Muscloskeletal senescence: a moving target ready to be eliminated
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Aging is the prime risk factor for the broad-based development of diseases. Frailty is a phenotypical hallmark of aging and is often used to assess whether the predicted benefits of a therapy outweigh the risks for older patients. Senescent cells form as a consequence of unresolved molecular damage and persistently secrete molecules that can impair tissue function. Recent evidence shows senescent cells can chronically interfere with stem cell function and drive aging of the musculoskeletal system. In addition, targeted apoptosis of senescent cells can restore tissue homeostasis in aged animals. Thus, targeting cellular senescence provides new therapeutic opportunities for the intervention of frailty-associated pathologies and could have pleiotropic health benefits.

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Loss of cell-intrinsic and cell-extrinsic integrity perturbs musculoskeletal rejuvenation during aging
Aged individuals can deteriorate exceptionally fast after the onset of complications affecting the musculoskeletal system. Tissue erosion due to life-long mechanical and biological stress can ultimately result in pathologies such as osteoporosis, sarcopenia, and osteoarthritis, and contribute to frailty [1]. While not all elderly people develop the same age-related diseases, virtually everyone will experience musculoskeletal complications sooner or later. To extend, and possibly even restore, healthy life expectancy in old age, it is essential to understand the cellular changes underlying musculoskeletal decline. Tissue regeneration by stem-cell differentiation is critical in overcoming the relentless day-by-day damage to the musculoskeletal system. In young tissues, differentiation proceeds without much hindrance unless one exercises excessively or suffers undue levels of stress. However, during aging, the number and function of adult stem cells declines [2,3]. For example, Pax7-expressing satellite stem cells, can replace damaged muscle fibers [4]. Removing Pax7-positive cells from mice impairs muscle regeneration after injury [5], whereas increased availability of these cells enhances muscle repair [6].

In addition to cell-intrinsic regulation, muscle stem cell regenerative capacity also depends intimately on the microenvironment. During aging, the levels of inflammation chronically increase, an affect known as inflammation [7]. Evidence for this is provided by studies showing that muscle stem cells (satellite cells) from aged mice become more fibrogenic, a conversion mediated by factors from the aged systemic environment [8]. In contrast, frailty is reduced by the JAK/STAT inhibitor Ruxolitinib, which reduces inflammation in naturally aged mice [9]. Stem-cell impairing cues do not necessarily have to come from local sources but can travel over a distance. Heterochronic parabiosis experiments showed that transfusion of old blood impairs stem cell function in young recipient mice [10], while the transfer of young blood factors restoring muscle regeneration and muscle stem-cell activation in aged animals [11]. Therefore, there is a great interest in developing methods to interfere with the age-associated pro-inflammatory signaling profile. The question is how? To address this question, cellular senescence has recently gained attention as a potential candidate for intervention.

Signaling noise by senescent cells impedes tissue homeostasis during aging
As we age, each cell in our body accumulates damage. Earlier in life, this damage is usually faithfully repaired [12], but over time more and more damage gets left behind. This can trigger a molecular chain of events, resulting in chromatin remodeling and the entry of cells...
Senescent cells persist for prolonged periods of time and eventually accumulate during aging [15]. This also means there is a gradual and, importantly, ever-present build-up of deleterious molecules. Thus, senescence can have continuous detrimental effects on tissue homeostasis during aging. That senescent cells are a direct cause of aging was proven beyond a doubt in studies in which senescent cells were genetically or pharmacologically removed. In these studies, both rapidly and naturally aged mice maintained healthspan for much longer, or even showed signs of aging reversal [16,17,18,19]. Factors secreted by senescent cells can induce pluripotency in vivo [20]. As such, these can impair normal stem cell function by forcing a constant state of reprogramming, something we dubbed a ‘senescence — stem lock’ [13]. This is supported by observations that factors secreted by senescent cells induce pluripotency in vivo [20]. Age-associated inflammation may thus deregulate normal stem cell function at different levels, for instance by preventing stem cells from producing differentiated daughter cells. Due to the constant secretion of SASP factors, senescent cells could thus impair local and distant stem cell function and differentiation in times of need. Here, we will highlight the interplay between senescence, the SASP and stemness in the individual musculoskeletal compartments: muscle, bone and cartilage.

