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The Achilles' Heel of Senescent Cells: From Transcriptome to Senolytic Drugs

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Summary

The healthspan of mice is enhanced by killing senescent cells using a transgenic suicide gene. Achieving the same using small molecules would have a tremendous impact on quality of life and burden of age-related chronic diseases. Here, we describe the rationale for identification and validation of a new class of drugs termed senolytics, which selectively kill senescent cells.

By transcript analysis, we discovered increased expression of pro-survival networks in senescent cells, consistent with their established resistance to apoptosis. Using siRNA to silence expression of key nodes of this network, including ephrins (EFNB1 or 3), PI3K δ , p21, BCL-xL, or plasminogen activated inhibitor-2, killed senescent cells, but not proliferating or quiescent, differentiated cells. Drugs targeting these factors selectively killed senescent cells.

Dasatinib eliminated senescent human fat cell progenitors, while quercetin was more effective against senescent human endothelial cells and mouse BM-MSCs. The combination of dasatinib and quercetin was effective in eliminating senescent MEFs. *In vivo*, this combination reduced senescent cell burden in chronologically aged, radiation-exposed, and progeroid *Ercc1*^{- Δ} mice.

In old mice, cardiac function and carotid vascular reactivity were improved 5 days after a single dose. Following irradiation of one limb in mice, a single dose led to improved exercise capacity for at least 7 months following drug treatment. Periodic drug administration extended healthspan in *Ercc1*^{- Δ} mice, delaying age-related symptoms and pathology, osteoporosis and loss of

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intervertebral disc proteoglycans. These results demonstrate the feasibility of selectively ablating senescent cells and the efficacy of senolytics for alleviating symptoms of frailty and extending healthspan.

Introduction

Aging is the leading risk factor for the chronic diseases that account for the bulk of morbidity, mortality, and health costs (Goldman *et al.* 2013). A fundamental aging mechanism that likely contributes to chronic diseases and age-related dysfunction is cellular senescence (Kirkland 2013b; Kirkland 2013a; Tchkonja *et al.* 2013; Kirkland & Tchkonja 2014). Senescence refers to the essentially irreversible growth arrest that occurs when cells are subjected to potentially oncogenic insults (Tchkonja *et al.* 2013). Even though senescent cell abundance in aging or diseased tissues is low, achieving a maximum of 15 percent of nucleated cells in very old primates, senescent cells can secrete pro-inflammatory cytokines, chemokines, and extracellular matrix proteases, which together constitute the senescence-associated secretory phenotype or SASP (Herbig *et al.* 2006; Coppé *et al.* 2008; Kuilman *et al.* 2008). The SASP possibly contributes to the association between senescent cell accumulation and local and systemic dysfunction and disease. Consistent with a role for cellular senescence in causing age-related dysfunction, clearing senescent cells by activating a drug-inducible “suicide” gene enhances healthspan and delays multiple age-related phenotypes in genetically-modified progeroid mice (Baker *et al.* 2011). Interestingly, despite only clearing 30 percent of senescent cells, improvement in age-related phenotypes is profound. Thus, interventions that reduce the burden of senescent cells could ameliorate age-related disabilities and chronic diseases as a group (Tchkonja *et al.* 2013; Kirkland & Tchkonja 2014).

Despite their harsh internal and external microenvironments, senescent cells are viable. They survive even though they have active DNA damage responses, heightened metabolic flux, and increased local levels of SASP inflammatory cytokines and other factors that are able to

induce apoptosis. Indeed, senescent cells are better able to withstand stresses such as serum deprivation than non-senescent cells (Wang 1995; Fridman & Lowe 2003). *In vivo*, senescent cells appear to be removed by the immune system (Xue *et al.* 2007), rather than apoptosis or necrosis. Therefore, we hypothesized that: 1) anti-apoptotic, pro-survival mechanisms could be up-regulated in senescent cells and 2) interfering with these protective mechanisms might achieve selective elimination of senescent cells. Based on these hypotheses, here we identified several clinically used drugs that induce apoptosis preferentially of senescent cells *in vitro* and *in vivo*, leading to improved cardiovascular function and exercise endurance, reduced osteoporosis and frailty, and extended healthspan in several murine systems.

Results

The senescent transcriptome and anti-apoptotic pathways

We first tested our hypotheses by comparing gene expression in senescent *vs.* non-senescent cells using transcript array analysis (Fig. 1A-C). We focused on fat cell progenitors, or preadipocytes, since they are arguably one of the most abundant types of senescent cells in humans (Tchkonia *et al.* 2010). Cultures were split and senescence induced in half of the cells using 10 Gy of ionizing radiation. Twenty-five days later, gene expression was measured using Affymetrix arrays and differential expression patterns identified by principal component analysis (see Supplemental Methods for details). Overall patterns of gene expression differed substantially between senescent and non-senescent preadipocytes isolated from the same subjects (Fig. 1A). Analyses of gene categories indeed revealed up-regulation of negative regulators of apoptosis (Fig. 1B) and anti-apoptotic gene sets (Fig. 1C) in senescent compared to non-senescent cells (see also Supplemental Fig. 8).

Senolytic siRNAs

We next employed RNA interference to identify potential “senolytic” targets. We used the following rationale for the selection of senescence-associated genes to target with siRNAs. 1) Senescent cells rely on anti-apoptotic, pro-survival defenses to a greater extent than non-senescent cells. 2) Senescent cells have much in common with cancer cells, such as active DNA damage responses (Ghosal & Chen 2013), except senescent cells do not divide. Thus pro-survival pathways that when inhibited are known to drive cancer cell apoptosis might be good targets, as long as the pathway is not linked to cell proliferation. 3) Similarly to cancer cells, senescent cells are metabolically active, with increased glycolysis (Dorr *et al.* 2013). Thus senescent cells may be more dependent on pathways that protect against metabolically-induced apoptosis than are non-senescent cells. 4) Dependence receptors promote apoptosis unless they are prevented from doing so by the presence of their ligands (Goldschneider & Mehlen 2010). Thus senescent cells may rely more on dependence receptor ligands than non-senescent cells, as is the case in cancer cells (Goldschneider & Mehlen 2010; Xi *et al.* 2012). Therefore, we targeted anti-apoptotic pathway elements by RNA interference, including the dependence receptor ligands and metabolic pro-survival transcripts identified in oncology. Importantly, targets identified by this rationale have the potential to yield senolytics that also will have anti-tumor effects.

Of the 39 transcripts selected for knock-down by siRNA transfection, at least 17 may have affected the viability of senescent cells more than non-senescent cells (Supplemental Table 1). Of these, six triggered senescent cell death, but had little effect on proliferating, non-senescent cells in two human cell types, preadipocytes (Fig. 1D&F) and endothelial cells (Fig. 1E&G). Interestingly, the senolytic transcripts included components of ephrin survival-regulating dependence receptor mechanisms (Goldschneider & Mehlen 2010), ephrin ligand (EFN) B1, and EFNB3, as well as cyclin-dependent kinase inhibitor 1A (p21), plasminogen activated inhibitor-2 (PAI-2), the phosphatidylinositol-4,5-bisphosphate 3-kinase delta catalytic subunit

(PI3KCD), a PI3K family member involved in regulating multiple cellular functions, including survival (Datta *et al.* 1999; Osaki *et al.* 2004), and BCL-xL, which regulates mitochondrial-dependent apoptosis and is the target of certain anti-cancer drugs (Minn *et al.* 1999; Leech *et al.* 2000).

Interfering with expression of EFNB1 or 3, PI3KCD, p21, BCL-xL, or PAI-2 indeed significantly reduced the viability (ATPLite intensity; Fig. 1D) and survival (crystal violet; Fig. 1F and Supplemental Fig. 6) of senescent but not proliferating human abdominal subcutaneous preadipocytes. Reducing EFNB2 or 4 or PI3K isoforms other than PI3KCD had less or no effect (Supplemental Fig. 2C; Supplemental Table 1). siRNA transfection efficiencies and extent of mRNA knock-down were similar in senescent and proliferating preadipocytes (Supplemental Fig. 1). Results were confirmed using second, distinct siRNAs or by Western immunoanalysis (Supplemental Figs. 2A,B,&F). While proliferating human umbilical vein cells (HUVECs) tended to be generally susceptible to siRNAs under the conditions used, senescent HUVECs were more susceptible to EFNB1 and BCL-xL siRNAs than non-senescent cells (Fig. 1E&G). EFNB1 or 3, PI3KCD, p21, BCL-xL and PAI-2 siRNAs also interfered with viability of preadipocytes made senescent by serial subculturing compared to non-senescent cells (Supplemental Fig. 2D) and did not interfere with viability of quiescent, differentiated preadipocytes (Supplemental Fig. 2E). Results were confirmed using crystal violet to measure cell survival (Fig. 1G; Supplemental Fig. 6).

