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ORIGINAL ARTICLE

Differential GFP Expression Patterns Induced by Different Heavy Metals in *Tg(hsp70:gfp)* Transgenic Medaka (*Oryzias latipes*)

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Abstract Heat shock protein 70 (Hsp70) is one of the most widely used biomarker for monitoring environment perturbations in biological systems. To facilitate the analysis of *hsp70* expression as a biomarker, we generated a *Tg*(*hsp70:gfp*) transgenic medaka line in which green fluorescence protein (GFP) reporter gene was driven by the medaka *hsp70* promoter. Here, we characterized *Tg*(*hsp70:gfp*) medaka for inducible GFP expression by seven environment-relevant heavy metals, including mercury, arsenic, lead, cadmium, copper, chromium, and zinc. We found that four of them (mercury, arsenic, lead, and cadmium) induced GFP expression in multiple and different organs. In general, the liver, kidney, gut, and skin are among the most frequent organs to show induced GFP expression. In contrast, no detectable GFP induction was observed to copper, chromium, or zinc, indicating that the transgenic line was not responsive to all heavy metals. RT-qPCR determination of *hsp70* mRNA showed similar induction and non-induction by these metals, which also correlated with the levels of metal uptake in medaka exposed to these metals. Our observations suggested that these heavy metals have different mechanisms of toxicity and/or differential bioaccumulation in various organs; different patterns of GFP expression induced by different metals may be used to determine or exclude metals in water samples tested. Furthermore, we also tested several non-metal toxicants such as bisphenol A, 2,3,7,8-tetrachlorodibenzo-p-dioxin, 4-introphenol, and lindane; none of them induced significant GFP expression in *Tg*(*hsp70:gfp*) medaka, further suggesting that the inducibility of *Tg*(*hsp70:gfp*) for GFP expression is specific to a subset of heavy metals.

Keywords Transgenic fish · Medaka · Hsp70 · Heavy metals · Biomonitoring · Toxicology

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Introduction

Heat shock protein (HSPs) are highly conserved proteins to respond to environmental stressors and they are commonly found in all organisms from bacteria to high vertebrates (Mukhopadhyay et al. 2003). HSPs were initially found to respond to thermal assault but were also observed to be elevated by other environment stressors such as chemical insult, oxidative stress, and ultraviolet light (Morimoto 1998). Many of the HSPs are present constitutively in cells and play essential roles in maintenance of protein homeostasis such as protein folding, aggregation, and trafficking. Under stress conditions, HSPs are upregulated to protect the cells against proteotoxic effect. Hsp70 family is the most conserved and the largest of all HSP families. Many chemicals, especially heavy

metals such as arsenic and cadmium, have shown to upregulate *hsp70* in organisms examined. Since it is one of the prominent genes that are upregulated in environmental perturbations, it has been often advocated to be used as a biomarker in environmental monitoring (Mukhopadhyay et al. 2003; Bierkens 2000; Yoshimi et al. 2009). Assays such as real time PCR, Western blot, and transfected cells culture with a *hsp70* promoter-driven reporter gene have been commonly used to determine the level of Hsp70, but these assays are generally labor-intensive, costly, and lack of physiological context like in vivo models.

In recent years, several transgenic fish with fluorescence protein reporters have been established for pollution detection because of its easy, on site, and real-time detection of fluorescence signal in living cells and organisms (Blechinger et al. 2002; Chen et al. 2010; Kurauchi et al. 2008; Kusik et al. 2008; Salam et al. 2008; Wu et al. 2008; Zeng et al. 2005; Ng and Gong 2013). Transgenic fish, like an *in vivo* system, reflects not only the bioaccumulation of toxicants but also the toxicity to targeted organs. For example, the olfactory system has been found to be affected by cadmium exposure in the hsp70-eGFP transgenic zebrafish (Blechinger et al. 2002). We recently also developed *gfp* transgenic medaka line under the medaka *hsp70* promoter, named as *Tg*(*hsp70:gfp*), when we tested the efficiency of maize Ac/Ds transposon system in generation of transgenic medaka (Ng and Gong 2011). However, the suitability of this transgenic line for biomonitoring aquatic toxicants has not been characterized. Upregulation of *hsp70* by heavy metals exposure such as cadmium and arsenic has been demonstrated in various aquatic organisms (Olsvik et al. 2011; Roy and Bhattacharya 2006; Schill et al. 2003; Elyse Ireland et al. 2004). The exact mechanism of how heavy metals exposure activates *hsp70* upregulation remains unclear; however, evidence of activation of HSF-1 signaling pathway by heavy metal exposure and possible cross-talk with metal response element pathway has been reported (Uenishi et al. 2006; Koizumi et al. 2013). In the present study, we exposed *Tg*(*hsp70:gfp*) fry to seven selected heavy metals including mercury, arsenic, lead, cadmium, copper, chromium, and zinc, all of which are listed as priority pollutants by EPA (United States Environmental Protection Agency). *Tg*(*hsp70:gfp*) fry responded to four of them (mercury, arsenic, lead, and cadmium) with inducible green fluorescence protein (GFP) expression in multiple organs, but not to the other three (copper, chromium, and zinc). Interestingly, the pattern of induced GFP expression varied by different heavy metals, which may provide a basis for predicting or excluding contaminated chemicals based on the pattern of GFP fluorescence in this transgenic line.

Materials and Methods

Transgenic Line and Fish Husbandry

Hd-rR medaka strain was obtained from National Institute for Basic Biology, Okazaki, Japan, through the National BioResource Project (NBRP Medaka), Japan (Sasado et al. 2010). Husbandry of medaka fish was based on Masato et al. (2009). Staging of medaka embryos and fry was based on Iwanatsu (2004). Generation of transgenic medaka *Tg(hsp70:gfp)* using a 2.0-kb medaka *hsp70-1* (referred as *hsp70* thereafter) promoter with the aid of maize Ac/Ds transposon system has been described previously (Ng and Gong 2011). All experimental protocols were approved by Institutional Animal Care and Use Committee of National University of Singapore (Protocol 079/07).

