Aging Cell (2012) **11,** pp675–682 Doi: 10.1111/j.1474-9726.2012.00832.x

Rapamycin slows aging in mice

John E. Wilkinson,[1] Lisa Burmeister,[2] Susan V. Brooks,[3] Chi-Chao Chan,[4] Sabrina Friedline,[2] David E. Harrison,[5] James F. Hejtmancik,[6] Nancy Nadon,[7] Randy Strong,[8] Lauren K. Wood,[3] Maria A. Woodward[9] and Richard A. Miller[2]

[1]Unit for Laboratory Animal Medicine and Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA
[2]Department of Pathology and Geriatrics Center, University of Michigan, Ann Arbor, MI 48109-2200, USA
[3]Department of Molecular and Integrative Physiology and Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109-2200, USA
[4]Histology Core, National Eye Institute, NIH, Bethesda, MD 20892-2510, USA
[5]The Jackson Laboratory, Bar Harbor, ME 04609, USA
[6]Ophthalmic Genetics and Visual Function Branch, National Eye Institute, NIH, Bethesda, MD 20892-2510, USA
[7]Division of Aging Biology, National Institute on Aging, Bethesda, MD 20892, USA
[8]Geriatric Research, Education and Clinical Center and Research Service, South Texas Veterans Health Care System, Department of Pharmacology, and Barshop Institute for Longevity and Aging Studies at The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[9]Department of Ophthalmology, W. K. Kellogg Eye Center, Ann Arbor, MI 48105, USA

Summary

Rapamycin increases lifespan in mice, but whether this represents merely inhibition of lethal neoplastic diseases, or an overall slowing in multiple aspects of aging is currently unclear. We report here that many forms of age-dependent change, including alterations in heart, liver, adrenal glands, endometrium, and tendon, as well as age-dependent decline in spontaneous activity, occur more slowly in rapamycin-treated mice, suggesting strongly that rapamycin retards multiple aspects of aging in mice, in addition to any beneficial effects it may have on neoplastic disease. We also note, however, that mice treated with rapamycin starting at 9 months of age have significantly higher incidence of testicular degeneration and cataracts; harmful effects of this kind will guide further studies on timing, dosage, and tissue-specific actions of rapamycin relevant to the development of clinically useful inhibitors of TOR action.

Key words: interventions; longevity pathology; TOR.

Introduction

Rapamycin has been found, in five laboratories (Chen *et al.*, 2009; Harrison *et al.*, 2009; Anisimov *et al.*, 2010, 2011; Miller *et al.*, 2011), to extend mouse lifespan. These observations are consistent with two broadly distinct explanations: rapamycin might (i) slow aging or (ii) retard lethal neoplasia. The idea that rapamycin slows aging per se is consistent with the observation that genetic or pharmacological inhibition of TOR (mTOR, target of rapamycin, FRAP1), the kinase target of rapamycin, can extend lifespan in worms and flies (Vellai *et al.*, 2003; Jia *et al.*, 2004; Kapahi *et al.*, 2004), in which lifespan is not typically limited by neoplasia, and with the observation that several aspects of aging are retarded in mice lacking S6K, a TOR target that mediates some of its effects on cell proliferation and stress resistance (Selman *et al.*, 2009). The idea that rapamycin extends mouse lifespan principally by blocking tumor development is consistent with studies showing rapamycin-mediated growth inhibition of many forms of cancer (Hidalgo & Rowinsky, 2000). Cancer is frequently the cause of death in both control and rapamycin-treated mice, although the rapamycin-treated mice are older at the time of death, suggesting that this agent may be extending lifespan by delaying cancer incidence or slowing its progression (Harrison *et al.*, 2009; Miller *et al.*, 2011). The two classes of explanation are not mutually exclusive: rapamycin might both retard neoplasia and also slow aspects of aging that are not reflections of neoplastic disease.

To test the idea that rapamycin retards aging in mice, we evaluated a series of age-sensitive outcomes in 20–22-month-old mice, that is, at an age where relatively few mice in the control or rapamycin-treated cohorts have died. We used a genetically heterogeneous mouse model with a wide range of age-related pathologies rather than a few strain-specific characteristics, including tests of the adrenals, heart, liver, tendons, eye, lung, and reproductive organs, toward the goal of testing the idea that rapamycin might slow aging effects on many tissues and thus by inference slow the aging process per se. We also focused on lesions that are, for the most part, unrelated to cancer biology and also unlikely to lead to death in mice, because we wished to mitigate effects prior to 22 months and because testing the general hypothesis requires evaluation of tissues above and beyond the neoplastic lesions that usually lead to death of these mice. We evaluated mice treated with a dose of rapamycin, 14 ppm in food, that has been shown to extend lifespan, as well as two other doses (4.7 and 42 ppm) for which lifespan studies are currently in progress. Although our main goal was to see whether age-sensitive changes occurred more slowly in rapamycin-treated mice, the data set also gave us an opportunity to look for harmful side effects of rapamycin treatment, side effects that might complicate attempts to use strategies of TOR inhibition to slow aging in clinical settings.

Results

Male and female mice exposed to rapamycin at doses of 4.7, 14, or 42 ppm from age 9 months were euthanized at 22 months of age for a detailed necropsy analysis. Young (4 month) control mice from the same genetically heterogeneous cross and old control mice without rapamycin treatment were evaluated in parallel by a board-certified veterinary pathologist (JW) who was blind to the rapamycin status. We noted four classes of lesions that were age-sensitive and in which the aging effect was opposed by rapamycin in males, females, or both sexes.