Based on potential associations among the genes targeted by senolytic siRNAs, we tested if the gene products could be components of a common pro-survival signaling network (Fig. 1H). Network analysis of these proteins using the STRING database suggested interaction of the anti-apoptotic proteins that we identified in siRNA assays.

Candidate senolytic drugs *in vitro*

We next tested if drugs that target gene products that protect senescent cells from apoptosis are senolytic *in vitro*. Of 46 agents tested, dasatinib (D) and quercetin (Q) showed particular promise in clearing senescent cells. D is a multi-tyrosine kinase inhibitor used for treating cancers (Montero *et al.* 2011) that interferes with E2F-mediated repression of apoptosis (Chang *et al.* 2008; Xi *et al.* 2012). D preferentially reduced viability and caused cell death in senescent human preadipocytes, but was much less effective on senescent HUVECs (Fig. 2A). Note that by day 3, proliferating preadipocytes increased by 2-5 fold in number vs. day 0 in the presence of D. The viability of non-dividing senescent preadipocytes from the same subjects decreased by 30-40% in the presence of 50 nM or higher D, indicating selective reduction in the viability of senescent cells. Q, a natural flavonol, inhibits PI3K, other kinases and serpinases (Olave *et al.* 2010; Bruning 2013). In contrast to D, at low concentrations Q reduced the viability and caused cell death of senescent HUVECs to a greater extent than proliferating cells, but was less effective on preadipocytes (Figs. 2B). Note that at 10 μ M Q, non-senescent HUVECs achieved a 2-3 fold increase in cell number between days 0 and 3, while parallel cultures of senescent cells were reduced by 50%, indicating selective killing of senescent cells. The combination of D+Q afforded selective killing of both senescent preadipocytes and endothelial cells (Fig. 2C-F). By day 3, the viability of non-dividing senescent preadipocytes exposed to D+Q was reduced by ~70% compared to day 0, while non-senescent, proliferating cells had increased by 2-4 fold. By day 3, the viability of senescent HUVECs treated with 10 μ M Q and 100 nM D was reduced by ~50% compared to day 0. Parallel cultures of non-senescent, proliferating HUVECs increased in number by 1.5 fold over the same time. This suggests that the combination of D+Q might selectively target a broader range of senescent cell types than either agent alone. In both senescent and non-senescent cultured preadipocytes, D and Q reduced expression of the anti-apoptotic regulator PAI-2 (Figs. 2G&H).

Dasatinib and quercetin reduce senescent cells *in vivo*

In anticipation of testing D+Q in a preclinical model, the drugs were tested for the efficacy in reducing the viability of senescent murine cells. The combination of D+Q led to a significant reduction in the viability of senescent, C₁₂FDG positive, primary mouse embryonic fibroblasts (MEFs) compared to either drug alone (Fig. 3A). Likewise, Q alone or D+Q caused a significant reduction in the viability of senescent bone marrow-derived murine mesenchymal stem cells (Fig. 3B). These data and those in Fig. 2 demonstrate that both D and Q are able to selectively kill senescent cells in two species albeit with distinct cell type specificity. We tested if D+Q administered by oral gavage was senolytic *in vivo*. We initially tested D+Q in old mice (>24 months-old), since senescent cell burden increases in fat tissue with aging and both preadipocytes and endothelial cells contribute to senescent cell burden with aging in fat (Tchkonia *et al.* 2010). A single dose of D+Q (D: 5 mg/kg body weight and Q: 50 mg/kg by oral gavage here and in the following studies), a drug ratio that was most effective in senescent MEFs (data not shown), reduced SA-βgal⁺ cells (Fig. 3C) and p16 mRNA (Fig. 3D) in fat from old mice within 5 days. D+Q also reduced p16-positive cells in liver from old mice (Fig. 3E-F). As with AP20187, the drug that activates selective killing of cells expressing p16 in transgenic *INK-ATTAC* mice (Baker *et al.* 2011), not all senescent cells were removed by D+Q. Following irradiation of one leg of wild type mice, a single treatment with D+Q reduced p16 expression in muscle (Fig. 3G) and SA-βGal⁺ cells in fat (Fig. 3H&I) at the site of localized ionizing radiation exposure in these mice.

Effects of senolytic agents on cardiovascular function in old mice

Cellular senescence is associated with cardiovascular dysfunction in humans (Tchkonia *et al.* 2013; Kirkland & Tchkonia 2014), a major cause of morbidity and mortality in the elderly. While only mild cardiac dysfunction has been reported in old mice (Dai *et al.* 2009; Roos *et al.* 2013), substantial impairment in vascular reactivity is observed in aged mice (Roos *et al.* 2013).

We tested if treating 24 month-old mice with D+Q would improve cardiac ejection fraction (the fraction of heart volume pumped during each heart contraction) and vascular responses to acetylcholine, nitroprusside, or U46619 (endothelium-dependent relaxation, smooth muscle vascular reactivity to nitric oxide, and smooth muscle contractile function, respectively (Roos *et al.* 2013)). To allow time for senescent cells to apoptose and exclude potential “off-target” effects of the drugs on non-senescent cell types, which require continued presence of the drugs, for example through direct vasoactive/antioxidant effects or through changing NAD⁺ (Chen & Pace-Asciak 1996; Ajay *et al.* 2006), we gave a single dose of the drugs and waited 5 days before assaying cardiac function. D and Q are cleared within 48 hours of the last dose (Luo *et al.* 2006; Piantelli *et al.* 2006).

Despite the fact that mice are relatively resistant to development of age-related systolic dysfunction, treatment of 24 month old mice with a single dose of D+Q significantly improved left ventricular ejection fraction (Fig 4A) and fractional shortening (Supplemental Table 3), effects that were mediated by reductions in end systolic cardiac dimensions (Fig. 4C) but not cardiac preload (Fig. 4B) alteration in cardiac mass (Supplemental Table 3). Consistent with previous reports from our group and others (Roos *et al.* 2013; Gioscia-Ryan *et al.* 2014), carotid arteries from aged mice displayed markedly impaired vascular relaxation in response to endothelium-dependent and -independent vasodilators compared to young mice. While D+Q elicited somewhat variable but statistically significant improvements in vascular endothelial function (Fig. 4D), a complex amalgam of nitric oxide, endothelium-derived hyperpolarizing factors, and other vasoactive substances (Feletou & Vanhoutte 2006), D+Q yielded physiologically important and consistent improvements in vascular smooth muscle sensitivity to nitroprusside (Fig. 4E). Interestingly, senescent cell clearance did not alter smooth muscle contractile function (Fig. 4F). Collectively, these data suggest that senescent cells are likely to exert deleterious effects on cardiovascular function with chronological aging, and that acute clearance of senescent cells may be a novel therapeutic approach to improve cardiovascular

function and reduce morbidity and mortality from cardiovascular disease in the elderly.

Effects on treadmill exercise capacity in mice after single leg radiation exposure

To test further the hypothesis that D+Q function through elimination of senescent cells, we tested the effect of a single treatment in a mouse leg irradiation leg model. One leg of 4 month old male mice was radiated at 10 Gy with the rest of the body shielded. Controls were sham-radiated. By 12 weeks, hair on the radiated leg turned grey (Fig. 5A) and animals exhibited reduced treadmill exercise capacity (Fig. 5B). Five days after a single dose of D+Q, exercise time, distance, and total work performed to exhaustion on the treadmill tended to be higher in the mice treated with D+Q than vehicle (Fig. 5C). Senescent markers were reduced in muscle and inguinal fat 5 days after treatment (Figs. 3G-I). At 7 months after the single treatment, exercise capacity was significantly better in the mice that had been irradiated and received the single dose of D+Q than vehicle-treated controls (Fig. 5D). D+Q treated animals had endurance essentially identical to that of sham-radiated controls. The single dose of D+Q had no effect on endurance 7 months later in sham-radiated controls vs. vehicle. Thus, a single D+Q dose 7 months previously led to sustained improvement in function, consistent with the ability of D+Q to clear damaged senescent cells acutely, resulting in enhanced later-life physical endurance.

