

Distinct tumor suppressor mechanisms evolve in rodent species that differ in size and lifespan

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Summary

Large, long-lived species experience more lifetime cell divisions and hence a greater risk of spontaneous tumor formation than smaller, short-lived species. Large, long-lived species are thus expected to evolve more elaborate tumor suppressor systems. In previous work, we showed that telomerase activity coevolves with body mass, but not lifespan, in rodents: telomerase activity is repressed in the somatic tissues of large rodent species but remains active in small ones. Without telomerase activity, the telomeres of replicating cells become progressively shorter until, at some critical length, cells stop dividing. Our findings therefore suggested that repression of telomerase activity mitigates the increased risk of cancer in larger-bodied species but not necessarily longer-lived ones. These findings imply that other tumor suppressor mechanisms must mitigate increased cancer risk in long-lived species. Here, we examined the proliferation of fibroblasts from 15 rodent species with diverse body sizes and lifespans. We show that, consistent with repressed telomerase activity, fibroblasts from large rodents undergo replicative senescence accompanied by telomere shortening and overexpression of p16^{Ink4a} and p21^{Cip1/Waf1} cycline-dependent kinase inhibitors. Interestingly, small rodents with different lifespans show a striking difference: cells from small shorter-lived species display continuous rapid proliferation, whereas cells from small long-lived species display continuous slow proliferation. We hypothesize that cells

of small long-lived rodents, lacking replicative senescence, have evolved alternative tumor-suppressor mechanisms that prevent inappropriate cell division *in vivo* and slow cell growth *in vitro*. Thus, large-bodied species and small but long-lived species have evolved distinct tumor suppressor mechanisms.

Key words: body mass; cancer; evolution; lifespan; senescence.

Introduction

Replicative senescence was first described in human cells over 40 years ago (Hayflick & Moorhead, 1961). The major mechanism underlying replicative senescence is progressive telomere shortening, which occurs with every cell division in cells lacking telomerase activity (Harley *et al.*, 1990; Shay & Wright, 2000). Replicative senescence is thought to be an antitumor mechanism that limits cell proliferation (Campisi, 2001; Sedivy, 2007) and contributes to long cancer-free human lifespan. Replicative senescence also occurs in the fibroblasts of several nonhuman primates (Steinert *et al.*, 2002; Herbig *et al.*, 2006; Gardner *et al.*, 2007), and large farm animals such as cow (Hornsby *et al.*, 1986), sheep (Davis *et al.*, 2005), and horse (Argyle *et al.*, 2003), (reviewed in Davis & Kipling, 2005; Gorbunova & Seluanov, 2008). But we still know little about senescence, or the lack thereof, in other mammalian species.

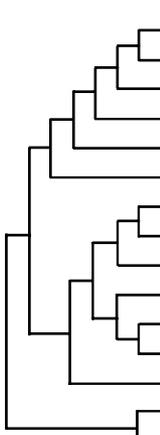
Senescence of mouse cells differs from replicative senescence of human cells in many ways (Sedivy, 1998; Wright & Shay, 2000; Parrinello *et al.*, 2003). First, mouse somatic cells express telomerase and have very long telomeres (Prowse & Greider, 1995). Second, when cultured under 20% oxygen, mouse fibroblasts slow their growth after approximately ten population doublings (PDs), but after a few days immortal clones emerge and continue rapid growth (Parrinello *et al.*, 2003). Such spontaneous immortalization does not occur in cultures of human fibroblasts (Wright & Shay, 2000). Third, under physiological oxygen concentration, mouse fibroblasts do not display senescent phenotype (Parrinello *et al.*, 2003). Thus, 'senescence' of mouse cells appears to be a stress response, which is more akin to stress-induced premature senescence than replicative senescence (Sherr & DePinho, 2000; Wright & Shay, 2000; Parrinello *et al.*, 2003).

The difference in telomere biology between humans and mice is intriguing. We recently undertook a study aiming to understand the evolutionary basis of this difference. To test the hypothesis that somatic repression of telomerase activity might have evolved as a tumor-suppressor adaptation in large or long-lived species, we analyzed telomerase activity in fresh tissues of 15 rodent species with diverse lifespans and body masses

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	Maximum lifespan (yr)	Adult body mass (g)	Replicative senescence	Cell growth rate (slope)	Animals examined
Capybara	15	55 000	yes	na	4
Guinea pig	10	728	no	0.4117	5
Paca	16	9 000	yes	na	4
N. American porcupine	23	8 600	yes	na	3
Chinchilla	17	642	no	0.1732	3
Naked mole-rat	28	35	no	0.1226	4
House mouse	4	30	no	0.2885	5
Norway rat	5	400	no	0.3786	5
Mongolian gerbil	6	53	no	0.3192	4
Deer mouse	8	21	no	0.3668	5
Golden hamster	4	105	no	0.3043	4
Muskrat	10	1 362	no	0.2106	4
American beaver	24	20 250	yes	na	4
E. American chipmunk	10	96	no	0.1984	3
Eastern grey squirrel	24	533	no	0.1145	8

Fig. 1 Phylogeny and phenotypic data for 15 rodent species. The tree topology is based on molecular phylogenies inferred from Martin *et al.* (2000), Michaux *et al.* (2001), Murphy *et al.* (2001), Montgelard *et al.* (2002), Adkins *et al.* (2003), and Steppan *et al.* (2004). Body mass and maximum lifespan data are obtained from Nowak (1999), Turturro *et al.* (1999), Buffenstein & Jarvis (2002), de Magalhaes *et al.* (2005), and Weigl (2005). The data on replicative senescence and growth rates are derived from the growth curves shown in Fig. 2. The growth curve for each species represents an average of at least five independent cultures.

(Seluanov *et al.*, 2007). Our comparative analysis showed that telomerase activity coevolves with body mass but not lifespan. The largest rodents, capybara and beaver, showed a near complete repression of telomerase activity, as in humans. In contrast, all small rodents showed high levels of telomerase activity in somatic tissues. Surprisingly, telomerase activity was detected in somatic tissues of both short- and long-lived small species, including the longest-lived rodents, the naked mole-rat and the Eastern gray squirrel.