Skeletal muscle: an intrinsic interplay between senescence and stemness

Several reports link senescence to muscle aging and muscle stem cell dysfunction. For example, expression of the major senescence marker p16INK4A prevents tissue regeneration by satellite cells after damage [21**]. Fast-aging BubR1H/H mice develop sarcopenia, and after genetic removal of senescent cells, they showed a reduction in kyphosis and an increase in muscle fiber diameter, findings suggestive of reduced sarcopenia [16]. Likewise, senescence of muscle stem cells occurs in muscles of mice with distinct dystrophinopathies, such as Duchenne muscular dystrophy or Steinert’s diseases [22–25].

The skeletal muscle stem cell niche is a candidate through which senescent cells may exert their deleterious effects. Interleukin 6 (IL-6) is a pleiotropic cytokine that can be released by inflammatory cells and by muscle fibers (acting as a myokine). IL-6 is also a major component of the SASP [14], and has been shown to regulate the transition of satellite cells from a quiescent to an activated state [26]. This is beneficial upon acute tissue stress, where IL-6 is transiently released by growing myofibers to activate satellite cells and thereby stimulate myogenesis [26]. However, the chronic IL-6 signaling caused by senescence during aging would have very detrimental effects on muscle function. Indeed, muscle atrophy is linked to high IL-6 levels in patients with inflammatory diseases such as cancer [27]. In addition, persistent IL-6 expression was shown to increase muscle degradation in combination with other circulating factors in mice [28,29]. Interestingly, when IL-6 receptors were blocked in mice with ectopic IL-6 expression, atrophy could be attenuated, indicating a direct regulation of muscle wasting by IL-6 [30]. Chronic IL-6 signaling causes protein degradation in muscle, explaining age-related muscle wasting [31]. Additionally, IL-6 dependent muscle degradation may be linked to stem cell function. For example, senescence induction after muscle injury can promote Pax7 positive unipotent cells to undergo reprogramming and regain pluripotency [32**]. This process is dependent on IL-6 secreted by the senescent cells. Further underscoring the role between the senescent niche and stemness in the muscle is provided by elegant work employing a system in which the four Yamanaka stem cell factors, Oct4, Sox2, Klf4 and c-Myc (OSKM) were transiently expressed in vivo. This resulted in a marked reduction in senescence, SASP factors as IL6 and improved recovery in muscle injury experiments [33*]. Together, this supports a model we postulated previously that because senescence increases locally during aging hotspots are formed of high IL6 concentrations. This can cause neighboring cells to become pluripotent. However, due to the chronic nature of the SASP, senescent cells provide a continuous source of IL6 causing these cells remain permanently locked in a pluripotent state and rendering them unable to rejuvenate the tissue after injury [13*] (Figure 1).
The myokines released by muscle cells not only signal to stem cells, but also attract immune cells that can facilitate tissue repair and regulate immune cell function. IL-15 is released by muscle cells in response to exercise and promotes survival of NK cells [37,38]; in contrast, NK cells are inhibited by IL-6 and TNFα [39]. An age-related decrease in muscle mass could therefore lead to a decrease in IL-15 and thereby a decrease in the number of NK cells, an effect aggravated by an increase in systemic IL-6 levels (Reviewed in [40]). Importantly, NK cells are natural eliminators of senescent cells [41]. Muscle atrophy during aging thus adds to the build-up of senescence by reducing the ability of the immune system to clear senescent cells. This, in turn, further accelerates muscle loss and age-related frailty. Studies are underway to determine whether anti-senescence treatment can overcome muscle loss. Aging is the greatest risk factor for most chronic diseases, and mechanistic links between aging and disease are starting to emerge. Several studies show an involvement of cellular senescence, and in particular, muscle stem cell senescence, in distinct types of muscular dystrophies. In Myotonic dystrophy type 1 (DM1 or Steinert’s disease), entry into senescence of human satellite cell-derived myoblasts correlates with a lower proliferative rate than age-matched controls and has been causally implicated in the progressive atrophy and degeneration of DM1 muscles [22,23]. Similarly, cellular senescence traits have been described in mdx mice, a widely used model of Duchenne muscular dystrophy (DMD), correlating with poor regenerative capacity [24,25,42]. Premature cellular senescence also underlies myopathy in a mouse model of limb-girdle muscular dystrophy [43]. Whether interference in cellular senescence can provide a therapeutic approach for these muscle diseases is unknown.