Liver degeneration

The occurrence of lipid filled vesicles at multiple foci within the hepatic parenchyma (multifocal macrovesicular lipidosis) was noted in 67% of the old male control mice, but in none of the young controls ($P < 0.001$ for age effect). Representative images of this lesion are shown in Fig. S1A,B. This lesion was seen in only 20% of the old females (nonsignificant compared with young females). A comparison among the four

Correspondence
Richard A. Miller, Department of Pathology and Geriatrics Center, University of Michigan, BSRB Room 3001, Box 2200, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA. Tel.: +734 936 2122; fax: +734 647 9749;
e-mail: millerr@umich.edu

Accepted for publication *3 May 2012*

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

Aging Cell

groups of old male mice (rapamycin at 0, 4, 14, and 42 ppm) showed a significant effect ($P = 0.02$, trend test for ordered categories). As shown in Fig. 1A, rapamycin leads to a dose-dependent decline in the incidence of liver degeneration in male mice, and the effect is dose dependent.

Atypical nuclei in cardiac myocytes

Twenty three per cent of young mice and 70% of old control mice had abnormalities of nuclear size and chromatin conformation in the myocardium (both sexes combined). Figure S1C,D shows representative sections, characterized by enlarged round or oval nuclei with clumped chromatin; more severe lesions include large multinucleated cells. The age effect, comparing young to old controls, was significant at $P = 0.001$. Rapamycin doses between 4.7 and 42 ppm were equally effective at lowering the incidence of these atypical nuclei (Fig. 1B), diminishing the frequency to 40/80 = 50% compared with 21/30 among old controls. This difference is significant by the one-sided Fisher's Exact Test at $P = 0.047$.

Endometrial cystic hyperplasia

This lesion, that is, the growth of multiple large cysts within the uterine lining, was seen in 1/15 (7%) of young female controls, and in 13/15 (87%) of old female controls ($P < 0.001$). Representative sections are shown in Fig. S1E,F. Neither of the two lower rapamycin doses produced a significant diminution of the incidence of this lesion, but the highest rapamycin dose did lower the incidence (8/15 = 53%, $P = 0.05$ compared with old controls; see Fig. 1C).

Adrenal tumors

Adrenal tumors were seen in 1/30 young mice (3%) and in 6/30 old control mice (20%), with both sexes combined. The incidence among rapamycin-treated mice was 5/80 = 6%, and this proportion differs from that of the old control group at $P = 0.04$ by the one-sided Fisher Exact Test. A test for trend among ordered groups (rapamycin doses of 0, 4.7, 14, 42 among the old mice) showed a significant trend at $P = 0.03$. Figure 1D shows the proportion of mice with adrenal tumors. It is worth noting that adrenal tumors rarely contribute to death in UM-HET3 mice; only 1/31 control mice, and 0/40 rapamycin-treated mice were judged to have died because of adrenal tumors in an end-of-life necropsy series (Harrison *et al.*, 2009).

Figure S1G,H shows representative sections.

The necropsy analysis showed significant age-dependent increases in adrenal hyperplasia (males only), adrenal telangectasia (i.e., dilation of blood vessels in the adrenal medulla), ovarian cyst (females only), thyroid cold follicles (i.e., large follicles filled with colloid that do not respond to thyroid-stimulating hormone), and lung tumor (Fig. 2); rapamycin-treated mice had a trend toward lower incidence rates for four of these five lesions, but the difference between untreated and rapamycin-treated

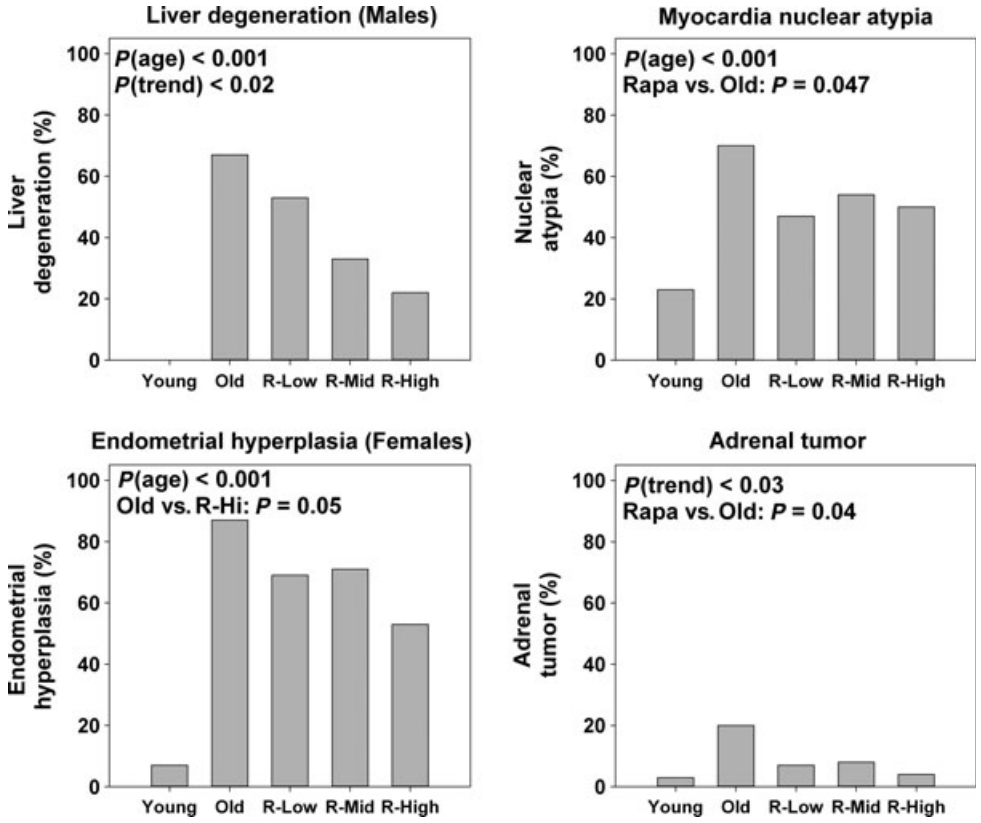

Fig. 1 Incidence of liver degeneration (males only), myocardial nuclear atypia, endometrial hyperplasia (females only), and adrenal tumor in young, old, and rapamycin-treated mice. Group sizes were as follows: young, old, and Rapa-Low, 15 of each sex; Rapa-Mid 14 females and 12 males; Rapa-High, 13 females and nine males. Fisher's Exact Test was used to evaluate the significance of age effects (young vs. old untreated), to compare Rapa (all doses) vs. old (untreated), or to compare old untreated vs. Rapa-high as indicated in the figure panels. Cuzick's nonparametric test for trends (Cuzick, 1985) was used to evaluate significance of differences among the four groups of old mice at rapamycin doses (zero, low, mid, or high).