Here we address several questions raised by our previous study. First, does the repression of telomerase activity in the somatic tissues of large rodents mean that their fibroblasts will display replicative senescence? Second, the presence of telomerase activity in somatic tissues of small long-lived rodents is puzzling: these animals remain largely tumor-free during their long lives despite the increased cancer risk of persistent telomerase activity. We therefore asked if fibroblasts from these species display the same rapid proliferation in culture as fibroblasts from short-lived, cancer-prone mice.

To answer these questions we studied replicative lifespan of fibroblasts from 15 rodent species. First, we show that fibroblasts from large rodent species that lack telomerase activity enter typical replicative senescence, as expected. Second, we demonstrate that fibroblasts from small short-lived rodent species maintaining telomerase activity lack replicative senescence, and display rapid continuous growth. Last, and most surprising, we show that fibroblasts from small *long-lived* rodents also lack replicative senescence and instead display unusually slow growth. We hypothesize that cells of small long-lived rodents have evolved alternative tumor-suppressor mechanisms – ones distinct from regulation of telomerase activity – that protect them from cancer *in vivo* and slow their proliferation *in vitro*.

Results

Rodent species display three distinct patterns of *in vitro* cell proliferation

To examine the growth characteristics of primary fibroblasts from long- and short-lived rodent species, we isolated primary lung and skin fibroblasts from 15 species with diverse lifespans and body masses (Fig. 1). Tissues were obtained from at least three young adults of each species, and one lung and one skin fibroblast culture was established from each animal. In total, at least five primary fibroblast cultures were analyzed for each species, as some cultures were lost at early stages due to contamination (Fig. 1).

Cells were passaged for 100 to 200 days under physiological oxygen concentration (3%) to avoid inducing premature senescence due to oxidative stress (Parrinello *et al.*, 2003). Cell numbers were counted at every passage, and growth curves were determined for each species (Fig. 2). The growth curves of skin and lung fibroblasts were similar; therefore the skin and lung cell data were combined for each species. Qualitatively, the fibroblast growth curves of rodent species fell into three groups: (1) replicative senescence (Fig. 2A); (2) continuous fast growth (Fig. 2B); and (3) continuous slow growth (Fig. 2B).

To understand the cell biological basis of these contrasting fibroblast growth curves, we next studied telomere biology in these cells. Telomerase activity and telomere length were examined at early and late cell passages. Telomerase activity was assayed using a modified telomeric repeat amplification protocol (TRAP) (Sztatmari & Aradi, 2001) (Fig. 3). The TRAP assay measures the extension of the telomeric sequence by telomerase in whole cell extracts. Fibroblasts from beaver, porcupine, and

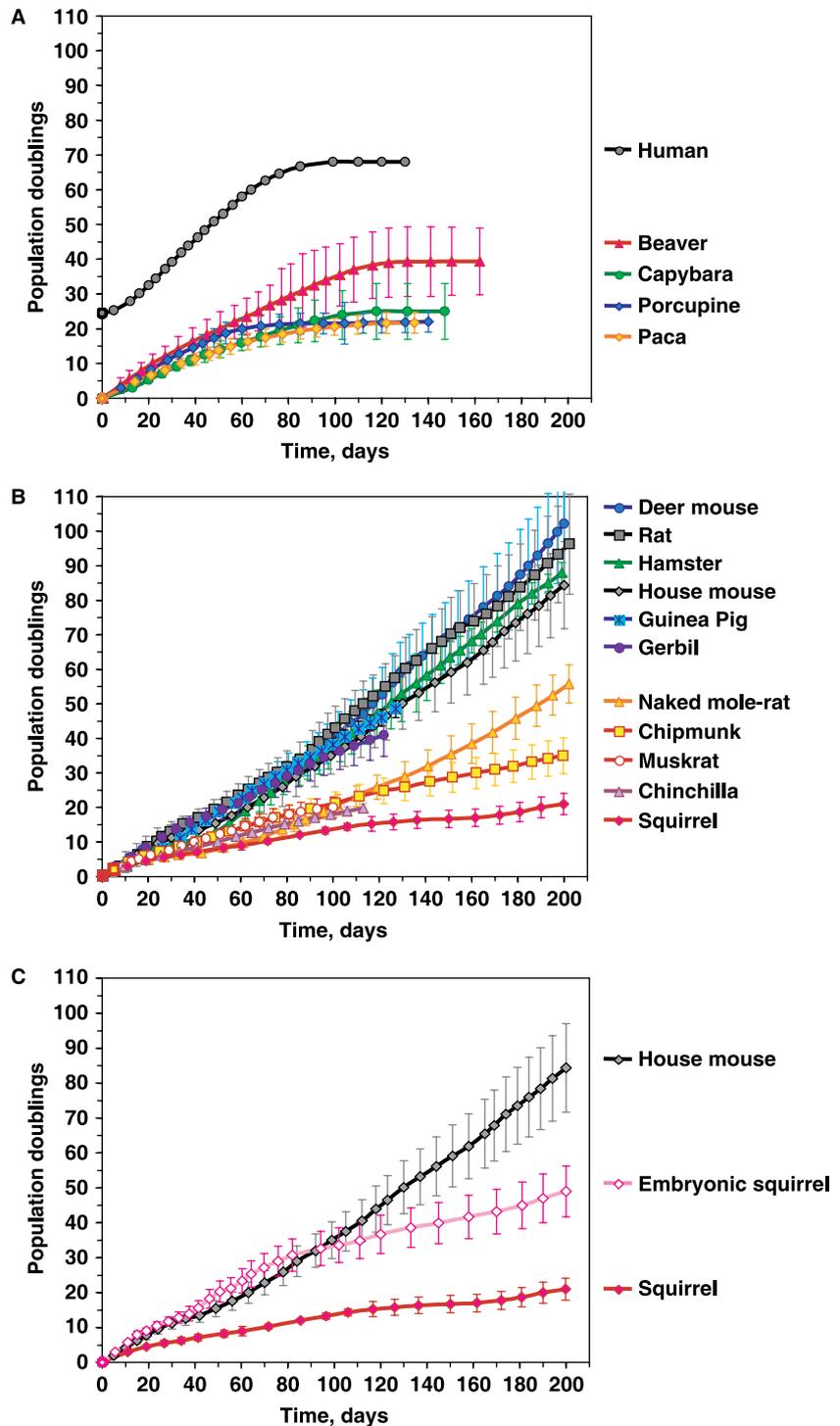


Fig. 2 Growth curves of primary rodent fibroblasts. Cells were isolated from skin and lung tissue and grown at 3% oxygen. The number of donors for each species is shown in Fig. 1. (A) Species that use replicative senescence. Each curve is an average of at least five independent cultures, and error bars show standard deviation (SD). Beaver cultures entered senescence at $PD39 \pm 10$; porcupine and paca cultures entered senescence at $PD22 \pm 3$; and capybara cultures senesced at $PD25 \pm 8$. Human primary fibroblasts culture HCA2 is shown as a reference. (B) Rodents that do not use replicative senescence fall into two groups: fast growing and slow growing. Each curve is an average of at least five independent cultures, and error bars show SD. (C) Growth of gray squirrel embryonic fibroblasts (SEFs). Cells grow rapidly for the first 30 PD, and then slow down and attain the growth rate of adult cells. SEFs were isolated from four embryos derived from two pregnant females.

