Divergence of sperm and leukocyte age-dependent telomere dynamics: implications for male-driven evolution of telomere length in humans

Kenneth I. Aston 1, Steven C. Hunt 2, Ezra Susser 3,4, Masayuki Kimura 5, Pam Factor-Litvak 3, Douglas Carrell 1,6,7, and Abraham Aviv 3,5,*

1Andrology and IVF Laboratories, Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT 84108, USA 2Cardiovascular Genetics Division, University of Utah School of Medicine, Salt Lake City, UT 84112, USA 3Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY 10032, USA 4New York State Psychiatric Institute, New York, NY 10032, USA 5The Center of Human Development and Aging, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA 6Department of Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, UT 84112, USA 7Department of Physiology, University of Utah School of Medicine, Salt Lake City, UT 84112, USA

*Correspondence address. University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 185 South Orange Ave Newark, NJ 07103, USA. Tel: +1-973-972-5280; +Fax: 1-973-972-5576; E-mail: avivab@umdnj.edu

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ABSTRACT: Telomere length (TL) dynamics in vivo are defined by TL and its age-dependent change, brought about by cell replication. Leukocyte TL (LTL), which reflects TL in hematopoietic stem cells (HSCs), becomes shorter with age. In contrast, sperm TL, which reflects TL in the male germ cells, becomes longer with age. Moreover, offspring of older fathers display longer LTL. Thus far, no study has examined LTL and sperm TL relations with age in the same individuals, nor considered their implications for the paternal age at conception (PAC) effect on offspring LTL. We report that in 135 men (mean age: 34.4 years; range: 18–68 years) on average, LTL became shorter by 19 bp/year (r = −0.3; P = 0.0004), while sperm TL became longer by 57 bp/year (r = 0.32; P = 0.0002). Based on previously reported replication rates of HSCs and male germ cells, we estimate that HSCs lose 26 bp per replication. However, male germ cells gain only 2.48 bp per replication. As TL is inherited in an allele-specific manner, the magnitude of the PAC effect on the offspring’s LTL should be approximately half of age-dependent sperm-TL elongation. When we compared the PAC effect data from previous studies with sperm-TL data from this study, the result was consistent with this prediction. As older paternal age is largely a feature of contemporary humans, we suggest that there may be progressive elongation of TL in future generations. In this sense, germ cell TL dynamics could be driving the evolution of TL in modern humans and perhaps telomere-related diseases in the general population.

Key words: sperm telomere length / leukocyte telomere length / paternal age / telomere evolution

Introduction

Modern societies are experiencing a demographic shift in which both parents conceive their first child at an older age (Office of National Statistics, 2002; Hamilton et al., 2005; Bray et al., 2006). It is well established that the relative risk for aneuploidy and other chromosomal abnormalities is increased in children of older mothers and that risks for rare conditions such as achondroplasia and Marfan syndrome are increased in children of older fathers (Tarin et al., 1998; Kuhnert and Nieschlag, 2004). Moreover, recent studies have shown that older paternal age at conception (PAC) is not only associated with rare conditions but also with relatively common neurodevelopmental disorders in the offspring (Malaspina et al., 2001; Reichenberg et al., 2006; Frans et al., 2008; Saha et al., 2009). Thus, the general consensus is that an older PAC might adversely affect the offspring. However, recent epidemiological studies examining telomere length (TL) in leukocytes suggest that an older PAC might also exert a potentially beneficial effect on offspring. That is because offspring of older fathers display a longer leukocyte TL (Unryn et al., 2005; De Meyer et al., 2007; Njajou et al., 2007; Kimura et al., 2008; Arbee et al., 2011) and a longer LTL is associated with resistance to atherosclerosis and increased survival in the elderly (Aviv, 2012). While TL undergoes age-dependent attrition in leukocytes, which are typically used to study human telomere dynamics in replicating
Materials and Methods

 Subjects

Sperm/blood donors (n = 135) were recruited at two sites. Donors at the University of Medicine and Dentistry of New Jersey, New Jersey Medical School were medical students, faculty and staff. Donors at the University of Utah included men previously enrolled in a therapeutic sperm donation program, and volunteers recruited from the general population and the University andrology clinic. All donors signed written informed consents approved by the review boards of the University of Utah School of Medicine and the University of Medicine and Dentistry of New Jersey, New Jersey Medical School.

