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Turning back time with emerging rejuvenation strategies

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Abstract

Ageing is associated with the functional decline of all tissues and a striking increase in many diseases. Although ageing has long been considered a one-way street, strategies to delay and potentially even reverse the ageing process have recently been developed. Here, we review four emerging rejuvenation strategies—systemic factors, metabolic manipulations, senescent cell ablation and cellular reprogramming—and discuss their mechanisms of action, cellular targets, potential trade-offs and application to human ageing.

Ageing represents a major risk factor for many chronic conditions, including cardiovascular disease, diabetes, cancer, arthritis and frailty[1,2]. Once considered irreversible, ageing is in fact remarkably malleable. Indeed, inhibition of high-nutrient-sensing pathways (for example, the insulin–insulin-like growth factor (IGF) and mechanistic target of rapamycin (mTOR) pathways) and activation of low-nutrient-sensing proteins (for example, 5′ AMP-activated protein kinase (AMPK) and sirtuins) extend lifespan in various model organisms[3,4]. Diet-based interventions, such as dietary restriction, and pharmacological interventions, including the mTOR inhibitor rapamycin, improve aspects of ageing even when administered late in life[5-10]. A key question is whether ageing of cells, tissues and organisms can be reversed or ‘rejuvenated’ rather than simply delayed.

A host of age-associated features have been identified, with a subset being potential drivers of the ageing process (extensively reviewed elsewhere[1,2]). At the molecular level, ageing hallmarks comprise DNA damage, epigenetic alterations, telomere attrition, protein aggregation and accumulation of aberrant mitochondria and lysosomes[1,2]. At the cellular

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and organismal level, ageing features include cellular senescence, stem cell exhaustion, deregulated nutrient sensing and chronic low-grade inflammation[1,2]. Various rejuvenation strategies that target these hallmarks have recently emerged and they fall into four broad categories: systemic (blood) factors, metabolic manipulations, senescent cell ablation and cellular reprogramming. Although these approaches seemingly target very different ageing features[11-15], a central question is whether they share common mechanisms of action. This Review discusses these four rejuvenation strategies and how they improve health and lifespan. We also address several key questions: which hallmarks of ageing are targeted by each strategy and are there commonalities in their modes of action? Does the rejuvenating effect come with trade-offs? Ultimately, can rejuvenation strategies be used to improve human health and longevity and target age-associated diseases?

Blood factors as targets for rejuvenation

Heterochronic parabiosis studies, in which the circulatory systems of a young mouse and an aged mouse are fused, have provided compelling evidence that blood factors influence organismal ageing (Fig. 1, Table 1 and Supplementary Table 1). Heterochronic parabiosis was initially shown to revitalize muscle stem cells in naturally aged mice, reversing the age-dependent decline in stem cell activation and number and improving their age-associated differentiation bias[16,17]. Since then, heterochronic parabiosis has been shown to enhance muscle, liver, brain and heart function of aged mice[17-24], by boosting the function of both stem and differentiated cells[17-23]. Sharing blood circulation with a young mouse also reduces genomic instability in the aged mouse[20] and reverses age-associated gene expression signatures[25]. Blood factors, rather than blood cells, seem to play a major role in these rejuvenating effects[24-26]: direct injection of young blood plasma (devoid of cells)[25] or of human umbilical cord plasma (also devoid of cells)[26] into aged mice can recapitulate several aspects of heterochronic parabiosis, notably the increase in neurogenesis and improvement of cognitive functions[25,26] (Table 1 and Supplementary Table 1). These observations raise the possibility that blood factors (for example, proteins, metabolites, lipids and exosomes) could be used to reverse aspects of the ageing process, perhaps even in humans.

How does young blood revitalize aged organs and tissues? Young blood may contain pro-rejuvenation factors, or it could dilute or inhibit pro-ageing factors in aged blood (Fig. 2). The pro-rejuvenation effect of young blood on the liver, muscle and brain is less pronounced than the pro-ageing effect of aged blood on these tissues[27], suggesting the presence of potent pro-ageing factors in aged blood. Indeed, systemic pro-ageing factors have been identified through heterochronic parabiosis, including eotaxin (also known as CCL11)[21] and β_2-microglobulin[23]. The levels of eotaxin and β_2-microglobulin increase with age, and these factors inhibit neurogenesis and cognition in young mice[21,23]. Whether blocking pro-ageing blood factors improves tissue function in aged mice remains to be shown, but aged β_2-microglobulin knockout mice exhibit enhanced neurogenesis and cognitive functions compared to age-matched wild-type mice[21,23]. Other systemic signalling pathways have been implicated in mediating the pro-ageing effect of aged blood[16,17,28-31]. For example, heterochronic parabiosis reverses the excessive Wnt signalling underlying the differentiation bias of aged muscle stem cells[16,29]. Furthermore, systemic attenuation of transforming

growth factor-β signalling improves age-dependent decline in neurogenesis and myogenesis[30], and inhibition of interferon signalling partially ameliorates neurogenesis and cognitive function in aged mice[31]. Thus, several pro-ageing factors have been identified in aged blood.

Identifying rejuvenation factors in young blood has been more difficult. Heterochronic parabiosis can restore the decreased Notch signalling that underlies the decline in muscle stem cell activation and number[17,28], although the specific systemic factor (or factors) remains unclear. Growth/differentiation factor 11 (GDF11) was initially identified as a circulating factor whose levels decrease with age but are restored through heterochronic parabiosis[18]. Exogenous GDF11 can rejuvenate heart function[18,32] and improve muscle and neural stem cell functions in aged mice[20,22] (Table 1 and Supplementary Table 1). However, subsequent studies reported no beneficial effects of GDF11 on heart and muscle stem cell function[33,34], and injection of GDF11 can induce cachexia[35]. Thus, although GDF11 may have beneficial effects under specific conditions, it is unlikely to be a universal mediator of rejuvenation. Another potential rejuvenating blood factor whose levels decrease with age is the hormone oxytocin[36]. Its systemic administration improves muscle regeneration by enhancing muscle stem cell activation and/or proliferation in aged mice[36] (Table 1 and Supplementary Table 1). As oxytocin is known for its role in social bonding[37], it could potentially link social environment and ageing. Finally, TIMP2, a metalloproteinase inhibitor, was identified in human umbilical cord plasma[26] and its levels have been shown to decrease with age in both mice and humans[26]. Injection of human cord blood induces hippocampal neurogenesis and improves learning and memory in naturally aged mice[26], effects that are attenuated by TIMP2 depletion[26]. Moreover, administration of exogenous TIMP2 can improve cognitive function in aged mice, pointing to TIMP2 as a key rejuvenating factor[26] (Table 1 and Supplementary Table 1).