**Bone: senescence distorts the balance between resorption and formation**

During aging, there is an increase in senescence in the bone. This, in turn, can lead to changes in bone density. Bone consists of multiple cell types, including osteoblasts that form bone, osteoclasts that break down bone tissue, and osteocytes that make up the majority of bone cells (reviewed in [44]). Out of the various cell types that are affected, the main SASP producing cells are senescent osteocytes [45]. Osteocytes are known to influence
osteoblast and osteoclast function [46], and SASP factors secreted by osteocytes, such as IL-1 and MMP13, increase osteoclast differentiation and thereby increase bone resorption to cause the age-related bone loss associated with osteoporosis [47–49]. The conditioned medium of senescent cells can decrease osteoblast function in vitro and promote osteoclast activity [50]. Furthermore, inhibition of senescence induction stimulates osteogenesis and prevents osteoporosis [51]. These observations indicate a causal role of senescence in disrupting the balance between bone formation and resorption, leading to osteoporosis (Figure 2).

Bone stem cell function during aging is likely influenced by secreted SASP factors. Osteoblasts have a relatively short lifespan and are derived from mesenchymal stem cells in the bone marrow (BMSCs), periosteum and elsewhere [52]. BMSCs can give rise to both osteoblasts and adipocytes [53]. This balance is heavily influenced by the microenvironment [54], and during osteoporosis oxidative stress and inflammatory cytokines influence BMSCs to favor adipogenesis over osteogenesis [55,56]. Therefore adipose tissue accumulation is a hallmark of osteoporosis and is linked to senescence in the microenvironment. Furthermore, BMSCs show a reduced differentiation capacity during aging. For example, serum from aged individuals inhibits differentiation of BMSCs into osteoblasts [57]. Additionally, BMSCs can become senescent during aging, secreting SASP proteins and promoting osteoclast activity [58,59]. Overall, these observations indicate that targeting senescent cells in bone would likely improve bone stem cell function.

There are several mouse models that show accelerated aging and are known to have an increased number of senescent cells, such as mice with DNA repair or telomerase deficiency; such mice often show osteoporosis and other musculoskeletal affilictions [60,61]. They are therefore ideal model organisms for studying the effect of senescence in these disorders. For example, Klotho-deficient mice show accelerated senescence and a wide variety of age-related diseases, including osteoporosis. When these mice were crossed with p16<sup>ink4a</sup> knockout mice, osteoporosis was attenuated [61], indicating that senescent cell ablation can potentially prevent this deterioration. Indeed, osteoporosis was delayed in naturally aged INK-ATTAC mice when senescent cells, which continuously develop, were ablated twice a week. Moreover, these mice had an improved microarchitecture and strength [62**]. The reduction of senescent cells likely leads to a lower level of inflammation in the bone. This then reduces the formation of osteoclasts and prevents bone degradation. Indeed, in INK-ATTAC mice, bone resorption was lowered and bone formation improved. In conclusion, senescent cell removal prevents age-related bone loss in mice.

**Cartilage: senescence-associated chronic inflammation perturbs cartilage regeneration**

Articular cartilage — a flexible connective tissue that protects the ends of bones within a joint — affords smooth surfaces with low friction for movement, and facilitates transmission of loads to the underlying bone. This tissue mainly consists of extracellular matrix produced by chondrocytes, the cell type present in cartilage. The regenerative potential of cartilage after damage is limited, possibly because the tissue contains a low number of mesenchymal stem cells [63]. Furthermore, like muscle stem cells, these stem cells are less able to regenerate damaged tissue with age. This is in part due to intrinsic MSC aging and senescence induction [64,65], but is also due to the altered tissue microenvironment and chronic inflammation [66]. Additionally, chondrocytes can express stemness markers in osteoarthritis [67,68]. Again, inflammatory factors promote a chronic dedifferentiated state and thereby prevent tissue repair during aging [69]. Altogether, this leads to thinning of cartilage during aging, resulting in stiffness and pain in the joints that are characteristic of osteoarthritis [70] (Figure 3).

A causal role of senescence in osteoarthritis was shown by transplanting senescent cells into mouse joints, resulting in pain and morphological changes indicative of osteoarthritis [71*].
Furthermore, chondrocytes show an age-related increase in senescence, and during osteoarthritis pro-inflammatory cytokines such as the prominent SASP factor IL-1 induce excess expression of matrix metalloproteinases (MMPs), leading to cartilage loss [72]. Increased levels of circulating SASP factors such as IL-6 are linked to frailty and risk of osteoarthritis [73]. Additionally, in a mouse model of osteoarthritis, overexpression of SIRT6 prevents senescence induction and concurrent inflammation, thereby reducing cartilage degeneration [74]. This finding indicates that eliminating senescent cells from cartilage would attenuate osteoarthritis and improve joint function, especially since chondrocyte death does not seem to drive cartilage damage in response to injury [75]. Several studies have examined the effect of senescent cell removal on osteoarthritis development. For example, osteoarthritis was surgically induced in mice through anterior cruciate ligament transection (ACLT) in the knee joint. In this model, genetic removal of senescent cells delayed the development of osteoarthritis, evidenced by reduced inflammation in the knee joint and an increase in cartilage development, indicating better joint function [76]. The mice had less pain after the senescent cells were removed. Furthermore, osteoarthritis occurs naturally in aged INK-ATTAC mice, and cartilage degeneration was attenuated after removal of senescent cells in this model.