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

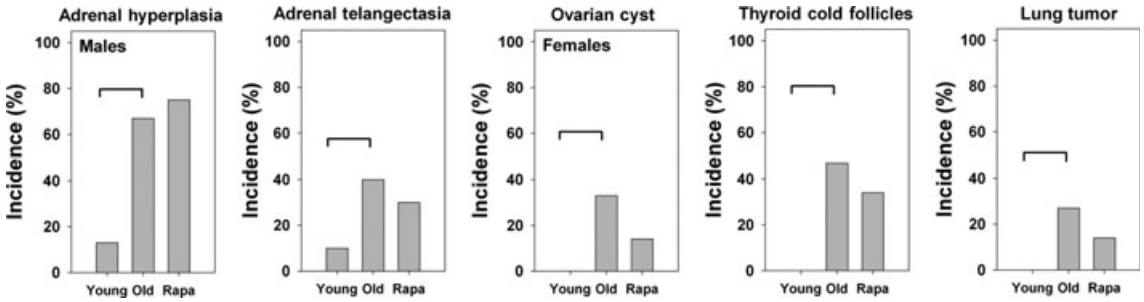

Fig. 2 Incidence of adrenal hyperplasia, adrenal telangectasia, ovarian cyst (females only), thyroid cold follicles, and lung tumor in young, old, and rapamycin-treated old mice (pooled across rapamycin doses). The bracket in each figure indicates a significant difference between young and untreated old mice by Fisher's Exact Test.

mice was not statistically significant in any of these cases. Figure S2 shows stained sections of these four forms of pathology.

Age effects on tendon

Tendons stiffen and lose elasticity in a region-dependent manner during aging in mice and humans (Wood *et al.*, 2011). To see whether rapamycin treatment slows this age-associated change, tibialis anterior (TA) tendons were dissected from mice euthanized at 22 months of age and evaluated for maximum tangent modulus (an index of resistance to stretching) and for hysteresis (an index of the extent to which tendons recover their original length as they return to an unstretched condition). The proximal section of the TA tendon (i.e., the section closest to the muscle and furthest from the bone attachment site) showed a significant age-dependent increase in maximum tangent modulus and decrease in hysteresis comparing young to old control mice (Fig. 3), and both age effects were significantly smaller in rapamycin-treated mice.

Thus, in summary, rapamycin significantly reduced the incidences of liver degenerative change, myocardial nuclear abnormalities, endometrial hyperplasia, and nonlethal adrenal tumors in 22-month-old mice, diminished the effects of aging on the biomechanical properties of tendon, and produced a numerical decrease in four of five other age-sensitive necropsy findings that did not achieve statistical significance.

Rapamycin increases cataract severity

Cataract severity was scored on a scale of 0–3 by slit lamp examination in unanesthetized mice at 20 months of age by an ophthalmologist (MW) who was unaware of the treatment condition. Scores for left and right eyes were highly correlated, and an average score was thus used as an index of cataract severity for each mouse. No cataracts were seen in young controls (Score = 0). Cataract scores increased by 20 months in females ($P = 0.0002$ compared with young), with a similar trend in males ($P = 0.06$). In the old mice (20 months), mean cataract score was significantly higher in females (0.61 ± 0.14, $N = 24$) than in males (0.10 ± 0.05, $N = 24$; $P = 0.002$), and so rapamycin effects were evaluated separately in each sex. The results (Fig. 4), comparing rapamycin doses of 0 (old controls), 4.7, 14, and 42 ppm, showed a significant effect for dose-dependent increase in both males ($P = 0.014$) and females ($P = 0.001$). When old control mice are excluded to confine the analysis to mice that received at least some rapamycin, the trend test is still significant for females ($P = 0.01$), but not for males.

Sections of three control eyes and four eyes from rapamycin-treated mice were evaluated histologically. Figure S3 illustrates the histological findings. Only the lenses were affected in ocular sections. Both control and rapamycin-treated eyes had evidence of mild posterior subcapsular changes, but only the specimens from the rapamycin group had evidence of cortical cataract, characterized by globular degeneration, that is, aggregation of lens crystallins (blue arrows, upper panels); posterior epithelial migration, especially below the bow region (curve arrows, upper right panel); and evidence of calcification (black arrows, lower panels). Although this survey is limited to a small number of specimens, it provides an initial suggestion of TOR-dependent processes that might contribute to maintenance of lens transparency in the second half of the lifespan.

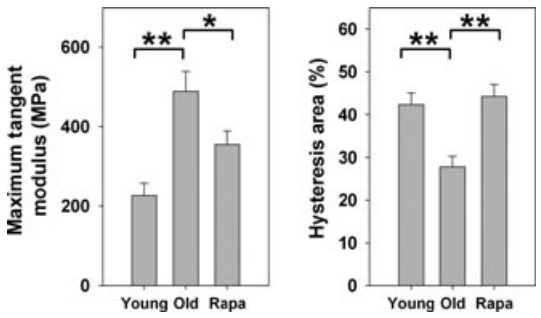

Fig. 3 Maximum tangent modulus (left) and hysteresis area (right) for tibialis anterior tendons. Bars show mean ± SEM for $N = 6$ young, 9 old, and 17–20 old rapamycin-treated mice. Young values differed from old at $P = 0.002$ for each endpoint by *t*-test. Old differed from rapamycin treatment group at $P = 0.036$ for maximum tangent modulus and at $P < 0.001$ for hysteresis area.