These studies suggest the presence of both pro-ageing and anti-ageing factors in the blood, which can be targeted to reverse age-related decline in multiple tissues. However, many open questions remain. Which cell types secrete these factors and could these cells be targeted to achieve similar effects? Do circulating factors drive rejuvenation of all tissues or do they have tissue-specific action? Comprehensive analysis of the response of multiple organs to blood factors will be required to address this question. Testing the interaction between individual factors, including pro-ageing and rejuvenation factors, could identify the main contributors and allow for combinatorial treatments to revitalize tissues and organs. A central question is whether young blood or specific blood factors can extend organismal lifespan. Although initial studies using young blood in aged mice or GDF11 in progeroid mice reported little effect on overall lifespan[38,39], a thorough investigation of the effect of blood factors on mammalian lifespan will be important.

Metabolic-induced rejuvenation

Long-term dietary restriction extends healthspan and lifespan across several species[12,40]. Less-restrictive diet regimens and drugs that mimic the metabolic effects of dietary restriction also have beneficial effects on lifespan[5,8-10,41,42]. Until recently, it was unclear whether these interventions could reverse ageing features in aged individuals. Initial studies

on short-term dietary restriction (5 days to 12 weeks) in middle-aged or old-aged mice revealed improved function in multiple tissues, including muscle, bone, liver, brain, vasculature and immune system[5,7,43] (Fig. 1, Table 1 and Supplementary Table 1), consistent with the possibility that dietary interventions could indeed reverse functional decline. Here, we focus on dietary interventions or mimics that are initiated at middle age or later and on their potential rejuvenation effects on ageing hallmarks.

The periodic fasting-mimicking diet (FMD) consists of cycles of very low caloric intake for 4 days, repeated twice per month, with ad libitum feeding in between[5]. When initiated in 16-month-old mice, FMD reverses age-associated haematopoietic differentiation bias, increases hippocampal neurogenesis and improves hippocampus-dependent memory[5] (Table 1 and Supplementary Table 1). FMD also increases median lifespan and decreases cancer incidence and inflammatory diseases, including ulcerative dermatitis[5]. Some of the beneficial effects of FMD are probably mediated by an increased proliferative capacity and number of stem cells[5]. The refeeding portion of FMD may play a key role in this, as it results in a boost in cell proliferation[5]. Whether FMD also improves tissue function by selecting against dysfunctional cells is unclear. The proliferation boost following refeeding may favour youthful cells, diluting out damaged ones and improving overall tissue function. Given that FMD (and the ketogenic diet, discussed below) reduces cancer incidence[5,9], such regimens may also select against cancerous or precancerous cells.

The ketogenic diet involves the same caloric intake as a normal diet but with reduced carbohydrate consumption. This diet mimics many of the metabolic changes occurring in mice under dietary restriction or fasting[8,9,44]. Both fasting and a ketogenic diet decrease blood glucose levels and increase ketone body levels and fatty acid oxidation[8,9,44] (Table 1 and Supplementary Table 1). Interestingly, alternating between a ketogenic and a control diet weekly in middle-aged mice improves recognition memory and midlife survival[8]. A non-cyclic ketogenic diet also increases median lifespan and improves motor function in aged mice while decreasing cancer incidence[9]. Although not explicitly stated in these studies, some measurements are similar or better post-treatment compared to those at the time of treatment initiation, suggesting not only a delay but also a reversal of the measured features[9,10]. Thus, manipulating diet content may constitute an effective approach for reversing ageing hallmarks and may be easier to implement in humans than long-term dietary restriction.

How do these diet regimens rejuvenate aged tissues? Nutrient-sensing pathways, including mTOR and insulin–IGF signalling, could play a key role[3,45-47] (Fig. 2). Periodic FMD was proposed to act by reducing insulin–IGF1 signalling and inhibiting the activity of mTOR and protein kinase A[6]. Short-term treatment (6 weeks) with rapamycin, an mTOR inhibitor, improves haematopoietic stem cell function in aged mice (although not to the level of 2-month-old mice) and extends lifespan[48] (Table 1 and Supplementary Table 1). The insulin and mTOR signalling pathways are also known to regulate autophagy[3,45-47], and FMD can indeed counteract the decline in autophagy-related proteins in ageing muscle[5]. This points to an important role of mTOR in mediating the beneficial effects of these regimens and raises the possibility that mTOR inhibitors could be used to rejuvenate ageing tissues. Although the effect of rapamycin on lifespan is well established[45], whether it is a rejuvenating

compound remains debated. A comprehensive assessment of ageing phenotypes following long-term (1 year) treatment of young and aged mice showed that rapamycin improves several features, including memory and learning[49]. However, it also ameliorates some of these features in young mice, suggesting that it may have age-independent positive effects[49].

Similarly to periodic FMD, the ketogenic diet also inhibits mTOR and insulin–IGF signalling[8,9]. Interestingly, although a short-term ketogenic diet (1 month) does affect the expression of genes related to insulin signalling and fatty acid synthesis, an extended ketogenic diet (14 months of cyclic diet) does not affect these genes[8]. Thus, repeated cycles may become less effective on signalling pathways[8]. Ketogenic effects could be mediated by increased circulating β-hydroxybutyrate levels, a ketone that inhibits histone deacetylases and may thereby link metabolism, epigenetics and rejuvenation[8,9]. Hence, β-hydroxybutyrate could represent an effective longevity and rejuvenating compound[50,51].

Other nutrient-sensing pathways could also be involved in the rejuvenation effects of dietary regimens (Table 1 and Supplementary Table 1). For example, metformin, which increases AMPK activity[42,47], preserves mitochondrial function and decreases inflammation when administered starting at middle age[41]. Resveratrol, which can activate sirtuins (and other nutrient-responsive pathways), also improves cognitive and renal function and reduces inflammation in rodents when initiated at mid-to-late life[42,52-54]. Whether these improvements represent a true reversal of pre-existing ageing phenotypes remains an open question.