**Targeting senescence to counteract age-related frailty**

The encouraging results observed upon genetic elimination of senescent cells have important implications for the treatment of musculoskeletal deterioration. Since senescence is thought to play a significant role in the progression of age-related frailty, anti-senescence drugs can be predicted to benefit patients with musculoskeletal disorders (Table 1).

Currently, drugs that target inflammatory cytokines are tested in patients with musculoskeletal diseases. For example, several strategies for IL1 inhibition in osteoarthritis have been explored. These therapies include IL1 receptor antagonist proteins (IRAP), monoclonal antibodies targeting free IL1 or the IL1-receptor, and an inhibitor of IL1β production called Diacerein (reviewed in [77]). Most of these therapies show a trend of pain reduction versus placebo. However, these results were often not statistically significant, possibly due to the short half-life of the antagonist proteins or blocking antibodies. Only Diacerein treatment has shown significant anti-inflammatory effects and pain reduction in most studies [77]. Treatment of mdx dystrophic mice with the NAD+ precursor nicotinamide riboside (NR) prevented senescence of muscle stem cells, and this rejuvenated their regenerative capacity [24]. The Notch pathway is chronically activated in severely dystrophic muscles of mdx mice double mutant for dystrophin and utrophin, and blocking this pathway with the γ-Secretase inhibitor DAPT reduced stem cell senescence and the histopathological features of DMD [42]. Importantly, abolition of p16INK4a, which accumulates abnormally in satellite cells of DMD muscles, partially restores early growth arrest and reduces senescence in vitro [22], reinforcing the idea that...
this mechanism might participate in the impaired regeneration of DM1 muscles. Notably, the regenerative deficit of satellite cells from dystrophic muscles resembles that of geriatric mice, which also show p16INK4a-induced senescence and can be rejuvenated by silencing of the gene encoding p16INK4a [21**]. Overall, these studies show limited effects, and the long-term safety of these drugs and/or genetic approaches has yet to be assessed. However, it is unlikely that essential molecules and pathways such as Notch or p16INK4a can be targeted systemically without severe secondary effects. In addition, these strategies are aimed at reducing symptoms and do not treat the underlying causes of disease progression. Removal of senescent cells is expected to reduce these inflammatory proteins while preserving stem cell function and is therefore expected to be safer and have more long-lasting effects.

The results obtained after genetic removal of senescent cells prompted a search for therapeutically applicable anti-senescence compounds. A small number of these compounds have been discovered, with varying degrees of success. One example is Navitoclax, a BCL2 family inhibitor. In the musculoskeletal system, Navitoclax was found to decrease the expression of cytokines that promote osteoclast activity in vitro, such as IL-1α and MMP-13 [58]. Furthermore, muscle stem cells isolated from naturally aged, Navitoclax-treated mice showed improved clonogenicity [78].

A major challenge when developing anti-senescence therapies is to avoid toxicity to healthy non-senescence cells. It is therefore important to identify the unique characteristics of senescent cells that can be targeted by a therapeutic compound. Senescent cells often express persistent nuclear damage foci called DNA-SCARS (DNA Segments with Chromatin Alterations Reinforcing Senescence) that contain DDR proteins such as 53BP1, γH2AX and activated p53 [79]. These DNA-SCARS play a role in maintaining permanent growth arrest and are critical for SASP expression. In addition, we recently showed that the transcription factor FOXO4 resides within PML bodies fused to these persistent damage foci [19*]. Here, FOXO4 binds p53 and prevents p53-dependent apoptosis. In order to disrupt this interaction and to induce apoptosis, we prospectively generated a D-Retro-Inverso peptide mimicking the FOXO4 p53-binding domain. This peptide, FOXO4-DRI, causes the release of p53 to the cytoplasm, where p53 indeed induces apoptosis in a transcription independent manner. Indeed, in vivo use of FOXO4-DRI shows promising results. For these experiments we made use of XpdTTD/TTD mice that show accelerated aging and age-related ailments such as osteoporosis and are therefore an ideal model for musculoskeletal diseases [60]. FOXO4-DRI treatment improved overall fitness and renal function in these mice, including an improved running wheel performance [19*], an especially promising result for the treatment of musculoskeletal diseases. FOXO4-DRI showed around 10 fold selectivity for eliminating senescent vs. control cells. While enough for experiments in rodents, translation to the clinic requires further improvement to eliminate toxicity, which would be intolerable in this setting. Such efforts are now underway in our laboratory.