Rapamycin increases testicular degeneration in male mice

The necropsy series illustrated in Figures 1 and 2 found one age-dependent change that was more severe in rapamycin-treated mice than in old controls, that is, testicular degeneration in males (Fig. 5). Testicular degeneration is characterized by a progressive loss of spermatids, spermatocytes, differentiating spermatogonia and primary spermatogonia. The degeneration follows an orderly loss of all stages of spermatogenesis from the most mature to least differentiated cells. Lumens of tubules often contain multinucleate giant cells, dead cells from different stages, and debris. Vacuolation of immature sperm cells is also prominent in many of the degenerating tubules. The lesion begins in small foci and progresses to involvement of all tubules. Degeneration was seen in 2/15 control males (13%), but in 30/36 (83%) of males in the rapamycin-treated groups ($P < 0.001$), with full effects apparent even in those mice receiving the lowest rapamycin dose. The degree of degeneration was recorded on a scale of 0–4. Analysis of variance among the four groups of 20-month-old mice showed a significant difference among groups ($P < 0.0001$), and a post hoc Sidak test showed that the order of groups was Old < R-Low = R-Mid < R-High.

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

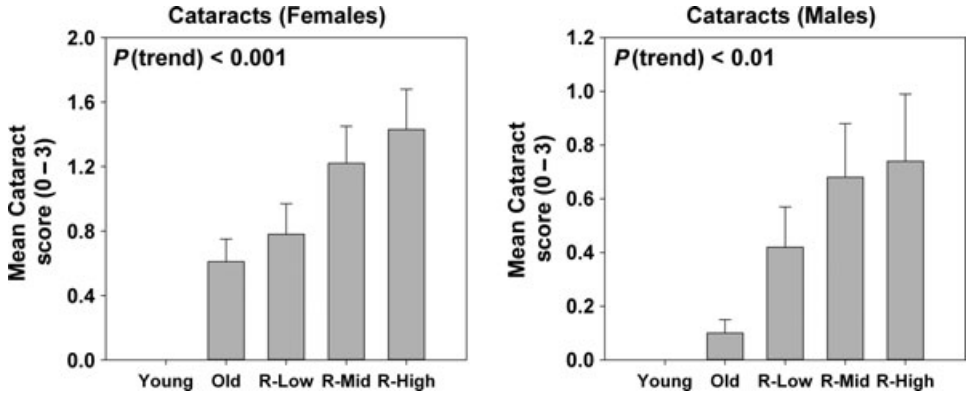

Fig. 4 Cataract severity in mice evaluated by slit lamp examination at 20 months of age. Each eye was scored on a scale of 0–3, and the mean value of the left and right eye was used as the index of severity for each mouse. Bars shown mean ± SEM for groups of 25–29 female mice or 17–30 male mice. Significance of the trend among four groups of 20-month-old mice (old, Rapa-low, Rapa-mid, and Rapa-high) was evaluated by the nonparametric method of Cuzick (Cuzick, 1985).

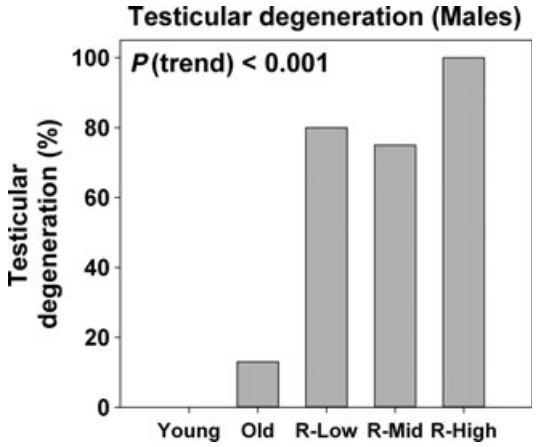

Fig. 5 Testicular degeneration in males. Group sizes as in Fig. 1, with statistical evaluation as in (Cuzick, 1985).

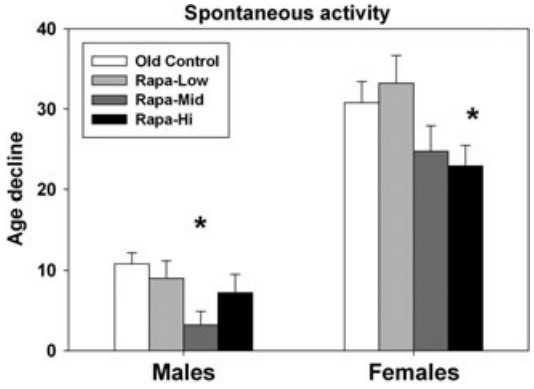

Fig. 6 Age-associated decline of spontaneous in-cage activity, calculated as a change score ($\times 10^{-3}$) for each mouse tested at both 7 and 18 months of age. Bars show means ± SEM, and asterisks indicate significant effects of rapamycin treatment at $P < 0.05$, as estimated using a two-factor ANOVA with Site (UT, UM, TJL) and Rx (rapamycin or untreated) as the predictor variables.

Spontaneous activity

We have previously reported (Miller *et al.*, 2011) that rapamycin, at 14 ppm, retarded the age-related loss of spontaneous activity between 7 and 18 months of age, but this change was seen only in male mice, and with significant site-to-site variation among mice housed at the University of Michigan (UM), the University of Texas Health Science Center in San Antonio (UT), and the Jackson Laboratory (TJL). In this study, we have measured spontaneous activity at both ages in independent cohorts of mice in the context of an ongoing longevity study at rapamycin doses of 4.7, 14, and 42 ppm. A change score, calculated as activity at 18 months minus activity at 7 months, was calculated for each animal for which scores at both ages were available, and mean values are shown in Fig. 6, pooling across the three test sites. For males, rapamycin at the middle dose, 14 ppm, led to a significant diminution of the age-related decline in spontaneous activity, replicating the published study in an independent cohort. Each of the other two rapamycin doses also reduced the age effect, but not to a significant degree. In females, the highest rapamycin dose, 42 ppm, led to a significant reduction in the age effect on spontaneous activity. There was considerable site-to-site variation (not shown), but in aggregate the pooled data suggest that rapamycin may retard age-dependent changes in activity. The basis for the change in movement with age is not well understood in rodents or humans and seems likely to involve a variety of changes in motivation, weight, muscle strength, and hormonal tone, but can be interpreted as one measure of integrated physiological function whose rate of decline seems to be rapamycin sensitive.