Extension of healthspan by periodic treatment of progeroid *Ercc1*^{-Δ} mice with senolytics

To demonstrate that treatment with D+Q can extend healthspan, we used the *Ercc1*^{-Δ} mouse model of accelerated aging. These mice, which model human XFE progeria, have features resembling accelerated aging with a lifespan of 6 months (Dolle *et al.* 2011) and spontaneously develop progressive age-related chronic degenerative diseases (Gregg *et al.* 2011). MEFs deficient in ERCC1 or bone marrow-derived MSCs from *Ercc1*^{-Δ} mice have increased senescence and are more susceptible to elimination by D+Q (Fig. 3A&B). *Ercc1*^{-Δ}

mice were treated with 5 mg/kg D plus 50 mg/kg Q weekly by oral gavage or vehicle only (10% PEG400 in water) beginning at 4-6 weeks of age. Symptoms associated with aging were measured biweekly by an investigator blinded as to the treatment groups. Animals were euthanized after 10-12 weeks of treatment and molecular and histopathological endpoints measured. D+Q resulted in reduced expression of senescence markers in several tissues (Supplemental Fig. 9). This correlated with a significant reduction in a composite score of age-related symptoms (Fig. 6A&B), including kyphosis, dystonia, tremors, loss of grip strength, coat condition, ataxia, urinary incontinence, impaired gait, hind limb paralysis, and poor body condition (Supplemental Fig. 10). This reduction in symptoms indicates an extension of healthspan due to both the delay in onset of symptoms and attenuation of their severity (Fig. 6B). In particular, the mice showed reduced dystonia and delayed onset of ataxia and gait disorders (Fig. 6C and Supplemental Fig. 10). In addition, quantitative computed tomography (pQCT) of lumbar spine demonstrated improved bone parameters in 16 week-old *Ercc1*^{-Δ} mice treated with D+Q compared to animals treated with vehicle only (Fig. 6D). Similarly, the level of proteoglycans in the nucleus pulposus of the intervertebral disc, a marker of age-related disc degeneration, was significantly increased in mice treated with D+Q, suggesting that treatment with D+Q can slow age-related dysfunction even of a relatively avascular tissue (Fig. 6E). Finally, sections of liver, kidney, and the femoral bone space were stained with H&E and scored for age-related pathology by two pathologists blinded to the treatment groups. Composite pathology scores for sibling groups revealed reduced pathology in most animals treated with D+Q compared to siblings treated with vehicle only (Fig. 6F). Remarkably, the sibling groups identified as having the most dramatic differences in pathology are identical to those identified as having the greatest difference in aging score (Fig. 6B and Supplemental Fig. 11), demonstrating a close correlation between pre- and post-mortem endpoints. Taken together, these data demonstrate that periodic treatment with senolytics is sufficient to reduce the burden of senescence markers, reduce frailty, and extend healthspan significantly.

Discussion

We previously demonstrated that the healthspan of transgenic mice can be enhanced by selectively killing senescent cells using a transgenic suicide gene (Baker *et al.* 2011). The identification of approaches to remove damaged, senescent cells would have a tremendous impact on quality of life and burden of age-related chronic diseases. To identify agents able to kill senescent cells, we hypothesized that senescent cells, like cancer cells, are dependent on anti-apoptotic pathways to ensure their survival following stress and damage. Based on this hypothesis, here we have demonstrated that senescent cells indeed are susceptible to selective clearance by targeting pro-survival mechanisms using siRNAs and drugs, even at doses insufficient to kill normal proliferating or differentiated quiescent cells. This observation opens up new approaches to develop clinically relevant small molecules or biologics that selectively eliminate senescent cells from non-genetically modified individuals, acting as senolytic agents. The prototype senolytic agents identified here, dasatanib and quercetin, have the ability to alleviate multiple aging phenotypes, as would be predicted if they truly act by eliminating senescent cells (Kirkland 2013a; Kirkland & Tchkonja 2014).

Interfering with expression of the ephrin dependence receptor ligands, EFNB1 or EFNB3, induced selective loss of senescent cells. Ephrin receptors are the largest family of receptor tyrosine kinases (Boyd *et al.* 2014). Together with ephrin ligands, these receptors coordinate tissue and organ patterning, cell positioning, and cell survival during development and tissue turnover in a cell type-specific manner (Xi *et al.* 2012). Ephrin B ligands, which span cell membranes, can act as both ligands and receptors, making contact with ephrin receptors on adjacent cells. EFNB ligands can participate in dependence networks that constrain both the cells they are located on and adjacent cells from undergoing apoptosis (Furne *et al.* 2009). Ephrin signaling has been linked to cellular senescence: EFNB3 overexpression can induce p21, PAI-1, the SASP, and SA- β Gal activity during wound healing (Hafner *et al.* 2005). Interfering with expression of EFNB3 in cancer cells can induce apoptosis and disrupt pro-

survival networks (Stahl *et al.* 2013). Similar to cancer cells, here we demonstrated that silencing EFNB ligands induces apoptosis selectively in senescent as compared to non-senescent cells.

Silencing EFNB3 expression down-regulates AKT in cancer cells (Stahl *et al.* 2013). AKT is involved in regulating FOXO1 and mTOR, among other key pro-survival and metabolic homeostasis mechanisms (Chandarlapaty 2012). PI3K is upstream of AKT and the PI3KCD (catalytic subunit δ) is specifically implicated in the resistance of cancer cells to apoptosis. PI3KCD inhibition leads to selective apoptosis of cancer (Cui *et al.* 2012; Xing & Hogge 2013). Consistent with these observations, we demonstrated that siRNA knockdown of the PI3KCD isoform, but not other PI3K isoforms, was senolytic in preadipocytes (Supplemental Table 1).

p21 and PAI-1, both regulated by p53, have been implicated in protection of cancer and other cells from apoptosis (Gartel & Radhakrishnan 2005; Kortlever *et al.* 2006; Schneider *et al.* 2008; Vousden & Prives 2009). We found that p21 siRNA is senolytic (Fig. 1D+F) and PAI-1 siRNA and the PAI-1 inhibitor, tiplaxtinin, also may have some senolytic activity (Supplemental Fig 3). We found that siRNA against another serine protease inhibitor (serpine), PAI-2, is senolytic (Fig. 1D+F). Like PAI-1, PAI-2 can protect against apoptosis (Tonnetti *et al.* 2008; Delhase *et al.* 2012). EFNB1 and 3 expression appears linked to that of BCL-xL, PI3KCD, p21, PAI-1, and, indirectly, PAI-2, in published reports and by bioinformatics analysis (Fig. 1H)(Hafner *et al.* 2005), suggesting these transcripts may be networked, an hypothesis that merits further study. Consistent with this hypothesis are our findings that D alone reduced the level of p21 protein, and furthermore D plus Q reduced p21, PAI-2, and BCL-xL (Fig. 2G&H).

D and Q are both approved for use in humans and appear to be relatively safe. Interestingly, imatinib, which is very closely related to D, was not senolytic, at least in preadipocytes (Supplemental Fig. 4). D and Q are promiscuous, like many drugs that affect signaling pathway kinases either directly or indirectly. Despite this, they appear to have more senolytic activity against some types of senescent cells than others and overall they appear to

work better in combination than individually. Thus one strategy to follow in developing future senolytic agents will be to use promiscuous agents or combinations of drugs to target anti-apoptotic networks. Alternatively, additional senolytic agents could be developed by deconvoluting the mechanisms through which D, Q or other senolytics exert their effects. Importantly, since we found that senescent cells originating from different types of cells vary in susceptibility to RNA interference and pharmacological interventions, it may be feasible to design drug strategies focused on specific indications by clearing senescent cells arising from particular cell types or in specific organs.