capybara had no detectable telomerase activity. Early passage paca fibroblasts were telomerase negative but started to express weak telomerase activity at late passages. Fibroblasts from all other animals expressed high levels of telomerase activity.

Telomere length was examined using the terminal restriction fragment (TRF) method (Fig. 4). Beaver, porcupine, and capybara displayed telomere shortening with increasing passages, which is consistent with the absence of telomerase activity in

these cells. Paca telomeres did not shorten. Cells from the species that express telomerase activity did not show appreciable telomere shortening during *in vitro* culture.

Inbred mouse strains have been reported to have longer telomeres than outbred strains (Bickle *et al.*, 1998; Hemann & Greider, 2000; Manning *et al.*, 2002). Since our collection included inbred as well as wild-caught house mice and Norway rats we compared telomere biology in these animals (Fig. S1).

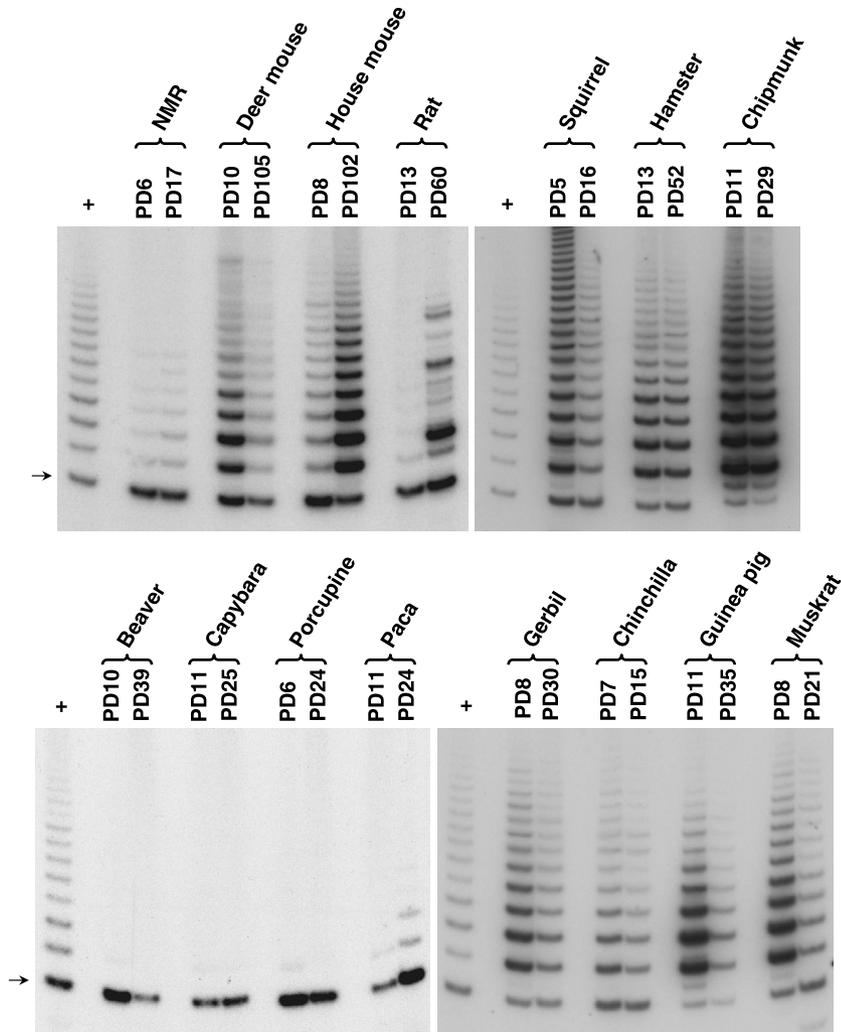


Fig. 3 Telomerase activity in early and late passage rodent fibroblasts. Telomerase activity was examined using modified telomeric repeat amplification protocol (TRAP) assay. Lanes labeled with '+' contains a sample of telomerase-positive human cancer cell line. Arrow indicates a primer dimer, which is not dependent on telomerase activity. NMR, naked mole-rat. The mouse sample shown is from a of 129/Sv \times BL6 F1 hybrid. The rat sample shown is from a Fischer F344 rat.

Fibroblasts of both inbred and wild-caught animals expressed telomerase activity, but the activity was somewhat higher in wild-caught animals. In agreement with the previous reports telomeres of the wild-caught mouse were shorter than telomeres of inbred mouse. However, telomeres of the wild-caught rat were longer than the telomeres of inbred rat. Importantly, cell growth rates did not differ between inbred and wild-caught animals, indicating that using inbred mice and rats does not confound the analysis of cell growth characteristics.

Fibroblasts from large species undergo replicative senescence

Fibroblasts from beaver, porcupine, capybara, and paca entered replicative senescence (Fig. 2A). Beaver cells senesced at PD39 \pm 10 (population doublings mean \pm range), and porcupine and paca cells senesced at PD22 \pm 3, and capybara cells senesced at PD25 \pm 8. Senescent rodent fibroblasts attained flat and enlarged morphology typical of senescent human cells, and showed positive SA- β -galactosidase staining (data not shown). As discussed above, beaver, porcupine, and capybara fibroblasts

expressed no detectable telomerase activity (Fig. 3), and senescence in these cells was accompanied by telomere shortening (Fig. 4), strongly suggesting that the observed replicative senescence is mediated by telomere shortening. Telomere length at the time of senescence was not uniform across the species. None of the beaver, porcupine, or capybara cultures spontaneously immortalized. Paca fibroblasts did not express telomerase activity at early passages, but later became telomerase positive. Interestingly, two of five paca cultures formed immortal clones after being kept in senescence for 3 weeks. Thus, replicative senescence in paca is less stringent than in beaver, porcupine, and capybara.