Rapamycin levels in peripheral blood

To confirm the idea that rapamycin levels in serum would be proportional to the dose of rapamycin provided to the mice, a separate group of UM-HET3 animals was placed on diets with 4.7, 14, or 42 ppm rapamycin at age 3–4 months of age, with 12–14 mice/group, and serum was taken 5 months later for rapamycin quantification using methods described in (Harrison *et al.*, 2009). Figure 7 shows rapamycin levels in individual mice. Average values for the three groups were, respectively, 6.5 ± 1.1 (mean ± SEM), 13.4 ± 2.6, and 57.5 ± 13.2. Thus, serum rapamycin levels rose in proportion of dose of this agent in chow, though with considerable variation among mice.

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

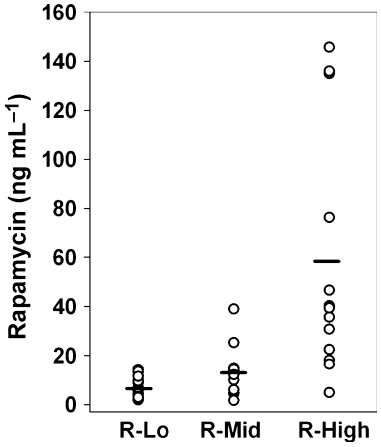

Fig. 7 Rapamycin levels in blood of mice tested at age 8–9 months after consumption of rapamycin for 5 months. Each symbol shows an individual mouse, and horizontal bars indicate means.

Discussion

Our results show that rapamycin-treated mice are delayed in the development of many forms of age-related pathology, including degenerative changes in the liver and heart, proliferative lesions of endometrium and adrenal gland, and alterations in tendon elasticity. The age-dependent decline in spontaneous activity was also diminished in rapamycin-treated mice, although the effective dose differed between males and females. In addition, five other lesions, affecting lung, adrenal gland, ovary, and thyroid, increased in incidence between 4 and 22 months of age, and four of these five showed a trend toward lower frequency in rapamycin-treated mice than in untreated controls, though these effects did not reach statistical significance. Taken together, these findings support the hypothesis (Blagosklonny, 2006) that the aging process is delayed by rapamycin in genetically heterogeneous mice.

Neoplastic disease is responsible for over 80% of the deaths of rapamycin-treated mice in our longevity population (Miller *et al.*, 2011), suggesting that longevity extension in these mice might reflect inhibition of multiple forms of neoplastic disease, rather than an effect on the aging process per se. Our new data do not disprove this idea; rapamycin may well both slow multiple aspects of aging and also have a direct anti-tumor effect, with the latter effect predominantly responsible for lifespan extension in these cancer-prone mice. It is also possible that the reduced cancer incidence could itself be a consequence of delayed aging, through mechanisms still to be defined. The finding that rapamycin started at 9 months of age does not provide significantly greater longevity effect than this drug started at 20 months (Miller *et al.*, 2011) also suggests that the major impact on lifespan is via modulation of processes that operate only after 20 months of age, with induction or progression of neoplasia an obvious suspect. Demographic analyses of our longevity cohorts (Miller *et al.*, 2011) have shown that rapamycin, at 14 ppm, produces a significant change in both parameters of the Gompertz distribution, that is, both age-dependent and age-independent effects on mortality risk, each of which might involve direct or indirect effects on cancer cell biology and host defenses against cancer. It will be of interest to determine whether rapamycin extends lifespan in strains of mice in which deaths because of cancer account for a smaller fraction of lethal illnesses and reduce age-related diseases and functional declines in other vertebrate species.

The idea that TOR inhibition might lead to a delay in aging, and thus in many age-associated dysfunctions, is consistent with work in invertebrate organisms. Mutations that diminish TOR function extend lifespan in flies (Kapahi *et al.*, 2004) and nematodes (Vellai *et al.*, 2003; Jia *et al.*, 2004). Inhibition of TOR also extend lifespan in models of both replicative and chronological lifespan in yeast (Kaeberlein *et al.*, 2005; Powers *et al.*, 2006). There is also good evidence for diminished TOR activity, at least in liver, of mice in which the aging process has been decelerated either by mutation of genes needed for GH and IGF-1 production (Sharp & Bartke, 2005), or by caloric restriction (Sun *et al.*, 2009). Evidence (Selman *et al.*, 2009) that deletion of ribosomal S6 protein kinase 1, an important mediator of TOR action, not only extends lifespan in female C57BL/6 mice, but also slows age-related changes in T cells, behavioral traits, and bone structure is also consistent with our observations of rapamycin-treated mice. Evidence is also beginning to accumulate that rapamycin can reverse or retard age-related change in clinically relevant model systems, including studies of B cell production and influenza vaccine responses (Chen *et al.*, 2009), and of β-amyloid and tau accumulation in CNS tissue (Caccamo *et al.*, 2010).

Some of the specific histopathology endpoints used as markers of aging in this study deserve further comment. The age-dependent liver lesion seen in controls was a multifocal to diffuse, centrilobular, macrovesicular lipidosis. Macrovesicular lipidosis can be induced by a number of xenobiotics, although xenobiotics generally induce a mixed change with both microvesicular and macrovesicular lipidosis (Thoolen *et al.*, 2010). In our study, rapamycin blocked this age effect, reducing the incidence of hepatic lipidosis in a dose-dependent manner in males. Increased expression of liver enzymes involved in the detoxification of xenobiotics has been observed in long-lived mutant mice with defects in growth hormone production (Amador-Noguez *et al.*, 2007), and we have unpublished data that mRNA for these enzymes is elevated within 3 months of rapamycin treatment in UM-HET3 mice (M. J. Steinbaugh and R. A. Miller, unpublished data). Resistance to the effects of xenobiotic hepatotoxins may be a common theme in both genetic and pharmacological models of slowed aging.

