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Sex-dependent telomere shortening, telomerase activity and oxidative damage in marine medaka *Oryzias melastigma* during aging

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ABSTRACT

Marine medaka *Oryzias melastigma* at 4 months (young), 8 months (middle-aged) and 12 months old (senior) were employed to determine age-associated change of sex ratios, sex hormones, telomere length (TL), telomerase activity (TA), telomerase transcription (*omTERT*) and oxidative damage in the liver. Overall, *O. melastigma* exhibited gradual senescence, sex differences in longevity (F > M), TL (F > M) and oxidative damage (F < M) during aging. In females, the plasma E2 level was positively correlated with TL (TRF > 5 kb), TA and *omTERT* expression ($p \leq 0.01$), and negatively correlated with liver DNA oxidation ($p \leq 0.05$). The results suggest high levels of E2 in female *O. melastigma* may retard TL shortening by enhancing TA via *TERT* transcription and/or reducing oxidative DNA damage. The findings support TL shortening as a biomarker of aging and further development of accelerated TL shortening, abnormal suppression of TA and excessive oxidative DNA damage as early molecular endpoints, indicative of advanced/premature aging in marine medaka/fish.

1. Introduction

The marine medaka *Oryzias melastigma* is increasingly recognized as a seawater model fish for molecular toxicology (Kong et al., 2008; Kim et al., 2016). It has been widely applied for studying reproductive toxicology and immunotoxicology in the marine environment. Many molecular endpoints have shown potential as biomarkers indicative of early reproductive impairments (Tse et al., 2015; Lai et al., 2015, 2016; Wang et al., 2016) and immune dysfunction (Bo et al., 2011, 2012; Ye et al., 2012, 2016). Conversely, very little is known about marine medaka regarding other Darwinian fitness traits such as growth and aging. Impaired growth and/or premature aging induced by environmental stresses may reduce the survival fitness of individuals. The subsequent impact on population sustainability is an important concern for ecological risk assessment. Recently, in marine medaka and orange-spotted grouper we demonstrated that muscle telomerase activity (TA) was related to growth rate and confounded by sex during aging (female > male) (Peterson et al., 2015). We also demonstrated in the freshwater medaka *Oryzias latipes* that sex differences in telomere length (TL, female > male) and longevity (female > male) exist in fish during aging (Gopalakrishnan et al., 2013). This, however, remains to be verified for *O. melastigma*. The potential of TL and TA as molecular biomarkers of aging has not been explored previously in fish.

Progressive shortening of telomeres is an important mechanism in the aging process. Telomeres are specialized DNA-protein complexes capped at the ends of linear eukaryotic chromosomes (Meyne et al., 1989; Blackburn, 1991). In humans and medaka, the telomeric DNA sequence consists of tandem repeats of 5′-TTAGGG-3′, with a single-stranded G-rich 3′ overhang, known as a G-tail (Moyzis et al., 1988; Meyne et al., 1989; Blackburn, 1991; Au et al., 2009). Telomere shortening occurs with successive cell divisions in eukaryotic cells due to the end replication problem (Watson, 1972; Aviv et al., 2005) as well as oxidative DNA damage (von Zglinicki et al., 1995; von Zglinicki, 2002).

The loss of telomere length (TL) can be replenished by the enzyme telomerase (Greider and Blackburn, 1985). Telomerase is a ribonucleoprotein enzyme composed of two sub-units: telomerase reverse transcriptase (TERT) and telomerase RNA (TR). TERT is a catalytic sub-unit with reverse transcriptase activity (Lingner et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997), while TR serves as the RNA template for new telomeric DNA synthesis (Feng et al., 1995; Cech, 2000). In fish, telomerase activity (TA) together with expression of *TERT* mRNA and protein were ubiquitous in somatic, reproductive and regenerative tissues (reviewed in Peterson et al., 2015). Muscle TA in marine medaka was associated with PCNA cell proliferation *in vivo* (Kong et al., 2008; Peterson et al., 2015).