We next tested whether replicative senescence in beaver, porcupine, capybara, and paca cells is accompanied by up-regulation of p16^{Ink4a} (p16) and p21^{Cip1/Waf1} (p21) cyclin dependent kinase inhibitors (CDKIs), as in human cells. Up-regulation of p16 is believed to signal cell stress, while up-regulation of p21 is more specific to telomere-mediated senescence (Herbig *et al.*, 2004). Young and senescent beaver, capybara, paca, and porcupine fibroblasts were subjected to Western blot analysis with antibodies to conserved regions of p16 and p21 (Fig. 5). Each

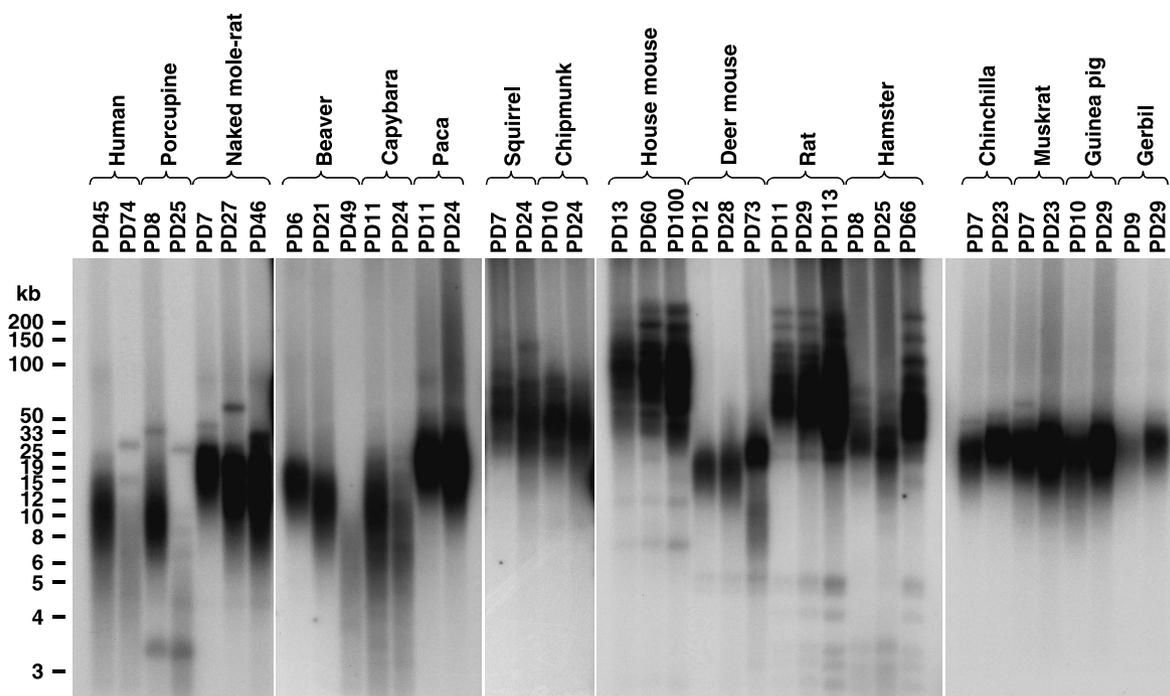


Fig. 4 Telomere length in early and late passage rodent cultures. Telomere length was analyzed using a terminal restriction fragment assay as described in Experimental procedures. Five micrograms of genomic DNA is loaded in each lane. The mouse sample shown is from a 129/Sv × BL6 F1 hybrid. The rat sample shown is from a BN rat.

protein was tested with two different antibodies, to confirm the protein identity. p16 and p21 proteins are not highly conserved, and the sizes varied among different species. p16 was strongly up-regulated in senescent beaver, paca, and porcupine fibroblasts. A very faint band was visible in senescent capybara cells, which could result from either a low amount of p16 or from poor antibody affinity. p21 was up-regulated in senescent cells of all four species. These results further confirm that large rodents use telomere-mediated replicative senescence.

The common feature of the four rodent species, beaver, porcupine, capybara, and paca, that use replicative senescence is their large body mass (Fig. 1). We therefore statistically evaluated whether the presence of replicative senescence correlates with body mass or lifespan across all 15 rodent species. These analyses reveal that replicative senescence is correlated with body mass ($t = -5.87$, d.f. = 13, $p < 0.0001$) but not with maximum lifespan ($t = -2.06$, d.f. = 13, $p = 0.0606$; Fig. 6). To correct these analyses for phylogenetic nonindependence, we analyzed phylogenetically independent contrasts (Felsenstein, 1985; Harvey & Pagel, 1991; Purvis *et al.*, 1994). These analyses confirm that the evolutionary change in body mass is correlated with the evolution of replicative senescence ($t = 4.86$, d.f. = 2, $p = 0.0398$) while change in the lifespan is not ($t = 1.93$, d.f. = 2, $p = 0.1939$).

In mammals, body mass correlates positively with longevity (Harvey *et al.*, 1989; Austad & Fischer, 1991; Austad, 2005). It would therefore be interesting to test whether evolutionary change in body mass correlates with replicative senescence independently of lifespan (Speakman, 2005). However, since in

our data set there are only three phylogenetically independent transitions from absence of replicative senescence to the presence of senescence, we do not have the statistical power to perform the appropriate multivariate analyses.

Fibroblasts from small long-lived species have a slow proliferation rate in culture

Fibroblasts from house mouse, deer mouse, Norway rat, hamster, guinea pig, gerbil, naked mole-rat, muskrat, chipmunk, chinchilla, and Eastern gray squirrel grew continuously in culture and did not enter replicative senescence (Fig. 2B). Consistent with the absence of replicative senescence in these species, their fibroblasts express telomerase activity (Fig. 3) and do not display appreciable telomere shortening with increasing passages (Fig. 4).

