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Review

Questioning causal involvement of telomeres in aging

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ABSTRACT

Multiple studies have demonstrated that telomere length predicts mortality and that telomeres shorten with age. Although rarely acknowledged these associations do not dictate causality. I review telomerase knockout and overexpression studies and find little support that telomeres cause aging. In addition, the causality hypothesis assumes that there is a critical telomere length at which senescence is induced. This generates the prediction that variance in telomere length decreases with age. In contrast, using meta-analysis of human data, I find no such decline. Inferring the causal involvement of telomeres in aging from current knowledge is therefore speculative and could hinder scientific progress.

Contents

1. Introduction

1.1. Telomere biology

Telomeres are repetitive DNA sequences at the ends of linear chromosomes and serve to maintain chromosome integrity (Riethman, 2008). Additional properties have made telomeres a focus in the biology of aging: (i) telomeres shorten at each cell division due to incomplete replication of their ends; (ii) they are shortened by oxidative damage; and (iii) when telomeres reach a critical length, cells enter a senescent state and cell division ceases. This latter property has been demonstrated in now classic experiments, showing that telomere length predicts the *in vitro* replicative capacity of human fibroblasts and that over-expressing telomerase—the enzyme that can reverse transcribe telomeric sequence—immortalizes fibroblast cell cultures (Bodnar

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et al., 1998; Rudolph et al., 1999). These experiments suggest the possible causal involvement of telomeres in the aging process and this hypothesis has increased in popularity since the finding that telomere length predicts human mortality (Boonekamp et al., 2013; Cawthon et al., 2003) and that, *in vivo*, human telomeres shorten during aging (Müezzinler et al., 2013).

1.2. Association is not causality, and no causality without association

It is rarely acknowledged in telomere biology that such associations do not necessarily dictate causality. Of course in principle associations can never show causality, and experimental evidence is a starting point. However when epidemiological associations are very strong and are corroborated by experimental evidence from animals or other supporting evidence, such as in the relationship between smoking and lung cancer (Cornfield et al., 2009), causality can be deduced. Such inference is useful interpreting data in terms of biological mechanism and for policy, but can also be harmful when such causality is prematurely inferred or assumed. This can lead to reduced or misfocused research effort to uncover the mechanisms actually responsible for the associations that are reported or false inference of the associated biology. In the biology of aging it is tempting to infer causality from biomarkers of aging because aging mechanisms remain so elusive. It is therefore not surprising that causality of telomere length in aging is often inferred from associations or from the available experimental evidence.

Such inferences span different fields of biology working on telomeres with the following quotes to illustrate this. Within evolutionary ecology: "When telomeres reach a critically short length. . .contributes to the decline in tissue function in later life" (Herborn et al., 2014). "..our study provides good evidence for a mechanistic link between telomere erosion and reduced organism longevity under natural conditions" (Bize et al., 2009). Mechanistic research: "Genetically modified animal models have established causal links between telomere loss, cellular senescence, and organismal aging" (López-Otín et al., 2013). "These findings demonstrate that short telomeres have a direct impact on longevity in mammals" (Vera et al., 2012). Epidemiology: "We propose that telomere length is not only associated with atherosclerosis and longevity but is also a causal determinant of both" (Aviv et al., 2015). Genetics: "Our findings support a causal role of telomere-length variation in some age-related diseases" (Codd et al., 2013). Psychiatry, reasoning from an association with telomere length: "..links of childhood maltreatment with psychopathology and associated medical problems could be due to effects of stress on cellular aging." (Tyrka et al., 2010).

These quotes cannot objectively summarize the dominating opinion in telomere biology, however they do highlight that causality is often deduced from the current literature. Here I question such causal involvement of telomeres in aging using a critical review of the mechanistic evidence and a formal meta-analysis of a key prediction when a causal link between telomeres and mortality is assumed.

2. Results

2.1. Mechanistic evidence

I review the telomerase knockout and overexpression studies that are often cited and hailed as providing the necessary evidence for the causal involvement of telomere biology in aging. I collated studies on the effects on longevity and conclude that, together; the results are surprisingly mixed and provide weak support (Fig. 1). In the cases where lifespan changed as predicted in response to the manipulation of telomerase, the effect on telomere length was either outside the normal range of telomere shortening or telomere elongation was not conclusively demonstrated, thereby limiting any strong conclusions. The conclusion that natural genetic variation in telomeres causes aging would need to be supported by shortening manipulations that fall within the range seen across the lifespan in unmanipulated individuals.

