), IGFBP-3 (**P*=0.0011), CCL2 (**P*=0.0116), GM-CSF

(*P=0.0001), IL-6 (*P=0.0030) Sen CM *vs*. CM. Conditioned media applied to cycling-competent CPCs and the following analyses performed; (**c**) Quantification of $BrdU^{pos}$ (green) CPCs per total nuclei and representative immunocytochemical images (*P=0.001 Sen CM *vs*. UM, *P=0.0002 Sen CM *vs*. CM). (**d**) $p16^{INK4A\text{-}pos}$ (red) CPCs per total nuclei (*P<0.0001 Sen CM *vs*. UM, *P<0.0001 Sen CM *vs*. CM). (**e**) SA-β-gal^{pos} (blue) CPCs per total nuclei (*P<0.0001 Sen CM *vs*. UM, *P<0.0001 Sen CM *vs*. CM). (**f**) $\gamma H2AX^{pos}$ (red) CPCs per total nuclei (*P=0.0006 Sen CM *vs*. UM, *P<0.0001 Sen CM *vs*. CM). Nuclei stained in blue by DAPI. In each scatter graph, data points represent individual measurements and error bars represent SEM. Statistical analysis by one-way ANOVA with Tukey's multiple comparison test.

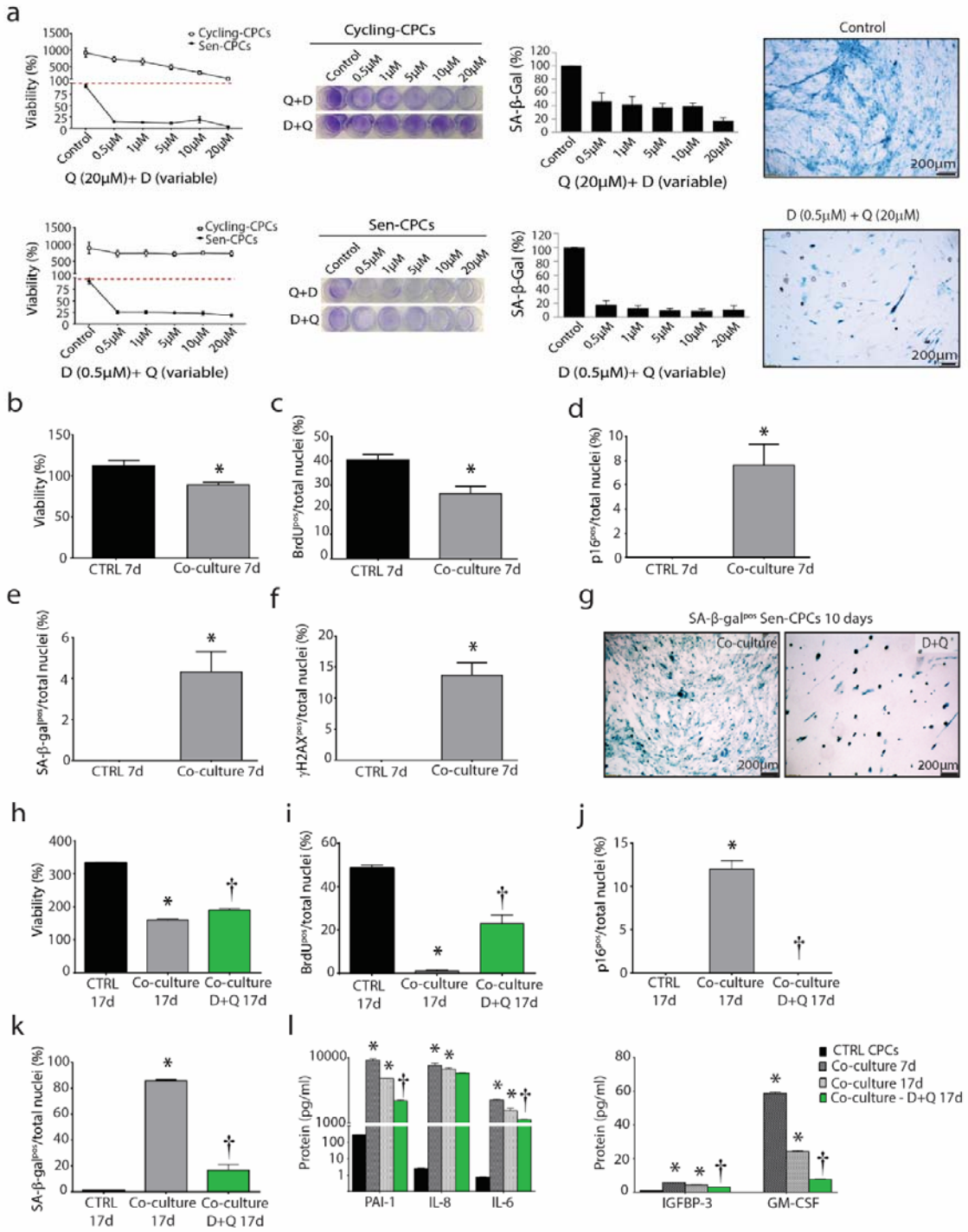

Figure. 5. Senolytic clearance abrogates the SASP. (**a**) Quantification of % viability and % SA-β-gal staining of cycling-competent CPCs and Senescent-CPCs exposed to various concentrations of D+Q for 3 days. Representative crystal violet (cell viability) and SA-β-gal (cell senescence) staining. (**b-f**) Quantification of 7 days of co-culture of cycling-competent CPCs with Senescent-CPCs for (**b**) viability (**P*=0.0068); (**c**) BrdU