

## Lagomorphs (rabbits, pikas and hares) do not use telomere-directed replicative aging in vitro

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### Abstract

Telomere shortening is used for replicative aging in primates and ungulates but not rodents. We examined telomere biology in rabbits to expand the comparative biology of telomere-directed replicative senescence within mammals. The order Lagomorpha consists of two families; Leporidae and Ochotonidae. We examined telomere biology in species representing three leporid genera (European White Rabbit, Black-tailed Jack Rabbit, and Swamp Rabbit) and the monotypic ochotonid genus (North American Pika). Of the leporids one species was a laboratory strain and the others were wild caught. The leporids neither exhibited cellular senescence after sustained periods in culture nor displayed detectable telomerase activity. Continued culture was possible because of their extremely long telomeric arrays. Immunofluorescence showed robust telomere signals at chromosome ends and significant internal chromosomal staining in some instances. Pika was unique in displaying endogenous telomerase activity throughout time in culture. These results show that it is unlikely that lagomorphs use telomere shortening and replicative senescence as a tumor protective mechanism.

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### 1. Introduction

A growing body of evidence has implicated telomere length as an important prognostic indicator and marker of human aging in vivo (Aviv and Aviv, 1999; Brouillette et al., 2003; Cawthon et al., 2003). In spite of this very little is known about telomere biology in other mammalian orders. Species representing the orders Cetartiodactyla, specifically artiodactyls (e.g. deer and sheep), and Primates have been shown to display telomere shortening and replicative aging which can be prevented by telomerase expression when grown in culture (Zho et al., 2002; Steinert et al., 2002; Cui et al., 2002). Of the species so far examined, members of the

Rodentia (rats and mice) do not exhibit telomere-directed replicative aging whereas the situation in Carnivora (dogs) is less clear (Sherr and DePinho, 2000; Wright and Shay, 2000; Forsyth et al., 2002; Nasir et al., 2001; Zaucha et al., 2001).

There is a wide variety of evidence indicating that rodents do not use replicative aging as a counting mechanism to suppress the progression of cancers. Rodents express telomerase in most of their adult tissues, tumors form normally during the first generation in mice lacking telomerase, such mutant mice do not exhibit significant abnormal phenotypes for several generations during which progressive telomere shortening is occurring in vivo, it takes many hundreds of cell divisions in the absence of telomerase for rodent cells lacking telomerase to eventually enter crisis due to telomere shortening, the culture “crisis” that has been called senescence in normal mouse cells is due to inadequate culture conditions, and normal rodent cells are immortal in

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culture if put in specially formulated media or appropriate conditions (reviewed in 7,8). In order to determine if the lack of telomere-directed replicative senescence was unique to Rodentia, we examined the telomere lengths and proliferative capacity of species contained within the closely related order Lagomorpha.

The order Lagomorpha contains two families, the Leporidae and the Ochotonidae (Stock, 1976). Phylogenetic placement of the Lagomorpha remains controversial with two prevalent alternative groupings (Murphy et al., 2001; Graur et al., 1996; Misawa and Janke, 2003). The first of these is as part of the cohort Glires with sister order Rodentia (Murphy et al., 2001). The alternative grouping places Lagomorpha closer to Scandentia (Tree Shrews) and Primates than to Rodentia (Graur et al., 1996). Leporids themselves are not only of significant commercial importance as livestock, game, and companion animals, they are also important in many diverse areas of biomedical research. Despite this, very little is known about the telomere biology of these mammals.