Promoter Sequence Analysis

A 2.0-kb medaka *hsp70* promoter based on medaka genome sequence (ENSORLG 00000000233 or AF286875.1) was cloned by genomic PCR and used for generation of *Tg(hsp70:gfp)*. The 2.0-kb sequence was presented in Fig. S1A and searched for relevant DNA responsive elements using Vector NT1 (Invitrogen). The basal transcription factor binding region or the TATA box was located at −620 bp upstream from the translation start codon. As the minimal sequence required for HSF1 trimer binding includes two inverted repeats of the pentameric sequence, nGAAn (Akerfelt et al. 2010), five such putative heat shock elements (HSEs) were identified within the 2-kb region upstream of the translation start codon. In addition, one putative metal response element (MRE:TGCRCnC; Kimura et al. 2009) and one putative electrophile response element (EpRE: GTGA CnnnGC; Rushmore et al. 1991), both in opposite direction, were also identified within the 2-kb sequence. The distribution of these responsive elements was schematically presented in Fig. S1B. The sequence from −1,983 to −1 upstream of translational start codon was used to construct the plasmid pDs(HSP70-EGFP) for establishing *Tg(hsp70:gfp)* transgenic medaka (Ng and Gong 2011).

Heat Shock Treatment

For heat shock treatment, five *Tg*(*hsp70:gfp*) fry of 1–3 days post-hatching (dph) were transferred to a flask containing 10 ml of pre-warmed egg medium (0.006 % *v*/*w* sea salt, Red Sea) in 37 °C water bath for 2 h. The control group was treated similarly in a 28 °C incubator for the same duration. After heat shock, the fry were then transferred into 3.5 mm petri dishes with 10 ml of egg medium and incubated at 28 °C for another 4 h before image capture.

Chemical Exposure

All chemicals used for exposure were purchased from Sigma-Aldrich. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), bisphenol A, and lindane were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), while 4-nitrophenol, mercury(II) chloride, cadmium chloride, sodium arsenate dibasic heptahydrate, copper(II) sulfate (pentahydrate), zinc sulfate, potassium dichromate, and lead(II) nitrate were dissolved in MilliQ water to make stock solutions. Stock solutions were diluted appropriately with egg medium to make up the final concentrations of exposure chemicals. One to 3 dph fry of *Tg(hsp70:gfp)* was used for chemical exposure since by then most organs were fully developed yet the abdomen wall remained transparent for easy observation of internal organs. Five or more fry were placed in each well of a 6-well dish, containing 5 ml of waters with or without testing chemicals at various concentrations at 28 °C. Preliminary exposure experiments with wild type 1–3 dph larvae were performed for each chemical and the highest concentrations used for transgenic fry exposure was those for causing 30–50 % mortality at 24 h exposure. In general, the medaka fry showed no adverse phenotype at low concentrations while high concentrations of some metals might cause edema, bended trunk, swimming difficulty, etc. Hemizygous transgenic embryos and fry were used in all chemical exposures for consistency in genetic background. Each experiment was repeated for at least three times. After 24 or 48 h of exposure, the fry were examined for GFP expression and image capture.

Observation of Fluorescent Signals

Fry were first anaesthetized by 0.1 % 2-phenoxyethanol (Sigma-Aldrich) and positioned in 3 % methylcellulose (Sigma-Aldrich) before observation. Images of the fry were observed and captured using a fluorescence microscope (Axiovert 200M, Zeiss) equipped with a digital camera (Axiocam HRc, ZEISS).

Reverse Transcription-Quantitative PCR (RT-PCR)

Total RNA was extracted from chemical-treated and untreated medaka fry. Two micrograms of DNase-treated total RNA was used for synthesizing first strand cDNA using an oligo-dT primer and SuperScript^{TM}II Reverse Transcriptase according to the manufacturer's protocol (Invitrogen). The cDNA samples were used for qPCR analysis using Lightcycler-FastStart DNA Master SYBR Green 1 and Lightcycler480 (Roche Applied Science) according to the manufacturer's instruction. Both *hsp70* and *β-actin* (internal control) mRNAs were measured and $-\Delta\Delta$Ct was used to represent the relative expression level of the genes. The statistical comparison of the relative mean expression level for *hsp70* gene between test and control groups was performed using Student's *T* test with *P* value <0.05 being considered significant. The primer pair for *hsp70* gene was: forward, 5′ CACAAAGTCATCCAGCAAAC; reverse 5′ TCAGTCCACCTCCTCAATAG. The pair for *β-actin* was: forward, 5′CCGTGACATCAAGGATAAGCT; reverse 5′ TCGTGGATACCGCAAGATTCC.

Determination Of Heavy Metal Uptake

Medaka fry were treated with 600 μg/l $HgCl_2$, 100 μg/l $Na_2HAsO_4 \cdot 7H_2O$, 200 μg/l $CaCl_2$, 1 mg/l $CuSO_4.7H_2O$, 100 mg/l $K_2Cr_2O_7$, 5 mg/l $ZnSO_4$, and 10 mg/l $Pb(NO_3)2$, respectively, for 24 h. Following the treatment, the treated and untreated medaka were collected and washed with Milli-Q water. They were placed into glass jars and stored at −80 °C until analysis. For analysis of heavy metal contents, medaka were dried for 24 h at 105 °C. A microwave digestion system (Milestone, ETHOS ONE SK-10 and SK12 Segmented Rotors) was used to prepare for the sample analysis. A sample of 0.2–0.5 g was placed in vessels (TFM Teflon Vessels), and 7 ml of 65 % (*w*/*v*) HNO_3 and 1 ml of 30 % (*w*/*v*) H_2O_2 were added until the predigestion was completed. The microwave digestion system was set up at the operating temperature of 190 °C. Afterwards, the samples were filtered (0.45 μm nylon syringe filter) and the residues were made up the volumes to 20 ml with deionized water. The calibration standard solutions for all targeted heavy metals were prepared by using Accu Trace Reference Standard solutions with a purity of 99.8 %. After appropriate dilutions of stock standard solutions, a five level calibration curve was prepared. Duplicate method blanks were processed and analyzed alongside the samples to monitor any loss or cross contamination. The concentrations of As, Cr, Cd, Cu, Hg, Pb, and Zn in fish tissues were determined by using ICP-MS (Agilent 7700 Series).