Ablation of senescent cells to restore tissue youthfulness

Cellular senescence is a cell-intrinsic mechanism induced by stress that prevents propagation of damaged cells[55,56]. Initially identified as a barrier against tumour development[56], senescence is now known to be involved in tissue remodelling during embryogenesis[57,58], wound healing[59,60] and ageing[61-70]. Senescence markers include senescence-associated β-galactosidase activity, the cell-cycle inhibitors $p16^{INK4a}$ and $p21^{CIP1}$, and many secreted inflammatory factors (collectively referred to as the senescence-associated secretory phenotype (SASP))[55,56,71]. Senescent cells are heterogeneous[72,73] and do not always exhibit all markers, and, conversely, some markers are also present in non-senescent cells[71,74]. Senescent cells accumulate in ageing tissues across organisms, including primates and rodents, and in age-related pathologies, such as atherosclerosis and Alzheimer's disease[75-79]. Accordingly, senescence has long been thought to contribute to organismal ageing[56], although whether it is a cause or consequence is only starting to be resolved. Indeed, mouse models and compounds that trigger senescent cell elimination have revealed that targeting senescent cells can reverse or delay aspects of the ageing process[61-70] (Fig. 1).

The first evidence that senescent cells can actively contribute to ageing came from genetically modified mice that allow for inducible elimination of p16-positive cells in the context of a progeroid disease[63]. In INK-ATTAC transgenic mice that express a drug-inducible form of caspase 8 under the *Cdkn2a* (which encodes $P16^{Ink4a}$) promoter, drug administration triggers caspase-8-mediated apoptosis in p16-positive cells[63]. In a progeroid mouse model (*BubR1*), caspase-8-mediated ablation of p16-positive cells starting from early

life delays the onset of age-associated features, including loss of fat and skeletal muscle and cataract development[63]. Even later in life, ablation of p16-positive cells reduces age-associated fat and skeletal muscle loss[63]. Follow-up studies in naturally ageing mice showed that the removal of p16-positive cells starting from 12 months of age (midlife) through to 18 months of age attenuates age-associated decline in adipocyte, kidney and heart function[62] (Table 1 and Supplementary Table 1). Importantly, the removal of p16-positive cells from midlife to end of life extends median lifespan by 24–27%[62]. Similar health benefits were observed with another model. In p16-3MR mice that express thymidine kinase from herpes simplex virus under the *Cdkn2a* promoter, administration of the thymidine kinase substrate ganciclovir initiates apoptosis of p16-positive cells[59]. In both mouse models, apoptosis induction in p16-positive cells late in life for at least 3 weeks improves liver, kidney, bone and adipocyte metrics and function[61,64,69]. Although not specifically stated, some metrics seem to be better after treatment than at initiation[61,64], suggesting that senescent cell ablation may reverse ageing features. However, high p16 expression has also been observed in non-senescent cells, notably macrophages[71,74]. Hence, the beneficial effects of this intervention might partially be due to targeting macrophages, which are known to change with age[1,2,80]. Despite the promise of these initial findings, more remains to be learned about the optimal times for treatment initiation and duration for maximal effects, and about specificity to senescent versus immune cells.

These initial proof-of-concept studies spurred the field to identify compounds that can relatively specifically kill senescent cells based on their unique molecular profiles. Several classes of such ‘senolytic’ drugs have been identified, including Bcl protein family inhibitors (for example, navitoclax, also known as ABT263)[65], kinase inhibitors (for example, dasatinib and quercetin)[68], heat shock protein 90 inhibitors (for example, 17-DMAG)[66] and inhibitors of the p53–MDM2 interaction (for example, UBX0101)[67,81]. Dasatinib and quercetin[68] and 17-DMAG[66] improve healthspan in the *Ercc1*$^{-/-}$ progeroid mouse model. In naturally aged mice, senolytics enhance cardiovascular, vascular, bone, liver and physical functions (dasatinib and quercetin)[64,68,69,82], revitalize haematopoietic and muscle stem cell populations (ABT263)[65], enhance cartilage regeneration (UBX0101)[67] and even extend median lifespan (dasatinib and quercetin)[70] (Table 1 and Supplementary Table 1). A forkhead box protein O4 peptide (FOXO4-DRI) also has senolytic effects. This peptide blocks the sequestration of p53 by FOXO4, which seems to be senescence specific, thus allowing p53 activation and cell death in senescent cells[61]. FOXO4-DRI restores fitness, fur density and kidney function in both progeroid (*Xpd*$^{TTD/TTD}$) and naturally aged mice[61] (Table 1 and Supplementary Table 1). Whether FOXO4-DRI acts on all types of senescent cells without targeting healthy cells, a common challenge for senolytic drugs[14,68,83-85], remains to be determined. Many senolytics were initially identified as cancer drugs because cancer cells exploit similar anti-apoptotic pathways, notably overexpression of Bcl family proteins[84,86]. Thus, some beneficial effects of senolytics may originate from the elimination of precancerous and cancerous cells.

How does the removal of senescent cells rejuvenate tissues and extend lifespan? Senescence could contribute to the decline in tissue homeostasis and function by inducing a permanent cell-cycle arrest in proliferative cell populations. Senescence of reparative stem and progenitor cells may lead to a decline in tissue regenerative potential. Senescence could also

act through SASP, which promotes local and systemic inflammation[55,56]. SASP factors could contribute to stem cell exhaustion or dysfunction, infiltration and alteration of immune cells, insulin resistance, damage of tissue structure and even propagation of the senescent phenotype in neighbouring cells[56,87]. Elimination of senescent cells can revive stem cell populations in naturally aged mice[65,88] (Fig. 2), and p16 depletion resets ageing features in aged muscle stem cells[89]. Moreover, SASP inhibition by the Janus kinase 1/2 inhibitor ruxolitinib reduces systemic and adipose tissue inflammation and increases insulin sensitivity in naturally aged mice[69,88,90]. Senescent cell removal can delay cancer development, which could be a source of the observed lifespan extension in mice[62]. Elucidating the mechanisms by which senolytics ameliorate tissue function will be important in identifying additional senolytic compounds and in determining how best to use them. Importantly, senescent cells can have beneficial effects, for example, by facilitating tissue repair after injury and preventing tissue fibrosis[59,60,91]. Identifying mechanisms that distinguish between the beneficial and harmful effects of senescence could help to identify therapeutic strategies to specifically target the latter.