Here we provide descriptions of telomere biology for species contained within the order Lagomorpha and show that they are extremely unlikely to use telomere shortening to limit replicative aging. Prolonged tissue culture without the onset of growth arrest was observed in all species examined; *Oryctolagus cuniculus* (European White Rabbit), *Lepus californicus* (Black-tailed Jack Rabbit), *Sylvilagus aquaticus* (Swamp Rabbit), and *Ochotona princeps* (North American Pika). All species displayed long telomeres at chromosomal ends when visualized with either electrophoresis or in situ hybridization. The amount of interstitial (not at telomere ends) telomeric sequence localization varied between species. Only one species examined displayed endogenous telomerase activity, Pika, and this activity was present throughout culture. These results show that both Leporid and Ochotona share some telomeric characteristics of Muridae species and that very long telomeres and the lack of replicative aging are not unique to either mice or inbred laboratory strains.

## 2. Materials and methods

### 2.1. Cell lines

*O. cuniculus* (European rabbit, New Zealand White laboratory strain) cells were recovered from approximately 6-month-old male abdominal skin tissue which was minced and trypsinised for 30 min at 37 °C, and cultured as described (Forsyth et al., 2003). *O. princeps* (North American Pika), *L. californicus* (Black-tailed jack rabbit), and *S. aquaticus* (Swamp rabbit) cells were recovered from minced and explanted ear snips from wild trapped animals. Age ranges for wild caught animals were not determined at point of capture. Outgrowths were pooled and cultured as for European White Rabbit. Cells were passaged when

Table 1

The average and maximum ages of the lagomorph species from which the primary cell lines used in this study were established

Species	Average age (years)	Maximum age (years)
European White Rabbit	5–6 <sup>a</sup>	18 <sup>b</sup>
Black-tailed Jack Rabbit	1–5 <sup>c</sup>	5–6 <sup>d</sup>
Swamp Rabbit	1–2 <sup>e</sup>	3–4 <sup>f</sup>
North American Pika	3–4 <sup>g</sup>	4–7 <sup>h</sup>

Maximum ages reflect those attainable in captivity whereas the average age is a reflection of lifespan in the wild. The maximum age for the European White Rabbit reflects a single recorded instance.

<sup>a</sup> Spector, S. William (Eds.), Handbook of Biological Data, W.B Saunders Company, London and Philadelphia, 1956, p. 182.

<sup>b</sup> Guinness World Records, 2004.

<sup>c</sup> <http://ladywildlife.com/animal/blacktailedjackrabbit.html>.

<sup>d</sup> Ballenger, L. 1999. “*Lepus californicus*” (On-line), Animal Diversity Web. Accessed December 06, 2004 at [http://animaldiversity.ummz.umich.edu/site/accounts/information/Lepus\\_californicus.html](http://animaldiversity.ummz.umich.edu/site/accounts/information/Lepus_californicus.html).

<sup>e</sup> <http://museum.nhm.uga.edu/gawildlife/mammals/lagomorpha/Leporidae/saquaticus.html>.

<sup>f</sup> [http://www.ncwildlife.org/pg07\\_WildlifeSpeciesCon/Profiles/rabbit-marsh.pdf](http://www.ncwildlife.org/pg07_WildlifeSpeciesCon/Profiles/rabbit-marsh.pdf).

<sup>g</sup> <http://www.bio.davidson.edu/people/vecase/Behavior/Spring2001/Spring01/Marques/marques.html>.

<sup>h</sup> <http://wildlife.state.co.us/Education/mammalsguide/pika.asp>.

approaching confluency and maintained using 1:8 split ratios.

### 2.2. Telomere length analysis

Genomic DNA (1 µg) was extracted at multiple passage points and digested with a cocktail of restriction enzymes (AluI, CfoI, HaeIII, HinfI, MspI, and RsaI) as described previously (Forsyth et al., 2003). Briefly, digested DNA samples were resolved using pulsed field electrophoresis parameters on a Mapper system (Biorad). The conditions applied were 1–50 kb (Pika), 1–50 kb (Swamp Rabbit),