Results

Selection of Tg(*hsp70:gfp*) for Biomonitoring Studies

Previously, we have obtained 10 transgenic founders for *Tg(hsp70:gfp)* transgenic medaka (Ng and Gong 2011). Most of their F1 progeny have variable constitutive GFP expression in the trunk muscles and notochord in addition to the high, constitutive GFP expression in the lens from 3 dpf in all transgenic families. There were only two transgenic lines without constitutive GFP expression (except for that in lens) and preliminary experiments showed that both transgenic lines had identical inducible expression patterns by mercury and cadmium. In general, different transgenic lines may have different constitutive GFP expression pattern, but the inducible GFP expression by the same metal is quite consistent

among different lines. In this study, after careful comparison and characterization, we selected one line, *Tg(hsp70:gfp)1.1* [referred as *Tg(hsp70:gfp)* thereafter] for further studies as it had no constitutive GFP expression except for that in the lens, which is beneficial for the identification of transgenic offspring. This stable line has a single transgene insertion in its genome as demonstrated by genomic Southern Blot hybridization (Ng and Gong 2011). We also observed maternal GFP expression in the yolk in early embryos derived from female transgenic parent. However, GFP expression was reduced gradually to almost none when the embryos reached 6 dpf. Nevertheless, for all tests in the present study, male transgenic fish was used to cross wild type females to obtain hemizygous transgenic embryos and no constitutive GFP expression was observed in early embryos except for that in the lens.

Induction of Ubiquitous GFP Expression by Heat Shock

The inducibility of *Tg(hsp70:gfp)* transgenic fry was first tested by heat shock at 37 °C for 2 h. All heat-shocked *Tg*(*hsp70:gfp*) fry were observed to have elevated GFP expression in muscle and gills at the end of heat shock. Four hours after heat shock, all heat-shocked *Tg*(*hsp70:gfp*) fry appeared to express GFP in many other organs. Relatively strong GFP expression was observed in muscle, gills, and skin, while weak GFP expression was observed in peritoneal organs such as liver, heart, and gut (Fig. 1b, d). No induced GFP expression was observed in the control group of transgenic fry at 28 °C at any time (Fig. 1a, c). These observations indicated that *Tg*(*hsp70:gfp*) responded to heat shock and GFP was inducible in most organs.

GFP Induction by Mercury Chloride

One to 3 dph *Tg(hsp70:gfp)* fry were treated with mercury chloride from 200 to 800 μg/l for 24 h for observation of GFP induction. GFP fluorescence was observed weakly in the kidney and showed spotty pattern in the gills in some of the fry at the lowest tested concentration (200 μg/l). Besides kidney and gills (Fig. 2d, f) , GFP expression was also observed in other organs such as the liver, weakly in skin and notochord (Fig. 2b, d, f) at concentrations higher than 200 μg/l (Fig. 2g). As quantified in Fig. 2g, the number of fry that expressed GFP in kidney, liver, and skin increased with dosage. However, dosage-dependent effect was not apparent in other organs such as gills and notochord.

GFP Induction by Cadmium Chloride

One to 3 dph *Tg(hsp70:gfp)* fry were treated with cadmium chloride from 25 to 400 μg/l for 24 h for GFP induction. The kidney (Fig. 3b) seemed to be the most sensitive organs since the highest number of fry showed GFP fluorescence in the kidney in all concentration groups (Fig. 3g). The liver was the next sensitive organ to express GFP (Fig. 3b, f). For example, at the lowest tested concentration (25 μg/l), about 40 % of the fry expressed detectable GFP in the kidney and 6.7 % of the fry expressed GFP in the liver. At 50 μg/l, the number of fry expressing GFP in the kidney and liver increased to 75 and 41.7 %, respectively (Fig 3g). Higher concentrations at 100 μg/l and above led to GFP expression in other organs such as olfactory pits and skin (Fig. 3b, d) at a relatively weak level. Induced GFP expression in olfactory pits has no apparent dosage dependence (Fig. 3g).

GFP Induction by Sodium Arsenate

We treated the *Tg(hsp70:gfp)*fry (1–3 dph) with sodium arsenate from 12.5 to 200 μg/l for 24 h. The lowest effective concentration of sodium arsenate for observation of visible GFP induction in this transgenic line (Fig. 4g) was between 12.5 and 25.0 μg/l as 26 % of the fry at 25.0 μg/l showed GFP induction in the liver while no expression was observed at 12.5 μg/l (Fig. 4g). In concentrations from 50 to 200 μg/l,

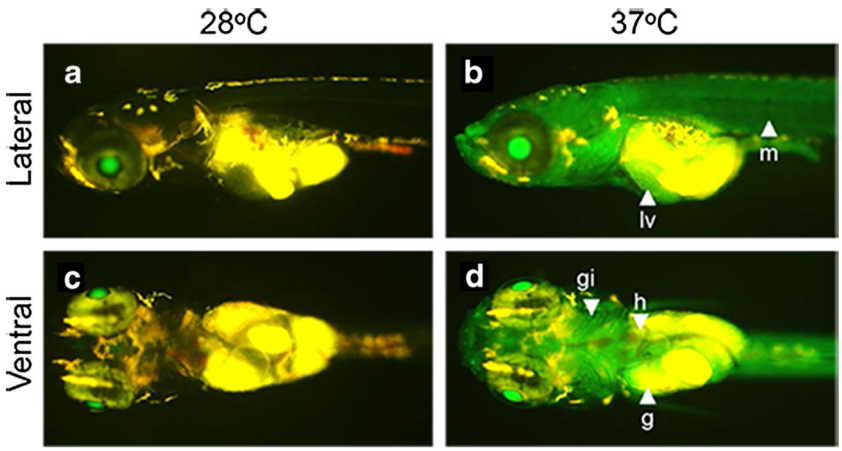

Fig. 1 Heat shock induced GFP expression in *Tg*(*hsp70:gfp*) fry. One to 3 dph *Tg*(*hsp70:gfp*) fry were heat-shocked at 37 °C for 2 h and images were taken 4 h after heat shock for GFP fluorescence under a fluorescent microscope. **a**, **c** Image of a representative fry under control condition at 28 °C in *lateral* (**a**) and *ventral views* (**c**). **b**, **d** Image of a representative fry after heat shock in *lateral* (**b**) and *ventral views* (**d**)

