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GENERATION OF *mt:egfp* TRANSGENIC ZEBRAFISH BIOSENSOR FOR THE DETECTION OF AQUATIC ZINC AND CADMIUM

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Abstract: Zebrafish embryo toxicity testing has become a popular method for detecting environmental pollutions. However, the present research showed that zebrafish embryos exhibited no visible paramorphia, malformation, or mortality when exposed to heavy metals in a range above environmental standard limits, indicating that zebrafish embryos are an imprecise model for monitoring environmental heavy metals concentrations above regulatory limits. Aiming to obtain a biosensor for aquatic heavy metals, a metal-sensitive vector including zebrafish metallothionein (MT) promoter and enhanced green fluorescent protein (EGFP) was reconstructed and microinjected into 1-cell stage zebrafish embryos. The authors obtained an *mt:egfp* transgenic zebrafish line sensitive to aquatic zinc and cadmium. A quantitative experiment showed that zinc and cadmium treatment significantly induced the expression of EGFP in a dose- and time-dependent manner. In particular, EGFP messenger RNA levels increased remarkably when exposed to heavy metals above the standard limits. The results suggest that the transgenic zebrafish is a highly sensitive biosensor for detecting environmental levels of zinc and cadmium. *Environ Toxicol Chem* 2016;9999:1–8. © 2016 SETAC

Keywords: Transgenic zebrafish Biomonitoring Zinc Cadmium Water quality criteria

INTRODUCTION

The issue of heavy metal pollution is drawing increasing attention worldwide [1,2,3]. Excessive heavy metals present a hazard and potential risk to dynamic homeostasis, antioxidant defenses, and growth progress in humankind, animals, and plants [4,5,6,7]. For example, a certain dosage of zinc (Zn) and cadmium (Cd) results in developmental defects, including head and eye hypoplasia, cardiac edema, yolk sac deformities, hypopigmentation, altered axial curvature and rudimentary tails, and even mortality of zebrafish (*Danio rerio*) larvae [8].

Metallothionein (MT) has been regarded as one of the most remarkable biomarkers in several fish species following heavy metal exposure [9,10]. Metallothionein transcript levels in livers are positively related to the average pollution index in the muscles of wild crucian carp (*Carassius auratus auratus*). Significantly positive relationships were also found between Cd dosages and MT abundance in rare minnow [11]. Metallothionein is also a potential biomarker of acute Cd exposure in yellow catfish *Pelteobagrus fulvidraco* (*Siluriformes*) [12]. Zebrafish MT expression is reported to be significantly induced by Zn^{2+} and Cd^{2+} [13]. Recently, it was reported that ZnO nanoparticles also led to induction of MT in zebrafish embryos [14].

The biomarker potential of MT to heavy metals is attributable mainly to the special gene structure. The zebrafish metallothionein gene (*mt*) shares the same structure with analogues in vertebrates. The basic structure includes flanking sequences and untranslated regions on both ends and 3 exons and 2 introns [15]. The promoter region includes metal response elements (MREs), protein binding site AP-1, and the TATA box, responding to heavy metal exposure. Metal response elements from different *mt* genes are structurally relevant, with the same core sequence of 5′-TGCRCN-3′ (R represents purine, and N represents any nucleotides) of various copy numbers [16], resulting in the binding affinity to heavy metals.

Transgenic zebrafish are widely used to detect toxicity of heavy metals [17], endocrine disruptors [18], organic pollutants [19], and even drugs [20]. In the present study, we aimed to generate a transgenic zebrafish biosensor to detect trace amounts of aquatic heavy metals, employing enhanced green fluorescence protein fused with metallothionein promoter as the biomarker. The transgenic biosensor was designed to have relatively high sensitivity to detect low environmental levels of heavy metals. The present biosensor system is meant to determine whether the level of heavy metals exceeds the regulatory limits rather than to precisely measure the concentrations.

MATERIALS AND METHODS

Materials

Zebrafish Tübingen and eukaryotic vector pEGFP-N3 were gifted from B. Zhang at Peking University (Beijing, China). Restriction enzyme *Ase*I was obtained from New England Biolabs. *Hind*III and other biochemical reagents were from Takara. All chemicals were analytical reagents.

Zebrafish maintenance and breeding

Large-scale feeding of zebrafish was maintained in an independent breeding system from Beijing ESEN, Environ-Science. The light:dark cycle (14:10 h) was controlled with an automatic timer. The cultivation water was regulated to 500 μS/cm and pH 7.0 by NaOH and $NaHCO_3$. The temperature of both the breeding room and the water was controlled at 28 ± 0.5 °C. The breeding protocol was carried out according to *The Zebrafish Book* [21].

