SUPPLEMENTARY TEXT

Selectivity of *ATTAC*-mediated cell clearance

ATTAC uses the first 2,617 base pairs of the endogenous mouse *p16*^{Ink4a} gene promoter to drive an *FKBP-Caspase 8* "suicide" gene and *EGFP* expression in senescent cells. This relatively small fragment lacked distal regulatory elements of the endogenous *p16*^{Ink4a} gene and was selected because of its high transcriptional activity in senescent relative to non-senescent cells[1]. Our earlier work in BubR1 progeroid mice demonstrated that only the tissues showing elevated expression of p16^{Ink4a} and other senescence markers (fat, skeletal muscle and eye) have elevated levels of FKBP-Casp8 and GFP transcripts[2]. Upon AP treatment, these tissues selectively showed an attenuated functional decline and decreased transcript levels of *FKBP-Casp8*, *GFP*, *p16*^{Ink4a}, and senescence markers, indicating that *ATTAC* targets senescent cells for elimination.

Robust induction of endogenous *p16*^{Ink4a} has also been observed outside the context of cellular senescence, for instance upon disruption of Rb, a frequent event in human cancers that drives neoplastic growth. To determine whether *ATTAC* might be co-activated and mediate cell death in this context, we employed SV40 large T antigen, a well-established inhibitor of Rb known to induce robust *p16*^{Ink4a} gene expression. A lentiviral expression system was used to express constitutively SV40 large T antigen in primary *ATTAC* MEFs. Western blot analysis confirmed that the endogenous *p16*^{Ink4a} gene was hyperactive in these MEFs (Extended Data Fig. 2a). However, we observed no co-induction of the *ATTAC* transgene (see Casp8 and GFP expression in revised Extended Data Fig. 2c), which correlated with a lack of induction of senescent cell markers. Furthermore, SV40 large T immortalized MEFs were resistant to AP-induced apoptosis (Extended Data Fig. 2b, c). Together, these data suggest that *ATTAC* is lacking *p16*^{Ink4a} promoter elements critical for driving *p16*^{Ink4a} transcription in the context of Rb loss, and is thus unlikely to eliminate cancer cells in which Rb is perturbed.

We repeated the above studies in MEF lines derived from *p16*^{Ink4a}-LUC[3] and 3MR mice[5]. The former MEFs are homozygous for a firefly luciferase knock-in at the endogenous *p16*^{Ink4a} locus and the latter MEFs contain a randomly inserted single copy of a bacterial artificial chromosome that spans the murine *Cdkn2a* locus and has a 3MR trimodal reporter gene (generating a fusion protein of Renilla luciferase, mRFP, and herpes simplex virus 1 thymidine kinase) inserted into *p16*^{Ink4a} exon 2 (see Extended Data Fig. 2d for a schematic representation of the differences in these models from *ATTAC*). In contrast to *ATTAC* MEFs, both *p16*^{Ink4a}-LUC and *3MR* MEFs induced transgene expression upon inactivation of Rb by SV40 LT antigen (Extended Data Fig. 2f and g). These data confirmed that induction of *p16*^{Ink4a} in non-senescent cells in the setting of Rb disruption depends on a *p16*^{Ink4a} promoter element located outside of the 2617 bp promoter fragment of *ATTAC*.

ATTAC was also not induced in peripheral blood T lymphocytes that robustly engage endogenous *p16*^{Ink4a} with aging without concomitant expression of multiple senescence

markers[4, 5] (Extended Data Fig. 2h), further implying that transgene induction is quite selective for senescence. However, these limited analyses certainly do not exclude the possibility that other *p16*^{Ink4a}-positive non-senescent cells engage *ATTAC* and die upon AP exposure. In the manuscript, we, therefore, referred to the cells eliminated by *ATTAC* as *p16*^{Ink4a}-postive cells rather than *p16*^{Ink4a}-positive senescent cells.

Lifespan and healthspan studies (supplementary information and discussion)

The central goal of this study was to explore the biological impact of senescent cells on health and lifespan, a longstanding unaddressed question. This study is a logical follow-up to our study that demonstrated a deleterious role for *p16*^{Ink4a}-positive senescent cells in premature aging phenotypes of progeroid BubR1 hypomorphic mice[2]. It is well established that lifespan and healthspan are very sensitive to very subtle manipulations. For example, minor changes in husbandry conditions or diet can have significant effects within the same animal facility. Furthermore, median lifespans of C57BL/6 mice (Extended Data Fig. 4c, d) and other mouse strains vary tremendously between test sites (up to 25-30%), even in settings where great effort is made to unify husbandry conditions across mouse facilities, such as the National Institutes of Aging (NIA) Interventions Testing Program (ITP)[6, 7]. Thus, instead of a narrowly defined optimal absolute lifespan, individual mouse strains seemingly have an optimal lifespan range that takes into consideration subtle differences in husbandry conditions as well as undefined, and thus uncontrollable, site-specific factors. With regards to husbandry conditions, our lifespan studies were conducted according to standard conditions (for details see methods section).