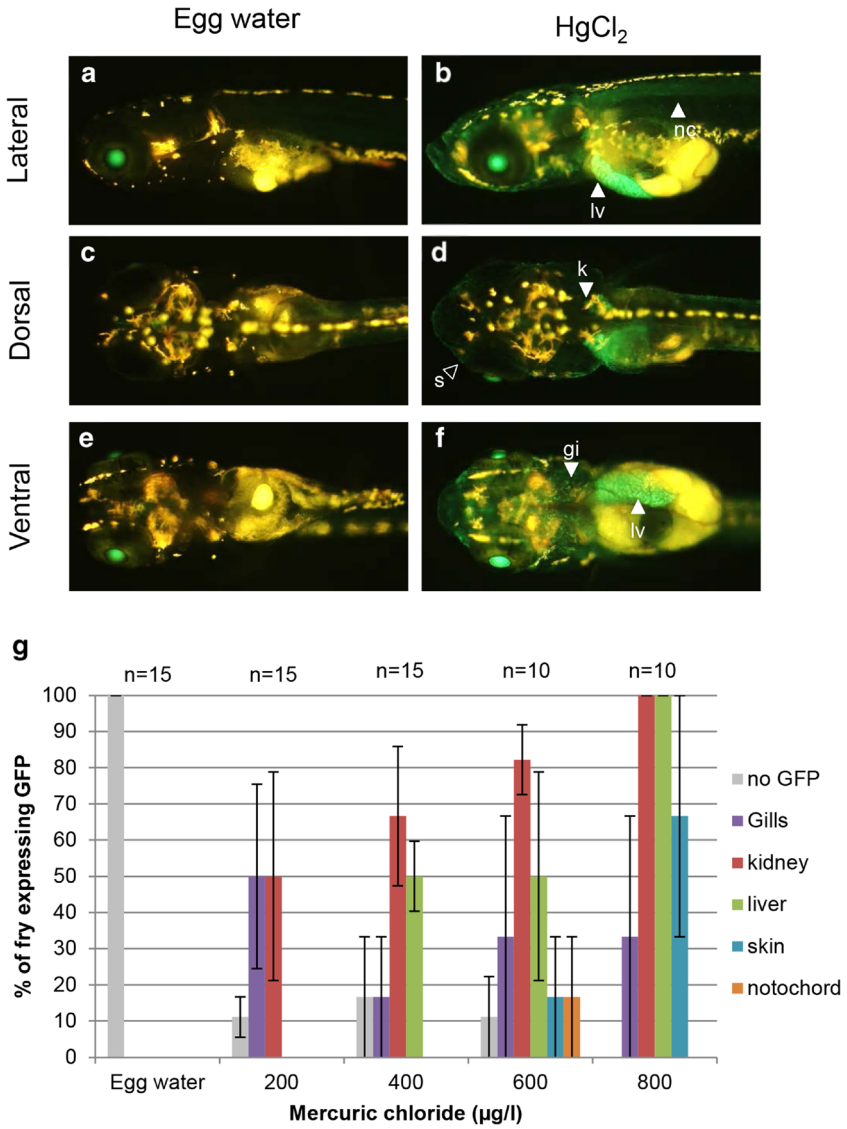

Fig. 2 GFP induction in mercury-treated *Tg*(*hsp70:gfp*) fry. One to 3 dph *Tg*(*hsp70:gfp*) fry were treated with various concentrations of mercury chloride for 24 h and images were taken at the end of treatment for GFP fluorescence under a fluorescent microscope. **a**–**f** Images of representative fry in 800 μg/l of mercury chloride for viewing GFP expression: untreated control fry in *lateral* (**a**), *dorsal* (**c**), and *ventral views* (**e**); treated fry in in *lateral* (**b**), *dorsal* (**d**), and *ventral views* (**f**). Organs with induced GFP expression in (**b**, **d**, **f**) are indicated by *arrowheads*. Abbreviations: *gi* gills, *k* kidney, *lv* liver, *nc* notochord, *s* skin. **g** Percentages of fry with induced GFP expression in different organs at various concentrations of mercury Chloride. *Error bars* represent standard errors

all fry were observed to express GFP in the liver (Fig. 4b, f). Besides that, there was an increasing numbers of fry expressing GFP in other organs such as gut, muscle, and skin (Fig. 4b, d, f) with increasing dosage (Fig. 4b, d, f). Generally, the GFP expression in most organs was also intensified in most fry when exposed to higher concentration of sodium arsenate.

GFP Induction by Lead Nitrate

One to 3 dph Tg(*hsp70:gfp*) fry were treated with lead nitrate from 0.1 to 10 mg/l for 24 h to determine GFP expression. GFP was induced in the kidney (Fig. 5b, d) of some treated fry (46.7–93.3 %) in all concentration groups (0.1–10 mg/l) but without an apparent dosage dependent effect (Fig. 5g). At 1 mg/l and more, GFP was also observed in the gut weakly (Fig. 5f) in increasing percentages (15.0–80.9 %) of fry as dosage increased (Fig. 5g).

Lack of GFP Induction by Copper, Chromate, and Zinc Metals

We also exposed transgenic Tg(*hsp70:gfp*) fry to three other heavy metals including zinc sulfate (0.625–10 mg/l), copper sulfate (0.0625–1 mg/l), and potassium dichromate (50–200 mg/l), using dosage up to the highest possible concentrations. Generally, no significant GFP induction was observed for these three metals after 24 h of exposure, although few of the surviving fry did express faint GFP in some gill epithelial cells at the highest concentration 1 mg/l of copper sulfate, where 40 % mortality was observed.

Correlation of GFP Expression with Endogenous *hsp70* mRNA Expression and Metal Uptake

To investigate whether GFP induction in *Tg(hsp70:gfp)* represents *hsp70* RNA induction in wild type medaka by the

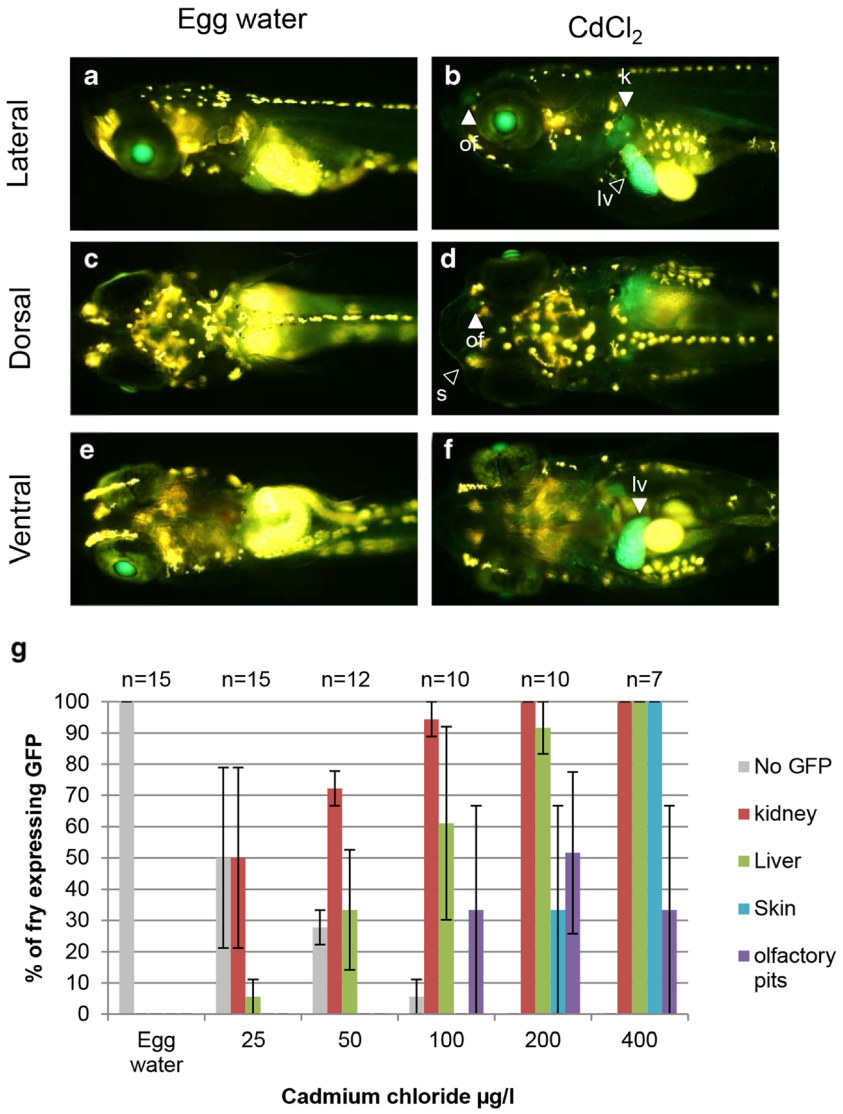

Fig. 3 GFP induction in cadmium-treated *Tg*(*hsp70:gfp*) fry. One to 3 dph *Tg*(*hsp70:gfp*) fry were treated with various concentrations of cadmium chloride for 24 h and images were taken at the end of treatment for GFP fluorescence under a fluorescent microscope. **a**–**f** Images of representative fry in 400 μg/l of cadmium chloride for viewing GFP expression: untreated control fry in *lateral* (**a**), *dorsal* (**c**), and *ventral views* (**e**); treated fry in in *lateral* (**b**), *dorsal* (**d**), and *ventral views* (**f**). Organs with induced GFP expression in (**b**, **d**, **f**) are indicated by *arrowheads*. Abbreviations: *k* kidney, *lv* liver, *of* olfactory pits, *s* skin. (**g**) Percentages of fry with induced GFP expression in different organs at various concentrations of cadmium chloride. *Error bars* represent standard errors

same set of metals, RT-qPCR was performed for wild type fry that was similarly treated with the seven metals tested in this study. As shown in Fig. 6a, *hsp70* mRNA was significantly induced by all of the four metals (mercury, cadmium, arsenic, and lead) that caused GFP induction; in contrast, no significant induction of *hsp70* mRNA was observed by exposure to the three GFP-negative metals, copper, zinc, and chromium. Thus, the observed GFP induction faithfully mimicked the induction of endogenous *hsp70* mRNA.

Furthermore, uptake of metals in medaka following a 1-day exposure was also measured in order to determine whether the lack of GFP induction was due to the uptake efficiency of exogenous metals in fish body. Medaka fish were then similarly treated with these metals and metal uptake was determined as described in the "Materials and Methods" section. The body level of these metals in the treated fish was compared with control fish in Milli-Q water and the fold increase was shown in Fig. 6b. It is clear that there was significant increase of metal uptake in fish body for cadmium, mercury, and lead, all of which also induced GFP expression in *Tg(hsp70:gfp)* medaka. In contrast, the three metals (copper, zinc, and chromium) that failed to induce GFP expression in *Tg(hsp70:gfp)* had quite minimum increase of body level of these metals. Thus, the chemical uptake data provided evidence that in vivo increase of metal concentration caused the GFP induction, One exception is arsenic that induced GFP expression in *Tg(hsp70:gfp)* but did not show significant uptake after arsenic exposure; the reason for this is not clear from the current experiment.

Lack of Significant GFP Induction by Non-Metal Chemicals

Tg(hsp70:gfp) fry was also exposed under similar conditions to several non-metal toxicants including 4-nitrophenol (2.5–

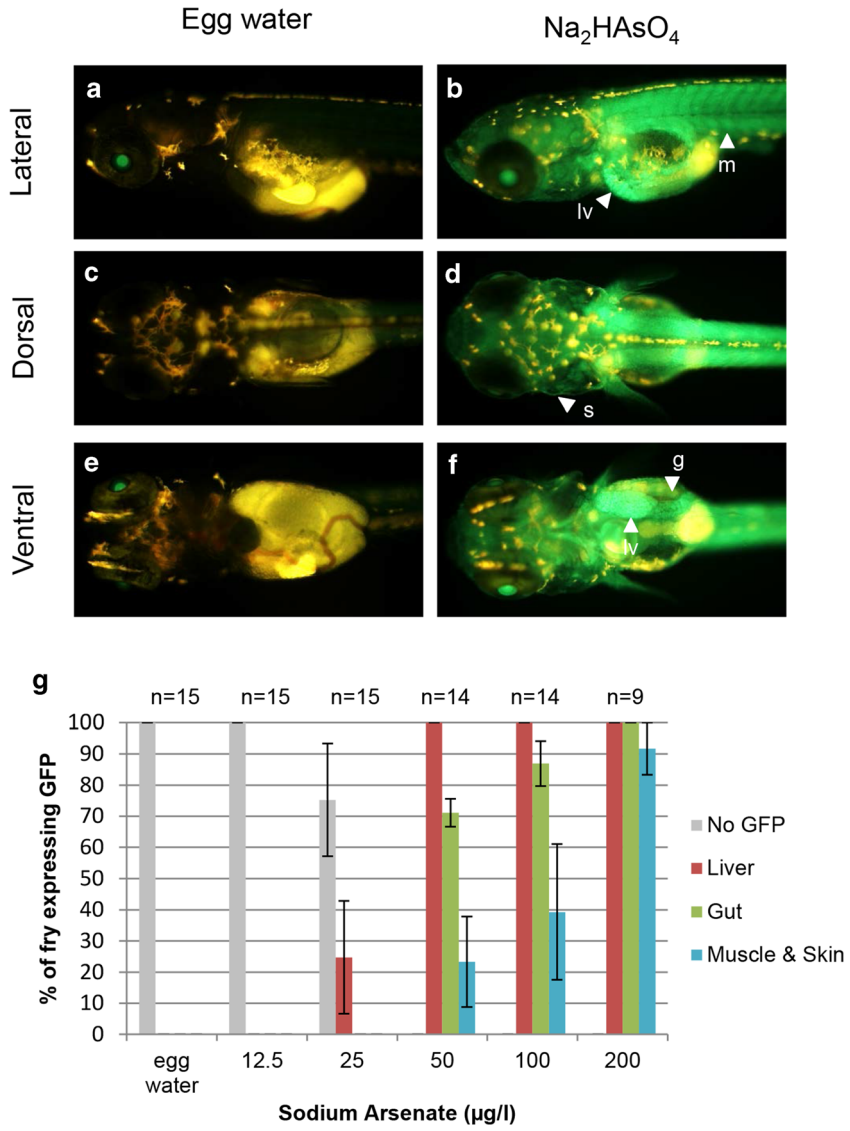

Fig. 4 GFP induction in arsenic-treated *Tg*(*hsp70:gfp*) fry. One to 3 dph *Tg*(*hsp70:gfp*) fry were treated with various concentrations of sodium arsenate for 24 h and images were taken at the end of treatment for GFP fluorescence under a fluorescent microscope. **a**–**f** Images of representative fry in 200 μg/l of sodium arsenate for viewing GFP expression: untreated control fry in *lateral* (**a**), *dorsal* (**c**), and *ventral views* (**e**); treated fry in in *lateral* (**b**), *dorsal* (**d**), and *ventral views* (**f**). Organs with induced GFP expression in (**b**, **d**, **f**) are indicated by *arrowheads*. Abbreviations: *g* gut, *lv* liver, *m* muscle, *s* skin. **g** Percentages of fry with induced GFP expression in different organs at various concentrations of sodium arsenate. *Error bars* represent standard errors

12.5 mg/l), bisphenol A (0.1–10 mg/l), TCDD (0.08–1.61 μg/l), and lindane (0.1–1 mg/l). For 4-nitrophenol, bisphenol A, and TCDD, no GFP expression was induced in the transgenic fry even after 48 h of exposure. We noted that the highest concentrations, 12.5 mg/l 4-nitrophenol and 10 mg/l bisphenol A, were lethal and all the fry in these groups died within 48 h yet no GFP expression from these fry was induced at any time. In lindane treatment, no obvious GFP expression was observed in any organ in the first 48 h of exposure. However, after 48 h exposure, some of the fry in the two highest concentration groups, 1 and 5 mg/l, showed very weak and discrete GFP expression in the truck region (Fig. S2A). GFP expression appears to be in discrete muscle fibers and such observation accounts for 17.6 and 50 % of the fry exposed in 1 and 5 mg/l concentration group, respectively (Fig. S2B).

Discussion

Metal Inducible Function of Response Elements in the Medaka *hsp70* Promoter

Carvan et al. (2000a) first proposed to generate transgenic fish for aquatic monitoring; this is achieved by using a reporter gene under control of selected responsive elements in the promoter and these responsive elements provide binding sites for specific transcriptional activators in response to certain environmental toxicants. Examples of such DNA motifs to respond to toxicants include aromatic hydrocarbon responsive elements, heavy metal responsive elements (MREs), HSEs, and EpREs (Carvan et al. 2000b). The 2-kb medaka *hsp70* promoter used to generate *Tg(hsp70:gfp)* medaka in this study contains five putative HSEs, one MRE, and one EpRE

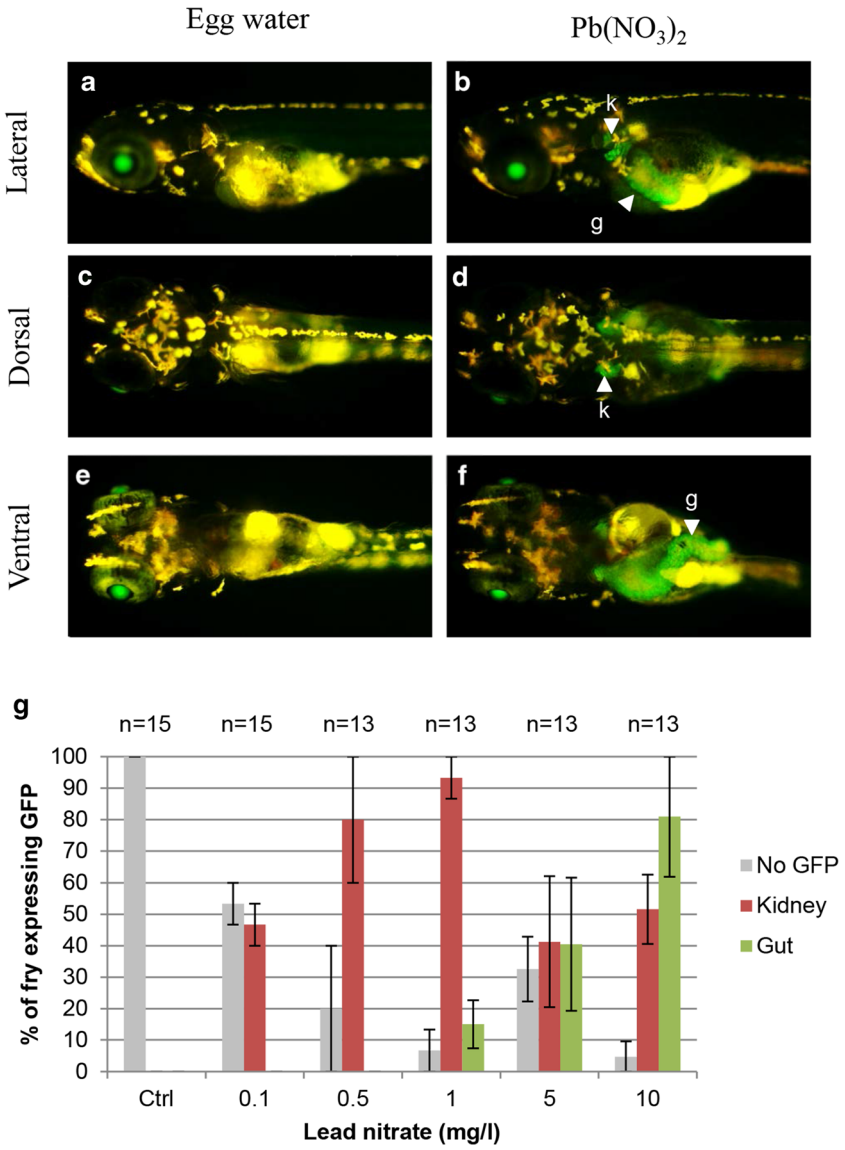

Fig. 5 GFP induction in lead-treated *Tg*(*hsp70:gfp*) fry. One to 3 dph *Tg*(*hsp70:gfp*) fry were treated with various concentrations of lead nitrate for 24 h and images were taken at the end of treatment for GFP fluorescence under a fluorescent microscope. **a**–**f** Images of representative fry in 10 mg/l of lead nitrate for viewing GFP expression: untreated control fry in *lateral* (**a**), *dorsal* (**c**), and *ventral views* (**e**); treated fry in *lateral* (**b**), *dorsal* (**d**), and *ventral views* (**f**). Organs with induced GFP expression in (**b**, **d**, **f**) are indicated by *arrowheads*. Abbreviations: *g* gut, *k* kidney. (**g**) Percentages of fry with induced GFP expression in different organs at various concentrations of lead nitrate. *Error bars* represent standard errors.

(Fig. S1). EpRE regulates the transcription via transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), while HSE and MRE are the binding sites for heat shock factor (HSF) and MRE-binding transcription factor-1 (MTF-1), respectively. Oxidative stress caused by heavy metal exposure is thought to induce the transcription of EpRE-regulated genes, which has been demonstrated by reporter gene expression driven by EpRE in transgenic zebrafish after mercury exposure (Kusik et al. 2008). Upregulation of HSF and MTF-1 activated genes after heavy metal exposure has been reported in various organisms, indicating that both HSEs and MREs are involved in response to heavy metal insults (Kusik et al. 2008; Huang et al. 2007; Wang and Fowler 2008). Furthermore, a recent study with detailed deletion analyses of the mouse *Hsp70* promoter has indicated that both the distal and proximal heat shock elements with HSF-binding activity are essential for the metal-responsive transcription of *hsp70* (Koizumi et al. 2013). Thus, these experimental data support the relevance of the responsive elements (HSE, MRE, and EpRE) in the medaka *hsp70* promoter and are consistent with our observation of heat shock and heavy metal inducibility. Our data also demonstrated that the 2-kb of medaka *hsp70* promoter is sufficient to offer the inducibility by heat shock and heavy metal in most, if not all, organs/tissues.

Differential GFP Expression Patterns Induced by Different Heavy Metals

In the present study, we found that GFP expressions were induced in various organs by four heavy metals: mercury, cadmium, arsenic, and lead (Figs. 2–5), demonstrating that the transgenic fry is responsive to these heavy metals at sublethal concentrations. Interestingly, difference in GPF expression pattern was observed among the four heavy metal

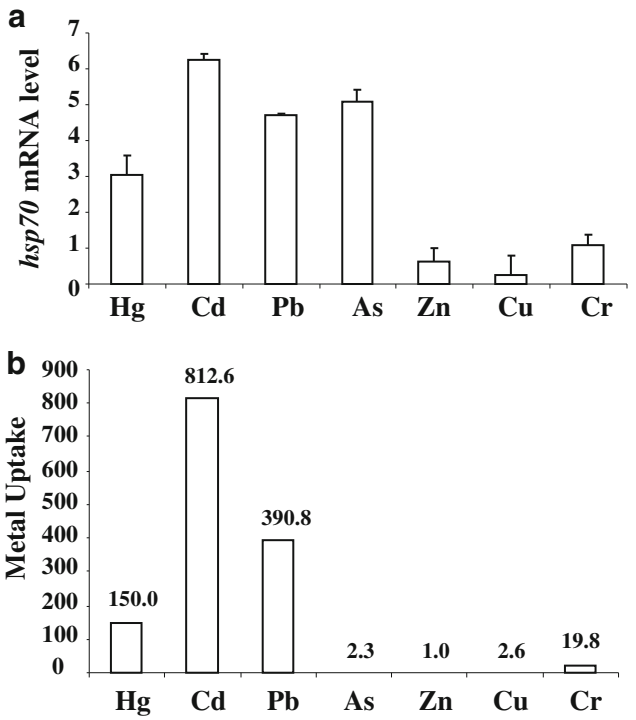

Fig. 6 Correlation of GFP expression with endogenous *hsp70* mRNA expression and metal uptake. Medaka fry were treated with 600 μg/l $HgCl_2$, 100 μg/l $Na_2HAsO_4 \cdot 7H_2O$, 200 μg/l $CaCl_2$, 1 mg/l $CuSO_4.7H_2O$, 100 mg/l $K_2Cr_2O_7$, 5 mg/l $ZnSO_4$, and 10 mg/l $Pb(NO_3)2$, respectively, for 24 h and then processed for measurement of endogenous hsp70 expression and metal uptake. **a** Induction of *hsp70* mRMA by various metals. Fold change relative untreated controls are shown. **b** Metal uptake in medaka fry treated with various metals. The fold change is comparison of total level of an individual metal between metal-treated and untreated fish, and the actual numbers of fold changes are indicated *above each bar*

treatments as summarized in Fig. 7. In general, the liver and kidney, followed by gut and skin, were the most frequent organs to express transgenic GFP in response to these heavy metals as evident from the strong GFP expression in high percentages of treated transgenic fry. These four organs are probably not only the most accessible organs for accumulation of heavy metals, but also major organs for detoxification (liver and skin) and osmoregulation (kidney and gills). It is interesting to note that many of the metals showed GFP induction in specific organs, such as muscle expression by arsenic, olfactory expression by cadmium, and notochord/gill expression by mercury. Thus, the difference in GFP expression patterns may provide a basis for distinguish different metals based on GFP expression. As GFP expression pattern may not be necessary to identify the metals in water samples tested, it should be valuable to exclude the presence of certain metals. For example, the lack of GFP induction in the kidney in the arsenic exposure and the lack of GFP expression in the liver in the lead exposure could be used to exclude the two metals based on GFP expression pattern. The expression of GFP transgene is likely reflective of endogenous *hsp70* expression and the pattern of GFP expression is likely affected by differential bioaccumulation of these metals in different organs as well as by the sensitivity of each organs in metal-induced *hsp70* expression. Consistent with this, it has been reported in numerous studies that the rate of accumulation of the same metal differs in different organs of fish and the order of accumulation in various organs is different for various metals under the same condition (Huang et al. 2007; Jabeen and Chaudhry 2010; Ebrahimi and Taherianfard 2010; Jarić et al. 2011). In the present study, we also tested the transgenic fish with other metals such as copper, zinc, and chromium but no GFP induction was observed, suggesting that this transgenic medaka line does not respond towards all types of metals.