Embryotoxicity of heavy metals

Working concentrations of aquatic Zn and Cd solution for the acute toxicity test were designed according to the People's

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Republic of China drinking water sanitary standard [22], sewage discharge standard [23], and integrated pollutant discharge standard [24]. The Zn gradients were 1.000 mg/L, 2.000 mg/L, 5.000 mg/L, and 10.000 mg/L Zn^{2+}. Cadmium gradients were 0.005 mg/L, 0.010 mg/L, 0.100 mg/L, and 0.350 mg/L Cd^{2+}. Zinc sulfate ($ZnSO_4$) and Cd chloride (2.5 hydrates; $CdCl_2 \cdot 2.5\ H_2O$) were purchased from the China National Pharmaceutical Group (Sinopharm). Working solutions of Zn and Cd were prepared by diluting stock solutions with cultivation water. The concentrations of metals for the embryo toxicity test were analyzed using inductively coupled plasma–mass spectrometry (ICP-MS, Agilent 7700x). Germanium (Ge) and rubidium (Rh) were used as internal standards. All glassware in the present study was acid washed to avoid contamination. All measurements were repeated 3 times.

Zebrafish embryos at 3 d, 5 d, and 10 d postfertilization (dpf) in similar size and good condition were collected for 6-h, 12-h, and 24-h acute toxicity tests with working concentrations of heavy metals. Thirty embryos were randomly exposed in each group with 3 replicates. The exposure temperature was $28 \pm 0.5\ ^{\circ}C$ under a 14:10-h light:dark cycle. During and after the exposure, the development and malformations were observed under a microscope (Olympus IX2).

Reconstruction of pEGFP-zMT

Primers zMT-1 (5′-GAATTCCAGAGAGACACTGCACACG-3′) and zMT-2 (5′-CGTTCACTCAGCTTGTGAATGAGT-3′) were designed and synthesized based on the promoter sequence of the zebrafish *mt* gene (GenBank AY305851.1). A zebrafish *mt* promoter sequence was cloned via a polymerase chain reaction (PCR) thermocycler (Bio-Rad C1000 Touch) with genomic DNA of 5-dpf embryos as a template. Afterward, the site of restriction enzyme *Ase*I (ATTAAT) was introduced into the zMT-1 primer sequence and the *Hind*III site (AAGCTT) into the zMT-2 sequence. Both promoter gene and vector pEGFP-N3 were digested with restriction enzymes *Ase*I and *Hind*III following ligation of T4 ligase according to the instruction manual. The recombinant plasmid pEGFP-zMT was transformed into *Escherichia coli* DH 5α; positive clones were screened with kanamycin. The 5 clones were further tested in PCR and double digestion of the extracted plasmids.

Microinjection

Tübingen strain zebrafish embryos were collected within 30 min postfertilization, washed 3 times with E3 buffer and immediately microinjected at 1-cell stage using micro injector (Harvard Apparatus PLI-90). The plasmids pEGFP-zMT for injection contained trace amount of phenol red. All injected embryos were cultivated in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) at 28.5 °C and observed under an Olympus fluorescence microscope.

Certification of transgenic zebrafish

The transgenic zebrafish founder and the offspring were kept under constant observation to detect the development and the green fluorescent protein (GFP) phenotype. We used n-(o-tolyl)-thiourea (PTU) to inhibit the formation of cytochrome after 20 h postfertilization (hpf). The embryos were removed from their chorions with a dilute solution of pronase (2 mg/mL in E3 medium, ~1 min, 28.5°C). Embryos were mounted for better observation with application of 3% methyl cellulose. We then traced the embryo development under a bright field and GFP phenotype emitting visible green light when excited by blue light (excitation maximum = 488 nm).

To verify the integration of pEGFP-zMT at the genomic level, an elaborate PCR was designed with zMT (E)-1 as the forward primer with EGFP-2 5′-AACTCCAGCAGGACCATGTGAT-3′ as the reverse primer. The EGFP-2 reverse primer was designed based on the enhanced green fluorescent protein (EGFP) sequence (GenBank U57609). Therefore, the amplicon would cover from the foreign gene zMT promoter to the reporter gene *egfp*. The genomic DNA of 5 dpf embryos and adult zebrafish (> 3 mo) expressing GFP was extracted for the template.

Reverse transcription PCR (RT-PCR) was conducted for further verification at the transcriptomics level. Primers were designed according to the *egfp* sequence: EGFP-1, 5′-CATCCTGGTCGAGCTGGACGGC-3′; EGFP-2, 5′-AACTCCAGCAGGACCATGTGAT-3′. The total RNA was isolated with RNiso Plus, and cDNA was synthesized separately with Recombinant DNase I according to the manual.