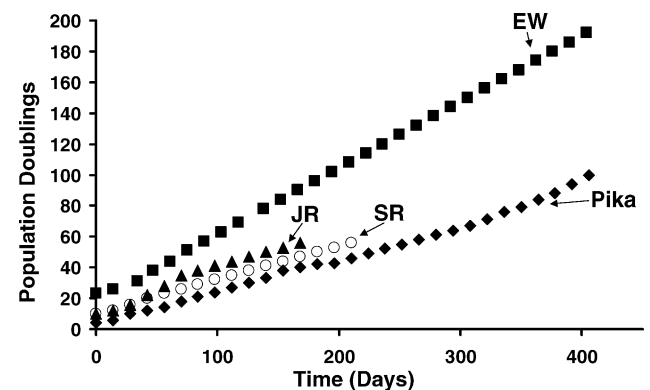


Fig. 1. Lagomorphs do not exhibit replicative senescence in vitro. Growth curves of European White (EW), Jack Rabbit (JR), Swamp Rabbit (SR), and Pika are represented by (■), (▲), (○), and (◆), respectively. Continued culture of cells derived from lagomorph species is possible because of their extremely long telomeres. However, although it remains possible that ultimately these cell lines may undergo sufficient telomere shortening to senesce, this point was not reached in this study. The x-axis represents time in culture (days) and the y-axis denotes population doublings (PD).

5–150 kb (Jack Rabbit), and 5–150 kb (European White). After denaturation and drying, samples were hybridized with radiolabelled  $(T_2AG_3)_3$  probes and visualized with a Storm 850 system (molecular dynamics).

### 2.3. Telomere *in situ* hybridization

Metaphase spreads were prepared using standard techniques as described elsewhere (Zho et al., 2002). Slides were then rehydrated, fixed in 4% formaldehyde/PBS for 2 min, dehydrated in an increasing alcohol series; air dried, denatured at 95 °C for 15 min and probed overnight at 37 °C in a humidified incubator. Slides were then washed 4 × 15 min in formamide buffer, 4 × 5 min in 0.025% Tween 20/PBS, and mounted in Vectashield with DAPI (Vector Laboratories Inc.). Metaphase cells were visualized and captured using an Axiocam fluorescence microscope (Zeiss) and Openlab 3.01 software (Improvision).

Phosphoramidate probes specific to telomeres and centromeres were a kind gift from Sergei Gryaznov at Geron Corp.

### 2.4. Telomere overhang strand hybridization

Genomic DNA (5 µg) was digested using a restriction enzyme cocktail (AluI, CfoI, HaeIII, HinfI, MspI, and RsaI) and preincubated o/n at room temperature with 5 fmol of radiolabelled  $T_2AG_3$  probe. Non-denaturing electrophoresis was performed at 130 V overnight at 4 °C using a 0.7% agarose gel followed by denaturation for 30 min at 4 °C and the gel dried face down on N+ membrane (Hybond). This denaturation melted the probe off the telomeric overhang so that it migrated out of the gel and bound to the membrane during drying. The dried gel was briefly wetted in order to separate the gel from the membrane. The nylon membrane was then air dried and exposed to reveal the location of the telomere G-rich overhang. The gel containing the now denatured telomeres was then neutralized and probed as