Whether candidate senolytics actually alleviate phenotypes through removing senescent cells or through possible off-target effects on non-senescent cells is an important and difficult issue to resolve. We first considered comparing effects of senolytics to those of removing senescent cells from *INK-ATTAC* mice. However, we felt that while this may indicate an association between phenotypic effects of removing senescent cells by candidate senolytic drugs and those of removal by activating a “suicide gene” in senescent cells, this approach would not establish cause and effect. Even if candidate senolytic agents had effects resembling those due to genetic clearance of senescent cells, and even if effects of the drugs were not additive to effects of genetic clearance, off target effects would still not be ruled out. For example, clearing senescent cells genetically could influence a critical effector protein also directly targeted by the drug, especially if studies involve continuous administration of drugs.

We also considered ruling out off-target effects by expressing constitutively active targets of the candidate senolytic drugs in senescent cells of genetically modified mice and determining if effects of the drugs are blocked in these animals. However, the targets of the senolytic agents we found have important functions in cell regulation, and constitutively expressing them would be anticipated to have many effects that could confound the experiment.

Instead, to start to rule out off-target effects, we examined if removing senescent cells has sustained effects for many weeks after the drug is no longer present. Apart from agents that

permanently alter cellular or tissue composition, such as antimicrobials, anti-cancer agents, extracellular matrix modifiers, or teratogens – there are few drugs known to exert a sustained effect long after the drugs are no longer present. Indeed, our results demonstrated that a single treatment of D+Q had phenotypic effects persisting far after the drug is no longer present. For example, the treadmill endurance in mice in which one leg had been radiated 3 months before a single dose of senolytics remained improved to the level of that in sham-radiated controls for 7 months after treatment with vehicle or D+Q. In addition, the senolytic treatment did not affect endurance in the sham-radiated controls. This long lasting effect is more consistent with a change in cellular or tissue composition, in this case a decrease in senescent cell burden, than an off-target effect on a metabolite, pathway, or physiological parameter that requires continued dosing with a drug.

An important observation is that senolytics appear to alleviate multiple types of dysfunction. The senolytic agents used here enhanced cardiac and vascular function in aging mice, reduced dysfunction caused by localized radiation, and alleviated skeletal and neurological phenotypes in progeroid mice. Remarkably, in some cases, these drugs did so with only a single course of treatment. In previous work, we and our collaborators found that genetic clearance of senescent cells slowed development of lordokyphosis, cataracts, and lipodystrophy in progeroid mice (Baker *et al.* 2011). Thus, the accumulation of senescent cells in association with a number of diseases, disabilities, and chronological aging may actually contribute to the causation and pathophysiology of these problems or their symptoms. Together with chronic, “sterile” inflammation, macromolecular dysfunction, and stem and progenitor cell dysfunction, cellular senescence may contribute to both aging phenotypes and increased susceptibility to a range of chronic diseases.

An advantage of alleviation of symptoms by a single or few doses of senolytics is that they might be given during periods of generally good health, for example before elective surgery or other circumstances where senescent cell generation could be beneficial. This may help both

to reduce side effects and still allow senescent cells to be generated when needed, for example during wound healing (Demaria, *et al.* 2014). This possibility merits further study in animal models. Additionally, since senescent cells do not divide, drug resistance would be expected to be less likely than is the case with antibiotics or cancer treatment, in which cells proliferate and so can acquire resistance (Tchkonia *et al.* 2013; Kirkland & Tchkonia 2014).

We view this work as a first step towards developing senolytic treatments that can be administered safely in the clinic. Several issues remain to be addressed, including some that must be examined well before the agents described here or any other potentially senolytic agents are considered for use in humans. For example, we found differences in responses to RNA interference and senolytic agents among cell types. Effects of age, type of disability or disease, whether senescent cells are continually generated (*e.g.*, in diabetes or high fat diet vs. effects of a single dose of radiation), extent of DNA damage responses that accompany senescence, sex, drug metabolism, immune function, and other inter-individual differences on responses to senolytic agents need to be studied.

Detailed testing is needed of many other potential targets and senolytic agents and their combinations. Other dependence receptor networks, which promote apoptosis unless they are constrained from doing so by presence of ligands, might be particularly informative to study, especially to develop cell type-, tissue-, and disease-specific senolytic agents. These receptors include the insulin, IGF-1, androgen and nerve growth factor receptors, among others (Delloye-Bourgeois *et al.* 2009; Goldschneider & Mehlen 2010). It is possible that more existing drugs that act against the targets identified by our RNA interference experiments may be senolytic. In addition to ephrins, other dependence receptor ligands, PI3K, AKT, and serpins, we anticipate that drugs that target p21, probably p53 and MDM2 (because they regulate p21 and serpins), BCL-xL, and related genes will also have senolytic effects. This is especially so since existing drugs that act through these targets cause apoptosis in cancer cells and are in use or in trials for treating cancers, including dasatinib, quercetin, and tiplaxtinin (Gomes-Giacoia *et al.* 2013;

Truffaux *et al.* 2014; Lee *et al.* 2015).

Effects of senolytic drugs on healthspan remain to be tested in chronologically aged mice, as do effects on lifespan. Senolytic regimens need to be tested in non-human primates. Effects of senolytics should be examined in animal models of other conditions or diseases to which cellular senescence may contribute to pathogenesis, including diabetes, neurodegenerative disorders, osteoarthritis, chronic pulmonary disease, renal diseases, and others (Tchkonia *et al.* 2013; Kirkland & Tchkonia 2014).

Like all drugs, D and Q have side effects, including hematologic dysfunction, fluid retention, skin rash, and QT prolongation (Breccia *et al.* 2014). An advantage of single doses or periodic short treatments is that many of these side effects would likely be less common than during continuous administration for long periods, but this needs to be studied. Side effects of D differ from Q, implying: 1) their side effects are not solely due to senolytic activity and 2) side effects of any new senolytics may also differ and be better than D or Q. There are a number of theoretical side effects of eliminating senescent cells, including impaired wound healing or fibrosis during liver regeneration (Krizhanovsky *et al.* 2008; Demaria *et al.* 2014). Another potential issue is cell lysis syndrome if there is sudden killing of large numbers of senescent cells. Under most conditions, this would seem to be unlikely, since only a small percentage of cells are senescent (Herbig *et al.* 2006). Nevertheless, this possibility needs to be tested.

Senescent cells have been identified at sites of pathology in multiple diseases and disabilities or may have systemic effects that predispose to others (Tchkonia *et al.* 2013; Kirkland & Tchkonia 2014). Our findings here give preliminary support for the speculation that these agents may one day find use for treating cardiovascular diseases, frailty, loss of resilience, including delayed recovery or dysfunction after chemotherapy or radiation, neurodegenerative disorders, osteoporosis, osteoarthritis, other bone and joint disorders, and adverse phenotypes related to chronological aging. Theoretically, other conditions that further study might indicate that senolytics could prove to alleviate include diabetes and metabolic

disorders, visual impairment, chronic lung disease, liver disease, renal and genitourinary dysfunction, skin disorders, and cancers (Kirkland 2013a; Kirkland & Tchkonja 2014; Tabibian *et al.* 2014). If senolytic agents can indeed be brought into clinical application, they could be transformative. With intermittent short treatments, it may eventually become feasible to delay, prevent, alleviate, or even reverse multiple chronic diseases and disabilities as a group, instead of one at a time.

Experimental Procedures

Preadipocyte isolation and culture

Detailed descriptions of our preadipocyte, HUVEC, MEF, and MSC culture methods are in Supplemental Data and publications (Tchkonja *et al.* 2007; Wang *et al.* 2012). The protocol was approved by the Mayo Clinic Foundation Institutional Review Board for Human Research.

Induction of cellular senescence

Preadipocytes or HUVECs were radiated at 10 Gy to induce senescence or were sham-radiated. Preadipocytes were senescent by 20 days after radiation and HUVECs after 14 days, exhibiting SA- β Gal positivity and SASP factor expression by ELISA (IL-6, MCP-1). Where indicated, senescence was induced by serially subculturing cells.