Generation selection

The transgenic zebrafish founder was actually chimeric as a result of the essence of microinjection technology. Initially, more than 1000 embryos were injected (Figure 1A); embryos expressing GFP were sorted out (Figure 1B) under fluorescence microscope at 48 hpf. After 3 mo, the adult transgenic zebrafish founder (F0) with heritable or non-heritable GFP phenotype was backcrossed (Figure 1C) with wild zebrafish. The positive F1 generation was screened (Figure 1D) and maintained for self-

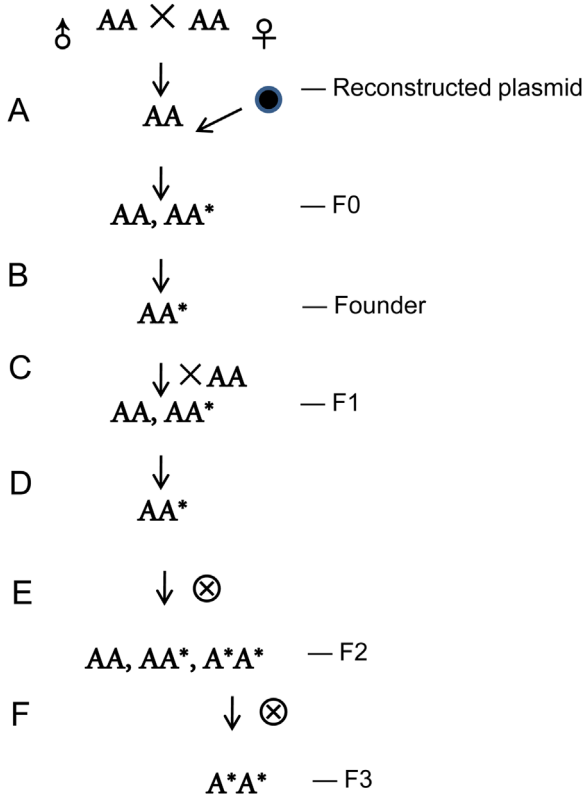

Figure 1. The crossbreeding process of transgenic zebrafish: (**A**) reconstructed plasmid (solid black circle) was injected into wild Tübingen embryos; (**B**) embryos expressing green fluorescent protein were sorted out under fluorescence microscope; (**C**) adult F0 generation was backcrossed with wild zebrafish; (**D**) positive F1 generation was screened under fluorescence microscope; (**E**) positive F1 generation with same phenotype self-crossed; (**F**) inbreeding of F2 homozygotes. F0 = transgenic zebrafish Founder, from which the positive (AA*) were selected; F1 = heritable zebrafish chimeras (AA*) emerged after backcross; F2 = homozygous zebrafish appeared after F1 self-crossing.

crossing (Figure 1E) after 3 mo. Thus, transgenic zebrafish founder was backcrossed with a wild type to acquire the F1 generation, whereas the F2 generation came from self-crossing in F1. Embryos with consensus phenotype from the same F2 zebrafish were collected as F3 (Figure 1F). As a supplementary measure, EGFP phenotype screening was adopted in a traditional crossbreeding process.

Real-time quantitative RT-PCR

Transgenic zebrafish F3 larvae (10 dpf) with coincident EGFP phenotype, similar size, and developmental synchrony were divided into 9 groups of 5 larvae each.

The larvae were exposed to aquatic heavy metal gradients for 6 h and 24 h; the 0.350 mg/L Cd ion solution was abandoned for its lethality. We washed the larvae with distilled water and isolated the total RNA immediately after the exposure was terminated. The first strand was synthesized via Reverse Transcriptase M-MLV (RNase H^-), following the instruction manual. Zebrafish β-actin gene (GenBank NM-131031) was named as reference gene with primers 5′-TCTGGTGATGGTGTGACCCA-3′ and 5′-GGTGAAGCTGTAGCCACGCT-3′. Primers of *egfp* were quoted from the previous literature [25] and GFP-1 was 5′-GACCACTACCAGCAGAACAC-3′, whereas GFP-2 was 5′-GAACTCCAGCAGGACCATG-3. Quantitative real time PCR (qRT-PCR) assays were conducted in Axygen PCR tube strips with SYBR Premix DimerEraser (DRR091S; Takara) using the relative standard curve method on a BioRad IQ5 Real-Time PCR Detection System under the optimized PCR conditions for each primer set. Initial denaturation came first at 95 °C for 5 s. Polymerase chain reaction amplifications were performed for 45 cycles with denaturation at 95 °C for 5 s, annealing at an optimum temperature of 60 °C for primers for 10 s, and extension at 72 °C at 10 s, followed by melt curve analysis. The fluorescent signal was collected at the extension phase, and data were analyzed by 2-way analysis of variance (ANOVA). Duncan's multiple range test was used to assess the differences between treatments at a significant level of 5% ($p < 0.05$).