2.1.1. Mouse models

Mice in which telomerase has been knocked out show reduced longevity, but this effect is only visible after multiple generations (Espejel et al., 2004; Rudolph et al., 1999), suggesting that telomeres have to become extremely short before they impinge on senescence *in vivo*. Such critically short lengths might never be reached in a normal mouse life. Telomerase knockout mice in generation 1, in which there is very limited modulation of lifespan, already exhibit severely shortened telomeres (Espejel et al., 2004; Rudolph et al., 1999). Knocking out telomerase shortens telomere length by about ~4–5 kb per generation (Blasco et al., 1997; Rudolph et al., 1999), whereas ~15 kb are lost over a wild-type mouse's lifetime (Vera et al., 2012). Thus when lifespan reduction does become apparent at generation 3 and beyond, telomeres in these telomerase-deficient mice have shortened by about as much as over the entire life of a normal mouse, yet these knock-out mice are still viable and their telomeres continue to shorten substantially over their lives prior to death (Rudolph et al., 1999). These results suggest that telomerase knockout models are not necessarily informative about the involvement of telomeres in normal wild-type aging, but are perhaps more appropriately seen as a model of telomerase-associated diseases, such as dyskeratosis congenita (Mitchell et al., 1999).

Another finding that is often used as evidence for the causal role of telomeres in aging is the fact that overexpressing telomerase in mice extends lifespan when selectively expressed at old age (Bernardes de Jesus et al., 2012). However, effects on actual telomere length are far from conclusive (Bernardes de Jesus et al., 2012). Doubts about actual increased telomere length *via* telomerase overexpression increase the possibility that the observed effects are mediated by the actions of telomerase beyond actual telomere length elongation (Cong and Shay, 2008). The sample sizes to verify telomere length elongation under telomerase overexpression (Bernardes de Jesus et al., 2012) are very low (3 or 4 per group) and the use of *t*-tests (that assume normality) over non-parametric equivalents is likely to be inappropriate for comparing proportions, such as the fraction of short telomeres that is often used as a measure. The lowest p value that can be reached with a sample of 3 per group using non-parametric alternatives is 0.05—the probability that all three values from one group are lower than the other by chance. This is strikingly different than some of the probabilities ($p < 0.01$) reported using similar sample sizes using *t*-tests on telomere length measures (Bernardes de Jesus et al., 2012), suggesting a strong reduction in p values due to inappropriately assuming normality. Moreover, the use of statistics on q-FISH data that treat each cell as an independent measurement (Bernardes de Jesus et al., 2012) is clearly incorrect and severely biased due to pseudoreplication, because the independent statistical unit is the individual mouse. In general, the causal contribution of telomeres to aging in inbred strains of laboratory mice can be questioned because these animals show markedly longer telomeres than their wild or outbred relatives, without living longer (Hemann et al., 2001; Miller et al., 2002). In a comparative context absolute length of telomeres is actually inversely related to lifespan in mammals, with the shortest telomeres amongst the longest-lived species, such as whales (Gomes et al., 2011). In addition, the claim that a drug, TA-65, increases telomere length, but does not affect overall mortality, also

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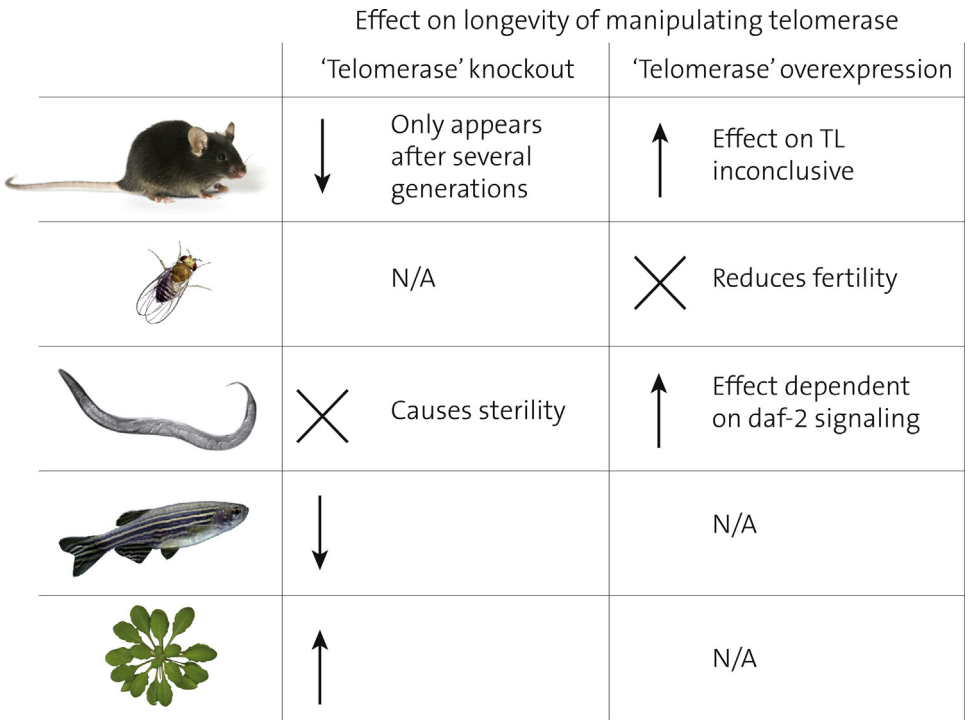