Reprogramming back to a youthful state

Cellular reprogramming is the conversion of terminally differentiated somatic cells into induced pluripotent stem cells (iPSCs)[92], for instance by the expression of the transcription factors OCT4 (also known as POU5F1), SOX2, KLF4 and MYC (OSKM)[92]. Cellular reprogramming allows for the generation of in vitro models to study ageing and age-associated diseases and the development of autologous stem cell therapies to replace ageing tissues[93-95]. Reprogramming also resembles to some extent the process of fertilization, during which the chronological age of the parent cells is effectively reset such that the resulting offspring has a normal lifespan[96]. Hence, cellular reprogramming has emerged as a potential rejuvenation strategy[15,96].

Reprogramming to pluripotency can erase several ageing features in vitro. iPSCs derived from aged cells show extended telomeres, improved mitochondrial morphology, number and fitness (ATP production and membrane potential) and restored nuclear morphology[15,95,97,98]. iPSC reprogramming of aged cells also resets heterochromatin marks and transcriptomic profiles[15,95,97,99]. After re-differentiation of these iPSCs into neurons or fibroblasts, transcriptomic changes, improvements in nucleocytoplasmic compartmentalization, nuclear morphology and (in the case of fibroblasts) proliferative potential largely remain in the rejuvenated state[95,97,99]. This suggests that the youthful state is not exclusive to pluripotency and can persist after re-differentiation. Although most age-associated phenotypes tested are reversed by in vitro reprogramming, iPSCs generated from aged human cells can retain a DNA methylation signature of their age, which can be erased with additional passaging[100]. Thus, some features of ageing may be harder to rejuvenate than others, and some aspects, such as pre-existing genetic mutations, cannot be reverted[94,100]. The ability to rejuvenate ageing traits may be specific to reprogramming to a pluripotent state because direct reprogramming to a differentiated state (for example, neurons) was less effective at erasing ageing marks[99]. Future studies should explore the extent and time course of molecular rejuvenation by iPSC reprogramming, to determine whether there is dependency between different age-associated features.

Recent studies using mouse models of doxycycline-inducible reprogramming factor (OSKM) expression have demonstrated that somatic cells can be reprogrammed to pluripotency in vivo[101-104], suggesting that the rejuvenating effects of cellular reprogramming might be recapitulated in an organism. A major limitation of initial studies was that persistent expression of OSKM led to teratoma formation[101-103]. Thus, an important step was to determine whether the rejuvenating aspect of reprogramming could be uncoupled from its dedifferentiating, teratoma-inducing properties[96]. Interestingly, this uncoupling was recently shown to be possible[105]. Short-term OSKM induction ('partial reprogramming') in fibroblasts from progeroid mice (*Lmna*^{G608G}) erased features of ageing, including DNA damage, dysregulation of histone marks, expression of senescence-associated genes and nuclear envelope abnormalities[105]. When applied in vivo, cyclic partial reprogramming (2-day induction with 5-day withdrawal) starting at 8 weeks of age extended both healthspan and lifespan (median ~30%, maximum ~20%) of these mice, without teratoma or cancer development[105] (Fig. 1). In vivo partial reprogramming applied to naturally ageing mice at midlife also improved glucose tolerance and the regenerative capacity of muscle and the pancreas after injury[105] (Table 1 and Supplementary Table 1). These observations underscore the potential of cellular reprogramming to rejuvenate cells and tissues in vivo, although more work is needed in the context of naturally aged mice. Indeed, some of the positive OSKM effects in muscle at midlife could be age independent, as the regenerative potential of muscle has not declined yet at this stage of life[36,106]. Whether partial reprogramming can reverse tissue decline in the absence of injury or disease and/or extend lifespan in naturally aged mice also remains to be determined.

How does cellular reprogramming rejuvenate aged cells and tissues? At the molecular level, epigenetic remodelling is a key factor in iPSC reprogramming[107,108], and histone modifications have been proposed to mediate the rejuvenating effects of partial reprogramming[105] (Fig. 2). At the tissue level, partially reprogrammed mice have increased numbers of muscle stem cells after injury[105]. Hence, enhanced regenerative capacity and stem cell function could contribute to the lifespan extension observed in the context of premature ageing[105]. Reprogramming could also act by eliminating dysfunctional cells in tissues or by diluting them through proliferation of healthy cells. The extent to which rejuvenating effects persist after in vivo reprogramming remains an important direction for future studies. Although some reprogramming-induced epigenetic and transcriptomic remodelling persists following doxycycline withdrawal[101], the increase of histone 3 lysine 9 trimethylation (H3K9me3) levels reverts within 8 days of withdrawal in vitro[105]. Thus, whether transient reprogramming leads to transient or persistent rejuvenation remains to be determined.

Common or distinct mechanisms of rejuvenation

One key question is whether the four rejuvenation strategies described above share modes of action or whether they use distinct mechanisms (Fig. 2). Common pathways could be harnessed to induce rejuvenation more directly, whereas differing ones could be targeted in combination to enhance it.

Inflammation.

Inflammation could be directly or indirectly affected by most rejuvenation strategies. Heterochronic parabiosis reduces inflammatory factors and pathways, such as eotaxin and interferon signalling[21,31]. FMD and dietary restriction (DR)-mimicking drugs have anti-inflammatory effects by suppressing the onset of senescence and the secretion of pro-inflammatory cytokines[109-111]. Senolytics could exert their beneficial effects by reducing inflammation, as senescent cells contribute to inflammation through SASP[56,87]. Finally, although age-associated activation of nuclear factor-κB signalling impairs cellular reprogramming[112], activation of innate immunity and inflammatory factors, such as interleukin-6 (IL-6), promote reprogramming[102,104,113-115]. These observations highlight inflammation as a critical target for rejuvenation strategies. Chronic inflammation (‘inflammaging’) has emerged as a key feature of ageing and age-associated diseases[1,2,116], and its genetic and pharmacological targeting has been shown to extend healthspan and lifespan across multiple species[117-121]. Interestingly, stimulation or blocking of hypothalamic nuclear factor-κB activity was shown to accelerate or decelerate ageing, respectively[122], suggesting a potential key role of the hypothalamus in modulating inflammation and ageing. Future studies should aim at investigating the interplay between rejuvenation strategies and inflammation, and exploring potential synergistic effects of rejuvenating compounds with anti-inflammatory drugs.

Nutrient-sensing pathways.