incorporation (**P*=0.0056); (**d**) $p16^{INK4A}$ (**P*=0.0025); (**e**) SA-β-gal (**P*=0.0025) and (**f**) γH2AX (**P*=0.0002). CTRL is cycling-competent CPCs alone. Data are means ± SEM. Statistical analysis by Student's t-test. (**g**) Representative SA-β-gal staining after clearance of Senescent-CPCs from co-culture by D+Q treatment. (**h-k**) Quantification of 17 days of co-

culture of cycling-competent CPCs with Senescent-CPCs or co-culture with D+Q treatment for (**h**) viability (**P*<0.0001 *vs*. CTRL 17d, †*P*=0.0001 *vs*. co-culture 17d); (**i**) BrdU incorporation (**P*<0.0001 *vs*. CTRL 17d, †*P*<0.0001 *vs*. co-culture 17d); **(j)** $p16^{INK4A}$ (**P*<0.0001 *vs*. CTRL 17d, †*P*<0.0001 *vs*. co-culture 17d) and (**k**) SA-β-gal (**P*<0.0001 *vs*. CTRL 17d, †*P*<0.0001 *vs*. co-culture 17d). CTRL is cycling-competent CPCs alone. Data are means ± SEM. Statistical analysis by a one-way ANOVA with Tukey's multiple comparison test. (**l**) SASP factor protein levels quantified by Luminex analysis from each treatment condition; PAI-1 (**P*<0.0001 *vs*. CTRL, †*P*=0.0003 *vs*. co-culture 17d); IL-8 (**P*<0.0001 *vs*. CTRL); IL-6 (**P*<0.0001 *vs*. CTRL); IGFBP-3 (**P*<0.0001 *vs*. CTRL, †*P*<0.0001 *vs*. co-culture 17d); GM-CSF (**P*<0.0001 *vs*. CTRL, †*P*<0.0001 *vs*. co-culture 17d). Data are means ± SEM. Statistical analysis was performed by a one-way ANOVA.