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Oxidative DNA damage accelerates telomere shortening (von Zglinicki et al., 1995, 2000; Honda et al., 2001). 8-hydroxydeoxyguanosine (8-OHdG) is one of the most abundant products of oxidative DNA damage because guanine (G) is the most easily oxidized nitrogenous base (Tardieu et al., 1998; Evans et al., 2004). Telomeric DNA contains G-rich tandem repeats (5′-TTAGGG-3′), rendering telomeres particularly sensitive sites for oxidative damage (Henle et al., 1999). When telomeres are critically shortened below a threshold level, the end of the chromosome becomes uncapped, and subsequent DNA damage response and signaling pathways are activated to induce replicative senescence or apoptosis (Cong et al., 2002; Smogorzewska and de Lange, 2004). Ample evidence from mammalian studies indicates that replicative senescence or apoptosis compromises tissue homeostasis and function that may eventually lead to organismal aging (Chen et al., 2007; for review, see Nalapareddy et al., 2008).

In mammals, TL is widely recognized as a biomarker of cellular aging. Accelerated TL shortening and elevated oxidative damage were evident in people subject to chronic stress (Epel et al., 2004; Shalev, 2012). Women that have experienced chronic life stresses have shorter telomeres compared to low stress women (Epel et al., 2004). The potential of these molecular endpoints as biomarkers of cellular aging has not been fully explored for fish. Given that the biology of aging is sex-dependent, in the present study, male and female *O. melastigma* were employed to study sex hormone (estrogen and testosterone) profiles and their relationship with liver TL, TA and oxidative damage during aging. The findings of this study shed light on the potential of further ecotoxicological research establishing these molecular endpoints as biomarker of aging in fish.

2. Materials and methods

Marine medaka *Oryzias melastigma* were obtained from the State Key Laboratory in Marine Pollution, City University of Hong Kong. Fish were maintained under optimal growth and breeding conditions in filtered and oxygenated 30‰ artificial seawater (using the DeepOcean sea salt) at 28 ± 2 °C under a 14:10 h light:dark cycle. For sex ratio determination, ca. 400 juvenile fish at 1-month old were randomly collected from the stock culture and maintained in a 500-l glass aquarium tank ($n = 3$ replicate tanks). In parallel, 20 pairs of male and female fish at 3 months old were kept in a 20-l aquarium tank for sampling at 4 months, 8 months and 12 months of age ($n = 3$ replicate tanks). The average life span of marine medaka reared under laboratory conditions is ca. 18–22 months (Au et al. unpublished data).

Fish were fed twice daily with hormone-free food flakes (e.g. the Flake Fish Food, Pentair aquatic ecosystems) in the morning and afternoon, and once in the evening with newly hatched brine shrimp *Artemia nauplii* (Lucky brand). For juvenile holding tanks, one tenth of the seawater was changed once a week. For adult culture tanks, 75% of the seawater was changed every other day to remove excess food and feces. Fish mortality was checked twice daily. Dead fish were sexed, recorded and removed immediately.

2.1. Blood collection for sex hormone analysis

The sex of fish is related to the plasma levels of 17α-estradiol (E2) and testosterone (T). Plasma sex hormones: E2 and T are measurable for marine medaka at 4 months old (young adult), 8 months old (mature adult) and 12 months old (aged adult). The procedures for blood collection and hormone analyses followed those described in Gopalakrishnan et al. (2013). Fish were first anesthetized by briefly chilling them in ice water. Excess water was removed from the fish and the body length and body weight of each individual were measured. The anesthetized fish was sacrificed by cutting the spinal cord, and 2 μl of whole blood was collected from the aorta near the spine. The blood from 3 fish was pooled as one sample. The levels of plasma E2 and T were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) using the Estradiol EIA kit (Cayman Chemical Company) and the Testosterone EIA kit (Cayman Chemical Company), respectively, according to the manufacturer's instructions. The detection limits of T and E2 are 6 and 1 pg ml^{-1}, respectively.