Similarity and Differences Between *Tg(hsp70:gfp)* Zebrafish and Medaka

Although, to our knowledge, there are no studies on the heavy metal exposure of *hsp70:gfp* transgenic medaka prior to our studies, there have been a few published reports on heavy metal exposures using *Tg*(*hsp70:gfp)* transgenic zebrafish (Blechinger et al. 2002; Hernandez et al. 2011). In *Tg*(*hsp70:gfp)* zebrafish in which zebrafish *hsp70* promoter is used, GFP expression is first detected in the gills, skin, and olfactory organ, followed by liver and pronephric ducts at 125 μM (23 mg/l) cadmium chloride, the highest concentration used in the study; Blechinger et al. 2002; Hernandez et al. 2011). This is in contrast to our observation in *Tg*(*hsp70:gfp*) medaka, GFP is expressed in a different sensitivity order: kidneys, liver, olfactory pits, and skins (Fig. 3). Nevertheless, the sensitivity of the two *Tg*(*hsp70:gfp)* lines in two different species is comparable since the lowest

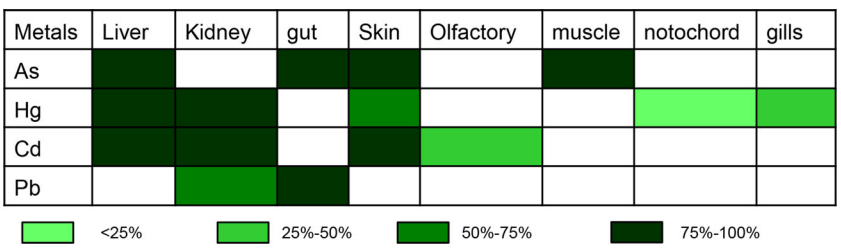

Fig. 7 Summary of induced GFP expression in various organs by arsenic (As), mercury (Hg), cadmium (Cd), and lead (Pb). Percentages of fry showing induced GFP expression in specific organs are indicated with different grades of *green colors*. *White boxes* represent 0 %

concentration to cause GFP expression in *Tg*(*hsp70:gfp)* zebrafish is 0.2 μM (36.6 μg/l) while in *Tg(hsp70:gfp)* medaka is 25.5 μg/l. Copper sulfate exposure has also been reported for the same *Tg*(*hsp70:gfp)* zebrafish (Hernandez et al. 2011). GFP expression is induced in gills and olfactory pits at 100 μm (6.3 mg/l) 24 h after a 2-h pulse exposure. Additional GFP expression in the liver, spinal cord, and brain has also been observed at higher concentrations. In contrast, our transgenic medaka did not appear to respond to copper insult even at a high lethal dosage (1 mg/l) after 24 h of exposure. While there is so far no available data on other metal treatment in *Tg(hsp70:gfp)* zebrafish, a complete comparison may be premature. However, the available data indicate that the two species are quite different in response to heavy metal treatments. It is not clear whether the difference is associated with specific transgenic strain or with the response of endogenous *hsp70* genes in the two species, more works will be required to clarify these.

Specificity and sensitivity of *Tg(hsp70:gfp)* medaka to respond environmental toxicants

Other than heavy metals, we also exposed the *Tg(hsp70:gfp)* fry to several other types of toxicants such as 4-nitrophenol, bisphenol A, TCDD, and lindane. Except for lindane treatment, no GFP expression was induced even at lethal dosage after two days of treatment. In contrast, TCDD could sensitively induce GFP expression in another transgenic line, *Tg*(*cyp1a:gfp*), at concentrations of 0.005 nM (1.61 ng/l; Ng and Gong 2013), while no GFP expression was detected in *Tg*(*hsp70:gfp*) medaka even up to 5 μM (1.61 μg/l) in which developmental defects such as curled tail have been observed. There was weak GFP expression in discrete muscle cells in the high concentration groups of lindane treatments, where deformities were observed, such as crooked and shrunken body with swimming difficulties, implying that muscles were significantly damaged. Thus, muscle damages caused by lindane perhaps induced a general stress in some muscle fibers leading to activation of the *hsp70* stress biomarker gene, and consequently, GFP expression in *Tg(hsp70:gfp)* fry. However, GFP expression in lindane-treated fry was rather weak as compared to the GFP expression induced by heavy metal treatments. Thus, it appears that *Tg(hsp70:gfp)* is only sensitively responsive to a subset of heavy metals including mercury, arsenic, lead, and cadmium.

Here we demonstrated that the *Tg(hsp70:gfp)* medaka fry showed detectable GFP induction by at least four metals: arsenic, mercury, cadmium, and lead. The lowest observed effective concentrations (LOECs) of these four metals were 5.5, 6.0, 62.6, and 147.8 μg/l for cadmium, arsenic, lead, and mercury, respectively, based on ion mass. Among these, the LOECs for cadmium and arsenic are about the maximum concentration level (MCL) set by United States Environmental Protection Agency for these two ions (5 μg/l for cadmium and 10 μg/l for arsenic), while the other two LOECs are significantly higher than EPA's MCL (2 μg/l for mercury and 15 for lead; http://water.epa.gov/drink/contaminants/index.cfm#two). Thus, the assays based on the current transgenic medaka line may be sufficiently sensitive for certain metals such as cadmium and arsenic for the safety of potable water. Although the LOECs for other metals are significantly higher than the EPA's MLCs, the GFP transgenic medaka still offer a convenient, economical, and potentially online sentinel system for aquatic environmental pollution. However, a major weakness of the GFP-based assay is the difficulty for quantification of GFP fluorescence and the problem can be partially overcome by quantification of the number of GFP positive individuals and/or by objective classification of strong/weak GFP expression as we previously described (Zeng et al. 2005; Ng and Gong 2013).

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