Aging changes and degeneration in cardiac myocytes were characterized primarily by nuclear atypia. Nuclei were enlarged, oval to round, hyperchromatic and occasionally clustered. Some of the nuclei had a dense central longitudinal bar similar to that characteristic of Anitschkow cells. Previous studies have associated nuclear atypia with cell cycle abnormalities and alterations in mitochondrial function, both known to increase with age (Ahuja *et al.*, 2007). A small number of our mice had evidence of myofiber degeneration and vacuolization, but the lesions were not associated with treatment.

Rapamycin led to significant inhibition of endometrial hyperplasia and adrenal tumors, and to statistical nonsignificant trends toward lower levels of adrenal telangectasia, thyroid follicles, and ovarian cysts. Cystic endometrial hyperplasia is the most common spontaneous lesion of the uterus in aged female mice (Davis *et al.*, 1999). Both the number and size of glands are increased and many of the glands are cystic and filled with serous fluid. The lesion is not preneoplastic. Cystic endometrial hyperplasia has been associated with changes in the estrous cycle (Davis *et al.*, 1999). Subcapsular hyperplasia of the adrenal cortex is also a common lesion in aging mice (Mohr *et al.*, 1996). In our UM-HET3 mice, this lesion was common and severe and included both Type A (spindle) cells and Type B (polygonal) cells. Subcapsular hyperplasia has been associated with alterations of hormones associated with aging (Mohr *et al.*, 1996). In addition, there was an increase in adrenal cortical adenomas, from 3% in young mice to 20% in aged controls (not statistically

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

significant), that were significantly lower in mice treated with rapamycin (Fig. 1D). An inhibition of adenoma and carcinoma incidence is consistent with other lines of evidence that rapamycin may inhibit neoplastic disease, although cancer of the adrenal very seldom leads to death in the UM-HET3 stock.

We have also observed that rapamycin-treated mice have more severe cataracts and have a higher incidence of testicular degeneration in males. Because TOR is involved in a very wide range of cellular responses to proliferative, nutritional, and stress signals, it is hardly surprising that blocking TOR signals throughout the lifespan may produce side effects that impair function in some tissues. Rapamycin started at 20 months of age produces equivalent lifespan extension, in UM-HET3 mice, as it does when started at 9 months of age (Miller *et al.*, 2011), and it may prove that the optimal ratio of benefit to harmful side effects will involve starting the drug in mice older than 9 months or may require an intermittent schedule in which months without drug exposure are alternated with months in which rapamycin is provided (Anisimov *et al.*, 2011). It is also still unclear as to whether the benefits of rapamycin treatment depend on TOR inhibition per se, or on compensatory changes in other pathways called into play by diminished TOR function; in the latter case, it may be possible to devise interventions that further improve the ratio of positive to negative effects on health.

An effect of rapamycin on cataract development has not, so far as we know, previously been noted in rodents or in humans. Rapamycin has been shown to diminish proliferation of lens epithelial cells in culture, to block migration of these cells in response to fibroblast growth factor, and to increase the ratio of Bax/Bcl-2 in these cells, perhaps predisposing them to apoptosis (Liu *et al.*, 2010; Wang & Wang, 2011), but whether these effects contribute to cataract development will require further evaluation.

Testicular tubular degeneration is a common lesion in aging males (Radovsky *et al.*, 1999). The lesion is characterized by degenerate and necrotic spermatogenic cells. Multiucleate giant cells are also a common feature. In advanced cases, only a few Sertoli cells and occasional spermatogonia remain. In our population, this lesion increased greatly in severity in rapamycin-treated males, although whether the mechanism of age-related change involves TOR function in one or more testicular cell types is not known. Spermatogenesis and fertility are impaired in men receiving rapamycin in the context of therapeutic transplantation (Zuber *et al.*, 2008).

To sum up, the claim that a particular gene or diet or drug slows aging should include evidence for retardation of age effects on proliferating cells, nonproliferating cells, and extracellular structures, because aging modulates the pace of decline in each of these domains. The conclusion that caloric restriction slows aging was based not only on lifespan data, but also in large part on evidence of this kind (Weindruch *et al.*, 1986; Weindruch & Sohal, 1997). Similarly, data showing that pituitary dwarf mice show slower rates of change in tumors, cognitive function, renal pathology, and cataracts (Flurkey *et al.*, 2001; Kinney *et al.*, 2001a,b; Ikeno *et al.*, 2003; Vergara *et al.*, 2004) have strengthened the case for delayed aging in these genetic models as well. In addition to its effects on the pace of lethal neoplasia (Harrison *et al.*, 2009; Miller *et al.*, 2011), rapamycin now seems to retard age effects on tendon, liver, myocardium, and endometrium (this report), and in bone marrow (Chen *et al.*, 2009). Although clearly a good deal of additional work will be needed to establish the range of age effects that are or are not modulated by rapamycin, it now seems safe to conclude that TOR inhibition does indeed slow aging in mice. The observation that inhibition of TOR can extend lifespan in worms, flies, and yeast, species in which neoplasia is not an important cause of death, is consistent with the idea that linkage of TOR-dependent processes to aging rate has deep evolutionary roots, even though the details by which age-dependent change increases mortality rate differ dramatically among these three species.