Microarray analysis

Microarray analyses were performed using the R environment for statistical computing (<http://www.R-project.org>). Array data are deposited in the GEO database, accession number GSE66236. Gene Set Enrichment Analysis (version 2.0.13)(Subramanian *et al.* 2005) was used to identify biological terms, pathways, and processes that were coordinately up- or down-regulated with senescence. The Entrez Gene identifiers of genes interrogated by the array were

ranked according to the *t* statistic. The ranked list was then used to perform a pre-ranked GSEA analysis using the Entrez Gene versions of gene sets obtained from the Molecular Signatures Database (Subramanian *et al.* 2007). Leading edges of pro- and anti-apoptotic genes from the GSEA were performed using a list of genes ranked by the Student *t* statistic.

Senescence-Associated β -Galactosidase activity

Cellular SA- β Gal activity was assayed using 8-10 images taken of random fields from each sample by fluorescence microscopy.

RNA methods

Primers are described in Supplemental Table 2. Cells were transduced with siRNA using RNAiMAX and harvested 48 hours after transduction. RT-PCR methods are in our publications (Cartwright *et al.* 2010). TATA-binding protein (TBP) was used as internal control.

Network analysis

Data on protein-protein interactions (PPIs) were downloaded from version 9.1 of the STRING database (Pubmed ID 23203871) and limited to those with a declared "mode" of interaction, which consisted of 80% physical interactions, such as activation (18%), reaction (13%), catalysis (10%), or binding (39%), and 20% functional interactions, such as post-translational modification (4%) and co-expression (16%). The data were then imported into Cytoscape (PMID 21149340) for visualization. Proteins with only one interaction were excluded to lessen visual clutter.

Mouse studies

Mice were male C57Bl/6 from Jackson Labs unless indicated otherwise. Aging mice were from the National Institute on Aging. *Erc*^{c74} mice were bred at Scripps (Ahmad *et al.*

2008). All studies were approved by the Institutional Animal Care and Use Committees at Mayo Clinic or Scripps.

Single leg radiation

Four month old male C57/Bl6 mice were anesthetized and one leg radiated with 10 Gy. The rest of the body was shielded. Sham radiated mice were anesthetized and placed in the chamber, but the cesium source was not introduced. By 12 weeks, p16 expression is substantially increased under these conditions (Le *et al.* 2010).

Vasomotor function

Rings from carotid arteries were used for vasomotor function studies (Roos *et al.* 2013). Excess adventitial tissue and perivascular fat were removed, and sections of 3 mm in length were mounted on stainless-steel hooks. The vessels were maintained in an organ bath chamber. Responses to acetylcholine (endothelium-dependent relaxation), nitroprusside (endothelium-independent relaxation), and U46619 (constriction) were evaluated.

Echocardiography

High-resolution ultrasound imaging was used to evaluate cardiac function. Short- and long-axis views of the left ventricle were obtained to evaluate ventricular dimensions, systolic function, and mass (Roos *et al.* 2013).

Treadmill endurance

As a measure of physical function, exercise capacity was determined on a motorized treadmill (LeBrasseur *et al.* 2009). Running time was recorded, and running distance (a function of time and speed of the treadmill) and work were calculated.

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Author Contributions

Mayo: TT, TP, and AKP performed animal studies and NG performed cell culture. YZ designed and performed drug screening, siRNA screening, and pathway analysis and contributed to preparation of the manuscript. HD performed confirmatory RT-PCR analyses. JM, CR, and GCV conducted the cardiac phenotyping study. SK and JF conducted the bone phenotyping study. NL and SO completed liver studies. NL contributed to the interpretation of frailty studies. MS contributed to manuscript preparation. JK and TT oversaw all experimental design, data analysis, and manuscript preparation.

Scripps: SJM performed the chronic treatment studies of *Ercc1*^{-Δ} mice with the help of ASB, TS, and DN. JZ, DN, and AUG analyzed the *Ercc1*^{-Δ} tissues for evidence of senescence. DC measured GAGs in the intervertebral discs from the *Ercc1*^{-Δ} mice. HFS and YYL performed the analysis of D&Q on senescent MEFs. AD performed the analysis of D&Q on senescent BM-MSCs. LJN, SJM, AUG, and PR contributed to the experimental design, data analysis, and preparation of the manuscript.

Other: YI and GB performed pathology studies. ML and AG performed microarray analysis. JW completed PPI network analysis.

Figure Legends

Fig. 1 Senescent cells can be selectively targeted by suppressing pro-survival mechanisms. **A:** Principal components analysis of detected features in senescent (green squares) vs. non-senescent (red squares) human abdominal subcutaneous preadipocytes indicating major differences between senescent and non-senescent preadipocytes in overall gene expression. Senescence had been induced by exposure to 10 Gy radiation (vs. sham radiation) 25 days before RNA isolation. Each square represents one subject (cell donor). **B and C:** Anti-apoptotic, pro-survival pathways are up-regulated in senescent vs. non-senescent cells. Heat maps of the leading edges of gene sets related to anti-apoptotic function, “negative regulation of apoptosis” (**B**) and “anti-apoptosis” (**C**), in senescent vs. non-senescent preadipocytes are shown (red = higher; blue = lower). Each column represents one subject. Samples are ordered from left to right first by proliferative state (N=8). The rows represent expression of a single gene and are ordered from top to bottom by the absolute value of the Student t statistic computed between the senescent and proliferating cells (*i.e.*, from greatest to least significance, see also Supplemental Fig. 8). **D-E:** Targeting survival pathways by siRNA reduces viability (ATPLite) of radiation-induced senescent human abdominal subcutaneous primary preadipocytes (**D**) and HUVECs (**E**) to a greater extent than non-senescent sham-radiated proliferating cells. siRNA transduced on day 0 against ephrin ligand B1 (EFNB1), EFNB3, phosphatidylinositol-4,5-bisphosphate 3-kinase delta catalytic subunit (PI3KCD), cyclin-dependent kinase inhibitor 1A (p21), and plasminogen activated inhibitor-2 (PAI-2) messages induced significant decreases in ATPLite-reactive senescent (solid bars) vs. proliferating (open bars) cells by day 4 (100, denoted by the red line, is control, scrambled siRNA). N=6; * $P < 0.05$; T tests. **F-G:** Decreased survival (crystal violet) intensity in response to siRNAs in senescent vs. non-senescent preadipocytes (**F**) and HUVECs (**G**). N=5; * $P < 0.05$; T tests. **H:** Network analysis to test links among EFNB-1, EFNB-3, PI3KCD, p21 (CDKN1A), PAI-1 (SERPINE1), PAI-2 (SERPINB2),

BCL-xL, and MCL-1.

Fig. 2 Dasatinib and quercetin target senescent cells. **A:** D is more effective in selectively reducing viability (ATPLite) of senescent preadipocytes than HUVECs. Preadipocytes and HUVECs were exposed to different concentrations of D for 3 days. The red line denotes plating densities on day 0 of non-dividing senescent (set to 100%) as well as proliferating non-senescent cells (also set to 100%). Preadipocyte data are means±SEM of 4 experiments in each of 4 different subjects. HUVEC data are means±SEM of 5 replicates at each concentration. **B:** Q is more effective in selectively reducing viability (ATPLite) of senescent HUVECs than preadipocytes. Proliferating and senescent preadipocytes and HUVECs were exposed to different concentrations of Q for 3 days. Preadipocyte data are means±SEM of 4 experiments in each of 4 different subjects. HUVEC data are means±SEM of 5 replicates at each concentration. **C:** Combining D and Q selectively reduced viability of both senescent preadipocytes and senescent HUVECs. Proliferating and senescent preadipocytes and HUVECs were exposed to a fixed concentration of Q and different concentrations of D for 3 days. Optimal Q concentrations for inducing senescent preadipocyte and HUVEC cell death were 20 and 10 μ M, respectively. Preadipocyte data are means±SEM of 4 experiments in each of 4 different subjects. HUVEC data are means±SEM of 5 replicates at each concentration. **D:** D and Q do not affect viability of quiescent fat cells. Proliferating non-senescent preadipocytes (proliferating), non-proliferating, non-senescent differentiated fat cells prepared from preadipocytes that had been exposed to differentiation medium (differentiated), and non-proliferating preadipocytes that had been exposed to 10 Gy radiation 25 days before to induce senescence (senescent) were treated with D+Q for 48 hours. N=6 preadipocyte cultures isolated from different subjects. * P <0.05; ANOVA. 100% indicates ATPLite intensity at day 0 for each cell type and bars, ATPLite after 72 hours. The drugs resulted in lower ATPLite in proliferating cells than in vehicle-treated cells after 72 hours, but ATPLite intensity did not fall