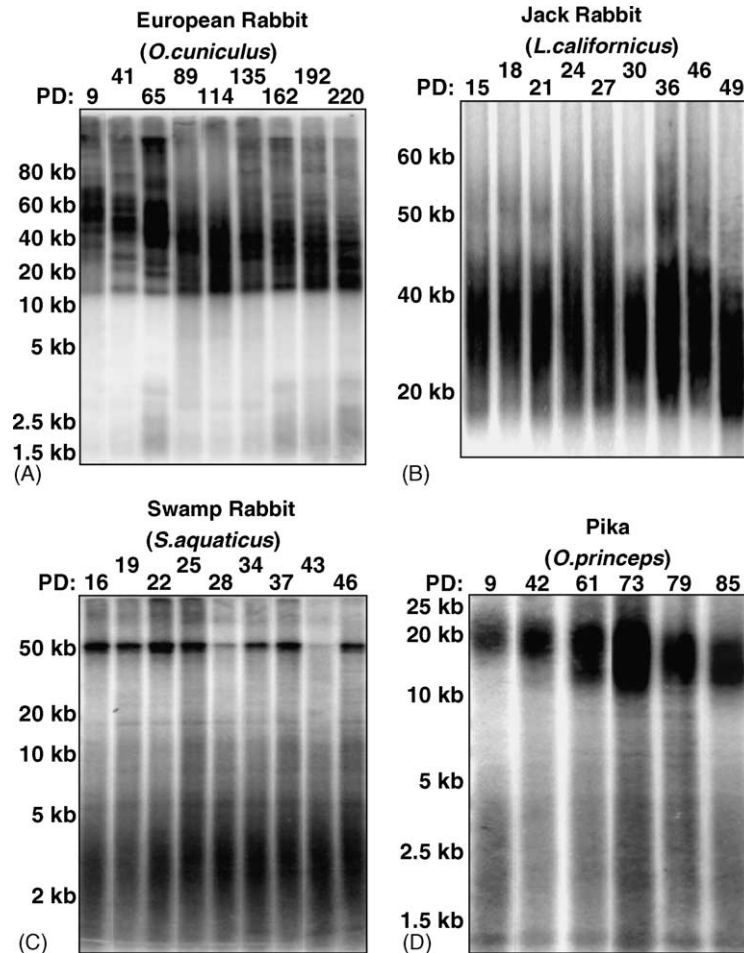


Fig. 2. Lagomorpha species display a variety of telomere lengths. (A–D) show FIGE gel analysis of telomeres for; *O. cuniculus* (European Rabbit), *L. californicus* (Jack Rabbit), *S. aquaticus* (Swamp Rabbit), and *O. princeps* (North American Pika), respectively. European rabbit telomeres are extremely long and heterogeneous whereas Jack Rabbit shows a somewhat more homogenous telomere population. Swamp Rabbit reveals two distinct signals at 2–5 kb and  $\geq 50$  kb. North American Pika also shows two predominant telomeric signals at 2.5 and 20 kb. Interstitial telomeric sequences contain restriction sites and appear as these 2–5 kb signals in Fig. 1C and D. Progressive PD and molecular weight markers are indicated on the upper x- and left hand y-axes, respectively, for each panel.

described above. Direct comparisons between the gel and membrane images allow those telomeric sequences present at chromosomal ends and thus containing single-stranded G-rich overhangs to be identified.

### 3. Results

#### 3.1. Proliferative capacity of Lagomorph fibroblasts in vitro

Primary cell lines were established from European White Rabbit, Black-tailed Jack Rabbit, Swamp Rabbit, and North American Pika (see Section 2). These species were selected due to availability and ease of collection. Although the cultures were established from different tissue sources, cells from European White, Jack Rabbit, and SR all appeared fibroblastic in appearance whereas Pika cells displayed a more cuboidal morphology. Average and maximal lifespan for species used in this study are recorded in Table 1. None of these lines underwent growth arrest after minimally, 6 months in culture (Jack Rabbit and Swamp Rabbit) and maximally, 18 months (European White and Pika) (Fig. 1). Asynchronous culture establishment times are reflected in the differences of time in culture. Continued culture without a slow down in doubling time or obvious clonal emergence of variants was observed in all species. All cell culture was performed in a reduced (2–5%) oxygen environment to minimize oxidative damage (Forsyth et al., 2003).

