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Telomeres shorten while *Tert* expression increases during ageing of the short-lived fish *Nothobranchius furzeri*

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ABSTRACT

Age research in vertebrates is often limited by the longevity of available models. The teleost fish *Nothobranchius furzeri* has an exceptionally short lifespan with 3.5 months for the laboratory strain GRZ and about 6 months for the wild-derived strain MZM-0403. Here we have investigated telomere length in muscle and skin tissue of young and old fish of both strains using different methods. We found age-dependent telomere shortening in the MZM-0403 strain with the longer lifespan, whereas the short-lived GRZ strain showed no significant telomere shortening with advanced age. Sequencing of the two main telomerase genes *Tert* and *Terc* revealed that both genes are highly conserved between the *N. furzeri* strains while there is little conservation to other fish species and humans. Both genes are ubiquitously expressed in *N. furzeri* and expression levels of *Tert* and *Terc* correlate with telomerase activity in a tissue-specific manner. Unexpectedly, the expression level of *Tert* is increased in aged muscle and skin tissue of MZM-0403 suggesting that telomeres shorten upon ageing despite increased *Tert* expression and hence high telomerase activity. We further conclude that the extremely short lifespan of the GRZ strain is not caused by diminished telomerase activity or accelerated telomere shortening.

1. Introduction

Telomeres are specialised structures at the chromosome ends, which in vertebrates consist of TTAGGG repeats and several associated proteins known as the shelterin complex (Meyne et al., 1989; Blackburn, 2001; de Lange, 2005). Apart from protecting the chromosome ends from fusions and from being recognised as double-strand breaks, the telomeres fulfill an important role in the replication of DNA ends. Due to the end-replication problem, 50–200 bp of telomeric DNA-repeats are lost during each replication cycle. This loss is mainly compensated by the enzyme telomerase that consists of two core components: a catalytic protein subunit (Tert, telomerase reverse transcriptase) and an RNA subunit (Terc, telomerase RNA component) that serves as a template for the addition of telomeric repeats (Greider and Blackburn, 1989). In humans, expression of the *TERT* gene is primarily detectable in highly proliferating cells and correlates with detectable levels of telomerase activity, while *TERC* is constitutively expressed (Feng et al., 1995; Avilion et al., 1996; Nakamura et al., 1997). At the cellular level it is well documented that human primary fibroblasts show no detectable telomerase activity and telomeres progressively shorten until telomeres reach a critical size and finally limit cell growth by either cellular senescence or crisis (Harley et al., 1990; Shay and Wright, 2007). This can be prevented by over-expression of *TERT* which leads to telomere elongation and immortalisation of the respective cells (Bodnar et al., 1998).

There are several human syndromes such as dyskeratosis congenita, aplastic anemia and idiopathic pulmonary fibrosis that are caused by mutations in the telomerase genes and are characterised by increased shortening of telomeres with age (Vulliamy et al., 2004; Yamaguchi et al., 2005; Tsakiri et al., 2007). Remarkably, mice without functional *Terc* gene develop many of the pathologies characteristic for dyskeratosis congenita patients. In particular, these *Terc*-deficient mice show extensive telomere shortening over several generations and decreased maximum lifespan (Blasco et al., 1997; Rudolph et al., 1999). These observations raised the question whether over-expression of the *Tert* gene could lead to extension of lifespan. However, mice with increased *Tert* expression suffer from a higher susceptibility to tumour formation. Yet, surviving cancer-free mice showed a small but significant lifespan elongation (Gonzalez-Suarez et al., 2001).