RESULTS

Embryotoxicology test to heavy metals

The measured values of Zn and Cd in exposure solutions were in good agreement with the nominal concentrations as shown in Table 1. During and after exposure, the growth and death of embryos were observed under the microscope. The embryo death was marked when the heart stopped beating. Under the condition of 0.350 mg/L Cd^{2+}, 3-dpf embryos assumed deformities and death when exposed for 6 h, 5-dpf embryos assumed deformities without death when exposed for 12 h, and 10-dpf embryos assumed no deformities until exposure for 24 h. Low concentrations (1.000 mg/L, 2.000 mg/L Zn^{2+}; 0.005 mg/L, 0.010 mg/L Cd^{2+}) of metals had no visible effect on the phenotype (Table 2). Therefore, the traditional fish embryo toxicity test judging by paramorphia, malformation, or mortality is not practicable to check whether or not the concentrations of heavy metals exceed the environmental regulatory limits. Embryos started showing symptoms of developmental deformities—including spinal curvature, pericardial cyst, delayed development (Figure 2), and even death—when the metal concentration increased (Table 2). Statistics showed that the embryo ratio of deformities and death went up with the increase of metal concentration (data not given). Resistance to a certain concentration varied when in different developmental stages.

Table 1. Nominal and measurement concentrations of heavy metals in exposure solutions[a]

Metal	Nominal concentrations (mg/L)	Measurement concentrations (mg/L)
Zn^{2+}	1.000	0.989 ± 0.063
	2.000	1.978 ± 0.215
	5.000	5.147 ± 0.277
	10.000	9.943 ± 0.642
Cd^{2+}	0.005	0.004 ± 0.001
	0.010	0.012 ± 0.001
	0.100	0.099 ± 0.008
	0.350	0.351 ± 0.032

[a]All values indicate mean ± standard deviation of 3 replicates. The exposure solutions were diluted from a single stocking solution.

Reconstructed plasmid pEGFP-zMT

A 789 base pair (bp) PCR product was cloned and sequenced. Results using the Basic Local Alignment Search Tool (BLAST) showed the cloned promoter was 98% similar to that of zebrafish *mt* gene (AY305851.1) published in the National Center for Biotechnology Information [26], indicating that we did clone a zebrafish *mt* promoter gene (Pmt). Elaborate analysis showed that the promoter included 4 MREs, 3 protein binding sites (AP-1), and a TATA box. Based on the sequence of *mt* promoter

Table 2. Embryotoxicology test to zinc and cadmium[a]

		Control	Zn^{2+} (mg/L)				Cd^{2+} (mg/L)			
Embryo	Exposure time	0.000	1.000	2.000	5.000	10.000	0.005	0.010	0.100	0.350
3 dpf	0 h	—	—	—	—	—	—	—	—	—
	6 h	—	—	—	—	—	—	—	—	⊕ ⊙
	12 h	—	—	—	⊕	⊕ ⊙	—	—	⊕	⊕ ⊙
	24 h	—	—	—	⊕ ⊙	⊕ ⊙	—	—	⊕ ⊙	⊕ ⊙
5 dpf	0 h	—	—	—	—	—	—	—	—	—
	6 h	—	—	—	—	—	—	—	—	—
	12 h	—	—	—	⊕	⊕ ⊙	—	—	⊕	⊕
	24 h	—	—	—	⊕	⊕ ⊙	—	—	⊕ ⊙	⊕ ⊙
10 dpf	0 h	—	—	—	—	—	—	—	—	—
	6 h	—	—	—	—	—	—	—	—	—
	12 h	—	—	—	—	—	—	—	—	—
	24 h	—	—	—	—	⊕	—	—	⊕	⊕ ⊙

[a]Symbols represent malformed embroys (⊕), including spinal curvature, pericardial cyst and delayed development, or dead embroys (⊙), with stop of heartbeat as the endpoint. Thirty embryos in each group repeated 3 times.
dpf = days postfertilization.

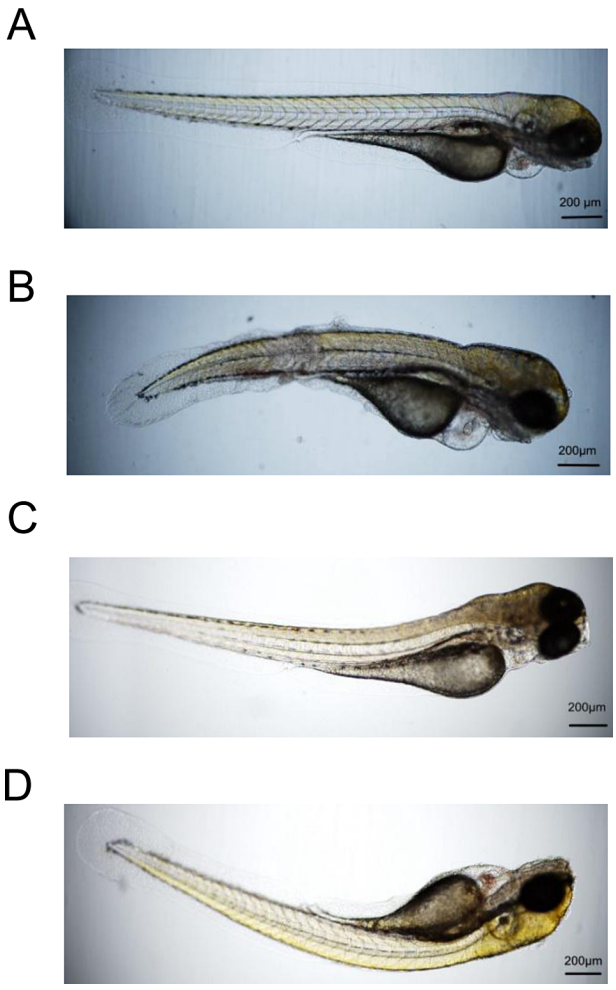

Figure 2. Symptoms of developmental deformities in toxicity test. Acute toxic assays of Zn^{2+} and Cd^{2+} on wild zebrafish embryos: (**A**) normal development; (**B**) development block; (**C**) spinal lordosis; (**D**) spinal kyphosis.

and plasmid pEGFP-N3, cleavage site *Ase*I was introduced into the 5′ end of Pmt and *Hind*III into 3′ end. Furthermore, the cytomegalovirus (CMV) promoter was deleted before Pmt DNA was subcloned, with no effect to the internal *mt* gene and *egfp* gene so that the plasmid pEGFP-zMT was reconstructed.

Both PCR and double digestion results verified the subclone of Pmt DNA in eukaryotic expression vector pEGFP-N3 with the CMV promoter deleted. Therefore, the newly reconstructed plasmid pEGFP-zMT had a sensitive *mt* promoter.

Transgenic zebrafish line

Transgenic zebrafish were certified via PCR and RT-PCR (Supplemental Data, Figure S1). After self-crossing of adult transgenic zebrafish F1 with similar phenotype traits of EGFP, F2 embryos were gathered and observed under a fluorescent microscope. The results suggested that embryos from most F1 parents expressed EGFP with various fluorescence intensity, among which some expressed the same phenotype as their parents, whereas others expressed strong EGFP or no fluorescence entirely. The 3 different GFP phenotypes corresponded to a ratio of 2:1:1, in accordance with Mendel's inheritance. It could be inferred that those with high fluorescence intensity were homozygotes.

After the crossbreeding process (Figure 1), we achieved F3 embryos with strong fluorescence. The fluorescence in the head was strong and clear as early as the somite stage, even in chorion (12 hpf; Figure 3A and B) and at the pharyngula period (24 hpf, Figure 3C and D). The fluorescence was clear around the body in the head, tail, and skin wrapped around the yolk and was much stronger as it developed (36 hpf; Figure 3E and F). The fluorescence in the eye and pericardium were also expressed strikingly (48 hpf, Figure 3G and H).

Genomic DNA was isolated from positive embryos. A 1550 bp amplicon was cloned, covering genes from Pmt to *egfp* reporter. The results demonstrated that pEGFP-zMT was indeed inserted into the zebrafish genomic DNA and replicated along with genome replication.

The RT-PCR results demonstrated the exogenous EGFP expression at the mRNA level. We extracted RNA from 5-dpf positive embryos. The first strand complementary DNA was reversely transcripted from RNA and used as template for PCR. The gel electrophoresis results, showing the clone of *egfp*, corresponded to the fluorescence microscopy findings.

EGFP expression changes of transgenic zebrafish after exposure to heavy metals

Strong and ubiquitous expression of *mt* gene is reported before hatching as a result of the maternal contribution [27]. Hence, it is difficult to judge the expression pathway of EGFP mRNA at the early development stage. The EGFP expression could be a result of the maternal contribution expression of the *mt* promoter or a result of the induction of heavy metals in the surroundings. We chose transgenic zebrafish embryos of 10 dpf for a quantitative experiment when specific expression of the *mt* promoter is dominant rather than ubiquitous. The qRT-PCR results showed that waterborne Zn above the regulatory limit (1.000 mg/L) induced a remarkable rise of EGFP messenger RNA (mRNA) in a dose-dependent manner (24-h group). When exposed to Zn concentrations above 2000 mg/L, the expression is 100 times the control (Figure 4A). Waterborne Cd at the regulatory limit (0.005 mg/L) did not induce the increase of EGFP mRNA, but Cd above the limit concentrations induced the significant expression of EGFP in a dose-dependent way (24-h group; Figure 4B). There was no significant difference of EGFP mRNA levels in all Zn concentrations in the 6-h group (Figure 4A). A significant increase of EGFP level was recorded when transgenic embryos were exposed to 0.1 mg/L Cd for 6 h (Figure 4B). No data about higher concentrations were recorded because the embryos died. When exposed for 24 h, the EGFP mRNA levels increased significantly under high concentrations of heavy metals (1.000 mg/L Zn^{2+}, 0.005mg/L Cd^{2+}). The expression was induced in a dose- and time-dependent manner.

DISCUSSION

In the present study, a recombinant plasmid pEGFP-zMT was constructed with a sensitive promoter Pmt and injected into 1-cell staged Tübingen zebrafish embryos. The obtained transgenic zebrafish were tested separately on the levels of genomics, transcriptomics, and phenotypes. Thus, we obtained the transgenic zebrafish founder pMT-Tü.

Embryotoxicology test

According to the People's Republic of China drinking water sanitary standard (GB 5749-2006) [22], total Cd dosage in drinking water should be less than 0.005 mg/L and total Zn dosage 1.0 mg/L. The urban sewage treatment plant emissions standards (GB 18918-2002) [23] state that the highest permissible effluent concentrations of total Cd and Zn are 0.01 mg/L and 1.0 mg/L, respectively, in urban sewage emissions; the integrated wastewater discharge standard (GB

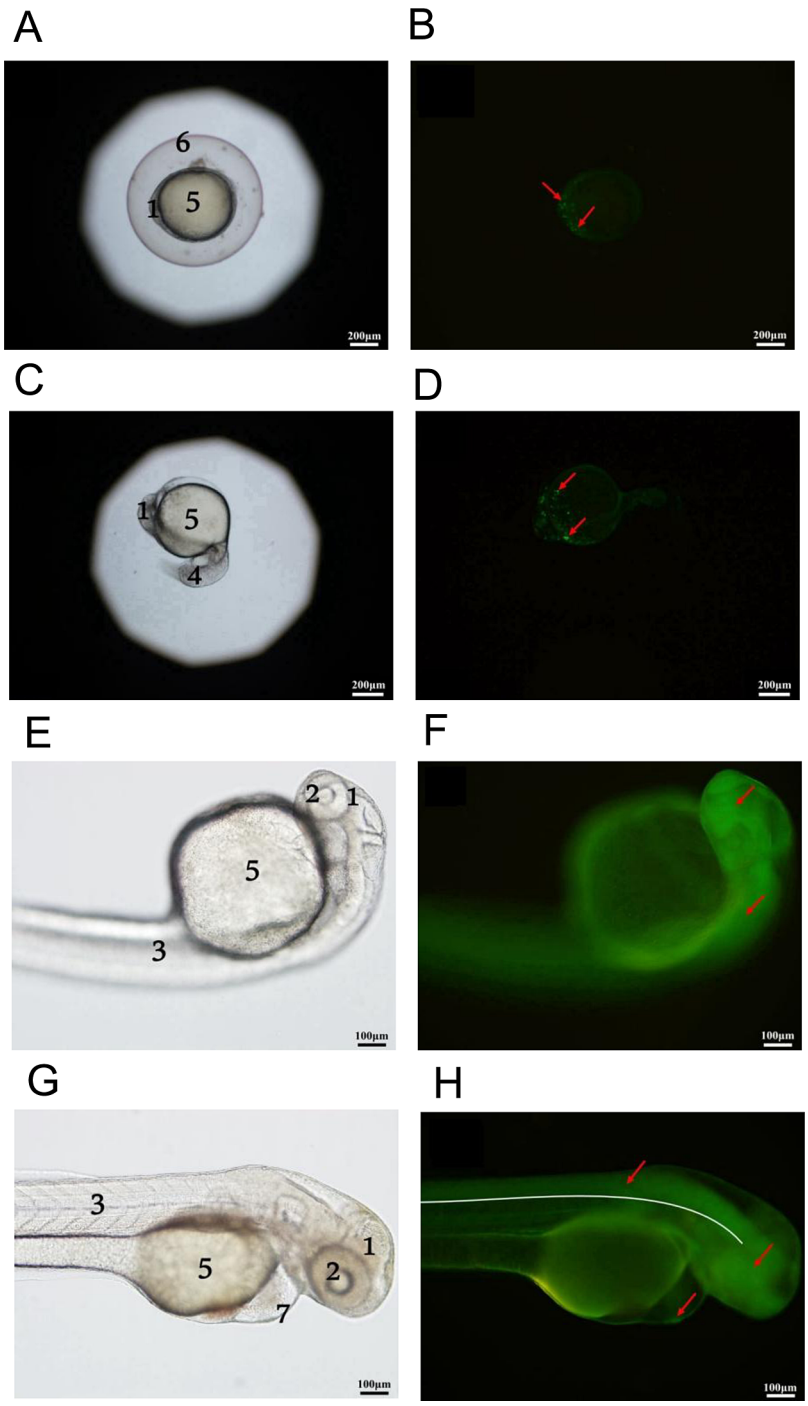

Figure 3. Green fluorescent protein (GFP) phenotypes of *mt:egfp* transgenic zebrafish: (**A,B**) embryos 12 h postfertilization (hpf); (**C,D**) embryos 24 hpf; (**E,F**) embryos 36 hpf; (**G,H**) embryos 48 hpf. Panels show the fluorescence images under blue light (**B, D, F**, and **H**) and the corresponding bright field images (**A, C, E**, and **G**). The white line represents craniocaudal axis. The red arrowhead represents green fluorescent protein. Arabic numerals represent main organs in zebrafish. 1 = head; 2 = eye: 3 = trunk; 4 = tail; 5 = yolk; 6 = chorion; 7 = pericardium.

