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Japanese medaka: A new vertebrate model for studying telomere and telomerase biology ☆

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ABSTRACT

A good understanding of telomeres and telomerase biology is crucial for unraveling mechanisms related to aging and cancer. However, *in vivo* vertebrate studies of telomere biogenesis and telomerase function have been limited by the development of appropriate animal model systems. The present study aims to demonstrate evolutionary conservation of telomerase in vertebrate species, supporting the potential application of fish as vertebrate model for studying telomeres and telomerase function. Comparison of genomic and protein information among vertebrate TERTs (*TE*lomerase *R*everse *T*ranscriptase), the Japanese medaka *Oryzias latipes* shares the highest similarity to that of the human than the other small size fish species studied (including pufferfish and zebrafish). The ubiquitous expression of TERT mRNA, the high constitutive level of telomerase activity, and the humanized telomere lengths further substantiate that Japanese medaka is an ideal vertebrate model for the study of telomere and telomerase-related mechanisms *in vivo*. Moreover, medaka exhibits fast, invariable growth and is able to provide a variety of useful developmental and reproductive endpoints for lifelong and multi-generational experiments. Our earlier and present findings support the use of medaka for studying organismal aging, tissue regeneration and carcinogenesis.

1. Introduction

Telomeres and telomerase have become a very important area of study, especially related to mechanisms of aging and cancer. Many reports have shown that the vast majority of human cancer cells express telomerase activity while normal cells are typically deficient in its expression (reviewed in Shay and Bacchetti, 1997). This lack of telomerase causes a decline in telomere length as normal cells divide, ultimately resulting in a cessation of growth known as cellular senescence (Hayflick, 1965). Expression of the catalytic subunit of human telomerase, hTERT, results in telomerase activation, telomere maintenance/elongation, and extension of cellular lifespan (Bodnar et al., 1998), indicating that telomere erosion is at least partially responsible for the onset of senescence.

The telomerase enzyme is minimally composed of 2 core components that facilitate telomere length maintenance and/or elongation, namely the *T*elomerase *R*NA (TR) and *TE*lomerase *R*everse *T*ranscriptase (TERT). The TR component serves as the templating sequence for the catalytic protein subunit, TERT, to synthesize telomeric tracts at the very ends of linear chromosomes, thereby extending telomere sequences to provide additional DNA for further replication by standard DNA polymerases. There are a number of additional accessory proteins that facilitate TR structure, telomerase holoenzyme assembly, and regulation of its telomere accessibility (reviewed by Collins, 2008).

In vivo vertebrate studies of telomere biogenesis and telomerase function have been limited by the development of appropriate animal model systems, with the majority of work having been done using inbred strains of laboratory mice (Blasco et al., 1997; Lee et al., 1998). Unfortunately, mice contain extraordinarily long telomere lengths, on the order of 2–5 times the lengths found in humans. Thus, it is imperative to discover additional vertebrate models for defining the mechanisms of telomere structure and function *in vivo*. We have recently explored the potential application of fish as an alternative system for studying telomeres and telomerase in aging, cancer, and tissue regeneration. There have been a number of studies assessing telomerase activity in fish e.g. channel catfish, pufferfish, rainbow trout, zebrafish, and Japanese medaka (Bradford et al., 1997; Klapper et al., 1998a; Barker et al., 2000, 2002; Kishi et al., 2003; Ossum et al.,

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2004; McChesney et al., 2005; Yap et al., 2005; Pfennig et al., 2008). However, few have attempted to determine the biological basis for telomerase activity in fish. One specifically linked the elevated telomerase activity levels with the increased lifespan observed in lobster (Klapper et al., 1998b). More recently, we have shown that lifespan is not related to telomere length and telomerase activity in aquatic species, but rather, the presence of telomerase is suggestive of the ability of these animals to regenerate amputated or injured tissues (Elmore et al., 2008).

We will show here that Japanese medaka is an ideal vertebrate model for the study of telomere-related mechanisms *in vivo*. The medaka genus *Oryzias* (Family Cyprinodontidae) consists of more than 14 species with a geographical distribution that covers most of Southwest Asia and East Asia (Iwamatsu et al., 1993). The small sized medaka fish (3–4 cm) have a genome that is about half the size of zebrafish and consist of 24 pairs of chromosomes (Sriramulu, 1959; Uwa et al., 1983; http://dolphin.lab.nig.ac.jp/medaka/; Kasahara et al., 2007). Sexual dimorphism is striking in medaka, and they breed and produce eggs continuously, completing their reproductive life cycle within 3 months under laboratory conditions, which makes medaka available for year-round experiments (Anken and Bourrat, 1998; Fujita et al., 2006). Medaka embryos hatch within 2 weeks after fertilization, and their larvae exhibit fast and invariable growth. The stages of normal development, from embryo to fry and young fish, have been identified for *Oryzias latipes* (Iwamatsu, 2004) and *O. melastigma* (Au, unpublished data).

Because medaka are easily maintained in the laboratory and provide a variety of useful developmental and reproductive endpoints for lifelong and multi-generational experiments, the freshwater species of *O. latipes* and the see-through (ST II) mutant of *O. latipes* have been rapidly established as vertebrate animal models for biomedical research and developmental genetics (Hardman et al., 2007, 2008; Kasahara et al., 2007). Over the past decade, *O. latipes* has also been used by various government agencies (e.g. the U.S. Environmental Protection Agency Office of Pollution Prevention and Toxics; Organization for Economic Co-operation and Development; European Commission) as a freshwater test species for ecotoxicological studies. Recently, *O. melastigma*, the seawater counterpart of *O. latipes*, has been successfully developed as a "universal" fish model for assessing multiple *in vivo* molecular responses to a variety of toxicants/stresses in the marine environment, including the study of *in vivo* response of TERT gene and protein expressions under hypoxia (Kong et al., 2008).