Fig. 1. Overview of the mechanistic evidence for a causal link between telomere length and longevity. Telomerase is the enzyme responsible for elongating telomeres. Consequently, a knockout of telomerase is expected to decrease longevity, whereas conversely overexpression is expected to increase longevity. Although telomere maintenance mechanisms and telomere functions differ between species (hence the quotation marks around 'telomerase') it is clear that effects on longevity are not universal and mixed. Up-arrow indicates an increase in longevity, down-arrow a decrease, a cross a null effect, N/A indicates that no study is available for this comparison. For further elaboration on the comments in the figure, refer to the main text.

contradicts a direct causal relationship between telomere length and mortality (de Jesus et al., 2011).

2.1.2. Other model species

Not only are studies on mice inconclusive, studies on other species also cast doubt on the universal causal involvement of telomeres in aging. Recently, telomerase-mutant zebra fish were shown to have strikingly shortened lifespans, but again the level of telomere attrition seen in these mutants is markedly higher than in old wild-type animals (Henriques et al., 2013), suggesting that the phenotype might not be representative of normal aging. Furthermore, telomerase-mutant *Arabidopsis* does not show a senescent phenotype and, despite developmental abnormalities, outlives wild-type plants (Fitzgerald et al., 1999; Riha et al., 2001). Longevity can also be uncoupled from telomere length in *Drosophila* (Walter et al., 2007) and in *Caenorhabditis elegans* (Meier et al., 2006; Raices et al., 2005) any life-extending effects of long telomeres are dependent on daf-16 signaling. Thereby demonstrating that this phenotype in *C. elegans* is not due to the negative mechanical consequences of critically short telomeres, but dependent on downstream molecular signaling pathways (Joeng et al., 2004).

2.1.3. Conclusion from current mechanistic evidence

From these studies I conclude that either telomeres are not causal in aging or, at least, that the underlying mechanisms are not universal, making unreliable any direct inference of causality from these model species to human biology. The recent finding that telomeres regulate DNA damage responses, independent of actual telomere length, questions further the role of telomere maintenance in cellular senescence (Fumagalli et al., 2012; Hewitt et al., 2012). Cell cycle progression is arrested when DNA-damage is not repaired, and persistent DNA-damage response foci are associated with cellular senescence. Such persistent DNA-damage response foci were found to be strongly localized at telomeres in human cell culture (Fumagalli et al., 2012; Hewitt et al., 2012) and increase with age in mouse liver and gut (Hewitt et al., 2012), and importantly, these effects are independent of the actual length of these telomeres (Fumagalli et al., 2012; Hewitt et al., 2012). This suggests that telomeres are signaling the current and past level of DNA-damage irrespective of their length, at odds with the assumption that critical shortening of telomeres causes cellular senescence.

2.2. Critical tests of causality using epidemiological evidence

2.2.1. The association of telomere length and mortality reduces with age

Epidemiological studies have demonstrated the associations between telomere length and mortality or disease, but epidemiological data also provide the opportunity to test the predictions that follow from the causal involvement of telomeres in aging. For instance, if telomere length determines mortality at old age, then there is a clear expectation that telomere length will more closely predict remaining lifespan at older *versus* younger ages. However, telomere length does not predict mortality in the very oldest (Bischoff et al., 2006; Martin-Ruiz et al., 2005) and a meta-analysis across the available literature has confirmed that the association between telomere length and overall mortality actually diminishes with age (Boonekamp et al., 2013).

2.2.2. Variance in telomere length should decline with age

Here, I test another specific prediction of the current hypothesis concerning the involvement of telomere length in aging (Blasco, 2007, 2005): there will be a critical length at which telomeres induce cellular senescence, aging and eventually death. Such a critical length of telomeres leads to the previously unrecognized prediction that variance in telomere length should strongly decrease with age. The individuals with the shortest telomeres would die off when their telomeres reached critically short lengths.