The insulin–IGF1, mTOR and AMPK pathways have been extensively studied in the context of longevity[1-3,45-47] and are key candidates for relaying rejuvenating effects. The anti-ageing diets discussed inhibit mTOR and/or elicit a drop in circulating insulin and IGF1 levels[5,8,9]. DR-mimicking drugs also inhibit insulin–IGF1 and mTOR signalling and activate AMPK[41,49,123]. However, evidence for the involvement of these pathways in heterochronic parabiosis, the elimination of senescent cells and cellular reprogramming is mostly circumstantial. The shared circulatory system and organs in parabiosis may affect glucose–insulin homeostasis and IGF1 signalling[124]. Moreover, the IGF1 and mTOR pathways promote senescent cell survival and regulate SASP[125-127], whereas AMPK pathway activation suppresses the development of senescence[128]. Finally, insulin–IGF1 signalling inhibits reprogramming[15,129,130], although the role of AMPK in cellular reprogramming is still debated[15]. An intriguing possibility is that nutrient-sensing pathways may be more important for delaying ageing than reversing it.

Epigenomic remodelling.

The epigenomic landscape of a cell reflects not only its identity but also its health and biological age[131-133]. Senescent cells exhibit a characteristic chromatin state[134,135], and their secreted factors (for example, IL-6) have been shown to induce epigenomic changes[136,137]. The rejuvenating effect of cellular reprogramming has been proposed to occur through epigenomic remodelling[105]. Moreover, dietary interventions and DR-mimicking drugs affect the epigenome[131,138,139], although whether these changes are necessary for rejuvenating effects is unclear. Finally, while chromatin changes have not yet been reported in the context of heterochronic parabiosis, chromatin changes could relay some effects[13]. Whether

restoring a youthful epigenome holds the key to a prolonged rejuvenated state is a compelling question.

Autophagy.

Autophagy, which includes the process of delivering damaged proteins and organelles to lysosomes for degradation, is key for cellular homeostasis[140] and could play an important role in mediating rejuvenation. Most diet regimens and DR-mimicking drugs induce autophagy[5,140,141], and the blood factor GDF11 was shown to enhance this process[20]. Senescent cell ablation could eliminate autophagy-deficient cells[142,143]. Finally, autophagy is also induced early in the reprogramming process[129]. Whether autophagy is necessary for the rejuvenation effects of cellular reprogramming remains unclear[15,129], but reactivation of the lysosome–autophagy pathway in aged stem cells improves their function[144-146]. These observations suggest a link between the lysosome–autophagy pathway and rejuvenation strategies, but the extent to which autophagy promotes rejuvenation remains to be explored.

Mitochondria.

Mitochondrial function could also be central to rejuvenation strategies. Cellular reprogramming increases mitochondrial fitness[98,147] and GDF11 can improve mitochondrial morphology and function[20]. Senescent cells have dysfunctional mitochondria with increased generation of reactive oxygen species, which in turn promote SASP and can induce senescence in neighbouring cells[148-150]. Mitochondria in senescent cells were recently suggested to have reduced ability to metabolize fatty acids, contributing to increased hepatic fat deposition with age and a decline in liver function[64]. Hence, the removal of senescent cells with poor mitochondrial function could be beneficial by reducing reactive oxygen species levels in the microenvironment and perhaps also by improving overall mitochondria function in ageing tissues and organs. However, rejuvenation strategies could also act by reducing mitochondrial function. Indeed, reduced mitochondrial activity extends lifespan in *Caenorhabditis elegans*, *Drosophila* and mice[151-155]. In addition, metformin, which inhibits mitochondrial function[141], can extend healthspan and/or lifespan in multiple organisms[41,141]. Future studies should explore how this organelle relays the rejuvenating effects of these different strategies.

These observations suggest that the four rejuvenation strategies could act through common molecular pathways. However, the degree to which these pathways are modulated and whether each strategy targets them directly or indirectly remain unclear. Investigating the regulation and sequential order of these pathways following each intervention will help to identify the mechanisms that are critical for restoring youthfulness and that could be targeted for greater effect. Different rejuvenation approaches could also act via diverse mechanisms, which could be combined to achieve synergistic effects. Broader conceptual questions also remain: is the rejuvenation process the direct opposite sequence of events that lead to ageing? Do rejuvenation strategies target the root cause of ageing or simply its consequences? Can these interventions affect overall lifespan?

Target cells for rejuvenation

Which cell types are primarily targeted by rejuvenation strategies and mediate their beneficial effects? Adult stem cells are an attractive candidate as they provide a renewable source of cells to repair damaged tissues (Fig. 2). Indeed, most rejuvenation approaches also improve stem cell functions[5,17,21,22,25,65,105,110,156], although whether these effects are direct or indirect remains unclear. The inherent plasticity of stem cells may make them more susceptible to the rejuvenating effects of cellular reprogramming, for example.

Stem cell state may also dictate susceptibility to ageing and rejuvenation. For example, quiescent stem cells exhibit increased age-related features compared to actively proliferating stem cells[146,157], raising the possibility that quiescent cells might benefit more from rejuvenation strategies. In fact, a proliferative state could itself reset ageing features (for example, DNA damage and protein aggregates) in stem cell populations[146,157]. In addition, these rejuvenation strategies could indirectly affect stem cells. For example, young blood was proposed to enhance neurogenesis in aged mice by improving endothelial cells and thereby the vasculature of the neural stem cell niche[22]. Moreover, although the senolyte ABT293 is thought to improve aged haematopoietic and muscle stem cells by eliminating senescent stem cells[65], it could also act by clearing senescent niche cells, such as endothelial cells and fibroblasts. In line with this notion, niche endothelial cells were shown to contribute to haematopoietic stem cell ageing, and transplantation of young endothelial cells could partially reverse these changes[158]. Thus, the primary target of rejuvenation approaches may be vascular and connective tissue cells. As these cells are present throughout the organism, targeting them may have broader organismal effects. Teasing apart the effects of rejuvenation strategies on different cell types and states will help efforts to improve tissue function and health and could identify strategies to simultaneously target both differentiated and stem cell populations for enhanced treatments.

Other attractive candidate cells for rejuvenation are senescent cells. Beyond their direct elimination by genetic means or senolytics, senescent cells may also be targeted by other rejuvenation strategies. The pro-ageing factor eotaxin[21] has been associated with senescence[159], potentially linking the beneficial effect of senescent cell ablation to changes in systemic factors. Moreover, FMD, DR and DR-mimicking drugs suppress senescence onset and pro-inflammatory cytokine levels[109-111,160]. Although speculative, it is also plausible that the proliferation bursts induced by FMD or partial reprogramming could dilute and/or trigger senescent cell clearance. Indeed, many of the age-associated features that are reverted by partial reprogramming are related to senescence[105]. Cellular reprogramming has been suggested to rejuvenate senescent cells[97]. However, the relationship between reprogramming and senescence is complex. Reprogramming factors can trigger cellular senescence[161,162]; conversely, senescence promotes cellular plasticity of neighbouring cells through SASP (for example, IL-6)[102,104,115,163]. In line with these observations, induction of reprogramming factors for 7 days results in more teratomas in aged mice than in young mice[102-104], possibly due to the presence of senescent cells in aged tissues. Finally, senescent cell removal using senolytic drugs or an inducible genetic system decreases in vivo reprogramming efficiency[115]. It will be interesting to elucidate the interplay between senolytic and reprogramming strategies for rejuvenation.

Potential trade-offs of rejuvenation

Ageing disrupts the balance of key biological processes that maintain organismal homeostasis and function. Hence, reversing it is not as simple as turning off these processes, but rather involves the need to restore a balance. For example, although age-associated senescence and/or chronic inflammation could impair tissue function, they are also critical for normal tissue repair and remodelling[59,60]. Accordingly, counteracting senescence and/or inflammation could reduce the ability of the organism to perform these processes (Fig. 1). Indeed, elimination of senescent cells impedes tissue repair and promotes tissue-specific fibrosis[59,60,91]. Similarly, DR-related interventions impair the immune response to infections and reduce wound healing[164], although refeeding after DR or DR-mimicking drugs can restore or even potentiate these responses[164,165]. DR regimens, when started too early, can also interfere with growth and fecundity and lead to amenorrhea and osteoporosis[12]. Importantly, excessive perturbation of a specific feature may ultimately lead to tumorigenesis and cancer progression. As senescence is a critical barrier against tumorigenesis[56], preventing its induction could increase cancer risk. Similarly, sustained expression of reprogramming factors could lead to tumour formation[101]. Senescent cells also exploit anti-apoptotic pathways, such as Bcl-2, that are important for the survival of healthy cells (for example, lymphocytes and platelets)[166-168]. Consequently, compounds that are used to target senescent cells (for example, pan-Bcl inhibitors) are also associated with gastrointestinal symptoms and haematopoietic system toxicity[83,84]. Hence, the risk/benefit ratio of these rejuvenation strategies must be taken into account before considering them as a viable anti-ageing treatment.

Future perspectives

There is now compelling evidence that the ageing process is plastic and that it is possible to revive aged cells and tissues. Although the four strategies discussed here have received much attention in recent years, other approaches may also turn out to have rejuvenating effects. Genetic perturbations such as the expression of telomerase in middle-aged and old-aged mice improves healthspan (for example, insulin sensitivity and osteoporosis) and extends median lifespan[169]. Similarly, life-long increased dosage of $p16^{INK4}$ and p53 can have beneficial effects to counter ageing[170-172]. Hence, inducible telomerase, p16 or p53 expression later in life could be future rejuvenation strategies. Environmental interventions that have benefits on healthspan and lifespan could also be leveraged for rejuvenation. For example, exercise improves hippocampal neurogenesis and muscle function in aged rodents[173-175]. Lowering core body temperature extends lifespan in invertebrates and African killifish[3,176-178] and even in mice[179]. Finally, the transfer of young microbiome in middle-aged killifish was recently shown to extend both healthspan and lifespan[180]. However, whether these potential strategies revert ageing hallmarks or delay the appearance of such characteristics remains to be tested. It will also be interesting to determine whether key organs or systems, such as the hypothalamus, orchestrate ageing in a centralized manner by integrating environmental inputs and secreting systemic factors[36,122,181]. These systems could then be targeted to achieve whole-organism rejuvenation.

The question also emerges of whether rejuvenation interventions, which were mainly tested in mice, may benefit human health and longevity (Fig. 1). Metabolic approaches have reached furthest in testing this possibility and have shown promise in benefiting humans. FMD in individuals ranging from 20 to 70 years of age was shown to improve physiological readouts that are altered with age, including body weight, blood pressure, cholesterol and IGF1 levels[5,182]. FMD and DR-mimicking drugs, such as metformin and rapamycin, can improve risk factors associated with age-related diseases, such as cancer, diabetes and cardiovascular disease[182-185]. Clinical trials are underway using metformin and rapamycin to target ageing[141] (ClinicalTrials.gov identifiers: NCT02432287 and NCT02874924) and rapamycin analogues are being tested in the elderly in the context of response to vaccination[165] and respiratory tract infection (ClinicalTrials.gov identifier: NCT03373903). Currently, there are no data showing beneficial effects of blood factors, senolytic drugs or reprogramming in humans. However, the levels of the pro-ageing blood factors eotaxin and β_2-microglobulin are increased in the plasma of elderly humans[21,23] and the rejuvenation factor TIMP2 is enriched in human umbilical plasma[26]. Moreover, most senolytic drugs identified can eliminate human senescent cells in vitro[61,65,66,68,85,186]. Similarly, cellular reprogramming can revert ageing features of human cells in vitro[95,97-100,105], raising the possibility that these approaches may also prove beneficial for human ageing. Indeed, some of these approaches are now being explored in the context of human age-associated diseases. For instance, young blood is being tested in Alzheimer's disease (ClinicalTrials.gov identifier: NCT02256306). Although the initial trial showed only a minor improvement[187], larger trials are underway to better assess efficacy. Several senolytics are currently used in the clinic as anticancer drugs[84,86] and are being tested on chronic kidney disease (ClinicalTrials.gov identifier: NCT02848131) and osteoarthritis (ClinicalTrials.gov identifier: NCT03513016). Initial findings are encouraging, but many challenges remain before these strategies can be used successfully in the clinic. The optimization of therapeutic dosage with minimal side effects will be key to translational efforts. It will also be critical to establish reasonable end points and robust biomarkers of healthy ageing to assess intervention efficacy.