 DNA extraction and TL measurements

DNA was extracted from whole blood using the Gentra Puregene Blood Kit (Qiagen, Inc., Valencia, CA, USA) according to manufacturer’s recommendations. Sperm DNA was extracted using the Genomic-tip 20/G Kit (Qiagen) based on the manufacturer’s protocol with modifications to sample preparation and cell lysis. Briefly, sperm cells were pelleted at 2000g for 3 min and washed once in PBS. Following the wash, the sperm pellet was resuspended in a 4 ml of a 1:3 mix of buffer C1:H2O and held on ice for 10 min. Sperm were again centrifuged at 4°C, 2000g for 15 min, and the pellet was subsequently resuspended in 1 ml of buffer C1:H2O, briefly vortexted, and centrifuged for an additional 15 min. Following centrifugation, the pelleted nuclei were resuspended in 1 ml of buffer G2 by vortexing, and 25 μl of proteinase K (20 mg/ml stock) and 80 μl DTT (1 M stock) were added followed by a 60 min incubation at 50°C. Following lysis of nuclei, part II of the manufacturer’s protocol was followed without modification.

 After verifying DNA integrity on 1% (wt/vol) agarose gel, TL was measured by the Southern blot method of the terminal restriction fragments, which were generated through digestion with restriction enzymes Rsa I and Hinf I in paired samples of sperm and leukocytes from the same donors (Kimura et al., 2010b). For leukocytes DNA was resolved in 0.5% while for sperm it was resolved on 0.3% agarose gels. A representative Southern blot is illustrated in Fig. 1. To calculate the mTRF length, we used the data points between 3 and 20 kb for blood and between 4 and 50 kb for sperm. A detailed description of the assay can be found in a recent methods paper published by several of the authors of the current manuscript (Kimura et al., 2010b).

 Statistical analysis

Both LTL and sperm TL were approximately normally distributed. Therefore, age-adjustment and regressions of TL on age were performed by linear regression. Pearson correlation coefficients were used to compare age-adjusted LTL with sperm TL. As there were no significant differences by race for either LTL or sperm TL, races were combined for analysis.

 Results

 Empirical findings

Table 1 shows the average and range of values for age, sperm TL and LTL of the 135 donors (84 from Utah; 51 from New Jersey) in this
study. The age range was 18–68 years, the LTL range of 4.9–9.2 kb and the sperm TL range of 9.6–18.8 kb. The Utah donors were predominantly white of European extraction (91.1%), while New Jersey donors were mainly white (64.7%) and African American (27.5%; Table II). Although sperm TL was longer in the New Jersey sample (the mean age of the New Jersey donors was older), after age-adjustment no statistical differences were observed in LTL between the Utah and New Jersey donors. There were no differences in telomere parameters between whites and African-Americans. There were also no differences in telomere parameters between individuals recruited from the Utah andrology clinic versus other donors.

Figure 2 displays the relations of sperm TL with age (top panel) and LTL with age (bottom panel). Both LTL and sperm TL showed considerable inter-individual variation, even for donors of the same age. However, while LTL showed progressive shortening with age, sperm TL showed progressive lengthening with age. The linear regression of sperm TL is described by $Y = 0.057x + 11.3$, while the linear regression of LTL with age is described by $Y = -0.019x + 7.55$. Stratifications by age into tertiles also showed that sperm TL was longer, while LTL was shorter in older men (Table III).

It is well established that TL is synchronized (positively correlated) among somatic tissues of the individual (Benetos et al., 2011; Granick et al., 2011; Smith et al., 2011). We therefore examined whether TL synchrony is displayed in leukocytes versus sperm. A positive correlation ($r = 0.33$) was observed between age-adjusted LTL and age-adjusted sperm TL (Fig. 3).

**Computational findings**

Based on the linear regression displayed in the top panel of Fig. 2, the sperm TL became longer at a rate of 57 bp/year. On average, the sperm TL in a 40-year-old donor was longer by 1140 bp than that of a 20-year-old donor. The estimated numbers of male germ cell replications are 150 at the age of 20 and 610 at the age of 40 indicating 460 replications in this 20-year span, corresponding on average to one cell division every 16 days, or 23 divisions per year (Crow, 2000). Thus, we can estimate the amount of TL lengthening per replication in the male germ cells as $1140/(610–150) = 2.48$ bp.

Similarly, based on the linear regression displayed in the bottom panel of Fig. 2, LTL was shorter by an average of 380 bp in a 40-year-old donor than in a 20-year-old donor. The estimated number of HSC replications between the ages of 20–40 years is 14.6 (0.86/year; Shepherd et al., 2004; Sidorov et al., 2009). Thus, we estimate the amount of TL shortening per replication of HSC as $380/14.6 = 26$ bp.