Lifespan and healthspan studies should ideally be carried out while exposing the animals to as little stress as possible[8]. For instance, the ITP prefers drug delivery via food or water over methods that require extensive animal manipulation such as repetitive injection[8]. Our approach for removal of senescent cells from transgenic *ATTAC* mice requires repeated administration of AP20187, which is an unstable compound that needs to be supplied by intraperitoneal (IP) injection (twice a week). It should therefore be emphasized that the intervention and control mice in our study were exposed to a greater frequency of manipulation stress than is typical in conventional longevity studies. In C57BL/6 *ATTAC* control males, repeated IP-injection of vehicle seemed to impact negatively the lifespan, as their lifespan is short of the lifespan range for unmanipulated C57BL/6 mice (see Extended Data Fig. 4c). In contrast, no such negative impact was observed in the corresponding female cohort because the lifespan of vehicle-injected C57BL/6 *ATTAC* females was within the normal range compared to eight other test sites (Extended Data Fig. 4d). Importantly, although vehicle-treated C57BL/6 *ATTAC* males lived shorter than unmanipulated C57BL/6 males, the primary cause of death, development of malignant tumors, remains unchanged. As is the case for unmanipulated C57BL/6 males, ~75-80% of vehicle-treated C57BL/6 *ATTAC* males have lymphomas, sarcomas or carcinomas at the time of death, without significant changes in tumor incidence and spectrum compared to AP-treated C57BL/6 *ATTAC* males, whose lifespan is extended to well into the normal range for unmanipulated C57BL/6 males. Based on these data, we believe the most plausible explanation for the shortened lifespan of vehicle-treated C57BL/6 *ATTAC* males is that repeated injections accelerate the progression of the neoplastic lesions that naturally

develop in this strain, causing the animals to die at an earlier than normal age. Consistent with this idea, several laboratories have demonstrated that repetitive stress can accelerate tumorigenesis in mice[9-11].

The diet used in the C57BL/6 *ATTAC* study consisted of 5% fat, which is the standard amount of dietary fat content used in lifespan studies. We fed mice of the C57BL/6-129Sv-FVB *ATTAC* study a diet containing 9% fat. Separate lifespan studies ongoing in the lab using unmanipulated wildtype mice of a similar genetic background (C57BL/6-129Sv) revealed that mice on this diet have a 25% shorter lifespan than those on a standard diet containing 5% fat (Extended Data Fig. 4a). Furthermore, these studies suggested that vehicle-treated C57BL/6-129Sv-FVB *ATTAC* mice on 9% fat had a median lifespan that is normal for this diet (Extended Data Fig. 4b): the median lifespans of vehicle-injected C57BL/6-129Sv-FVB *ATTAC* mice were nearly identical to unmanipulated wildtype C57BL/6-129Sv hybrid mice for both males and females. These data indicate that the lifespan of our vehicle-injected C57BL/6-129Sv-FVB control mice is normal for the diet that they were on, and that the 27% lifespan extension observed in AP-treated animals is not just bringing the lifespan for the strain back to normal but an actual lifespan extension for hybrid mice fed a 9% fat diet.

Our experimental system has limitations with regards to killing senescent cells, in that clearance of *p16*^{Ink4a}-positive cells by the *ATTAC* system was partial and tissue/organ selective. Possible explanations include bioavailability and volume of distribution of the drug (e.g. kidney), the level of transgene expression on a per cell basis, expression of anti-apoptotic proteins on a per cell basis, and other currently unknown effects. Therefore, lifespan extensions observed with the *ATTAC* model may well underestimate the effects of more efficient senescent cell clearance. It is important to keep in mind that this is different from lifespan studies using approaches that involve genetic, dietary or pharmacological interventions, which unlike our system, impact virtually all cells of the animal.

The aging field is now broadly recognizing that "normal aging" and "normal lifespan" are not single entities or reference values. There is an evolving understanding of "aging" by the research community and how to best use lifespan and healthspan measures as indices of this process[12]. Lifespan, in particular an increase in both mean and maximum longevity, has historically been the method through which an intervention has been deemed successful in "altering aging" [12, 13]. Interpretation of interventions that increase health and lifespan in terms of "slowing aging" is now more and more recognized as a highly complicated matter and a topic of continued debate in the field. Rather than speaking imprecisely about interventions being "anti-aging", they should instead be described as ameliorating specific age-related declines under defined conditions. Therefore, in the current study, we sought to investigate the role of senescent cells in a variety of robust, reproducible age-related changes. These include the age-associated increases in cancer, glomerulosclerosis, lipoatrophy, and cardiomyocyte hypertrophy, and decreases in cardiac stress resilience, spontaneous activity and exploratory behavior, all of which are strongly delayed by *p16*^{Ink4a}-positive cell clearance. Importantly, these beneficial effects appear to be independent of genetic background, sex or diet.

SUPPLEMENTARY TEXT REFERENCES

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