Experimental procedures

Mice

All the mice used in this project were of a genetically heterogeneous stock, called UM-HET3 in previous publications, produced by a cross between (BALB/cByJ × C57BL/6J)F1 mothers (JAX stock #100009) and (C3H/HeJ × DBA/2J)F1 fathers (JAX stock #100004). Each mouse in the population is thus genetically unique, and a full sib, with respect to nuclear genes, to all other members of the UM-HET3 stock. Husbandry and diet details were as described in earlier reports (Strong *et al.*, 2008; Miller *et al.*, 2011). The diet for controls and rapamycin-treated mice was irradiated Purina 5LG6, which contains 4% fat and 18% protein. In this study encapsulated rapamycin was administered, from 9 months of age unless otherwise indicated, to mice at doses of 4.7, 14, or 42 parts per million in food (calculated on the basis of the equivalent mass of unencapsulated rapamycin). The 14 ppm dose has previously been shown to extend lifespan when started at 9 or at 20 months of age (Harrison *et al.*, 2009; Miller *et al.*, 2011); a lifespan study at the 4.7 and 42 ppm dose levels is under way. Serological assessment of sentinel mice was conducted at least quarterly to evaluate the possibility of infection in the colonies, and all such tests were negative throughout the period of testing. All of the data presented in this report are from mice housed at the University of Michigan (UM), except that the data on spontaneous activity were obtained from equal numbers of mice at UM, the Jackson Laboratory (TJL, Bar Harbor, ME, USA), and the University of Texas Health Science Center (UT, San Antonio, TX, USA), and data on rapamycin levels in serum were obtained from mice originally produced at TJL and then shipped to UT for evaluation.

Necropsy procedures

Complete necropsies were performed on each mouse using standard established protocols (Harrison *et al.*, 2009). All grossly visible lesions were recorded. All major organs were dissected and fixed in 10% formalin. A standard set of tissues was processed, sectioned, and stained with haematoxylin and eosin from each mouse. Any gross lesions noted in the necropsy were also examined histologically. The standard set of tissues was determined by past experience and previous studies of aging mice (Mohr *et al.*, 1996; Harrison *et al.*, 2009; Miller *et al.*, 2011). Liver, spleen, pancreas, kidney, adrenal, heart, skeletal muscle, lung, thyroid, and reproductive tract were examined from each mouse. All lesions were recorded and specific lesions associated with aging were identified and scored based on severity.

Cataract assessment

Mice were tested at age 20 months for lens opacity using a handheld slit lamp after pupillary dilation with one drop of 1% tropicamide. Opacity was scored on a scale from 0 (no evidence of cataract) to 3 (severe) for each eye separately, and the mean score from the left and right eye was used as an index of cataract severity.

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

Tendon evaluation

Previous work has shown that mouse TA tendons stiffen and lose viscous properties in a region-dependent manner with old age, with the most pronounced changes occurring in the region nearest to the muscle (proximal region). Therefore, mechanical properties were determined for the proximal region of the tendons, as previously described (Wood *et al.*, 2011). Briefly, tibialis anterior (TA) muscle-tendon units (MTUs), consisting of the TA muscle, TA tendon, and 1st metatarsal, were extracted and mounted in our custom-made tensile tester and 25 μm polystyrene beads brushed along the tendon to serve as optical markers for strain measurements. The MTUs were immersed in PBS, and the cross-sectional area was determined at six points along the tendon. Tendons were subjected to a load–unload cycle of 10% grip-to-grip strain at a constant strain rate of 0.01 s^{-1}. Synchronized force and image recordings were obtained during testing, and bead positions were tracked using Metamorph software. Stresses and nominal strains were calculated for the proximal tendon region. The maximum slope of the stress–strain response during the loading phase, termed Maximum Tangent Modulus, was determined and taken as our measure of tendon stiffness. Hysteresis area, which is a measure of tendon viscous properties, was calculated as the difference between the area under the loading curve and the area under the unloading curve expressed as a percentage of the area under the loading curve.

Spontaneous activity

We used an Accuscan system, as in (Miller *et al.*, 2011), to evaluate activity. Total number of laser-beam breaks over a 50-h test interval was taken as the index of activity and a change score calculated for each mouse that had been measured at both 7 and 18 months of age. Experiments were performed in parallel at three test sites (UM, TJL, UT) and then pooled for analysis. For males, the data set included 113 control animals and 57–58 mice in each of the rapamycin groups. For females, the data included 142 controls and 74–82 mice in each of the rapamycin groups.

Statistical methods

We first tested in each case to see whether there was an age effect in males and in females. If we found an age effect in one sex only, then we evaluated rapamycin effects in that gender only. If there was an age effect in each sex, we then tested for rapamycin effects in the data pooled across sex. Effects of age on the frequency of specific lesions were evaluated using Fisher's Exact Test in comparisons of young to old controls. Effects of rapamycin on lesion frequency were evaluated by Fisher's Exact Test either by comparison of old controls to mice treated with one dose of rapamycin, or pooled across all rapamycin doses as described in the text. Cuzick's nonparametric test for trends (Cuzick, 1985) was used to evaluate significance of differences among the four groups of old mice at four levels of rapamycin (zero, low, mid, or high). Hypotheses about parametric data, such as the measures of tendon elasticity, were evaluated by unpaired *t*-test. Two-tailed tests were used throughout. Other statistical details are given in the text as appropriate.

Acknowledgments

This work was supported by grants AG022303, AG007996, and AG013283. We thank Vivian Diaz, Lynn Winkleman, and Mike Astle for expert technical assistance.

Author contributions

Drs. Harrison, Nadon, Miller, Strong, and Wilkinson designed the study. Dr. Wilkinson performed all of the pathology examinations and interpreted the necropsy results. Ms. Burmeister and Ms. Friedline conducted all of the animal work and coordinated sample distribution. Dr. Brooks and Ms. Wood conducted and interpreted the tendon assays. Dr. Woodward evaluated cataract status. Drs. Hejtmancik and Chan evaluated histological sections of the eyes. Dr. Miller did the statistical analysis and drafted the paper. All coauthors contributed to the final text of the manuscript.

Reference

Ahuja P, Sdek P, MacLellan WR (2007) Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol. Rev.* **87**, 521–544.

Amador-Noguez D, Dean A, Huang W, Setchell K, Moore D, Darlington G (2007) Alterations in xenobiotic metabolism in the long-lived Little mice. *Aging Cell* **6**, 453–470.

Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Antoch MP, Blagosklonny MV (2010) Rapamycin extends maximal lifespan in cancer-prone mice. *Am. J. Pathol.* **176**, 2092–2097.

Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV, Blagosklonny MV (2011) Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. *Cell Cycle* **10**, 12–15.