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**Table I** Age, sperm TL and LTL of donors

| Parameter | Utah (n = 84) | New Jersey (n = 51) | Combined (n = 135) |
|-----------|--------------|---------------------|-------------------|
| Age (years) | Mean 32.8, Min 20.0, Max 68.0, SD 9.1 | Mean 37.0, Min 18.0, Max 59.0, SD 11.5 | Mean 34.4, Min 18.0, Max 68.0, SD 10.2 |
| Sperm TL (kb) | Mean 12.3, Min 9.6, Max 14.8, SD 1.1 | Mean 14.8, Min 11.4, Max 18.8, SD 1.7 | Mean 13.2, Min 9.6, Max 18.8, SD 1.8 |
| LTL (kb) | Mean 6.9, Min 4.9, Max 8.3, SD 0.6 | Mean 6.9, Min 5.7, Max 9.2, SD 0.7 | Mean 6.9, Min 4.9, Max 9.2, SD 0.6 |

**Table II** Ethnicity of donors

| Ethnicity | Utah (%) | New Jersey (%) | Combined (%) |
|-----------|----------|----------------|--------------|
| White     | 91.1     | 64.7           | 77.1         |
| African American | 0.0     | 27.5           | 14.6         |
| Asian     | 6.7      | 2.0            | 4.2          |
| Hispanic  | 0.0      | 3.9            | 2.1          |
| Mixed ethnicity | 2.2 | 2.0           | 2.1          |
**Discussion**

Based on TL measurements in the sperm and leukocytes from the same individuals, while sperm TL undergoes age-dependent elongation, LTL displays attrition with age. Moreover, although TLs in both sperm and leukocytes are highly variable across individuals, partial synchrony in TL is observed between leukocytes and sperm within the individual; the longer the LTL, the longer the sperm TL. Wide inter-individual variation in LTL (Rufer et al., 1999; Barbieri et al., 2009) and sperm TL (Allsopp et al., 1992; Baird et al., 2006) has been previously observed. Yet synchrony in TL among somatic tissues, which is quite strong in the fetus (Youngren et al., 1998) and in the newborn (Okuda et al., 2002), is largely maintained later in life both in humans and other mammals (Benetos et al., 2011; Granick et al., 2011; Smith et al., 2011). These findings indicate that the variation stemming from telomere shortening with age due to different rates of replication is smaller than the wide inter-individual variation in TL at birth. Perhaps due to divergent trajectories with age of TLs in HSCs versus male germ cells, the correlation we observed between sperm TL and LTL, although highly significant ($P = 0.0001$), is much weaker ($r = 0.33$) than has been observed among somatic tissues, (Benetos et al., 2011; Granick et al., 2011; Smith et al., 2011).

What might be the mechanisms that explain the divergent age-dependent trajectories of TLs in leukocytes versus sperm? During extra-uterine life, the activity of telomerase, the reverse transcriptase that replenishes telomere repeats lost with replication, is largely repressed in somatic cells of humans and other large mammals (Gomes et al., 2011); telomerase repression also applies to HSCs (Broccoli et al., 1995; Chiu et al., 1996; Yui et al., 1998). In contrast, telomerase activity is robust in the testis and presumably male germ cells, although mature spermatozoa show no detectable activity of telomerase (Wright et al., 1996). Telomerase activity is also robust in testis of other large mammals (Nasir et al., 2001; Gardner et al., 2007). Therefore, when HSCs replicate, their TL undergoes progressive shortening, a phenomenon expressed as age-dependent LTL attrition. Growth and development entail rapid expansions in tandem with the growing soma of the HSC compartment, the hematopoietic progenitor cell compartment and the peripheral blood cell pool. These expansions require numerous symmetric replications of HSCs (dividing into two HSCs) and asymmetric replications of HSCs (dividing into one HSC and one hematopoietic progenitor cell; Morrison and Kimble, 2006), which cause rapid LTL shortening during the first two decades of life (Sidorov et al., 2009). HSC-TL attrition slows during adult life, and cross-sectional data of LTL versus age among adults are best fitted by a linear term in regression models, as shown in Fig. 2.

We propose two reasons for the divergence observed between sperm TL and LTL after birth. First, while telomerase activity is repressed in HSCs, the precursors to sperm in the male germline display robust activity of the enzyme (Wright et al., 1996; Riou et al., 2005). In telomerase-positive cells, the activity of the enzyme is usually calibrated to replenish the amount of telomere repeats clipped off with each cycle of replication. Thus, telomeres are maintained at a constant length. This explains the longer sperm TL than HSC-TL in adults, but it does not account for the age-related elongation of sperm TL. We therefore suggest that there is also a second reason for the observed divergence, that is, an ‘over-activation’ of telomerase in the male germ cells, such that with each replicative cycle of sperm precursors, there is a progressive lengthening of TL.

Based on data displayed in Fig. 2 and previous publications on the replication rate of the male germ cells (Crow, 2000), we suggest that the ‘over-activation’ of telomerase only slightly elongates TL per replication of the male germ cells (estimated value of 2.48 bp/replication in this study). In contrast, based on the replication rate of HSCs (Shepherd et al., 2004; Sidorov et al., 2009), TL in HSCs becomes notably shorter with each replication (estimated value of 26 bp/replication in this study). Given the large inter-individual variation in sperm TL, LTL and probably their rates of change, our calculations are only approximations, but they showcase the relative magnitudes of TL elongation in male germine versus TL shortening per replication in the hematopoietic system. They also underscore

### Table III Sperm TL and LTL of age-stratified groups

| Age group (tertiles; $n = 45$ in each group) | Mean LTL ± SEM | Mean sperm TL ± SEM | Beta | $P$ |
|--------------------------------------------|----------------|---------------------|------|-----|
| $\leq 28$                                  | $7.08 \pm 0.09$ | $12.59 \pm 0.26$    | 0.14 | 0.03|
| $>28-<37.5$                                | $6.91 \pm 0.09$ | $12.95 \pm 0.26$    | 0.04 | 0.42|
| $\geq 37.5$                                | $6.73 \pm 0.09$ | $14.18 \pm 0.26$    | 0.16 | 0.0006|

**Figure 3** Correlation between leukocyte and sperm TL within an individual.
that the considerable lengthening of sperm TL during adult life is driven more by the number of germ cell replications than the number of telomere repeats added per replication.

TL elongation with age in the male germ cells might explain the PAC effect on TL in the offspring (Unryn et al., 2005; De Meyer et al., 2007; Njajou et al., 2007; Kimura et al., 2008; Arbeev et al., 2011). A change in the genetic makeup of an individual’s somatic cells may affect the individual’s health but is not transmitted to offspring. However, change in the genetic makeup of the male germ cells due to age-dependent telomere elongation is likely to be transmitted to offspring, as ostensibly shown by the PAC effect on the offspring’s LTL. This would be entirely consistent with research showing that human telomeres are inherited in an allele-specific manner (Baird et al., 2003; Graakjaer et al., 2006). Therefore, it is only reasonable to propose that a longer sperm TL might be transmitted to offspring in ordinary Mendelian fashion. If so, we anticipate that its contribution to the overall LTL in the offspring would be approximately half of sperm TL elongation with age (since half of a child’s DNA is derived from the father, albeit slightly less DNA in sons than daughters due to the smaller size of the Y chromosome than the X chromosome). Based on the studies that used Southern blots, which generate TL data expressed in base pairs, the PAC effect on the offspring’s LTL amounts to ~20 bp/year (range: 12–38 bp/year; Unryn et al., 2005; De Meyer et al., 2007; Kimura et al., 2008; Arbeev et al., 2011). This is in the same range as half of the rate of sperm TL elongation displayed in Fig. 2, i.e. $57/2 = 28.5$ bp/year. Allowing for interindividual variation and measurement error, the result is quite consistent with our hypothesis.

Haldane (1937) was the first to posit the concept of male-biased mutations due to the numerous replications of the male germ cells compared with the female germ cells. This has led to the notion of ‘male-driven evolution’ of DNA sequences, particularly in higher primates (Shimmin et al., 1993; Makova and Li, 2002). Mediated through the numerous replications of the male germ cells during the life course, age-dependent telomere elongation in sperm suggests another form of male-driven evolution of DNA sequences in the context of telomere biology. Finally, genetic anticipation is an established phenomenon with respect to relatively rare human diseases involving germ cell mutations in telomerase (Vulliamy et al., 2004; Armanios et al., 2005). The outcome of these mutations is progressive telomere shortening in successive generations, which brings about manifestations of disease onset at a younger age. Older PAC is largely a feature of contemporaries (Shimmin et al., 1993; Makova and Li, 2002). Mediated through the numerous replications of the male germ cells during the life course, age-dependent telomere elongation in sperm suggests another form of male-driven evolution of DNA sequences in the context of telomere biology.

Authors’ roles
K.I.A. was responsible for sample collection and preparation, and assisted in data analysis and preparation of the manuscript. S.C.H. performed data analysis and assisted in manuscript preparation. E.S. assisted in data analysis and interpretation and manuscript preparation. M.K. assisted in sample preparation, performed all telomere assays and assisted in data analysis and manuscript preparation. PFL assisted in data analysis and interpretation and manuscript preparation. D.T.C. was involved with study design, data interpretation and manuscript preparation. A.A. directed the design of the study, data analysis and interpretation and prepared the manuscript.

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Conflict of interest
None declared.

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