These small-bodied species appear to fall into two groups according to cell proliferation rate (Fig. 2B). The first group includes species with rapid *in vitro* cell proliferation rate: house mouse, deer mouse, Norway rat, hamster, guinea pig, and gerbil. The second group includes species with slow *in vitro* cell proliferation rates: naked mole-rat, muskrat, chipmunk, chinchilla, and Eastern gray squirrel. The two groups differ statistically in lifespan (Mann–Whitney test, $P_{\text{MWU}} = 0.0097$), with the fast group containing shorter-lived species (mean lifespan \pm standard deviation = 6.2 ± 2.4 years) and the slow group containing longer-lived species (17.8 ± 8.1 years).

We next statistically evaluated whether fibroblast proliferation rate correlates with lifespan or body mass. Cell proliferation

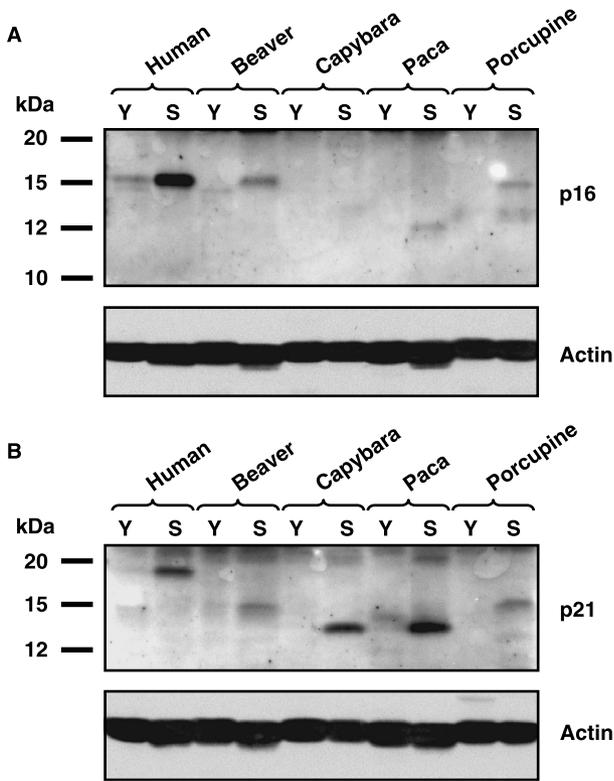


Fig. 5 Activation of p16 (A) and p21 (B) in senescent rodent fibroblasts. Western blot was performed using antibodies to conserved regions of the proteins. p16 and p21 are poorly conserved and molecular weights differ between species. Y, young cells; S, senescent cells.

rates for each species were estimated from the slopes of lines fitted to the data from the first 100 days of cell culture in Fig. 2(B). The slopes were derived from early passages because rodent cells may undergo aneuploidization and clonal selection upon extended passaging. Fibroblast proliferation rate showed a significant negative correlation with maximum lifespan ($F_{1,9} = 10.68, r^2 = 0.54, p = 0.0097$) but not with body mass ($F_{1,9} = 0.11, r^2 = 0.012, p = 0.746$) (Fig. 7). Phylogenetically independent contrasts also showed a significant negative correlation between fibroblast growth rate and maximum lifespan ($F_{1,9} = 6.7, r^2 = 0.428, P = 0.029$) but not body mass ($F_{1,9} = 0.31, r^2 = 0.033, p = 0.593$). To account for the potential contribution of the correlation between body mass and lifespan, we analyzed the residuals of phylogenetically independent contrasts. Evolutionary change in lifespan controlled for body mass was significantly related to growth rate ($F_{1,9} = 5.898, r^2 = 0.396, p = 0.038$). (The p -value from the latter analysis should, however, be treated with caution because the heterogeneity of variances assumption was violated and could not be remedied by transformation of the data.) Thus, all three analyses of uncorrected data, of phylogenetically independent contrasts, and of residuals of phylogenetically independent contrasts confirm a significant negative correlation between *in vitro* fibroblast growth rate and lifespan.

During *in vitro* culture, cells are forced into continuous cell division under nonphysiological conditions. Normal cells resist

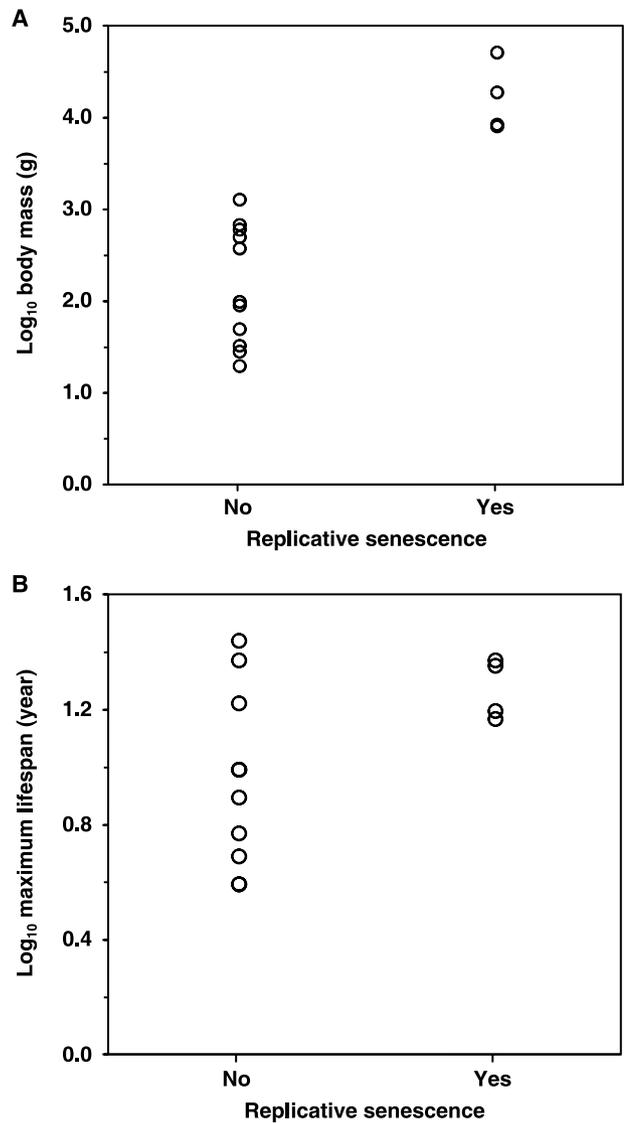


Fig. 6 Species that display replicative senescence have larger body mass. Relationship of replicative senescence with body mass (A), and maximum lifespan (B) was analyzed. Replicative senescence correlates with body mass ($t = -5.87, d.f. = 13, p < 0.0001$) but not with maximum lifespan ($t = -2.06, d.f. = 13, p = 0.06$). 'No' and 'Yes' indicate presence or absence of replicative senescence in the species. Plots use species data uncorrected for phylogeny.

this continuous growth by employing various antitumor mechanisms, such as replicative senescence. We propose that long-lived rodents, which do not use replicative senescence, have evolved alternative mechanisms to restrict cell proliferation that make these cells acutely sensitive to their environment, and slow their *in vitro* growth.

Squirrel embryonic fibroblasts have fast proliferation rate at early passages, but slow down later

To test whether cells from the third group of species are capable of rapid growth under our standard culture conditions, we

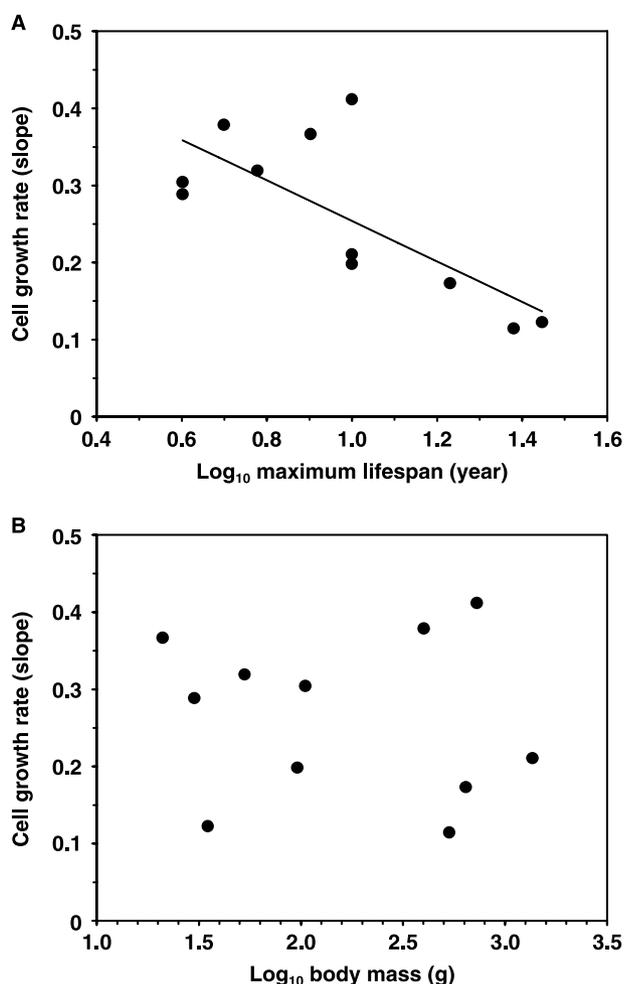


Fig. 7 Relationships of cell growth rate with maximum lifespan (A) and body mass (B). Cell growth rate correlates with maximum lifespan ($F_{1,9} = 10.68$, $r^2 = 0.54$, $p = 0.0097$) but not body mass ($F_{1,9} = 0.11$, $r^2 = 0.012$, $p = 0.746$). The analysis is done for the small-bodied species only, and the species having replicative senescence are not included. Plots use species data uncorrected for phylogeny.

examined the growth of embryonic fibroblasts. We chose Eastern gray squirrel because cells from this species had the slowest growth rate, and we were able to obtain two pregnant females. Squirrel embryonic fibroblasts grew rapidly, similar to mouse fibroblasts up to PD30, after which the proliferation rate slowed to a rate similar to that of an adult squirrel culture (Fig. 2C). We hypothesize that the growth control mechanisms, which restrict proliferation of squirrel cells are activated late during squirrel development. This result strongly suggests that the *in vitro* proliferation rates of adult cell cultures reflect physiological properties of these cells.

Discussion

Our study shows that fibroblast growth characteristics have coevolved with species body mass and lifespan. Previously, we showed that repression of telomerase activity in rodent tissues

coevolves with large body mass in rodents (Seluanov *et al.*, 2007). Here we extend these observations to include a correlation between larger body mass and the presence of replicative senescence: the largest rodents – beaver, capybara, porcupine, and paca – have evolved replicative senescence (Fig. 8). The sizes of these species suggest that, to the level of resolution of our study, body mass greater than 8000 g favors the evolution of replicative senescence (Fig. 1). Evolutionary increases in body mass increase cancer risk, as larger animals contain more cells in their body, and any cell may potentially turn cancerous. Increased mortality rate due to cancer then drives the adaptive evolution of repression of telomerase activity in species with large body mass. It has been predicted that larger animals should have multiple, redundant tumor-suppressor systems so that their cells require more steps for tumor formation (Graham, 1983; Nunney, 1999; Leroi *et al.*, 2003). Our data on rodents provide experimental support for this theory.

Our study is focused exclusively on rodent species and it remains unclear if the same rules apply to other groups of mammals. Telomere biology in mammals studied so far supports the model of coevolution of replicative senescence and body mass (reviewed in Gorbunova & Seluanov, 2008). For instance, large mammals such as cow, sheep, and horse do not express telomerase in somatic tissues, and their fibroblasts have finite lifespan in culture (Hornsby *et al.*, 1986; Argyle *et al.*, 2003; Davis *et al.*, 2005). Replicative senescence has also been documented in large primates such as chimpanzee, orangutan, gorilla, baboon, and several macaque species (Steinert *et al.*, 2002; Herbig *et al.*, 2006; Gardner *et al.*, 2007). An intermediate situation where fibroblasts do not express telomerase activity but their cultures fail to undergo growth arrest has been detected in smaller species, such as rabbits and the ring-tailed lemur (Steinert *et al.*, 2002; Forsyth *et al.*, 2005). A general trend seems to be that mammals with a body mass greater than 8000 g evolve stringent replicative senescence, mammals smaller than 2000 g do not use replicative senescence, and species with body mass between 2000 and 8000 g display a spectrum of intermediate phenotypes.

