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香港城市大學

Development of Transgenic Marine Medaka (*Oryzias melastigma*) as a Sentinel Species for Biomonitoring Estrogenic Endocrine Disruptors
搆建轉基因海水鯖鰿鱼(*Oryzias melastigma*)作為敏感物種來檢測雌激素類內分泌幹擾物質

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By

Chen Xue Ping
陳雪平

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Abstract of thesis entitled

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Chen Xueping

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Endocrine-disruptor (ED) is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently produces adverse health effects in an intact organism, or its progeny, or (sub) populations. Aquatic pollution by EDs, especially estrogenic EDs, has become one of the most serious environmental problems worldwide. The objective of this study is to develop a transgenic marine medaka (*Oryaizs melastigma*) for accurate and prompt detection of estrogenic EDs contaminated waters and screening of new EDs.

O. melastigma is scarcely studied and its embryonic development is not reported. The transparent chorion and embryo allow clear observation of the development of inner organs under light microscope. According to the diagnostic features used to stage the freshwater medaka (*O. latipes*), the embryonic developmental process of *O. melastigma* was divided into 39 stages and showed high morphological similarity to that of *O. latipes* with minor differences. Moreover, advantageous characteristics including small body size (3-4 cm), short life cycle (2-3 months), high prolificity (20-30 embryos daily per pair

under proper temperature, light cycle and feeding systems), and especially the habitability in both fresh water and sea water, make *O. melastigma* an ideal model for aquatic toxicology studies.

Teleost choriogenins, precursors of the inner layer subunits of egg envelope, are regarded as sensitive biomarkers for estrogenic pollutants. To select the more estrogen sensitive gene for transgenic study, full-length cDNAs—*omChgH* and *omChgL*—which encode the choriogenin H and L forms, respectively, were isolated from *O. melastigma* and their induced expression at different developmental stages were analyzed. 17β-Estradiol (E2; 10 μg/L)-dependent expression of *omChgH* and *omChgL* was observed starting at embryonic stage 34 and restricted to the liver. In hatchlings, E2 induction of *omChgH* was stronger than that of *omChgL*. Static exposure of adult fish to E2 (0, 1, 10, 100 and 500 ng/L), 17α-ethinylestradiol (EE2; 0, 1, 10, 100 and 500 ng/L), 4-nonylphenol (NP; 0, 1, 10, 100 and 200 μg/L) and bisphenol A (BPA; 0, 1, 10, 100 and 200 μg/L) in artificial seawater for 7 days resulted in dose-dependent induction of both genes in the liver. In the male livers, the sensitivity of *omChgH* to these estrogenic compounds was higher than that of *omChgL*; the lowest-observed-effect concentrations (LOECs) of E2, EE2, NP and BPA on *omChgH* were 10 ng/L, 10 ng/L, 100 μg/L and 100 μg/L, respectively, and on *omChgL* were 100 ng/L, 100 ng/L, 100 μg/L and 200 μg/L, respectively. All these observations highlighted the potential of using *omChgH* expression as a sensitive biomarker for estrogenic EDs in the developing *O. melastigma* embryos, juveniles and male adults.

Thus, *omChgH* genomic DNA sequence including ca. 5 kb 5'-upstream region and ca. 0.8 kb 3'-flanking region was cloned. *Cis*-regulatory activity analysis of different sizes of *omChgH* 5'-upstream region and the regulation effects of 3'-flanking region were analyzed

using microinjection techniques. Results showed that 750 bp 5'-upstream region from transcription initiation site had the highest promoter activity and homogenous 3'-flanking region was important for obtaining high promoter activity in *omChgH* transgenic studies.

Based on the *cis*-regulatory activity analysis of *omChgH* 5' and 3'-flanking region, a transgenic *O. melastigma* strain harboring the reporter gene green fluorescence protein (*GFP*) gene regulated by 758 bp *omChgH* 5'-upstream region and flanked by *omChgH* 3'-flanking region was established. In this strain, *GFP* transgene was expressed constitutively in the liver of mature female, but could also be induced from non-expression liver of embryos (since stage 34), juvenile and male fish in response to 17ß-estradiol (E2). GFP fluorescence quantification analysis using MetaMorph revealed that 0.63 nM E2 or 0.17 nM 17a-ethanylestradiol (EE2) significantly induced GFP expression in the livers of larvae after 24-h exposure and the responses were dose-dependent. Additionally, this strain was also observed to express GFP fluorescence after exposure to different estrogenic compounds at concentrations equal to or higher than 1.8 nM estrone (E1), 1.73 nM estriol (E3), 2000 nM 4-nonylphenol (NP), 4380 nM bisphenol A (BPA), 3670 nM genistein and 0.25 nM ethinylestradiol 3-methyl ether for 24 h. These results suggested the high estrogen sensitivity of this transgenic *O. melastigma* strain and its capability to monitor a wide range of estrogenic chemicals.

Further preliminary field study found that marine water samples collected from Hong Kong Victoria Harbor could induce GFP expression in the liver of transgenic larvae after exposure for 24 h. This result demonstrated the practical applicability of this transgenic *O. melastigma* strain for prompt *in vivo* biomonitoring of estrogenic activity of aquatic environment directly, and such high estrogen sensitivity has not been reported yet.

To conclude, this study successfully developed the first transgenic marine fish for biomonitoring estrogenic endocrine-disrupting pollutants. This strain showed quick response to a wide range of estrogenic compounds including weak EDs (e.g. NP, BPA and genistein), and could detect estrogenic activity of environmental water samples promptly and directly. All my findings indicate the great practical potential of this transgenic *O. melastigma* as a sensitive sentinel for simple, economic, rapid and accurate screening of new EDs and identification of estrogenic EDs contaminated waters. While the wide salinity adaptability to both freshwater and marine environment makes this transgenic strain more unique and powerful.

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