#### 3.2. Telomere analysis of lagomorphs

Relatively unlimited cell proliferation in culture is possible through two closely related mechanisms. Linear chromosomes can have telomeres that are sufficiently long so that telomere-induced senescence is not encountered until well after the 60–80 population doublings typically encountered with normal human somatic cells in culture. Alternatively, telomere length is maintained by the expression of endogenous maintenance mechanisms such as telomerase expression. Pulsed field electrophoresis revealed that telomeric patterns for each of the examined species were diverse and distinct (Fig. 2). In some instances differences in DNA concentration resulted in marked increases in apparent intensities between adjacent lanes, i.e. Fig. 2, Pika PD73. These variations do not affect final interpretations of telomeric structures. The inbred laboratory strain of European White had long and heterogeneous telomeres with lengths ranging from 10 to 80+ kb (Fig. 2A). Wild caught species also had very long telomeres. Jack Rabbit telomeres were in the range of 20–50 kb (Fig. 2B) while Swamp Rabbit telomeric repeats formed two distinct populations (Fig. 2C), a high molecular weight species of greater than 50 kb and a low molecular weight species predominantly present in the 2–5 kb range. Pika also had telomeres that resolved into two separate populations with

mean lengths of 20 and 2.5 kb (Fig. 2D). Telomerase activity was assayed in all lines by the TRAP assay (data not shown) (Wright et al., 1995). Pika was the only species that displayed endogenous telomerase activity. All species were tested at the earliest possible sample collection point.

Interstitial telomeric repeats usually diverge so that they contain multiple internal restriction sites and appear as small fragments on gels. True mammalian telomeres contain single-stranded G-rich 3' overhangs ( $(T_2AG_3)_n$ ) and can be identified by hybridization to telomeric C-rich ( $(C_3TA_2)_n$ ) probes under non-denaturing conditions where interstitial repeats remain double-stranded (Wei et al., 2002; Makarov et al., 1997). Fig. 3 shows that the shorter telomeric fragments seen in Swamp Rabbit and Pika were derived from interstitial repeats since they lack 3' overhangs.

#### 3.3. Telomere-directed in situ hybridization

To confirm the location of telomeres we performed in situ hybridization on metaphase spreads using both telomere and

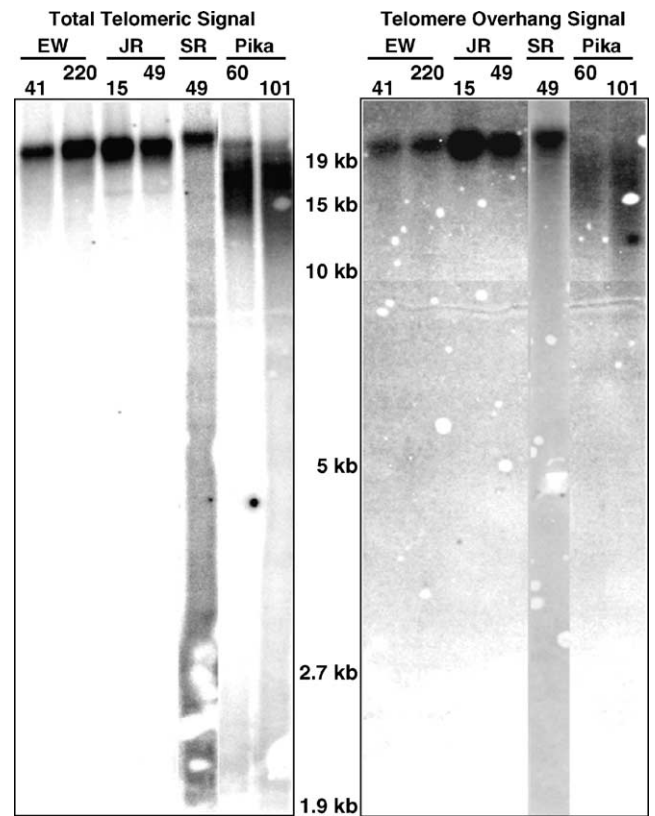


Fig. 3. Telomere size using overhang hybridization in Lagomorph species. Interstitial telomeric sequences lack the single-stranded G-rich overhang that is present at the ends of chromosomes. Direct comparison of the total telomeric signal from denatured DNA (left Panel) and telomere overhang specific signal (right Panel) reveals that the limit mobility signal represents the chromosomal ends. Progressive PD is indicated on the upper x-axis of each panel. Molecular weight markers are indicated on the central y-axis. EW (European White, *O. cuniculus*), JR (Jack Rabbit, *L. californicus*), SR (Swamp Rabbit, *S. aquaticus*), Pika (North American Pika, *O. princeps*). Samples were run on a normal rather than a FIGE gel as in Fig. 1 so that very high molecular weight species are not resolved.