below that at day 0. This is consistent with inhibition of proliferation, and not necessarily cell death. Fat cell ATPLite was not substantially affected by the drugs, consistent with lack of an effect of even high doses on non-proliferating, differentiated cells. ATPLite was lower in senescent cells exposed to the drugs for 72 hours than at plating on day 0. Since senescent cells do not proliferate, this indicates that the drugs decrease senescent cell viability. **E&F:** D and Q cause more apoptosis of senescent than non-senescent primary human preadipocytes (terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL] assay). **E:** D (200 nM) plus Q (20 μ M) resulted in 65% apoptotic cells (TUNEL assay) after 12 hours in senescent but not proliferating, non-senescent preadipocyte cultures. Cells were from 3 subjects; 4 replicates; $**P < 0.0001$; ANOVA. **F:** Primary human preadipocytes were stained with DAPI to show nuclei or analyzed by TUNEL to show apoptotic cells. Senescence was induced by 10 Gy radiation 20 days previously. Proliferating, non-senescent cells were exposed to D+Q for 24 hrs and senescent cells from the same subjects were exposed to vehicle or D+Q. D+Q induced apoptosis in senescent, but not non-senescent cells (compare the green in the upper to lower right panels). The bars indicate 50 μ . **G:** Effect of vehicle, D, Q, or D+Q on non-senescent preadipocyte and HUVEC p21, BCL-xL, and PAI-2 by Western immunoanalysis. **H:** Effect of vehicle, D, Q, or D+Q on preadipocyte on PAI-2 mRNA by PCR. N=3; $*P < 0.05$; ANOVA.

Fig. 3 Dasatinib and quercetin reduce senescent cell abundance in mice. **A:** Effect of D (250 nM), Q (50 μ M), or D+Q on levels of senescent *Ercc1*-deficient murine embryonic fibroblasts. Cells were exposed to drugs for 24 hours prior to analysis of SA- β Gal⁺ cells using C₁₂FDG. The data shown are means \pm SEM of 3 replicates, $***P < 0.005$; T-test. **B:** Effect of D (500 nM), Q (100 μ M), and D+Q on senescent BM-MSCs from progeroid *Ercc1*^{- Δ} mice. The senescent MSCs were exposed to the drugs for 48 hrs prior to analysis of SA- β Gal activity. The data shown are means \pm SEM of 3 replicates. $**P < 0.001$; ANOVA. **C-D.** The senescence markers, SA- β Gal and p16, are reduced in inguinal fat of 24 month-old mice treated with a single dose of senolytics

(D+Q) compared to vehicle only (V). Cellular SA-βGal activity assays and p16 expression by RT-PCR were done 5 days after treatment. N=14; means±SEM. ** $P<0.002$ for SA-βGal, * $P<0.01$ for p16 (T tests). **E-F:** D+Q treated mice have fewer liver p16⁺ cells than vehicle-treated mice. **E:** Representative images of p16 mRNA FISH. Cholangiocytes are located between the white dotted lines that indicate the luminal and outer borders of bile canaliculi. **F:** Semi-quantitative analysis of fluorescence intensity demonstrates decreased cholangiocyte p16 in drug treated animals compared to vehicle. N=8 animals/ group. * $P<0.05$; Mann Whitney U test. **G-I:** Senolytic agents decrease p16 expression in quadriceps muscles (**G**) and cellular SA-βGal in inguinal fat (**H-I**) of radiation-exposed mice. Mice with one leg exposed to 10 Gy radiation 3 months previously developed grey hair (Fig. 5A) and senescent cell accumulation in the radiated leg. Mice were treated once with D+Q (solid bars) or vehicle (open bars). After 5 days, cellular SA-βGal activity and p16 mRNA were assayed in the radiated leg. N=8; means±SEM, p16: ** $P<0.005$; SA β-Gal: * $P<0.02$; T tests.

Fig. 4 Effects of senolytic agents on cardiac (**A-C**) and vasomotor (**D-F**) function. D+Q significantly improved left ventricular ejection fraction of 24 month-old mice (**A**). Improved systolic function did not occur due to increases in cardiac preload (**B**), but were instead a result of a reduction in end-systolic dimensions (**C**; Supplemental Table 3). D+Q resulted in modest improvement in endothelium-dependent relaxation elicited by acetylcholine (**D**), but profoundly improved vascular smooth muscle cell relaxation to nitroprusside (**E**). Contractile responses to U46619 (**F**) were not significantly altered by D+Q. In panels D-E, relaxation is expressed as the percentage of the pre-constricted baseline value. Thus for Panels D-F, *lower* values indicate improved vasomotor function. N=8 male mice/ group. * $P<0.05$; A-C: T tests; D-F: ANOVA.

Fig. 5 Senolytic administration alleviates radiation-induced impairment in treadmill exercise endurance. **A-B:** One leg of 4 month old mice was radiated at 10 Gy. Three months later, hair

on the radiated leg had turned grey (**A**) and treadmill exercise capacity (**B**) was lower in radiated (N=13) than sham-radiated mice (N=14). ** $P < 0.002$; T-test. **C**: Five days after a single dose of D+Q, treadmill endurance was better than in vehicle treated controls. D+Q had no effect in sham-radiated controls. 7 sham-radiated vehicle, 7 sham-radiated D+Q, 6 radiated vehicle, 9 radiated D+Q mice. Bars represent means \pm SEM; * $P < 0.05$; ** $P < 0.001$; ANOVA; Tukey-Kramer Test. **D**: 7 months after the single dose of D+Q, treadmill endurance was again assayed. All groups ran on the treadmill on 4 occasions, each 1 week apart. Bars represent means \pm SEM of the average performance of each group on each of the 4 occasions when they ran (N=4). Endurance is shown as a function of the overall performance of all 4 groups on each occasion when mice ran (mean Joules per group/total Joules per all groups that day). * Different from the other groups; $P < 0.05$; ANOVA; Duncan's test.

Fig. 6 Periodic treatment with D+Q extends the healthspan of progeroid *Ercc1^{-Δ}* mice. Animals were treated with D+Q or vehicle weekly. Symptoms associated with aging were measured biweekly. Animals were euthanized after 10-12 weeks. N=7-8 mice per group. **A**: Histogram of the aging score, which reflects the average percent of the maximal symptom score (onset and severity) for each treatment group and is a reflection of healthspan (Tilstra *et al.* 2012). * $P < 0.05$ and ** $P < 0.01$ Student's t test. **B**: Representative graph of the age at onset of all symptoms measured in a sex matched sibling pair of *Ercc1^{-Δ}* mice. Each color represents a different symptom. The height of the bar indicates the severity of the symptom at a particular age. The composite height of the bar is an indication of the animals' overall health (lower bar better health). Mice treated with D+Q had delay in onset of symptoms (*e.g.*, ataxia, orange) and attenuated expression of symptoms (*e.g.*, dystonia, light blue). Additional pairwise analyses are found in Supplemental Fig. 11. **C**: Representative images of *Ercc1^{-Δ}* mice from the D+Q treatment group or vehicle only. Splayed feet are an indication of dystonia and ataxia. Animals

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treated with D+Q had improved motor coordination. Additional images illustrating the animals' gait and body condition are in Supplemental Fig. 10. **D:** Quantitative computed tomography (QCT)-derived bone parameters at the lumbar spine of 16-week-old *Ercc1*^{-Δ} mice treated with either vehicle (N=7) or drug (N=8). BMC = bone mineral content; vBMD = volumetric bone mineral density. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. **E:** Glycosaminoglycan (GAG) content of the nucleus pulposa of the intervertebral disc. GAG content of the NP declines with mammalian aging, leading to lower back pain and reduced height. D+Q significantly improves GAG levels in *Ercc1*^{-Δ} mice compared to animals receiving vehicle only. **P* < 0.05, Student's t test. **F:** Histopathology in *Ercc1*^{-Δ} mice treated with D+Q. Liver, kidney, and femoral bone marrow hematoxylin and eosin stained sections were scored for severity of age-related pathology typical of the *Ercc1*^{-Δ} mice. Age-related pathology was scored from 0-4. Sample images of the pathology are provided in Supplemental Fig. 13. Plotted is the percent of total pathology scored (maximal score of 12: 3 tissues x range of severity 0-4) for individual animals from all sibling groups. Each cluster of bars is a sibling group. White bars represent animals treated with vehicle. Black bars represent siblings that were treated with D+Q. The √ denotes the sibling groups in which the greatest differences in pre-mortem aging phenotypes were noted, demonstrating a strong correlation between the pre- and post-mortem analysis of frailty.

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FIGURE 1

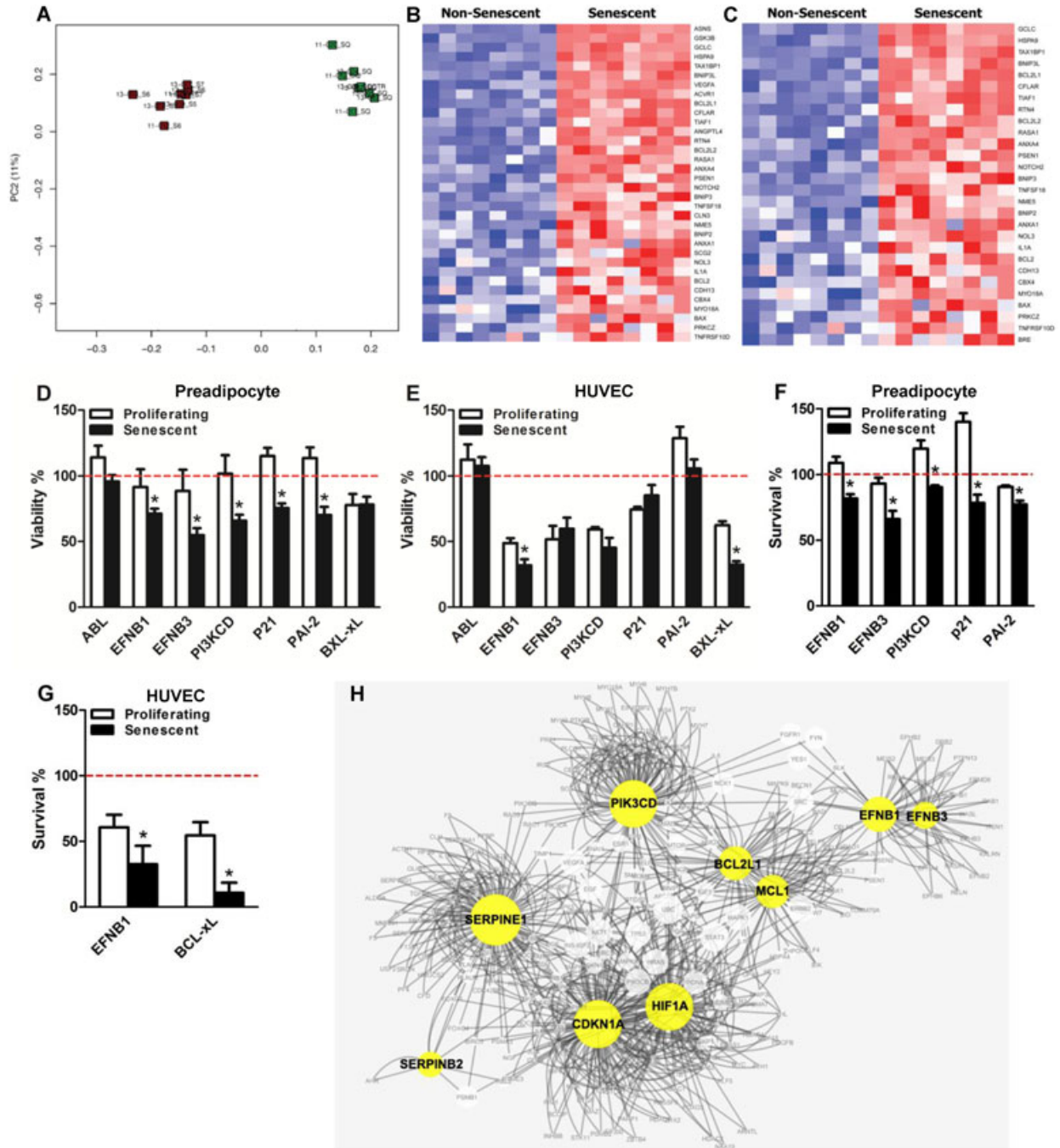


FIGURE 2

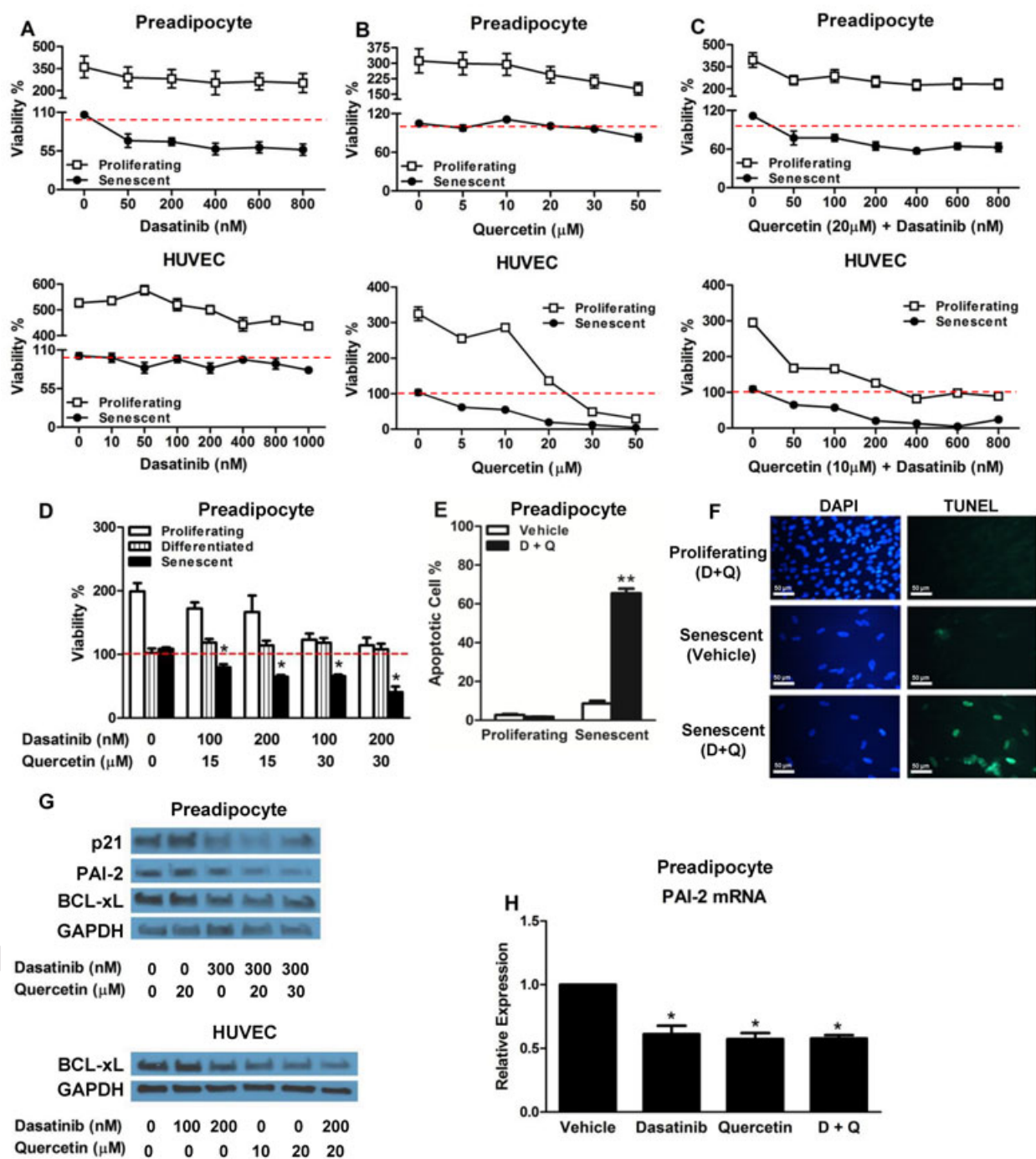


FIGURE 3

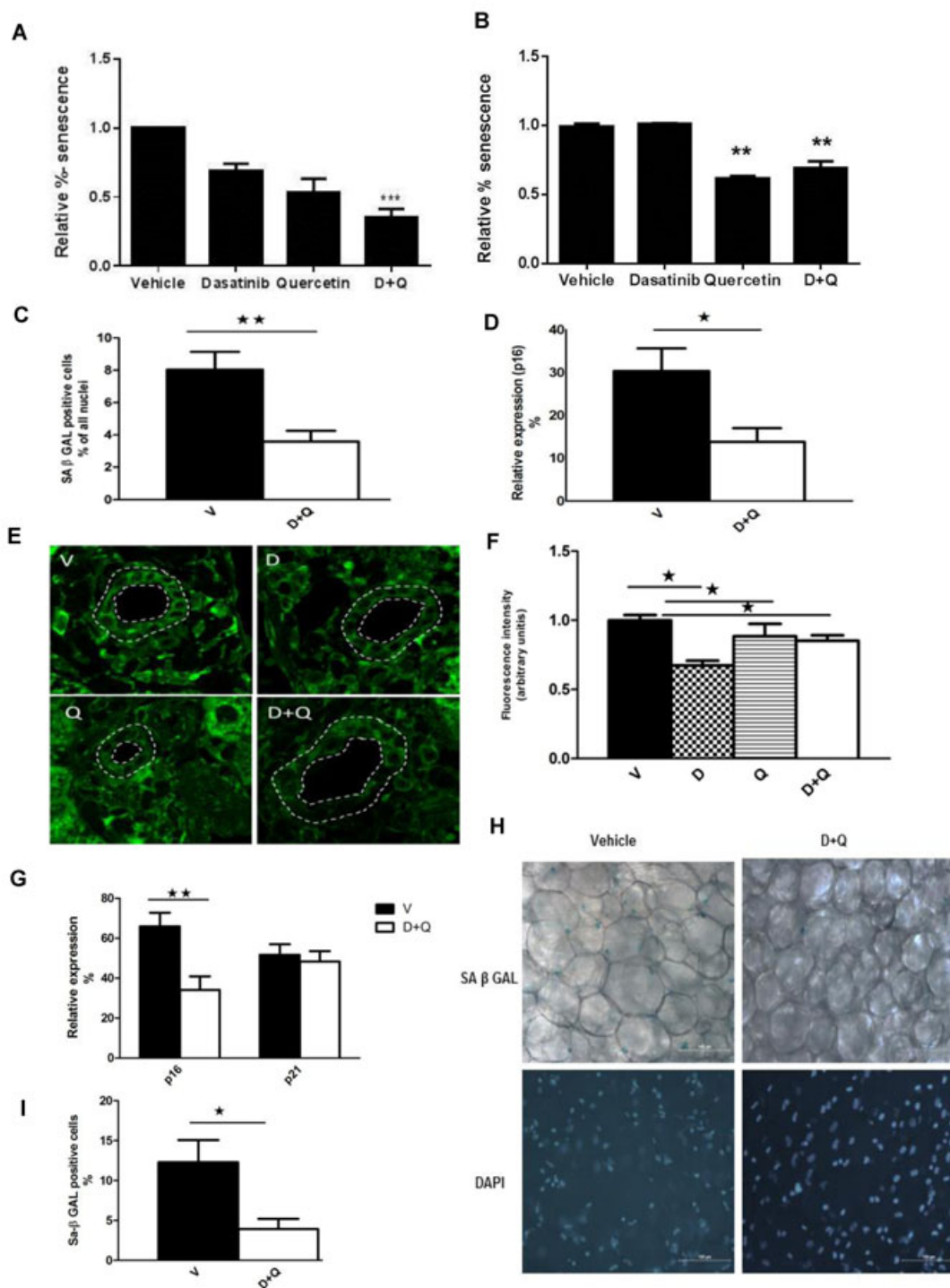