Even less is known about telomere biology in birds. Long-lived species, such as the storm petrel and common tern, express telomerase throughout their lives (Hausmann *et al.*, 2007). Both of these species are small with an adult body mass below 200 g. Telomerase activity has also been detected in the somatic tissues of a larger bird, the domestic chicken (Venkatesan & Price, 1998). However, chicken fibroblasts do not express telomerase and undergo a clear-cut replicative senescence (Ponten, 1970; Dinowitz, 1977). Little is known about telomere biology in the largest bird species such as ostrich or emperor penguin. The number of PDs before senescence has been correlated to maximum lifespan (Rohme, 1981), or when phylogenetic correction was applied, to species body mass (Lorenzini *et al.*, 2005). Our data set does not allow for such analysis as only four species undergo replicative senescence with three of them showing similar replicative lifespans.

Long lifespan, like body mass, is expected to increase cancer risk. It may therefore seem puzzling that the long-lived rodents

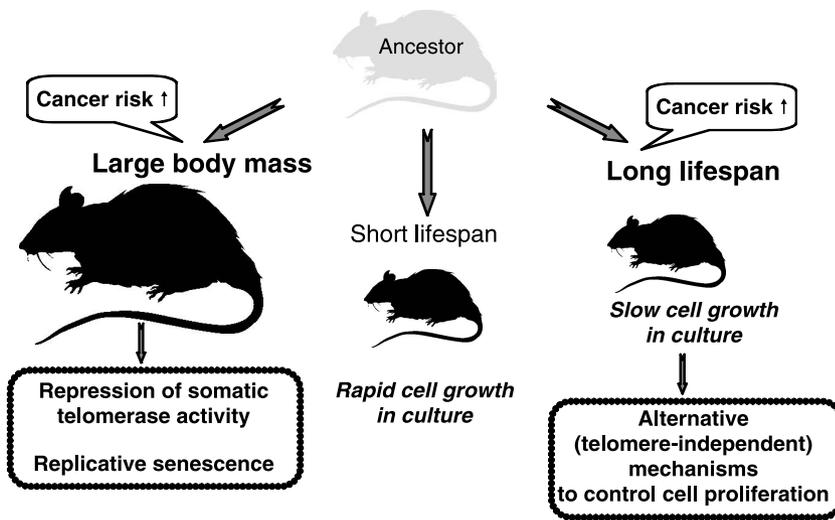


Fig. 8 Coevolution of body mass, lifespan and tumor-suppressor mechanisms. Increase in body mass leads to increased cancer risk and evolution of replicative senescence. Small, short-lived rodent species have lower cancer risk and fewer tumor suppressors. Fibroblasts from these species grow rapidly in culture. An increase in lifespan in small rodents also increases cancer risk and drives evolution of alternative tumor-suppressor mechanisms, which restrict cell growth *in vitro*.

in our study have not evolved replicative senescence. While being a potent tumor suppressor, replicative senescence has many tradeoffs, such as slower wound healing and less robust immune response. Furthermore, replicative senescence is only one of many possible tumor-suppressor mechanisms, and it is plausible that these species rely on other mechanisms to mitigate the cancer risk conferred by their long lifespan. Indeed we found that fibroblasts of small, long-lived species such as gray squirrel, naked mole-rat, chinchilla, musk-rat, and chipmunk exhibit a novel *in vitro* phenotype: their cells do not enter replicative senescence but instead proliferate slowly in culture (Fig. 8). We show that, for small rodent species that have not evolved replicative senescence, *in vitro* fibroblast proliferation rate negatively correlates with longevity. We hypothesize that the slow *in vitro* growth rate is a manifestation of those alternative tumor suppressor mechanisms that evolve in small, long-lived species lacking replicative senescence (Fig. 8).

Interestingly, embryonic squirrel fibroblasts, proliferated rapidly up to PD30, after which the culture slowed down and attained the adult growth phenotype. Thus, the growth control mechanisms that restrict proliferation of squirrel cells are characteristic of an adult but not embryonic cells. This scenario is reminiscent of the repression of hTERT expression during human embryonic development. Telomerase is expressed in early embryogenesis, but its expression is progressively shut off in later development (Bekaert *et al.*, 2004). Thus, the mechanisms that restrict proliferation of somatic cells are inactive during early development when they would otherwise interfere with rapid cell division.

In vitro culture forces cells to proliferate under nonphysiological conditions. *In vivo*, the majority of cells in adult tissues are nondividing. When placed in culture, cells are stimulated to divide by mitogens provided by fetal serum. The ability of cells to proliferate in culture dishes can be considered a measure of their tumorigenic potential. We hypothesize that long-lived rodents evolve mechanisms that make their cells acutely sensitive to any environmental or physiological imbalances, and arrest cell proliferation in inappropriate conditions. The same

mechanisms will prevent inappropriate cell division *in vivo*, protecting the organism from tumor growth and metastasis.

What are the potential cues that slow proliferation of adult fibroblasts of small long-lived rodents? Perhaps, it could be unrestrained mitogenic stimulation, disrupted cell-cell contacts, sensitivity to DNA damage, or some other as yet undetermined cue. It seems likely that these proliferation control mechanisms differ among species because, as can be inferred from rodent phylogeny (Fig. 1), they have evolved with slow aging independently at least three times. In-depth studies of the individual small long-lived species are required to understand the molecular mechanisms responsible for the different anticancer adaptations that have evolved among them.

In summary, our analysis of fifteen rodent species has uncovered an intricate picture of how increased cancer risk conferred by large body mass or long lifespan drives evolution of tumor suppressor mechanisms (Fig. 8). Body mass has previously been linked to the evolution of several characteristics such as repression of telomerase activity (Seluanov *et al.*, 2007), more efficient DNA repair (Promislow, 1994), and the number of PDs before senescence (Lorenzini *et al.*, 2005). Here we show that both body mass and lifespan contribute to the evolution of tumor suppressor mechanisms, but in two different ways. Large body mass coevolved with replicative senescence, while long lifespan is associated with evolution of alternative mechanisms that increase the sensitivity of the cells to growth conditions and slow cell proliferation in culture.