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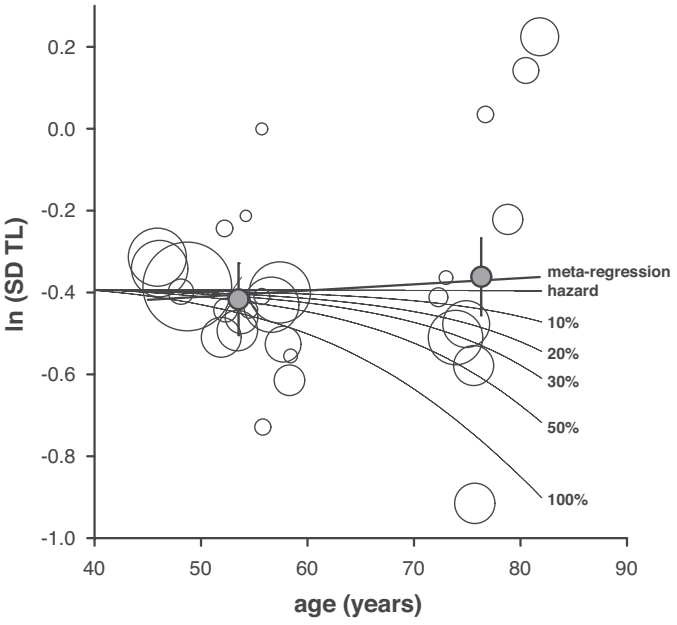

Fig. 2. Variance in telomere length does not decrease with age in humans. Plotted are the 29 estimates (across 16,384 subjects) of standard deviation of telomere length collected against the mean age of the subjects in each study. Bubble area represents the inverse of the sampling variance, the precision of a study, which is a function of sample size. Please refer to the supplementary material for the full dataset used. The bold line refers to the meta-regression and the closed dots with associated standard errors represent the meta-analysed standard deviation for young *versus* old subjects. Predicted reductions in variance from the simulations (see text) are plotted for a continuous hazard and for different proportions dying from telomere-related causes, as indicated.

This cut-off will generate a strong reduction in the variance in telomere length with age. Note, that due to this cut-off the reduction in variance is substantially stronger than a continuous association of mortality risk with telomere length. The crucial distinction here is whether individuals actually die from having critically shortened telomeres or whether there is an association between telomere length and mortality that is not causal.

To illustrate these predicted decline I simulated the predicted decline in variance in telomere length due to mortality from age 40 onwards using USA mortality records from 2005 to 2009 (Human Mortality Database, 2014), under two scenarios. In the first scenario, an association of telomere length with age-related mortality risk is modelled using the mean hazard ratio across studies from a recent meta-analysis (Boonekamp et al., 2013), and this generated only a very shallow, negligible, decrease in the variance of telomere length (Fig. 2). In the second scenario, I modelled a causal relationship between short telomeres and mortality risk, such that only the 10% of individuals with the shortest telomeres were at risk of dying from telomere-related causes. I simulated this under differential fractions of mortality caused by telomeres, ranging from 100% to 10%. The model predicts declines in the variance of telomere length across this whole range (Fig. 2). The results from these simulations are dependent on there being limited variation in telomere shortening amongst individuals and this assumption is supported by recent large studies (Benetos et al., 2013; Daniali et al., 2013; Ehrlenbach et al., 2009). The results also depend on the assumed initial variation in telomere length; I based my estimate of this variation on the overall standard deviation estimated in the meta-analysis (see below). I subsequently tested for these predicted declines in variance using a meta-analysis.

2.2.3. A meta-analysis: variance in telomere length does not decline with age in humans

For the meta-analysis, I collected data on standard deviation from a large meta-analysis on sex differences in telomere length in the general population (Gardner et al., 2014) and I enriched this set with data from two other recent meta-analyses (Boonekamp et al., 2013; Müezzinler et al., 2013). I selected samples from which the age at sampling was on average over 45 years, after which age-related mortality occurs. Moreover, I restricted the data to include only studies that used terminal restriction fragment (TRF) analysis (southern blotting) (Kimura et al., 2010). TRF analysis is the only method in which the means and standard deviations can be reliably compared between laboratories, because it uses a single standard across laboratories (a DNA molecular weight ladder) to calculate the mean telomere length per individual (Verhulst et al., 2015). However when I analysed the available data on qPCR, the other main method used to measure telomere length, the conclusions below did not change.