Blagosklonny MV (2006) Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. *Cell Cycle* **5**, 2087–2102.

Caccamo A, Majumder S, Richardson A, Strong R, Oddo S (2010) Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. *J. Biol. Chem.* **285**, 13107–13120.

Chen C, Liu Y, Liu Y, Zheng P (2009) mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. *Sci. Signal* **2**, ra75.

Cuzick J (1985) A Wilcoxon-type test for trend. *Stat. Med.* **4**, 87–90.

Davis JD, Dixon D, Herbert RA (1999) Ovary, oviduct, uterus, cervix, and vagina. In *Pathology of the Laboratory Mouse* (Maronpot RR, Boorman GA, Gaul BW, eds). St. Louis, MO: Cache River Press, pp. 409–443.

Flurkey K, Papaconstantinou J, Miller RA, Harrison DE (2001) Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proc. Nat. Acad. Sci. U.S.A.* **98**, 6736–6741.

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392–395.

Hidalgo M, Rowinsky EK (2000) The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* **19**, 6680–6686.

Ikeno Y, Bronson RT, Hubbard GB, Lee S, Bartke A (2003) Delayed occurrence of fatal neoplastic diseases in ames dwarf mice: correlation to extended longevity. *J. Gerontol. A Biol. Sci. Med. Sci.* **58**, 291–296.

Jia K, Chen D, Riddle DL (2004) The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. *Development* **131**, 3897–3906.

Kaeberlein M, Powers RW III, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK (2005) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193–1196.

Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S (2004) Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr. Biol.* **14**, 885–890.

Kinney BA, Coschigano KT, Kopchick JJ, Steger RW, Bartke A (2001a) Evidence that age-induced decline in memory retention is delayed in growth hormone resistant GH-R-KO (Laron) mice. *Physiol. Behav.* **72**, 653–660.

Kinney BA, Meliska CJ, Steger RW, Bartke A (2001b) Evidence that Ames dwarf mice age differently from their normal siblings in behavioral and learning and memory parameters. *Horm. Behav.* **39**, 277–284.

Liu H, Feng G, Wu L, Fu S, Liu P, Yang W, Zhang X (2010) The effects of rapamycin on lens epithelial cell proliferation, migration, and matrix formation: an *in vitro* study. *Mol. Vis.* **16**, 1646–1653.

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de CR, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, Sinclair D, Starnes JW, Wilkinson JE, Nadon NL, Strong R (2011) Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. *J. Gerontol. A Biol. Sci. Med. Sci.* **66**, 191–201.

Mohr U, Dungworth DL, Capen CC, Carlton WW, Sundberg JP, Ward JM (1996) *Pathobiology of the Ageing Mouse*. Washington, DC: ILSI.

Powers RW III, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S (2006) Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev.* **20**, 174–184.

Radovsky A, Mitsumori K, Chapin RE (1999) Male reproductive tract. In *Pathology of the Mouse*, (Maronpot RR, Boorman GA, Gaul BW, eds). St. Louis, MO: Cache River Press, pp. 381–407.

Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F, Woods A, Robinson IC, Schuster E, Batterham RL, Kozma SC, Thomas G, Carling D, Okkenhaug K, Thornton JM, Partridge L, Gems D, Withers DJ (2009) Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* **326**, 140–144.

Sharp ZD, Bartke A (2005) Evidence for down-regulation of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-dependent translation regulatory signaling pathways in Ames dwarf mice. *J. Gerontol. A Biol. Sci. Med. Sci.* **60**, 293–300.

Strong R, Miller RA, Astle CM, Floyd RA, Flurkey K, Hensley KL, Javors MA, Leeuwenburgh C, Nelson JF, Ongini E, Nadon NL, Warner HR, Harrison DE (2008) Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice. *Aging Cell* **7**, 641–650.

Sun L, Sadighi Akha AA, Miller RA, Harper JM (2009) Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age. *J. Gerontol. A Biol. Sci. Med. Sci.* **64**, 711–722.

Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey DE, Kaufmann W, Kuttler K, Deschl U, Nakae D, Gregson R, Vinlove MP, Brix AE, Singh B, Belpoggi F, Ward JM (2010) Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol. Pathol.* **38**, 5S–81S.

Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F (2003) Genetics: influence of TOR kinase on lifespan in *C. elegans*. *Nature* **426**, 620.

Vergara M, Smith-Wheelock M, Harper JM, Sigler R, Miller RA (2004) Hormone-treated Snell dwarf mice regain fertility but remain long-lived and disease resistant. *J. Gerontol. Biol. Sci.* **59**, 1244–1250.

Wang Z, Wang Z (2011) Effects of rapamycin on expression of Bcl-2 and Bax in human lens epithelial cells and cell cycle in rats. *J. Huazhong Univ. Sci. Tech.* **31**, 555–559.

Weindruch R, Sohal RS (1997) Seminars in medicine of the Beth Israel Deaconess Medical Center. Caloric intake and aging. *New Engl. J. Med.* **337**, 986–994.

Weindruch R, Walford RL, Fligiel S, Guthrie D (1986) The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. *J. Nutr.* **116**, 641–654.

Wood LK, Arruda EM, Brooks SV (2011) Regional stiffening with aging in tibialis anterior tendons of mice occurs independent of changes in collagen fibril morphology. *J. Appl. Physiol.* **111**, 999–1006.

Zuber J, Anglicheau D, Elie C, Bererhi L, Timsit MO, Mamzer-Bruneel MF, Ciroldi M, Martinez F, Snanoudj R, Hiesse C, Kreis H, Eustache F, Laborde K, Thervet E, Legendre C (2008) Sirolimus may reduce fertility in male renal transplant recipients. *Am. J. Transplant.* **8**, 1471–1479.

Supporting Information

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

Fig. S1 Representative sections from histopathological analysis.

Fig. S2 Representative sections from histopathological analysis.

Fig. S3 Representative sections of lens from the eye of a rapamycin-treated mouse with cataract.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

© 2012 The Authors
Aging Cell © 2012 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland