Development of the medaka *Oryzias melastigma* as a marine fish model for *in vivo* molecular toxicology

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ABSTRACT

Recently, there is an increasing trend of using small size fish as sentinel vertebrate species for (eco)toxicology and biomedical research. To this end, the zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), mosquito fish (*Gambusia affinis*), guppy (*Poecilia reticulata*) and Japanese medaka (*Oryzias latipes*) have been commonly used as freshwater fish models in ecotoxicological studies. Surprisingly, a fish model for assessing environmental stress in the marine environment has not been developed.

The marine medaka *Oryzias melastigma* (McClelland) has a number of attributes rendering it a potentially good marine fish for ecotoxicological studies. The *O. melastigma* is small and easy to culture and breed, and it completes the whole life cycle in seawater. The marine *O. melastigma* is similar to its freshwater counterpart *Oryzias latipes*, they both exhibit uniform growth which confers an additional advantage in using this species for ecotoxicological studies. Phylogenetically, this medaka species is closely related to the Japanese medaka *O. latipes*, of which the entire genome has been worked out recently. The anatomy, biology and nutritional requirements of *O. melastigma* are similar to that of *O. latipes*, which are well known and an atlas is available. In addition, much of the information on the physiology of *O. latipes* is also applicable for *O. melastigma*.

Notwithstanding, the use of small fishes for tissue-specific molecular analyses presents a major challenge. The quantity of a specific tissue available for analysis is often very limited, and isolation of such a small amount of tissue is often difficult and time consuming. However, this limitation can be overcome by using in situ hybridization (ISH) and immunohistochemistry (IHC) analyses on preserved whole fish tissues. Previous fixation protocols for adult small fish e.g. the Japanese medaka, zebrafish, guppy and mosquito fish were specifically designed for histopathologic
evaluation, only a few on immuno-localization studies using whole embryo or larvae, and there was no successful attempt for whole adult fish. Various technical problems are often associated with fixation and sectioning of relatively large-sized adult fish specimens (ca. 3 mm), mainly due to its heavy bony structures. Traditional decalcification of bony structures, using formic acid or EDTA, not only lead to poor RNA preservation, but are also time consuming (and may require up to 7 d). Until now, no protocols have been developed for parallel detection of mRNA and protein molecules in tissues of whole adult small fish.

In this study, we have successfully developed and optimized protocols for fixation and processing of whole adult marine medaka, enabling the production of whole fish tissue sections suitable for ISH and IHC analyses as well as histological evaluation. Moreover, with the recent advent of image analysis software, cost-effective procedures for stereological analysis (volume density, \( Vv \)) and color deconvolution have also been established in the present study for quantification of IHC and ISH signals (abundance and signal, respectively) on tissue sections. The development of quantification methods for ISH/IHC signals allows statistical analyses to be made on these \textit{in vivo} ISH (gene) and IHC (protein) data, which is a significant advancement in the application of whole fish model for molecular toxicology.

Telomerase is an enzyme involved in cell immortalization, carcinogenesis and tissue regeneration. The catalytic subunit telomerase reverse transcriptase (TERT) has been shown to regulate cell proliferation, mediate apoptosis, promote DNA repair and cell survival \textit{in vitro}, which suggest that TERT has a central role in controlling \textit{in vivo} cell growth and tissue homeostasis. In fish, TERT gene expression has been ubiquitously found in a variety of somatic tissues. In this study, we employed the \textit{omTERT} mRNA and protein, and Proliferating Cell Nuclei Antigen (PCNA, a protein marker for cell proliferation) as molecular endpoints to demonstrate the feasibility of
using ISH and IHC techniques to simultaneously localize and quantify in vivo expression levels of these gene and proteins in different tissues, including liver, gonad, kidney, gill, intestine and muscle, of a single marine medaka fish. Stereological analyzing results showed a significant positive relationship between omTERT mRNA and omTERT protein expression in male *O. melastigma*, and there was also a statistically significant correlation between PCNA, with omTERT mRNA as well as omTERT protein for both male and female fish.

Hypoxia has now become a pressing environmental problem in aquatic systems worldwide. Hypoxia has been reported to up-regulate TERT expression in liver of *O. melastigma*, which may perturb normal cell proliferation and apoptosis in hepatocytes. In this study, hypoxia was employed as a model stressor and the liver and gonads as model organs for studying the stress responses of omTERT mRNA (by ISH) and protein (by IHC), cell proliferation (by PCNA) and apoptosis (by the terminal dideoxynucleotidyl-mediated dUTP nick end labeling, TUNEL) in *O. melastigma*. Results of stereological analyses showed a significant induction of omTERT mRNA and a corresponding increase in omTERT protein in hepatocytes of hypoxic male fish as compared to the normoxic control. Additionally, an increase in PCNA-positive staining but a reduction of TUNEL apoptosis was also observed in the liver of hypoxic fish. By using color deconvolution method for detecting of signal intensity, ISH expression of omTERT mRNA was reduced in sperm cells of decreasing proliferative ability: spermatogonia > spermatocytes > spermatids, and absence in spermatozoa. Moreover, a significant reduction of omTERT mRNA expression was found in spermatocytes of hypoxia male *O. melastigma*. This may be useful to explain the observed arrest of spermatogenesis at spermatocytes of hypoxic males.

Findings of this study demonstrate that *O. melastigma* can serve as a good marine
fish model for (eco)toxicology. The whole adult medaka platform developed in this study has proven useful not only for histological evaluation, but also for spatial localization and quantification of in vivo molecular responses at the nucleic acid and protein levels simultaneously in different tissues/cells of the same individual. This whole fish tissue microarray approach serves as a novel and highly cost-effective tool for in vivo molecular toxicology.
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