Unanswered questions
As we highlighted here, the tissues of the musculoskeletal system are damaged by inflammation during aging. Cellular senescence, by driving a persistent inflammatory response, is a major contributor to these effects. However, it remains unclear which senescent cell types are the main producers of these pro-inflammatory factors. Aging of the musculoskeletal system is due to both local and systemic factors. For example, senescent cells transplanted into cartilage can independently cause osteoarthritis [71*]. On the other hand, systemically increased IL-6 levels are linked to muscle wasting, and the immune system also seems to be crucial in this process [28,29]. This systemic inflammation can be caused by many cell types. For example, adipose tissue significantly contributes to systemic inflammation [80]. Fat present in joints can produce factors that promote osteoarthritis [81]. In turn, cells of...
the musculoskeletal system also secrete systemic factors and influence overall tissue integrity. For example, muscle cells affect NK cells during aging and, as NK cells are responsible for clearance of senescent cells [41], these would also influence the systemic senescence burden. Since various anti-senescece compounds potentially kill distinct subsets of senescent cells, it is vital to know which cell type to target; knowledge about which senescent cells contribute most to musculoskeletal degeneration will ultimately guide the development of effective treatment. Anti-senescece therapy may also be beneficial for several incurable muscular dystrophies and for wasting, by reducing inflammaging and hence boosting the satellite cell regenerative functions. Interestingly, cellular senescence has been shown to mediate fibrotic pulmonary disease, and senescent cell ablation improves pulmonary function in this setting [82]. Most dystrophinopathies also feature increased muscle fibrosis [83], which aggravates disease progression by substituting muscle with scar tissue, and it is plausible that anti-senescent cocktails will also halt fibrosis and improve patient health status. Thus, elimination of senescent cells may have benefits for tissue repair by reversing several detrimental processes; however, it remains to be determined whether senescence should be blocked partially or totally or eliminated only once early potential stemness-related functions have been completed. The answers to these questions may not be easy to obtain, yet we are rapidly obtaining tools that allow manipulation of the senescence process (for removing senescent cells, neutralizing the SASP, or both processes). The final goal is to preserve stem cell benefits while minimizing the deleterious consequences of senescence.

It also remains unclear how tissues rejuvenate after senescent cell ablation and whether side effects or unexpected challenges will occur. For example, in addition to its potential to eliminate senescent cells, tissue engineering is being explored as a treatment for musculoskeletal diseases. In this scenario, stem cells are isolated and healthy tissue is generated ex vivo to replace damaged tissues such as cartilage and bone. For example, mesenchymal stem cells can be isolated and cultured on a biodegradable scaffold where they are stimulated with TGFβ to induce differentiation into chondrocytes [84]. This newly formed cartilage could then be used for surgical reconstruction of joints. However, a major challenge in tissue engineering is to prevent stem cell senescence [85]. It remains unclear whether similar issues will arise after senescence clearance. So far, tissue regeneration seems efficient after these cells are removed. For example, although cartilage has a weak regenerative potential, it is rejuvenated after senescent cells are removed. Tissue-specific stem cells are likely key to this regeneration. It is possible that the reduction of SASP proteins in the tissue microenvironment releases these cells from their ‘stem cell lock’, resulting in a restored regenerative potential. In addition, cells that are dedifferentiated due to senescence, such as chondrocytes, could help rejuvenate musculoskeletal tissue. In general, multiple factors likely contribute to this rejuvenation. Both local and systemic inflammation are expected to decline, affecting immune system functioning, natural senescent cell clearance, stem cell function, and tissue regeneration.

In conclusion, targeting senescence has the potential to prevent or reverse multiple age-related diseases and to reduce frailty. Furthermore, it seems likely that therapeutically applicable anti-senescent compounds will be available in the future. However, the toxicity of these drugs remains a major concern. Periodic treatments will likely be necessary to maintain possible beneficial effects and it is still largely unknown what the effect of multiple treatment rounds will be. Therefore, the timing and frequency of these treatments should be studied, as well as the long-term effect of senescence clearance on biological processes such as stem cell function.

Conflict of interest
PDK is co-founder, shareholder and consultant for Cleara Biotech B.V., the Netherlands.

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