Data were analysed using the natural logarithm of the reported standard deviation of telomere length, for which the sampling variance is known to be a function of sample size (Nakagawa et al., 2014), in a mixed model setting (with study included as random term) using the R package *metafor* (Viechtbauer, 2010). Separate models were fitted that also included covariates such as sex, the natural logarithm of mean telomere length (Nakagawa et al., 2014), the standard deviation of subject age in the study, and any combination of these. In none of these models did variance significantly decrease with age and, in general, the estimated slope was positive rather than negative (slope of age without any covariates: 0.0015 ± 0.0036 (s.e.), $p = 0.68$). The predicted reductions in the variance of telomere length are, however, not linear but depend on the increase in mortality with age (Fig. 2). The quadratic term for age, however, was also close to zero and far from significant (-0.0001 ± 0.0003, $p = 0.69$). Because there were no studies with mean ages between 59 and 71 years, the data could also be objectively dichotomised between young (<60 years) and old (>70 years). These two categories did not differ in telomere variance (difference 0.055 ± 0.095, $p = 0.56$), corroborating earlier analyses. These results, and earlier epidemiological evidence (Boonekamp et al., 2013), therefore suggest that telomere length is not a determinant of aging but rather a marker able to explain life expectancy and disease risk, for currently unknown mechanistic reasons.

3. Telomere length in the study of aging

The suggestion that telomeres are not causally involved in the aging process does not render telomere biology any less interesting. Assumptions of causality from correlational data or the, to date, restricted number of *in vivo* mechanistic tests (López-Otín et al., 2013), could, however, hinder progress in understanding telomere biology and mechanisms of aging in general. Inferring causality from current knowledge discourages further critical mechanistic experiments to test for causality and, potentially even more damaging, can lead to false conclusions concerning the biological significance of associations with telomere length. For example, interpreting associations of telomere length with lifestyle or detrimental psychological events as being associations with cellular senescence is potentially misleading.

Assuming the causality of telomeres in the biology of aging can also limit our understanding of the actual core mechanisms of aging. Investments in finding ways to elongate telomeres *in vivo* might be better spent on investigating the factors that contribute to differences in the rate of telomere shortening or the initial variance of telomere length at birth (Broer et al., 2013; Hjelmborg et al., 2015). The latter is especially interesting, considering that

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it might represent variation in telomere shortening in early life, with relatively constant telomere loss thereafter (Benetos et al., 2013; Daniali et al., 2013; Ehrlenbach et al., 2009). This would also explain why telomere length in early life is most predictive of life expectancy (Boonekamp et al., 2013; Heidinger et al., 2012), and fits with observations that events in early life have long-lasting effects (Tarry-Adkins and Ozanne, 2011), for which telomeres might then be a marker (Entringer et al., 2011).

Note that most studies in telomere biology, including most of those covered here, use mean telomere length of a cell or a population of cells. Whereas it has been suggested that a single critically short telomere in a cell can induce cellular senescence, potentially contributing to organismal senescence (Abdallah et al., 2009; Bendix et al., 2010; Hemann et al., 2001; Vera et al., 2012). There is considerable heterogeneity in telomere length across chromosomes and cells during ageing as was shown by the use of single telomere length analysis (STELA) (Baird et al., 2003; Kimura et al., 2007; Lin et al., 2014). Evidence from *in vitro* studies do suggest that such heterogeneity determines cellular senescence (Bendix et al., 2010; Hemann et al., 2001). It remains to be determined however whether *in vivo* the amount of critically short telomeres is a better predictor of aging-related mortality. Moreover, if this is the case, whether this is due to increased measurement precision of STELA (Martin-Ruiz et al., 2014; Verhulst et al., 2015) or because the measurement of single telomere lengths is closer to the actual mechanism determining senescence. In a study using another method of telomere measurement, flow FISH, variance of (median) telomere length did decrease with age (Halaschek-Wiener et al., 2008), in contrast with the presented meta-analysis, but with a markedly lower sample size of 181 compared to a total of 16,384 subjects in the meta-analysis presented here (see supplementary data). Note also that flow FISH is a relative measure, as qPCR, standardised to a single reference sample of know length, precluding quantitative comparisons of variances in telomere length between studies.

4. Conclusions

Telomere length is attractive as an integrative measure of somatic damage or past cell replication history, and can therefore provide an excellent way to further understand the core mechanisms of aging and early-life programming. The associations between telomere length and age-related disease and mortality should therefore inspire further research unravelling the factors contributing to between-individual differences in telomere length. Such studies can focus on mechanism, but, as also demonstrated in this paper, re-appreciation of epidemiological data has the potential to provide important new insights into the physiological *in vivo* functions of telomere length.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arr.2015.08.002.

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