These studies provide compelling evidence that the ageing process is malleable and that it is possible to revive aged cells, tissues and organs. They also raise the exciting possibility of translation to address human ageing and age-associated diseases. The coming years will undoubtedly see exciting developments in ongoing efforts to better understand, delay and potentially reverse ageing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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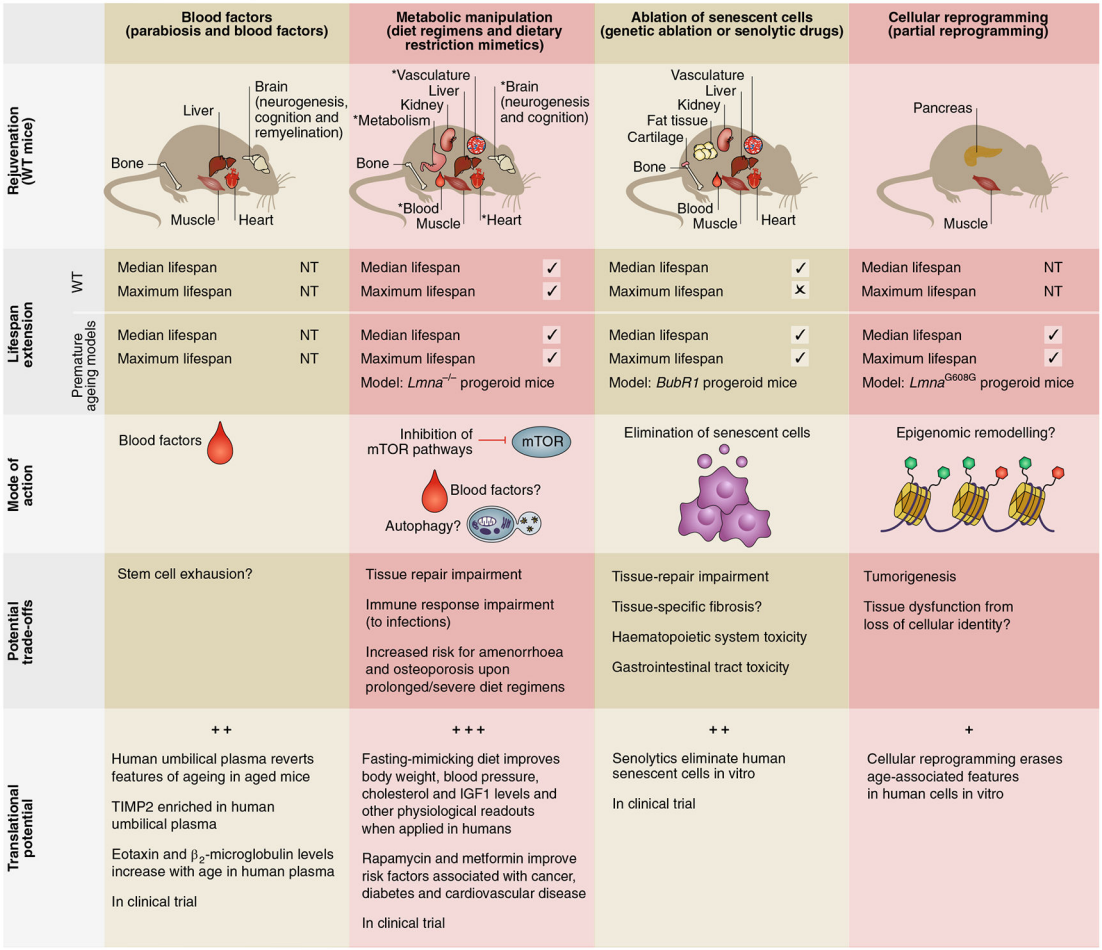

Fig. 1 | Comparison of emerging strategies for organismal rejuvenation and lifespan.
A comparison of the four emerging rejuvenation strategies: blood factors, metabolic manipulation, ablation of senescent cells and cellular reprogramming. The figure depicts the features that improve when treatment in mice is initiated at midlife or later. The top panel shows organs or tissues that exhibit a rejuvenated phenotype in wild-type (WT) mice. For rapamycin, features that have been shown to improve also in young mice following treatment are indicated with an asterisk (*). The effect on lifespan, proposed primary mode (or modes) of action and possible trade-offs of these strategies are also presented. Finally, the translational potential in humans is indicated by the increasing number of plus signs (+) based on present evidence in human ageing and current feasibility. NT, not tested. Question marks indicate possible modes of action and trade-offs. Figure adapted from ref. [188].

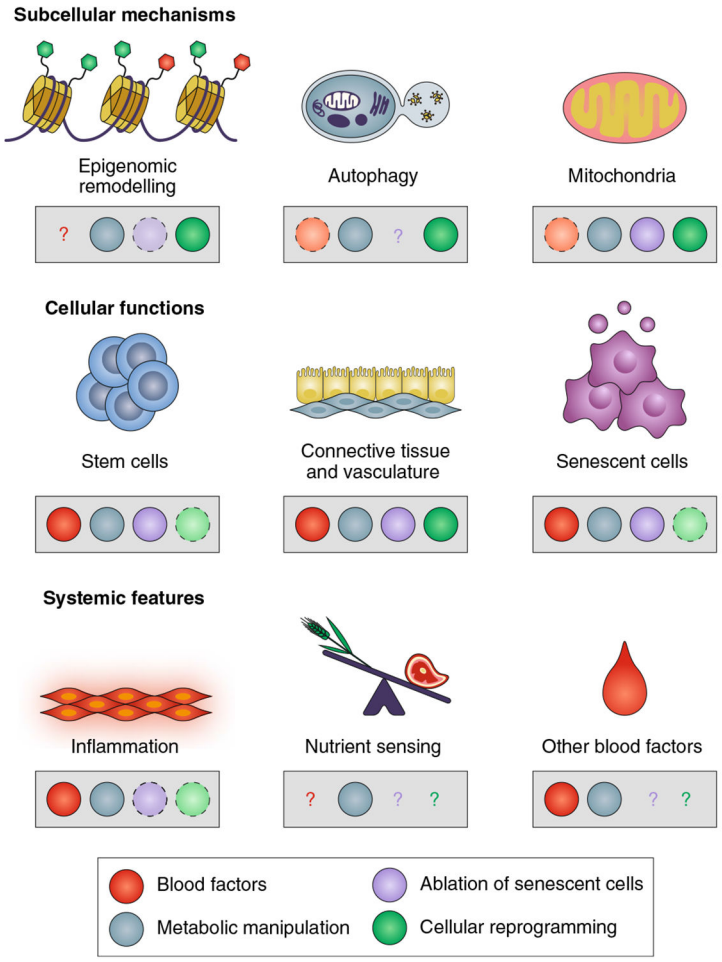

Fig. 2 | Potential common mechanisms and target cells of the rejuvenation strategies.
A comparison of the proposed underlying mechanisms of action and target cell types influenced by each of the rejuvenation strategies. These include subcellular mechanisms (for example, chromatin changes, induction of autophagy pathways and alteration in mitochondrial function), cellular functions (such as revival of stem cell populations, attenuation of the deleterious effects of senescent cells and changes in connective tissue cells (for example, endothelial cells, fibroblasts and adipocytes)) and intercellular features (for example, decrease in inflammation, perturbation of nutrient-sensing pathways and changes in blood factors). The circles below each feature are colour-coded for each rejuvenation strategy and represent the current level of evidence for the effect of the corresponding strategy on the feature. Solid/dark circles, strong evidence. Dotted/light circles, mostly indirect evidence. Question marks, no evidence as of now.

