Spermatozoa telomeres determine telomere length in early embryos and offspring

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Abstract

Offspring telomere length (TL) has been correlated with paternal TL, but the mechanism for this parent of origin-specific inheritance remains unclear. The objective of this study has been to determine the role of spermatozoa TL in embryonic telomere lengthening by using two mouse models showing dimorphism in their spermatozoa TL: *Mus musculus* vs *Mus spretus* and old vs young *Mus musculus*. *Mus spretus* spermatozoa displayed a shorter TL than *Mus musculus*. Hybrid offspring exhibited lower TL compared with *Mus musculus* starting at the two-cell stage, before the onset of telomerase expression. To analyze the role of spermatozoa telomeres in early telomere lengthening, we compared the TL in oocytes, zygotes, two-cell embryos and blastocysts produced by parthenogenesis or by fertilization with *Mus musculus* or *Mus spretus* spermatozoa. TL was significantly higher in spermatozoa compared with oocytes, and it increased significantly from the oocyte to the zygote stage in those embryos fertilized with *Mus musculus* spermatozoa, but not in those fertilized with *Mus spretus* spermatozoa or produced by parthenogenesis. A further increase was noted from the zygote to the two-cell stage in fertilized *Mus musculus* embryos, whereas hybrid embryos maintained the oocyte TL. Spermatozoa TL shortened with age in *Mus musculus* and the offspring from young males showed a significantly higher TL compared with that fathered by old males. These significant differences were already noticeable at the two-cell stage. These results suggest that spermatozoa telomeres act as a guide for telomerase-independent telomere lengthening resulting in differences in TL that persist after birth.

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Introduction

Telomeres are repetitive DNA sequences situated at chromosome ends that exert a predominant role in DNA protection (de Lange 2005). In somatic cells, telomeres shorten in every cell cycle and thus, telomere length (TL) determines the number of cell divisions before senescence. The importance of its protective role on the maintenance of genomic integrity is manifested by the association of several disorders with telomere dysfunction. Short telomeres or telomere attrition has been linked to cancer, dyskeratosis congenita, aplastic anemia, ataxia telangiectasia, Werner syndrome, Bloom syndrome, Fanconi anemia and Duchenne muscular dystrophy (Heiss *et al.* 1998, Oeseburg *et al.* 2010, Armanios & Blackburn 2012, Calado & Young 2012, Mourkioti *et al.* 2013).

In humans, offspring TL correlates strongly with paternal TL (Njajou *et al.* 2007, Atzmon *et al.* 2010), but the mechanisms responsible for this correlation remain unclear. In some cases, the paternal effect can be

attributed to different haplotypes of TERT, a component of telomerase, the enzyme responsible for telomere lengthening (Atzmon *et al.* 2010). However, if that was the general case, a similar heritability of TL should be expected from the paternal or maternal side, but paternal TL consistently show a stronger positive correlation with offspring TL compared with maternal TL (De Meyer *et al.* 2007, Njajou *et al.* 2007). Furthermore, offspring TL has also been linked to paternal age in mixed populations (Unryn *et al.* 2005, De Meyer *et al.* 2007, Kimura *et al.* 2008, Eisenberg *et al.* 2012), an approach that excludes the effect of different haplotypes, as similar haplotype frequencies are expected across groups of different ages. In these cases, the effect of paternal age seems to be mediated by the TL of the spermatozoa, as telomeres are longer in both spermatozoa and the offspring of old men compared to young men (Allsopp *et al.* 1992, Baird *et al.* 2006, Kimura *et al.* 2008). However, the notion that spermatozoa TL determine offspring TL currently lacks a mechanistic support.

TL has been proposed to be established during early preimplantation development, in the morula-to-blastocyst transition by a telomerase-dependent mechanism (Schaetzlein *et al.* 2004), and thereby without a role of spermatozoa TL. However, telomerase-null mice elongate their telomeres during the first embryonic cleavage division, well before the onset of telomerase expression, occurring in morula (Liu *et al.* 2007). This telomerase-independent elongation may occur by an alternative recombination based mechanism (Bryan *et al.* 1995), providing a molecular explanation of the effect of spermatozoa TL on the newborn TL, as the spermatozoon telomeres may act as a recombination template for oocyte telomeres. To test this possibility, we have analyzed the TL of preimplantation embryos in two models showing dimorphism in their spermatozoa TL: i) *Mus musculus* vs *Mus spretus* males and ii) old vs young *Mus musculus* males.

Results

In order to use a systems biology approach with substantial differences in TL between males, we fertilized *Mus musculus* oocytes with spermatozoa from two mouse species displaying a wide dimorphism in TL (*Mus musculus* and *Mus spretus*). As expected based on the well-known differences in telomeres observed in somatic cells (Kipling & Cooke 1990, Starling *et al.* 1990), the spermatozoa TL was significantly longer in *Mus musculus* males compared to *Mus spretus* (Fig. 1A). Natural breeding between *Mus spretus* males and *Mus musculus* females resulted in offspring with a shorter TL compared with age-matched pure *Mus musculus*, but significantly longer than *Mus spretus* (Fig. 1B). These differences were already established at the blastocyst stage (Fig. 1C), when TL is thought to be set by the expression of telomerase (Schaetzlein *et al.* 2004).

Subsequently, we determined whether a telomerase-independent mechanism played a role in the differences in embryonic TL by analyzing the changes in TL before embryonic genome activation occurs in embryos produced with spermatozoa carrying long telomeres (*Mus musculus*), short telomeres (*Mus spretus*) or produced by parthenogenesis (i.e., diploid embryos with genomes derived exclusively from maternal – oocyte – origin). A preliminary experiment observed that the sample collection protocol lead to similar DNA content (quantified by *Rn18S*) in oocytes, zygotes and two-cell embryos samples (Fig. 2A), allowing TL quantification by absolute qPCR. TL increased significantly from the oocyte to the zygote stage in fertilized *Mus musculus* embryos, but this increase was not noticed in parthenogenetic *Mus musculus* or embryos produced with spermatozoa with short telomeres (*Mus spretus*), suggesting that the telomeres in *Mus musculus* spermatozoa are longer than those present in the oocyte chromosomes (Fig. 2B). This hypothesis was confirmed

Figure 1 Telomere length in spermatozoa, offspring and embryos from *Mus musculus* and *Mus spretus*. (A) Relative telomere length in spermatozoa; (B) Relative telomere length in offspring; (C) Relative telomere length in blastocysts. Different letters indicate significant differences based on ANOVA ($P \leq 0.05$).

by comparing TL between *Mus musculus* spermatozoa and oocytes, which were found to be significantly longer in spermatozoa (Fig. 2C). At the two-cell stage, a further increase in TL occurs in fertilized *Mus musculus* embryos. In contrast, fertilized hybrid embryos maintained the TL of the oocyte, whereas parthenogenetic embryos increase their TL, although to a lesser extent than their fertilized counterparts (Fig. 2B).

Finally, we sought to determine whether the smaller differences in TL occurring between *Mus musculus* males at different ages played a role on offspring TL. The analysis of TL in mouse spermatozoa obtained from males of different ages revealed that, contrary to the situation observed in humans, spermatozoa telomere shorten with age (Fig. 3A), following the same tendency as somatic cells. Then, we analyzed whether these differences occurring in spermatozoa resulted in different TL in embryos and offspring. TL was significantly higher in two-cell embryos fathered by young males compared with those fathered by old males (Fig. 3B). Consistently, relative TL was significantly

Figure 2 (A) Absolute genomic DNA quantification in *Mus musculus* oocytes, zygotes and two-cell embryo samples; (B) Absolute telomere length in *Mus musculus* oocytes and zygotes and two-cell embryos obtained by fertilization with *Mus musculus* or *Mus spretus* spermatozoa or by parthenogenetic activation; (C) Relative telomere length in *Mus musculus* oocytes and spermatozoa. Different letters indicate significant differences based on ANOVA ($P \leq 0.05$).

higher in pups fathered by young males compared with those produced by old males (Fig. 3C).

Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co.

Mice

All experimental procedures were approved by the INIA ethical committee in animal research, according to European

legislation. All mice used in this experiment were maintained in an animal facility with controlled temperature and photoperiod ($23 \pm 1^\circ\text{C}$ and a ratio of 14 h light:10 h darkness) with regular access to water and food and allowed to feed and drink *ad libitum*.

Production of mouse embryos

Mus musculus and *Mus musculus* x *Mus spretus* F1 embryos were obtained by IVF following a previously described method with minor modifications (Martín-Coello *et al.* 2008). Spermatozoa were collected from the epididymis of either young (3 months) or old (1 year) *Mus musculus* C57CBAF1 mature fertile males or *Mus spretus* males and were pre-incubated in a 500 μl drop of human tubular fluid (HTF) (Quinn *et al.* 1985) medium supplemented with 2 mg/ml BSA under oil at 37°C in 5% CO_2/air for 15 min to allow sperm dispersion. Cumulus oocyte complexes were obtained from superovulated *Mus musculus* C57CBAF1 females (8–10 weeks old). Hormonal treatment consisted of an i.p. injection of 7.5 IU of equine

Figure 3 Telomere length in spermatozoa, offspring and embryos obtained from young and old *Mus musculus* fathers. (A) Relative telomere length in spermatozoa; (B) Absolute telomere length in two-cell embryos; (C) Relative telomere length in the offspring. Different letters indicate significant differences based on ANOVA ($P \leq 0.05$).

chorionic gonadotrophin (Folligon 500, Intervet) followed by 7.5 IU of hCG (Veterin Corion, Equinvest) 48 h later. Females were euthanized 14 h after hCG injection, their oviducts were removed and cumulus oocyte complexes were recovered from the ampulla, washed in HTF and co-incubated with spermatozoa at a final concentration of 1×10^6 spermatozoa/ml in 500 μ l of HTF medium for 4–5 h at 37 °C in 5% CO₂ in humidity saturated air. After IVF, the remaining cumulus cells and attached spermatozoa were removed by gentle pipetting, and the presumptive zygotes were washed in M2 medium before being placed in 30 μ l of KSOM media (Millipore) at 37 °C in a humidified atmosphere of 5% CO₂ in air. The protocol yielded better results (~80% cleavage) with *Mus musculus* sperm compared with *Mus spretus* (~30% cleavage), probably due to the protocol being optimized for *Mus musculus* spermatozoa.

Parthenogenetic embryos were obtained from mature oocytes collected 16 h after hCG injection as previously described (Kishigami & Wakayama 2007). Cumulus cells were dispersed in hyaluronidase solution and matured oocytes were activated by incubation in KSOM media supplemented with 10 mM SrCl₂, 2 mM EDTA and 5 μ g/ml cytochalasin B for 6 h (Kishigami & Wakayama 2007). After activation, oocytes were washed three times and then cultured in KSOM as described above.

Absolute TL evaluation in oocytes, zygotes and two-cell embryos

The differences in TL between oocytes, zygotes and two-cell embryos produced by IVF with semen from *Mus musculus* or *Mus spretus* or parthenogenesis were analyzed in ~30–40 samples per stage by absolute qPCR. The low amount of DNA in these samples precludes the use of relative qPCR (i.e., data normalization to an internal control). Absolute qPCR consisted of the direct comparison of cycle threshold (Ct) values between samples without data normalization to an internal control. The method is in essence the same as absolute qPCR by a standard curve, but, for clearer representation, the data were normalized to the group with the highest average Ct (i.e., shortest TL), following the method for Δ Ct described by (Schmittgen & Livak 2008). By this approach, the data represents the fold change in TL relative to the group exhibiting the shortest TL, whose fold-change value is 1, consistent with the data obtained by relative TL quantification. As absolute qPCR requires equal amounts of starting DNA and to compensate for the differences in ploidy between the different stages, zona-free oocytes, zygotes and one-cell parthenotes were frozen in groups of two (4c, excluding polar body/ies) and two-cell embryos were individually stored (4c). In order to test that this sample

collection protocol lead to similar DNA content in all samples, 20 additional samples of IVF-derived *Mus musculus* oocytes, zygotes and two-cell embryos were collected for genomic DNA quantification by qPCR as described below. Metaphase II oocytes were collected from the ampullae 19–20 h after hCG injection and had their cumulus cells removed by incubation in M2 media supplemented with hyaluronidase (300 μ g/ml). Zygotes were identified by the presence of two visible pronuclei and collected 10 h after IVF or 8 h after oocyte activation. Two-cell embryos were collected at 1.5 day of culture. In all cases, the zona pellucida was removed by incubating oocytes and embryos into a Tyrode's acidic solution at 37 °C for 30 s, in order to remove possible attached spermatozoa and polar bodies and to increase digestion efficiency. Zona pellucida-free oocytes and embryos were washed three times in PBS, snap-frozen in liquid nitrogen in 0.1 ml qPCR tubes (Corbett Research, Sydney, Australia) and stored at –80 °C until further analysis.

Samples were digested at 55 °C overnight with 8 μ l of a 100 μ g/ml proteinase K solution followed by proteinase K inactivation at 95 °C for 10 min. The total digested sample (8 μ l) was used to analyze TL by absolute quantification by qPCR in oocytes, zygotes and two-cell embryos samples using primers specifically designed for the telomere sequence (Cawthon 2002) (Table 1). Genomic DNA quantification was conducted in independent samples by absolute quantification of the genomic sequence *Rn18S* by qPCR, following the same sample digestion and cycling conditions than absolute TL quantification. qPCR was performed using a Rotorgene 6000 Real Time Cycler (Corbett Research) (94 °C 3 min followed by 40 cycles of 94 °C 10 s, 60 °C 30 s and 72 °C 30 s). The absolute Ct value was normalized to the highest Ct observed value.

Relative TL evaluation in spermatozoa, oocytes, blastocysts and tissue samples

The relative TL in sperm was determined on samples from five adult *Mus spretus* and five adult *Mus musculus* males. Spermatozoa were collected in M2 media from the cauda epididymis. Sperm cell concentration was determined and 5×10^6 spermatozoa were frozen after centrifugation and removal of the supernatant. To compare relative TL between *Mus musculus* spermatozoa and oocytes, three groups of 50 oocytes were obtained from superovulated *Mus musculus* females as previously described, and their TL compared with those from three groups of ~200 spermatozoa. Spermatozoa and oocytes DNA was obtained after overnight digestion in 30 μ l of proteinase K (1.25 μ g/ μ l) in Tris NaCl EDTA buffer (STE) and 5 μ l of 0.1 M dithiothreitol (DTT) at 55 °C followed

Table 1 Sequences of primers and amplicon size of the qPCR used for telomere length determination.

Gene	Primer sequence (5'–3')	Fragment size (bp)	Gene Bank accession no.
Telomere	F:CGGTTTGGTTGGGTTGGGTTGGGTTGGGTTGGGTT R:GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	79	NT_039202.7
<i>Rn18s</i>	F:AGAAACGGCTACCACATCCAA R:CCTGTATTGTTATTTTCGTCACACTACCT	91	NR_003278.1
<i>Env4</i>	F:TGCTTGGGCTCAGCAACATGG R:GACAGAATGCCTCATCTATCGT	260	Y12713

by phenol/chloroform extraction and then followed by isopropanol precipitation.

Blastocysts of *Mus musculus* embryos and hybrid *Mus spretus* (20 per group) were transferred into acidic tyrode to remove the zona pellucida and any attached spermatozoa. They were then washed three times in PBS and individually snap-frozen in liquid nitrogen in 0.2 µl Eppendorf tubes and stored at -80°C until analysis. Samples were digested with 8 µl of a 100 µg/ml proteinase K (Sigma, P8044) solution at 55°C overnight. After digestion, proteinase K was inactivated at 95°C for 10 min and 2 µl of the digested sample were used for qPCR as described below.

Renal tissue was collected post-mortem from five male and five female adult *Mus musculus* (C57BL/6J), *Mus spretus* (ESPRET/Ei) and hybrid *Mus spretus* (C57BL/6J x ESPRET/Ei) mice and were frozen until further analysis. A tail biopsy was obtained from the offspring of old and young *Mus musculus* males 10 days after birth. Telomeres have been reported to shorten at equivalent rates in somatic tissues of adults (Daniali *et al.* 2013). Genomic DNA was extracted from these samples according to standard protocols. Briefly, 30 µl of a dilution of proteinase K (1.25 µg/ul) in STE buffer was added to the frozen samples, incubated at 55°C overnight, then cooled to 4°C and diluted in a final volume of 300 µl of DNase-free water. After digestion, genomic DNA was extracted using a phenol-chloroform protocol followed by isopropanol precipitation and the pellet dissolved in 200 µl of DNase-free water and then diluted to a final concentration of 50 ng/µl.

Relative TL was determined using a real-time quantitative PCR method previously described (Cawthon 2002), which amplifies telomeric DNA with specially designed primers, with minor modifications (Bermejo-Alvarez *et al.* 2008). The values of telomere repeats were normalized to the amount of DNA present in the sample (determined by the amount of the multi-copy gene *Rn18S*) by the comparative Ct method. For relative TL analysis in oocytes and spermatozoa, the multi-copy gene *Env4* was used to normalize to the amount of DNA instead of *Rn18S*, as preliminary experiments yielded better amplification efficiencies for *Env4* in low-DNA concentrations. Two microliter of the extracted DNA were used for each 20 µl qPCR reaction using a Rotorgene 2000 Real Time Cycler (Corbett Research) (94°C 3 min followed by 40 cycles of 94°C 10 s, 60°C 30 s and 72°C 30 s). Primer sequences, fragment size and GeneBank accession numbers are shown in Table 1.

Statistical analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. One-way ANOVA (followed by multiple pair-wise comparisons using Tukey test) was used for the analysis of TL differences between groups.

Discussion

Telomerase-dependent mechanisms cannot explain the telomere lengthening occurring in the absence of telomerase (Liu *et al.* 2007) and the observed effects of spermatozoa TL in that of the offspring. Herein, we report that spermatozoa TL determine the TL of early

embryos before telomerase is expressed and, thereby, likely by an alternative lengthening of telomeres (ALT) (Bryan *et al.* 1995) where spermatozoa telomeres may act as guide. The epigenetic dynamics of preimplantation development provide a favorable landscape for ALT. The methylation levels of the oocyte genome are substantially lower than in somatic cells (Kobayashi *et al.* 2012, Smith *et al.* 2012) and an intense active demethylation occurs in the paternal genome after fertilization (Wossidlo *et al.* 2011), providing a low DNA methylation environment ideal for ALT (Gonzalo *et al.* 2006).

As TL seems to be inherited mostly from the paternal side (Unryn *et al.* 2005, De Meyer *et al.* 2007, Njajou *et al.* 2007, Kimura *et al.* 2008, Eisenberg *et al.* 2012), spermatozoon telomeres should play a predominant role on the recombination mechanism compared with oocyte telomeres, but the reason for this paternal-specific TL inheritance remains unclear. The longer TL observed in spermatozoa compared with oocytes can explain the exclusively paternal effect, as the paternally inherited telomeres could be used as a template or guide to lengthen the shorter maternally inherited telomeres during ALT. In agreement, oocytes telomeres were previously found to be surprisingly short compared with somatic cells (Liu *et al.* 2007). In contrast, another article reported longer TL for oocytes and female pronuclei compared with spermatozoa and male pronuclei (Turner & Hartshorne 2013). A possible cause for the discrepancies is that TL analysis in the later report was performed by qFISH and thereby the results may have been influenced by differences in hybridization efficiency due to the very distinct chromatin conformation in male and female gametes, especially when taking into account that metaphase plates cannot be obtained from gametes or pronuclea. Supporting this idea, sperm TL quantified by qFISH (Turner & Hartshorne 2013) are half of those obtained using telomere restriction fragment (TRF) analysis (Kozik *et al.* 1998, Baird *et al.* 2006, Kimura *et al.* 2008, Pickett *et al.* 2011). In further agreement with the notion of oocyte telomere being shorter than those of the spermatozoon, we observed that TL was shorter in parthenotes compared with those obtained by fertilization with *Mus musculus*. In the same trend, another article showed a shorter TL in parthenogenetic *Mus musculus* embryos compared to fertilized embryos when qPCR was employed, whereas qFISH yielded inconsistent results depending on the strain (Liu *et al.* 2007).

The precise mechanism by which spermatozoa telomere plays a direct role in embryonic TL remains unclear. It is tempting to think that spermatozoon telomeres serve as a template for lengthen oocyte's telomeres during syngamia, but a recent report refutes this idea: using a transgenic mouse model with tagged telomeres, the authors observed that the tag was transmitted to the offspring following a Mendelian

inheritance. This observation proves that telomeres are not copied from one chromosome to its homologous counterpart in the germline or preimplantation embryos as it occurs by ALT in somatic cells (Neumann *et al.* 2013). Thus, it seems that the ALT occurring in early embryos does not occur inter-telomerically (i.e., by copying the telomere of another chromosome), but intra-telomerically (i.e., by using its own sequence as template) (Muntoni *et al.* 2009), so the spermatozoon telomeres would serve as a guide to determine the final length rather than a template for ALT.

We have observed that the differences in early embryo TL originated from differences in spermatozoa TL were maintained through fetal development. That is, resulting offspring with diverse TL depended on the TL of the spermatozoa from which they were conceived. The offspring from older mice displayed a lower TL compared with that from younger mice, in agreement with the differences in spermatozoa TL. Similarly, the offspring from *Mus spretus* males exhibited shorter telomeres than that from *Mus musculus*. However, in the latter case, we acknowledge that although the differences observed at the two-cell stage are only dependent on spermatozoa TL, the differences observed later, after the onset of embryonic telomerase expression, may have been influenced by possible differences in activity or expression between *Mus musculus* and *Mus spretus* telomerases. Further support for the major role of spermatozoa TL in offspring TL comes from a study of TERT haploinsufficiency in mice, where the restoration of TERT was not able to restore TL in the offspring, thereby suggesting that other factors, such as sperm TL, determined the offspring TL (Chiang *et al.* 2010).

In conclusion, we provide evidence for a direct role of spermatozoa telomeres in the telomerase-independent telomere lengthening taking place during preimplantation development, where spermatozoa telomeres seem to act as guides for intra-telomeric ALT of the oocyte telomeres. The differences originated before the onset of telomerase expression are not compensated later in development and persist after birth, thereby providing a mechanistic explanation for the paternally mediated inheritance of TL observed in human studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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