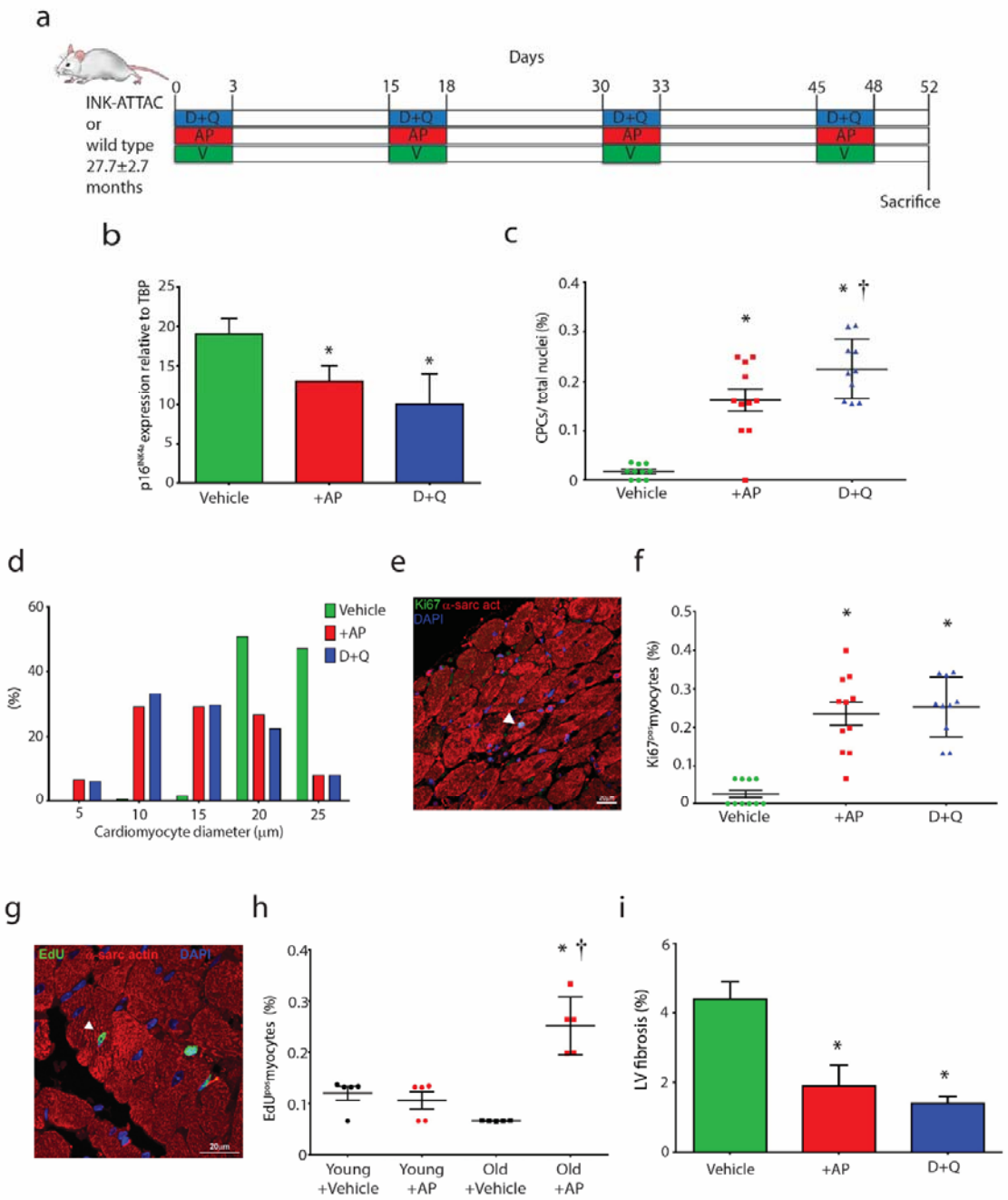

Figure. 6. Clearance of senescent cells leads to stimulation of CPCs and new myocyte formation in the aged heart (**a**) Experimental design for senescent cell clearance *in vivo* experiments. (**b**) Total $p16^{Ink4a}$ gene expression relative to TBP in aged treated hearts (**P*=0.0039, AP *vs*. Vehicle; *P=0.001, D+Q *vs*. Vehicle; n=5). (**c**) Quantification of CPCs (**P*<0.0001 *vs*. Vehicle, †*P*=0.0453 vs. AP, n=10-11). Data points represent individual mice and error bars represent SD. (**d**) Frequency distribution histogram of cardiomyocyte diameter (n=6). (**e**) A Ki67-positive (green) cardiomyocyte (red) in the LV of a 32 month INK-ATTAC D+Q-treated mouse. Nuclei are stained by DAPI in blue. (**f**) Quantification of $Ki67^{pos}$ myocytes per total myocytes (**P*=<0.0001 AP *vs*. Vehicle, **P*=<0.0001, D+Q *vs*. Vehicle, n=10). (**g**) An EdU-positive (green) cardiomyocyte (red) in the LV of a 22 month INK-ATTAC AP-treated mouse. Nuclei are stained by DAPI in blue. (**h**) Quantification of EdU^{pos} myocytes per total myocytes (**P*==<0.0001 Old+AP *vs*. Old+Vehicle, †*P*=<0.0001

Old+AP *vs*. Young+AP, n=5). (**i**) Quantification of LV fibrosis (**P*=0.0210 AP *vs*. Vehicle, **P*=0.0092 D+Q vs. Vehicle, n=3). Data are Mean ± SD. Statistical analysis by one-way ANOVA with Tukey's multiple comparison test.