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The biological implications of telomere length alterations have been studied in a variety of organisms. In rodents such as laboratory rats (*Rattus norvegicus*) and wild-derived mice (*Mus spretus*) mean telomere length of most analysed tissues is shortening with age. Interestingly, the rate of telomere shortening varies among tissues and highly proliferating tissues seem to undergo faster telomere shortening (Coviello-McLaughlin and Prowse, 1997; Cherif et al., 2003). The effects of telomere length on the post-mitotic nematode *Caenorhabditis elegans* is still under debate and data on an association of telomere length and lifespan have revealed opposing results (Joeng et al., 2004; Raices et al., 2005). Investigations on the effects of telomere length on human lifespan have shown an association in 60–75-year-old blood donors, but the lack of an association in >85-year-old individuals (Cawthon et al., 2003; Martin-Ruiz et al., 2005). Overall, telomere length seems to shorten in a variety of organisms with advanced age, although it is poorly understood how this process is regulated.

Despite the fact that teleost fishes are the most diverse group of vertebrates and that their natural lifespan ranges from 2 months in the pygmy goby *Eviota sigillata* to 200 years in the rockfish *Sebastes aleutianus*, very little is known about their telomere length and telomere homeostasis (Cailliet et al., 2001; Depczynski and Bellwood, 2005). It is, however, known that various fish species exhibit high telomerase activity in somatic tissues at all ages, which has been explained by the fact that fishes continuously grow throughout their life (Klapper et al., 1998; Kishi et al., 2003; Hatakeyama et al., 2008; Pfennig et al., 2008). It has been also shown that telomeres progressively shorten during ageing of the ricefish medaka (*Oryzias latipes*), the only fish species in which age-related telomere attrition has been studied so far (Hatakeyama et al., 2008). Recently, the two telomerase genes *Tert* and *Terc* have been identified in several fish species such as the Japanese pufferfish fugu (*Takifugu rubripes*), zebrafish (*Danio rerio*) and medaka (Yap et al., 2005; Lau et al., 2008; Pfennig et al., 2008; Xie et al., 2008). Both zebrafish and medaka are well-known model organisms, in particular for the analysis of developmental processes. However, their maximum captive lifespan of 4–5 years (Egami and Etoh, 1969; Gerhard et al., 2002; Hatakeyama et al., 2008) has limited their use for ageing studies.

The short-lived turquoise killifish (*Nothobranchius furzeri*) is emerging as an alternative model organism for age research (Genade et al., 2005; Terzibasi et al., 2007). *N. furzeri* is characterised by accelerated growth and an exceptionally short lifespan of about 3–6 months. This is the shortest reported maximum lifespan of a vertebrate in captivity (Valdesalici and Cellerino, 2003). The short lifespan is a consequence of its natural life cycle as *N. furzeri* lives in temporary pools in South Eastern Africa and eggs survive the dry season in the dried mud. After hatching the fish show an explosive growth, early sexual maturation and with advanced age typical ageing related features like a decline in learning/behavioural capabilities as well as expression of ageing biomarkers (Valenzano et al., 2006a, b). The laboratory strain of *N. furzeri* (GRZ) was collected in the Gona Re Zhou National Park of Zimbabwe in 1968 and has been maintained most of the time by only few fish breeders (Jubb, 1971). Recently, we have collected a new *N. furzeri* strain from a location in Mozambique that is about 300 km away from the Gona Re Zhou National Park. This strain lives longer and shows delayed expression of age-related markers compared to the GRZ laboratory strain (Terzibasi et al., 2008).

The aim of this study was to investigate the telomere length of *N. furzeri* and to analyse whether the short- and long-lived strains differ in telomere maintenance and whether there is any change in telomere length with age. Furthermore we compared *Tert* expression between young and aged animals of both strains. Our results indicate that despite increased *Tert* expression, telomeres shorten in the MZM-0403 strain with age, whereas no significant change in telomere length was observed in the GRZ strain. We conclude that telomere shortening and telomerase activity are not responsible for the extremely short lifespan of the short-lived GRZ strain.