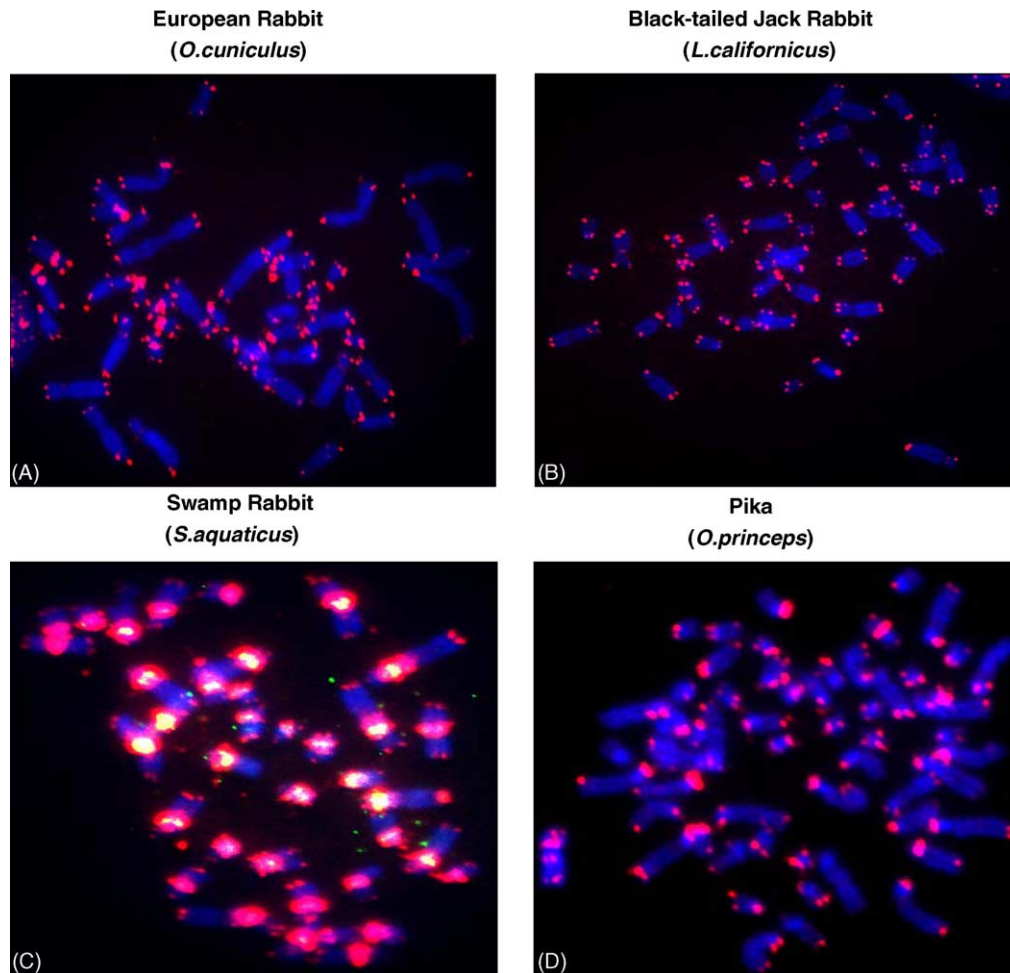


Fig. 4. Telomere repeat localization in Lagomorpha species. (A–D) Shows the distribution of telomeric repeats following in situ hybridization for; *O. cuniculus* (European Rabbit), *L. californicus* (Jack Rabbit), *S. aquaticus* (Swamp Rabbit), and *O. princeps* (Pika) respectively. In situ analysis shows telomere signals (red) at virtually all chromosome ends in all species examined as well as a significant amount of interstitial telomeric staining. A human centromeric probe (green) successfully hybridized in only one species examined, *S. aquaticus*, and colocalized (yellow) with the extremely large and abundant interstitial telomeric sequences (C).