The telomerase reverse transcriptase (TERT) catalytic subunit gene has been cloned for *O. melastigma* (Yu et al., 2006) and *O. latipes* (Pfennig et al., 2008). The omTERT protein was found localized strictly to the nucleus of all medaka tissues studied (Kong et al., 2008), which is consistent with telomerase's localization in human and mouse cells. In this study, we demonstrate that the TERT protein is conserved both at the sequence level and structurally from a variety of vertebrate species. We further demonstrate that medaka has a number of advantages over other laboratory fish (including pufferfish and zebrafish) for the following reasons: 1) medaka have human-like telomere length and ubiquitous telomerase expression in all tissues of male and female fish; 2) the medaka TERT gene and protein sequences show the highest homology to the human TERT component sequences; 3) zebrafish exhibit low but detectable telomerase activity in tissues and cell lines; and 4) there is a clear divergence of zebrafish TERT between fish and mammals species. Our goal is to outline the evolutionary conservation of telomerase in vertebrate species, showing the potential of medaka as a vertebrate model for studying telomeres and telomerase function in environmental toxicology, tissue regeneration, and organismal aging.

2. Materials and methods

Some disagreement exists about the naming and identification of the species in question. Our experimental fish *O. melastigma* is identified as a distinct species according to the medaka homepage maintained by Nagoya University (http://biol1.bio.nagoya-u.ac.jp:8000/melastig.html). If queried for *O. melastigma*, www.fishbase.org automatically returns *Aplocheilus panchax* (Aplocheilidae) as the preferred name. However, our type specimens notably differ from the specimen of *A. panchax* at fishbase.org. Therefore, until the controversy has been resolved, we prefer to identify the genus of our experimental fish as *Oryzias* and the species as *O. melastigma*.

2.1. TERT genomic organization and protein in fish

The genomic sequence of medaka *O. latipes* TERT gene was obtained from tBLASTn search in the medaka genome database at NIG DNA Sequencing Centre. The TERT gene sequence of zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*) were obtained from genome database at Ensembl Genome Browser and GenBank (Accession No. AY861384), respectively. Fish *TERT* mRNA sequences are available in GenBank for the (1) medaka *O. latipes* long variant (DQ248968) and short variant (DQ870623); (2) medaka *Oryzias melastigma* (DQ286654); (3) zebrafish *D. rerio* (NM_001083866); (4) pufferfish *T. rubripes* (AY861384); and (5) orange-spotted grouper *Epinephelus coioides* (DQ317442).

2.2. Telomerase activity

Tissues in 1-year old adult medaka *O. melastigma* ($n=10$ pooled for female/male) were dissected on ice and lysed in CHAPS-containing lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, pH 8.0, 0.5% (v/v) CHAPS, 10% glycerol, 0.1 mM PMSF, 5 mM β-mercaptoethanol) for 30 min. The protein extract was cleared by centrifugation at 16,000 g 4 °C for 30 min. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, California) using bovine serum albumin (BSA) as a standard. Telomerase activities in adult medaka, zebrafish, and its derived cell lines were determined by TRAPeze XL Telomerase Detection Kit (Chemicon, USA) or using the radioactive version of the TRAPeze kit (see McChesney et al., 2005; Elmore et al., 2008). Telomerase positive control (equivalent to 1000 cells, provided by the kit), heat-inactivated control of the gonad and spleen samples, minus telomerase control (contained lysis buffer only and with Taq Polymerase) and minus Taq polymerase control (contained lysis buffer and without Taq Polymerase) and serial dilution of TSR8 (control template) were included in every run. Each sample and control was run in duplicate. After amplification for the fluorescent-based assay, the telomeric products were mixed with 150 μL buffer containing 10 mM Tris–HCl pH 7.4, 0.15 M NaCl and 2 mM $MgCl_2$ and transferred to a clear 96-well plate. Excitation/emission of fluorescein (485 nm/538 nm) and sulforhodamine (585 nm/620 nm) signals were measured by *f*max in combination of SOFTmax PRO software (Molecular Devices). For the radioactive assay, telomerase activity was quantified according to the procedures described in Compton et al. (2006) with the aid of ImageQuant software after phosphorimager scanning (Molecular Dynamics, Sunnyvale, CA, USA).

2.3. Telomere length measurement by terminal restriction fragment (TRF) analysis

Genomic DNA was extracted from the livers of adult male and female *O. latipes* and *O. melastigma* (1-year old) and whole fry of *O. melastigma* (1-month old) using the Genomic-tip system (Qiagen, Germany). For each assay, 2 μg genomic DNA was digested to completion with RsaI and HinfI at 37 °C overnight. The digested DNA was resolved by electrophoresis on a 1% agarose gel, run in parallel with a λ HindIII/EcoRI molecular marker (Fermentas, Canada) and subsequently transferred to Hybond-XL membrane (GE Healthcare, USA) for overnight capillary transfer. The membrane was pre-hybridized with ExpressHyb™ Hybridization Solution (Clontech, USA) at 42°C for 30 min while 60 ng telomeric probe $(TTAGGG)_5$ was end-labeled with γ-^{32}P-ATP (3000 Ci/mmol, 10 mCi/mL from PerkinElmer,