Table 1 |

Summary of studies testing rejuvenation interventions at midlife or later in naturally ageing mice

Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
Blood factors				
Parabiosis	2–3, 19–26	Skeletal muscle (MuSC) and liver regeneration	Old-old and young-young parabionts	17
Parabiosis	4–6, 24–26	Muscle regeneration (MuSC and fibrosis)	Old-old and young-young parabionts	16
Parabiosis	3–4, 18–20	Neurogenesis and cognitive function	Old-old and young-young parabionts	21
Parabiosis	1–2, 10–12	Spinal cord remyelination	Middle-aged-middle-aged and young-young parabionts	24
Parabiosis	2, 23	Cardiac metrics	Old-old and young-young parabionts	18
Parabiosis	2, 15–16 or 21	Neurogenesis and cognitive function	Old-old and young-young parabionts	22
Parabiosis	2–3, 22–24	Muscle regeneration (MuSC) and function	Old-old and young-young parabionts	20
Parabiosis	3, 18	Synaptic plasticity and gene expression	Old-old parabionts	25
Parabiosis	3, 19	Bone regeneration	Old-old and young-young parabionts	19
Parabiosis	3, 18	Neurogenesis and cognitive function	Young-young parabionts	23
Young blood injection	18	Cognitive function and gene expression	Old blood	25
Human plasma injection (cord, young and elderly)	8–10, 13–14	Neuronal and cognitive functions and gene expression	Age-matched vehicle control, young (22 years of age) and old (66 years of age) human plasma	
TIMP2 administration	18	Synaptic plasticity and cognitive functions	Age-matched vehicle control	26
Oxytocin administration	2–4, 22–24	Muscle regeneration (MuSC and fibrosis)	Age-matched vehicle and antagonist (only young) control	36
GDF11 administration	23–24	Cardiac metrics	Age-matched vehicle control	18
GDF11 administration	21–23	Neurogenesis and cognitive function	Age-matched vehicle control	22
GDF11 administration	2–3, 22–24	Muscle regeneration (MuSC) and function	Age-matched vehicle control	20
GDF11 administration	23	Muscle regeneration (MuSC)	Age-matched vehicle control	34
GDF11 administration	24	Cardiac metrics and function	2 months of age, 3 months of age and age-matched vehicle treated	33
GDF11 administration	2, 22	Cardiac metrics and body weight	Age-matched vehicle control	32
Metabolic manipulation				
Short-term dietary restriction	5–8, 28–30	Vasculature metrics	Age-matched ad libitum	43
Short-term dietary restriction	2, 18	Skeletal muscle (MuSC)	Age-matched ad libitum	7
Fasting-mimicking diet	16	Organ size and regeneration	16 months of age and age-matched ad libitum	5

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Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
Fasting-mimicking diet	16	Immunosenescence	4 months of age, 16 months of age and age-matched ad libitum	5
Fasting-mimicking diet	16	Cognitive function	Age-matched ad libitum	5
Fasting-mimicking diet	16	Bone density	12 months of age and age-matched ad libitum	5
Fasting-mimicking diet	16	Cancer and inflammation	Age-matched ad libitum	5
Ketogenic diet	12	Physiological and metabolic metrics; physical, behaviour and cognitive functions	Age-matched ad libitum and low-carbohydrate non-ketogenic	
Ketogenic diet	12–14	Cognitive and motor function and frailty index	12 months of age and age-matched ad libitum	8
Ketogenic diet	12–14	Cognitive and motor function	12 months of age and age-matched ad libitum	8
Rapamycin	22	Immune system (HSC and immunity)	2 months of age and age-matched vehicle control	48
Rapamycin	4, 13, 20–22	Comprehensive organismal assessment (>25 tissues)	3–6 months of age and age-matched vehicle control	49
Metformin	12	Serum biomarkers	Age-matched ad libitum and dietary restricted	41
Metformin	12	Physical performance	Age-matched ad libitum	41
Metformin	12	Liver, muscle and gene expression	Age-matched ad libitum and dietary restricted	41
Resveratrol	12	Physiological metrics and gene expression	Age-matched untreated controls	54
Resveratrol	18	Renal function and histology	Age-matched untreated control	53
Ablation of senescent cells				
Ablation of p16-positive cells	18	Adipose tissue metrics	Age-matched wild-type treated	88
Ablation of p16-positive cells	12	Kidney, heart and adipocyte metrics and function	12 months of age and age-matched vehicle control	62
Ablation of p16-positive cells	24	Vasculature function	Age-matched vehicle control	82
Ablation of p16-positive cells	12	Cartilage degeneration	Age-matched vehicle control	67
Ablation of p16-positive cells	24	Fat accumulation in liver	Age-matched vehicle control	64
Ablation of p16-positive cells	12, 20	Bone metrics and loss	Age-matched vehicle control	69
Ablation of p16-positive cells	>25	Renal function	Age-matched vehicle control	61
Dasatinib + quercetin	24	Cardiac metrics and function	Age-matched vehicle control	68
Dasatinib + quercetin	24	Vasculature function	Age-matched vehicle control	82
Dasatinib + quercetin	24	Fat accumulation in the liver	Age-matched vehicle control	64
Dasatinib + quercetin	20	Bone metrics and loss	Age-matched vehicle control	69
Dasatinib + quercetin	20	Physical performance	Age-matched vehicle control	70
ABT263	21–22	Immune system (HSC) and muscle (MuSC) function	2 months of age and age-matched vehicle control	65

Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
UBX0101	2–3, 19–20	Cartilage regeneration	Age-matched vehicle control	67
FOXO4-DRI	24	Renal function and frailty	Age-matched vehicle control	61
Cellular reprogramming				
Transient reprogramming	12	Pancreas regeneration	Age-matched vehicle control	105
Transient reprogramming	12	Muscle regeneration	Age-matched vehicle control	105

An extended version of this table with more details regarding the method of administration or procedure, duration of intervention, mouse strain and sex is available as Supplementary Table 1 HSC, haematopoietic stem cell; MuSC, muscle stem cell.