Experimental procedures

Animal samples

Capybaras were obtained from Bio Fau Assesoria e Comercio (São Paulo, Brazil). Pacas were from the animal facility at São Paulo State University. Outbred multicolored guinea pigs were purchased from Elmhill Labs. Chinchillas were purchased from Molton Chinchilla Ranch. Naked mole-rats were from the colony of K.C.C. at Vanderbilt University (Nashville, TN, USA). Two mice

were 129/Sv × BL6 F1 hybrids, two mice were BL6, and one mouse was wild-caught in New York State. Two rats were BN, two rats were F344, and one rat was wild-caught in New York State. Outbred Mongolian gerbils CrI:Mon(Tum) and outbred hamsters CrI:LVG(Syr) were purchased from Charles River Laboratories. Beavers, deer mice, muskrats, chipmunks, and squirrels were wild-trapped in New York State. All animals used in this study were young adults. Exact age was known for laboratory animals and was estimated for wild-caught animals from body measurements and color. Live animals were euthanized according to the University of Rochester Animal Care and Use Committee guidelines. Care was taken to minimize pain and discomfort to the animals.

Cell isolation and culture

Primary rodent cells were isolated from lung and under-arm skin. We used cell isolation protocol adapted from S. Austad (University of Texas Health Sciences Center, San Antonio, TX, USA). Skin was shaved and cleaned with 70% ethanol. Tissue was minced and incubated in DMEM F-12 media (Gibco, Carlsbad, CA, USA) with collagenase (Liberase Blendzyme 3, Roche, Indianapolis, IN, USA) at 37 °C on a stirrer for 30–90 min. Dissociated cells were then washed, and placed in tissue culture dishes with DMEM F-12 media (Gibco) and antibiotics/antimycotic (Gibco). All cells except naked mole-rat were cultured at 37 °C, 5% CO₂, 3% O₂; naked mole-rat cells were cultured at 32 °C, 5% CO₂, 3% O₂. After the plates became confluent, cells were replated, and all subsequent culture was performed in EMEM media (ATCC) supplemented with 15% fetal bovine serum (Gibco), nonessential amino acids, 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin (Gibco). All the experiments were performed using the same batch of fetal bovine serum. To obtain cell growth curves, cells were replated regularly at 70–80% confluence and the numbers of cells plated and harvested were recorded and used to calculate the PDs.

Telomeric repeat amplification protocol

TRAP assay was performed according to a modified protocol described in (Szatmari & Aradi, 2001). Briefly, this protocol uses modified oligonucleotide sequences to ensure that the number of telomeric repeats present in the original telomerase products does not change during polymerase chain reaction (PCR) amplification. In the first step of the TRAP assay, substrate oligonucleotide is incubated with 0.5 µg of protein extract. If telomerase is present and active, telomeric repeats (GGTTAG) are added to the 3' end of the oligonucleotide. In the second step, extended products are amplified by PCR in the presence of hot dCTP. A human cancer cell line over-expressing telomerase was used as a reference in each assay.

Telomere lengths

Telomere length was analyzed by Southern blot using the TRF method. Genomic DNA was extracted from fibroblasts, 5 µg of

DNA was digested overnight with a mixture of *AluI*, *HaeIII*, *RsaI*, and *HinfI* (restriction enzymes that do not cut within telomeric repeat sequence), and separated using pulse field gel electrophoresis. Dried gels were hybridized with radiolabeled oligonucleotide containing telomeric sequence (TTAGGG)₃. Pulse field gels were 1% agarose, and were run in 0.5× TBE using a CHEF-DR II apparatus (Bio-Rad, Hercules, CA, USA) for 22 h at constant 150 V, using ramped pulse times from 1 to 10 s, with a ratio 1.0, at 8 °C.

Western blot

Cells were harvested, washed in PBS, and boiled in 1 × Laemmli sample buffer with protease inhibitors (Complete Mini, Roche) for 10 min with vortexing every 3 min. Extracts were incubated at room temperature for 2 min, and centrifuged at 10 000 g for 10 min. Protein concentrations in the extracts were measured according to modified Lowry procedure (RC DC Protein Assay, Bio-Rad) using bovine serum albumin as a standard. An aliquot (50 µg of proteins) from each sample was then separated on 12% to SDS/PAGE. After electroblotting, the nitrocellulose membrane was incubated with anti p16 or anti p21 serum as indicated, washed twice, and incubated with goat antirabbit antibodies conjugated to horseradish peroxidase. Identity of each protein was confirmed with two different antibodies: p16, ab14244 and ab17517; p21, ab14061 and ab7960 (Abcam Inc., Cambridge, MA, USA). Equal loading of samples was verified by hybridizing the same membranes with pan-Actin Ab-5 antibodies (Lab Vision, Fremont, CA, USA).

Statistical analysis

The phylogenetic relationships among the 15 species studied here were inferred from information from six studies (Martin *et al.*, 2000; Michaux *et al.*, 2001; Murphy *et al.*, 2001; Montgelard *et al.*, 2002; Adkins *et al.*, 2003; Steppan *et al.*, 2004), which were based on a sequence analysis of 22 genes. We used Felsenstein's method (Felsenstein, 1985) of analyzing phylogenetically independent contrasts to test for correlated evolution between replicative senescence, fibroblast proliferation rate, maximum lifespan, and body mass. We generated standardized independent contrasts for phenotypic values using the CRUNCH algorithm of the Comparative Analysis by Independent Contrasts program (Purvis & Rambaut, 1995). We were unable to infer branch lengths for the tree because no common molecular phylogeny exists of all 15 species. We therefore assumed equal branch lengths, consistent with a punctuational model of evolution (Purvis *et al.*, 1994). To test for correlated evolution, linear and multiple regression models forced through the origin were fit to independent contrasts (Harvey & Pagel, 1991). For all analyses, the statistical and evolutionary assumptions of the independent contrasts methods were met (Purvis & Rambaut, 1995). Normality was tested using the Shapiro–Wilk test implemented in R. In analyses of the uncorrected data, body mass was log-transformed to meet the assumption of normality. All statistical tests are two-tailed.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Comparison of telomere biology and cell growth kinetics in wild-caught and laboratory mice and rats. (A) Telomerase activity examined by TRAP assay in early and late passage fibroblasts. (B) Telomere length analyzed by terminal restriction fragment assay. (C) Growth curves of primary fibroblasts. Mouse and rat averages (Fig. 2B) are compared to the individual growth curves of the wild-caught mouse and rat. LF and SF correspond to lung and skin fibroblasts, respectively.

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