centromere-directed amide probes (Fig. 4). A robust signal at chromosomal ends and some interstitial staining was observed in European White and Jack Rabbit (Fig. 4A and B). The abundant interstitial sequences seen with electrophoresis in Swamp Rabbit and Pika were confirmed with in situ hybridization (Fig. 4C and D). This extremely abundant interstitial telomeric sequence staining overshadowed the positively stained telomere ends. Interestingly these sequences often co-localized with a human centromeric probe in Swamp Rabbit (Fig. 4C, co-localization is yellow).

#### 4. Discussion

This study forms the first description of the comparative biology of in vitro aging and telomere dynamics in lagomorph species. From analysis of telomere biology we have observed that lagomorphs do not appear to use

replicative aging. None of the representative species underwent telomere-directed replicative senescence or exhibited a premature growth arrest/crisis/“spontaneous immortalisation” process. This is similar to the behavior of rodent cells grown in low oxygen (Parrinello et al., 2003) although it should be noted that European White cells did not undergo senescence even when grown in the presence of ambient oxygen. The extreme length of lagomorph telomeres explains readily the ability of these cells to undergo long-term culture (Reddan et al., 1986; Xiang et al., 2000), even though some telomere shortening could be observed.

Inbred laboratory and wild species of mice vary greatly in their mean telomere size (Coviello-McLaughlin and Prowse, 1997; Kipling and Cooke, 1990). Inbred and wild leporid strains did not vary as widely, although there did appear to be some increase in heterogeneity in the laboratory rabbit strain. The species representing the monotypic ochotonid genus, Pika, was unique in that telomerase activity was

present throughout time in culture. This activity level was not sufficient to fully maintain the length of these long telomeres, as telomere shortening in the Pika cells was apparent with continued passages. It remains possible that a spontaneous upregulation event occurred during the establishment of this culture. We have not observed spontaneous telomerase expression in fibroblast cultures from any species that exhibits replicative aging (human, six other primate species, Indian muntjac), while such upregulation has been observed in species that do not exhibit replicative aging (Prowse and Greider, 1995). In some cases telomerase expression in vivo is lost once cells are put in culture (Venkatesan and Price, 1998). In *M. spretus* cultures, telomerase expression was initially low/absent but increased after telomeres had shortened in culture (Prowse and Greider, 1995). Interestingly, Pika exhibited the shortest telomeres of any of the lagomorph species examined. Although the diversity of species tested is yet too limited to draw firm conclusions, the lack of stringent repression of telomerase in cultured fibroblasts, especially in the presence of shorter telomeres, may be characteristic of species that do not use replicative senescence as a tumor-suppression mechanism. Rigorous proof of the lack of replicative aging requires many different criteria. These include germline elimination of telomerase components and the demonstration of lack of phenotypic effects within the first generation, and/or demonstration of telomerase activity in a variety of adult tissues that lack telomerase activity in humans. Such tests are relatively impractical in broad comparative studies. The present conclusion that lagomorphs do not use replicative aging is thus strongly supported but not rigorously proven.

Most strains of laboratory mice only live for approximately two years, while a European White rabbit has been reported to live for almost 20 years (Table 1). The failure of a species to use telomere-based replicative aging as an anticancer mechanism is thus not restricted to small short-lived animals.

In summary, we find that the lack of telomere-based replicative aging is not restricted to rodents but is probably also present in lagomorphs. Additional studies will be required in many different mammalian orders before the evolution of replicative aging can be understood.

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