2. Materials and methods

2.1. Fish strains and maintenance

The laboratory strain GRZ of *N. furzeri* was originally obtained from Marc Bellemans (http://users.pandora.be/marc.bellemans) and initially characterised by Valdesalici and Cellerino (2003). The *N. furzeri* strain MZM-0403 was collected during a field trip in Mozambique in 2004 and its characterisation is reported elsewhere (Terzibasi et al., 2008). Groups of 12–14 fish were kept in 40 l tanks at 26 °C under a light regime of 12:12 h light:dark and fed on red mosquito larvae (Chironomidae) ad libitum twice a day. Tank water was filtered using air-driven foam filters and half of the tank water was changed twice a week. All animal experiments were performed according to the "Principles of laboratory animal care" as well as to the current version of the German Law on the Protection of Animals.

2.2. DNA, RNA and protein extraction

Tissues for DNA and RNA isolation were collected from 10 to 12 individual fish of both *N. furzeri* strains, GRZ and MZM-0403 and from each age group (young GRZ and MZM-0403 = 5 weeks, aged GRZ = 13 weeks, and aged MZM-0403 = 21 weeks). Genomic DNA was prepared from muscle and skin using the QIAmp kit (Qiagen). 30 mg of each muscle and skin sample were taken for RNA isolation using the RNeasy kit (Qiagen). In addition, RNA and protein extracts for measuring telomerase activity were prepared from testis, ovary, muscle, liver, brain, eye, skin, gill and spleen of five different fish (MZM-0403) that were 15-week-old.

2.3. PCR amplification and sequencing

Total RNA and SuperScript II reverse transcriptase (Invitrogen) were used for cDNA synthesis. Based on gene sequences of other teleost fish species, degenerated primers were designed and used to amplify cDNA sequences from *N. furzeri* (Supplementary Table 1). To obtain full-length cDNA of the *Tert* gene, we used the Smart RACE kit (BD Biosciences) to amplify the 5′- and 3′-ends. All PCR products were cloned into the pGEM-T easy vector (Promega) and eight different clones of each product were sequenced from both ends using the BigDye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) and ABI 3730xl capillary sequencer (Applied Biosystems). Sequence assembly was carried out using the GAP4 module of the Staden Sequence Analysis Package (Staden, 1996). Nucleotide and amino acid sequences were aligned using NEEDLE (EMBOSS, version 3.0.0) (Rice et al., 2000).

2.4. Quantitative real-time PCR

Real-time PCR was used for gene expression analysis among different tissues, for comparison of gene expression between young and old tissues, and for measurement of the telomere content within genomic DNA samples. Real-time PCR was performed with the iCycler iQ detection system (Bio-Rad). PCR reactions were performed in 25 μl volume containing 10 nM fluorescein (Bio-Rad) for calibration and 0.75 μl Sybr Green I (5x stock solution, Sigma) for detection. All reactions were performed in triplicates and negative controls were always included. In case of expression analysis among different tissues, the cycle threshold (C_t) values were normalised to the mean C_t value of muscle tissue which showed the lowest expression levels. Fold changes describe the difference in expression level between muscle and each tissue. When comparing expression levels between young and old tissues, C_t values of the analysed genes were normalised to C_t values of the housekeeping gene *Tbp* (TATA box binding protein). Quantitative telomere PCR was carried out with 50 ng genomic DNA from muscle and skin. C_t values of telomere PCR products were normalised to two independent genomic loci (Cawthon, 2002; Callicott and Womack, 2006) and compared between samples from young and old animals.

2.5. TRAP assay

Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP) according to (Kim et al., 1994). Protein extracts from different *N. furzeri* tissues were prepared in 1× CHAPS lysis buffer (10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM ß-mercaptoethanol, 0.5% CHAPS, 10% glycerol) and 1 μl protein extract (200 ng/μl) was used for each reaction. As negative controls one sample from each tissue was treated with *RNase* prior to PCR amplification. Telomerase activity was allowed to proceed for 10 min at 25 °C, followed by 27 cycles of PCR amplification with the [γ-^{32}P]-ATP end-labelled TS primer and the reverse CX primer (Kim and Wu, 1997). The samples were loaded on a 12% non-denaturing polyacrylamide gel and run at 150 V overnight. The gel was dried and analysed using the phosphor imager FLA-7000 (Fujifilm). Signal