8978-1996) [24] specifies the highest permissible effluent concentration of total Cd is 0.1 mg/L, and the 3 grades of highest permissible effluent concentrations for total Zn are 2.0 mg/L, 5.0 mg/L, and 5.0 mg/L, respectively. All of the above stands were considered when designing the heavy metal concentrations in the present study.

Prehatched zebrafish embryos (1 dpf) are more resistant to Cd exposure than are posthatched larvae [8,28]; thus, we employed fully hatched larvae after 2 dpf for the exposure test rather than start the exposure immediately on freshly fertilized eggs. Furthermore, zebrafish treated in a later stage experience more severe morphological effects [29]; thus, larvae 3 dpf to 10 dpf were tested instead of freshly hatched embryos. Trunk abnormality is a predominant malformation in the present study, which is in agreement with previous reports. Spinal curvature is commonly observed after fish larvae are exposed to heavy metals [30,31]. Cheng et al. [8] attributed the spinal deformities to reduced formation of myosin heavy chain and myotome disorganization in the somites. Zhang et al. [32] hypothesized that the spinal curvature is related to the muscle or skeleton based on the broken, disorganized, and loosened array of muscle fibers. In addition, we found a high dosage of Cd and Zn blocked

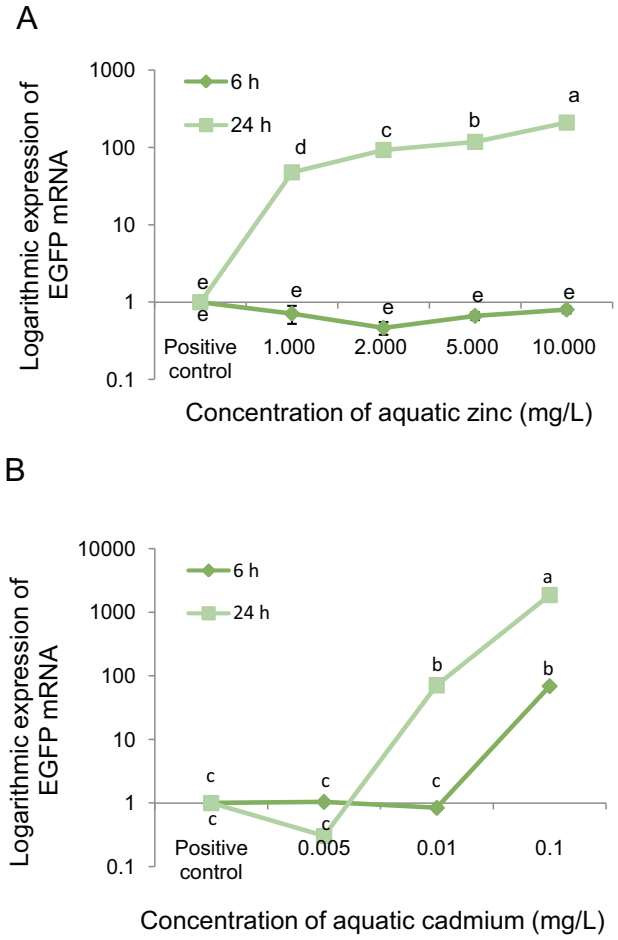

Figure 4. Enhanced green fluorescent protein (EGFP) messenger RNA (mRNA) levels of F3 embryos after exposure to heavy metals. Green fluorescent protein (GFP) expression in transgenic zebrafish F3 embryos after exposure to aquatic zinc and cadmium for 6 h and 24 h. (**A**) GFP mRNA level of transgenic embryos exposed to aquatic zinc solutions for 6 h and 24 h; (**B**) GFP mRNA level of transgenic embryos exposed to aquatic cadmium solutions for 6 h and 24 h. The positive control was transgenic zebrafish exposed without heavy metals. Results are expressed as the mean of 3 replicates ± standard deviation ($n = 5$). The mean value with different letters denotes significant difference at $p < 0.05$.

the development of transgenic larvae, which has been observed in other organisms including *Hydroides elegans* [33] and *Xenopus laevis* [34].