2.2. Liver isolation for multiple molecular analyses

The liver is a multi-functional organ involved in regulating body metabolism and homeostasis. Immediately after blood collection, the liver was carefully removed from the fish (kept under ice-cold phosphate-buffered saline) and cut into three equal portions. One set of the ⅓ liver portions from each of the three fish used for blood sampling were pooled as one replicate for RNA extraction (for *TERT* and *ERα* mRNA expression), the other ⅓ liver portions were pooled for protein extraction (for telomerase activity and protein oxidation) ($n = 15$ for each age group and each gender). For DNA extraction (for telomere length and DNA oxidation), the remaining ⅓ liver portions were pooled from 9 fish as one replicate to obtain a sufficient amount of genomic DNA to perform Southern blotting (>3 μg for each sample was needed) ($n = 5$ for each age group and each gender). Correlation analyses among different end-points was feasible for the same fish sample groups. The pooled liver samples were snap-frozen in liquid nitrogen and kept at −80 °C for further analysis.

2.3. Telomere length measurement by Southern blotting

The genomic DNA of pooled livers was extracted using DNeasy® Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. For each sample DNA precipitation was performed by incubating 400 μl of extracted genomic DNA in 40 μl of 3 M sodium acetate (pH 5.2) and 1 ml of ice-cold absolute ethanol. After washing in 70% ethanol and air-drying, the DNA pellet was re-suspended in 40 μl of nuclease-free water. The DNA extract was digested with *Hin*fI and *Rsa*I restriction enzymes into DNA fragments (New England BioLabs, USA) at 37 °C overnight. 3 μg of completely digested genomic DNA was loaded into a 1% agarose gel and gel electrophoresis was performed in 1× Tris-acetate-EDTA (TAE) buffer. The DNA samples were run in parallel with 10 μl of 0.1 μg/μl of GeneRuler™ DNA Ladder Mix (Fermentas) and 2 μl of 0.5 μg/μl of Lambda DNA/*Eco*RI + *Hin*dIII (Fermentas) as the DNA molecular weight markers. The DNA in the gel was then blotted onto a positively charged nylon membrane (Hybond XL, Amersham) by capillary transfer using 10× saline-sodium citrate (SSC) buffer.

To detect the DNA fragments with the telomere sequence (TTAGGG repeats), $TTAGGG_5$ oligonucleotide probes (Invitrogen) were DIG-labeled using DIG Oligonucleotide 3′-End Labeling Kit 2nd Generation (Roche Applied Science) according to the manufacturer's instructions. 10 μl of DIG-labeled $TTAGGG_5$ oligonucleotide probes were de-natured at 95 °C for 5 min using a PTC-200 Peltier Thermal Cycler (Bio-Rad), quenched on ice for 1 min and added to 10–15 ml of ExpressHyb solution in which the DNA blotted nylon membranes were incubated overnight at 42 °C. After hybridization, the membranes were washed before incubation in a blocking solution (Roche Applied Science) and then in anti-DIG-AP conjugate (Roche, 1:10,000 in blocking solution) for 2–4 h. Finally, the membranes were incubated for 5 min in the detection buffer (0.1 M Tris-HCl, pH 9.5 and 0.1 M NaCl) and in 10 ml of CDP-star (Roche, 1:200 in the detection buffer). The membrane was then exposed to Hyperfilm™ ECL (Amersham) in the dark for 5 min. The exposed films were scanned using a Duoscan HiD scanner (AGFA) with the Agfa FotoLook 3.60.00 software and saved as TIFF files. The files were analyzed using ImageJ (NIH, freely available at rsbweb.nih.gov/ij/) to determine the length distribution of the telomere restriction fragments (TRFs) in each sample. Quantification of the proportion of TRFs >5 kb, 5–4 kb, 4–3 kb, 3–2 kb and <2 kb was carried out using TeloRun (freely available at www4.utsouthwestern.edu/cellbio/shay-wright/

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