FIGURE 4

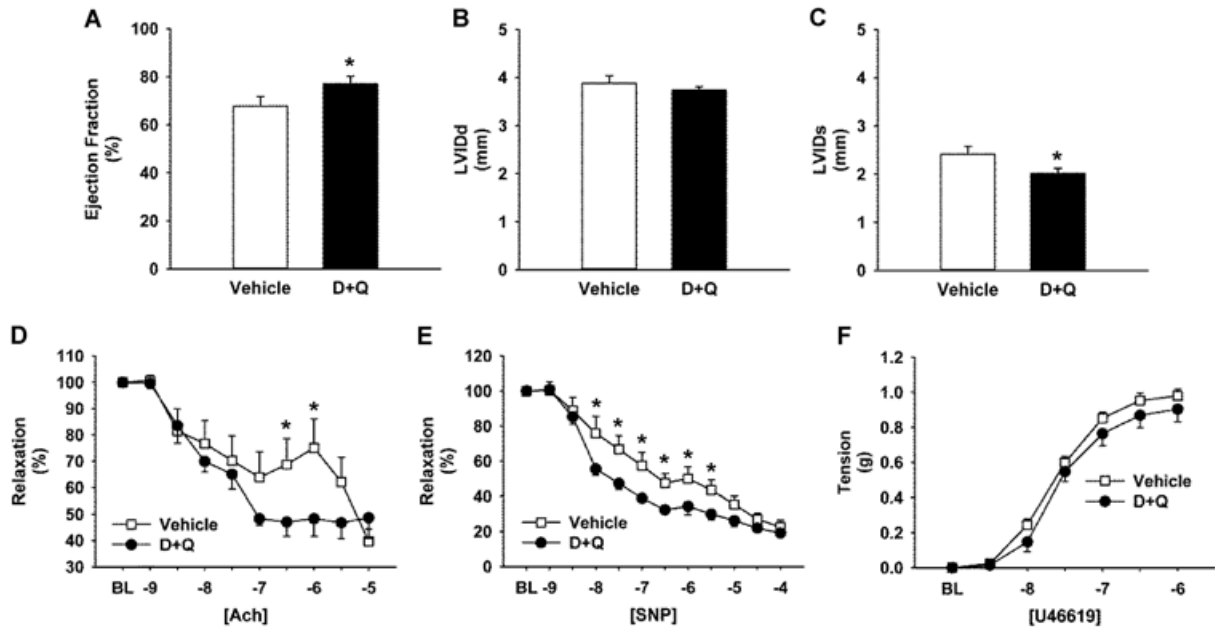
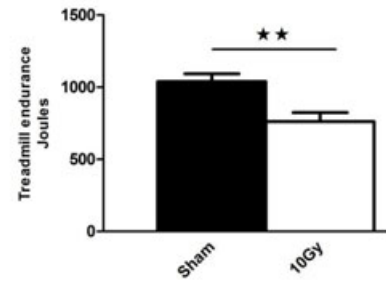


FIGURE 5

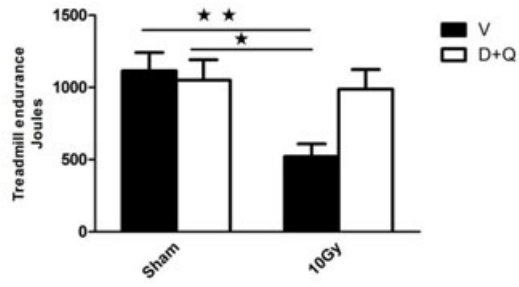
A



B



C



D

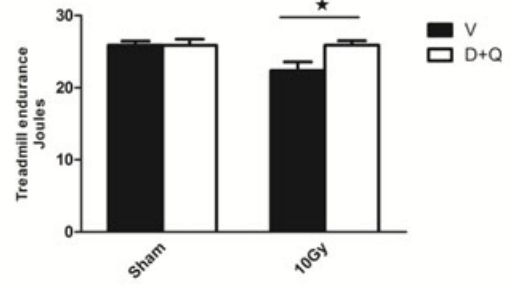


FIGURE 6