All embryos (3 dpf, 5 dpf, and 10 dpf) developed normally when the heavy metal concentrations were twice the regulatory limits, indicating that heavy metal concentrations within a certain range above the regulatory limits led to no malformations or mortality of embryos. According to Zhang et al. [32], however, even lower concentrations of Cd will increase the embryo mortality rate over an extended period of exposure time. These data indicate that heavy metals under some limits will damage organisms, although without obvious paramorphia. More seriously, joint treatment will increase the toxicity of Cd [35]. Thus quick, accurate judgment of aquatic heavy metal concentrations is difficult to achieve solely based on the various toxicological endpoints in acute toxicity tests. The toxic effects of molecular levels induced by trace amounts of heavy metals might require a longer time to be reflected on phenotypes. It is necessary to build a real-time biosensor system to monitor environmental heavy metals, especially at low concentrations. Because zebrafish have been a popular model in monitoring environmental contaminations and investigating ecotoxicology [36], we generated the *mt:egfp* transgenic zebrafish biosensor to determine whether the concentration of aquatic heavy metals exceed water quality standard limits.

EGFP expression of transgenic zebrafish after exposure to heavy metals

Waterborne Cd and Zn distribute quickly and induce an increase in the MT transcript abundance in many species' tissues. These elevated levels are kept for at least hours [12], resulting in a cumulative effect. This explains the time-dependent effect of aquatic Cd and Zn on EGFP, which is a theoretical foundation of our transgenic zebrafish biosensor, making the fluoresce expression sensitive and persistent.

A zebrafish embryo assay suggests that either Zn^{2+} or Cd^{2+} will bind to the metal-responsive transcription factor (MTF-1); the MTF-1 is then activated, transferred from cytoplasm to cell nucleus, and located on the metal response elements in the *mt* promoter. Thus the *mt* gene expression will be induced [37]. The MTF-1 is an 80-kD conservative protein including 6 Cys2-His2 Zn finger domains and other transactivation domains such as acid box domain, proline domain, and serine/threonine domain [38]. Many documents demonstrate that the MTF-1 plays a vital role in the metal-induction to the *mt* gene. Yet another view holds that Zn induces the formation of a co-activator complex, which contains MTF-1 and histone acetyltransferase p300; the latter plays an elementary role in activating the MT-I transcription [39]. Another recorded pathway is that heavy metals will lead to the dissociation of histone deacetylase (HDAC1) from the *mt* promoter, causing the histone hyper-acetylation and activation of MTF-1, which binds to MRE and induces the expression [40].

As indicated in the embryo toxicity test, 10-dpf embryos showed no visible abnormalities exposed even to heavy metal concentrations sufficiently higher than the regulatory limits. However, the transgenic zebrafish line in the present study had very high sensitivity to even environmentally relevant concentrations of aquatic Zn and Cd. The regulatory limit of aquatic Zn is 1.000 mg/L and is 0.005 mg/L for Cd. The EGFP mRNA levels increased remarkably when *mt:egfp* transgenic embryos were exposed to heavy metals above the regulatory limits for 24 h. Accordingly, the fluorescent intensity increased under a fluorescence microscope. Compared with metallothionein, using mRNA as a biomarker for metal detection, the *mt:egfp* transgenic zebrafish do not require the operator to have a background in molecular biology. The EGFP expression will be obtained directly under a microscope rather than complicated experimental technology. Therefore, transgenic biosensor is a sensitive and user-friendly method to check whether the concentrations of Zn or Cd exceed the regulatory limits. The present study's quantitative experiment showed that the GFP mRNA levels depended on the dose of Zn and Cd, making the transgenic zebrafish a potential biosensor for detecting waterborne Zn and Cd above the environmental standard limits.

Much literature has reported the EGFP-based biosensor in vivo. Fini et al. [41] generated a fountain flow cytometry based on EGFP transgenic *Xenopus laevis* tadpoles. The fountain flow cytometry system combines rapidity and in vivo measurements. However, the LED-illuminated fountain flow cytometry system is designed mainly for professionals because of its complex operation. Furthermore, the fountain flow cytometry system adapts to tadpoles. The amphibian will develop quickly into a large frog and fail to meet the requirements of the system, which

is against animal welfare and the 3Rs principle (reduction, refinement, and replacement). The present transgenic zebrafish biosensor overcame these disadvantages. First, the small size of adult zebrafish (4–5 cm) makes the *mt:egfp* transgenic biosensor available across the zebrafish's entire life span. Second, zebrafish are easily maintained as pet fish. With further improvement, the biosensor therefore promises to be an ornamental fish detector of heavy metals in residents' homes.

CONCLUSION

We generated *mt:egfp* transgenic zebrafish as a fluorescent biosensor in vivo with the *mt* promoter sensitive to environmental concentrations of Zn and Cd. Research data indicated the transgenic zebrafish F3 embryos showed dose- and time-dependent sensitivity to Cd and Zn. In particular, EGFP mRNA levels increased remarkably when exposed to heavy metals above the standard limits. The biosensor system is designed to determine whether the concentration of heavy metals exceeds the regulatory limits rather than to precisely measure the levels of Zn and Cd in environmental water. The results suggest that our transgenic zebrafish is a highly sensitive biosensor for detecting aquatic heavy metals in a cost-effective and user-friendly way.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3362.

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Data availability—Readers interested in receiving data should contact yanyanchun@caas.cn.

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