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Methyl Mercury Suppresses the Formation of the Tail Primordium in Developing Zebrafish Embryos

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The objective of this study was to characterize the mechanisms of action of the model environmental toxicant methyl mercury (MeHg) in the zebrafish embryo. Zebrafish embryos were exposed to MeHg, and the effective concentration and window of exposure were determined in wild-type and fluorescent reporter transgenic zebrafish embryos. Genes were systematically assessed for altered expression in response to MeHg by *in situ* hybridization. MeHg impairs development of the fin fold and the tail fin primordium. Alterations in transgene expression were noted at 6 μg/l MeHg, making this shh:gfp line the most sensitive biosensor of MeHg exposure. The matrix metalloproteases *mmp9* and *mmp13* and eight other genes are induced in the embryonic tail in response to MeHg. Our data suggest that MeHg impairs tail development at least partially by activation of the tissue remodeling proteases Mmp9 and Mmp13.

Key Words: **zebrafish; methyl mercury; mmp9; mmp13; caudal fin primordium; fin fold; biosensor genes.**

The teratogenic and neurotoxic effects of methyl mercury (MeHg) became sadly evident after large-scale poisonings in Japan and Iraq in the last century (Ekino *et al*., 2007). In the environment, inorganic mercury is rapidly transformed into methyl mercury, which then accumulates in the food chain. Populations with a high seafood consumption are at particular risk (Myers *et al*., 2007). As recognized in follow-up studies of the poisonings in Japan and Iraq, methyl mercury can act as a neurotoxicant in humans. Studies of animal models suggested that low level of MeHg affects specifically the motor and sensory systems (for review, see Ekino *et al*., 2007). Large-scale, long-term cohort studies of the effects on low-level intake of MeHg via the food chain were conducted in New Zealand, on the Seychelles and the Faroe islands (Davidson *et al*., 2006; Grandjean and Landrigan, 2006). The U.S. National Academy of Science—upon careful review of these and other studies—concluded that prenatal exposure to low levels of MeHg causes behavioral deficits (Grandjean and Landrigan, 2006, and references therein).

Long-term studies of human exposure represent one approach to assess the health risk emanating from low levels of MeHg. Other strategies relied on studies of animal models to understand the mechanisms how MeHg can affect development. Zebrafish embryos may not only be ecotoxicologically relevant models but may also aid in the elucidation of the molecular mechanism underlying the effects of low-level MeHg exposure in humans (Yang *et al*., 2009 and references therein). Zebrafish embryos develop completely outside the mother and were previously shown to be highly sensitive to MeHg exposure (Samson and Shenker, 2000; Yang *et al*., 2007), thereby providing a vertebrate model whose development is not complicated by the physiology of the mother as in mammals (Kimmel *et al*., 1995). Exposure of zebrafish embryos at levels between 20 and 30 μg/l MeHg led to characteristic flexures of the axis, impaired development of the fin fold, and caused embryonic or early larval death presumably due to cardiac malfunction (Samson *et al*., 2001). Doses between 10 and 20 μg/l were previously reported to show a delayed mortality syndrome and behavioral deficits such as impaired swimming activity and prey-capture performance (Samson *et al*., 2001). Morphological and behavioral end points are frequently not very informative with respect to underlying mechanisms of the toxic effect. In a toxicogenomic study using zebrafish embryos, we have identified 79 genes that were significantly upregulated in zebrafish embryos exposed to MeHg (Yang *et al*., 2007). Toxicants such as cadmium ($CdCl_2$), lead ($PbCl_2$), or arsenic (As_2O_3) showed related but distinct patterns of gene activation.

In this and previous studies (Samson and Shenker, 2000; Yang *et al*., 2007), it was noted that MeHg effects the development of the median fin fold. These effects appeared reminiscent of the phenotypes of mutant zebrafish with an impairment of the sonic hedgehog (Shh) signaling pathway (Hadzhiev *et al*., 2007). During larval stages, the tail develops from a primordium located in the ventral fin fold, a region that

coincides with a gap in the melanophore streak located immediately anterior to the posterior tip of the notochord. This region is fortuitously marked by expression of a transgene harboring upstream and downstream sequences of the *shh* gene of zebrafish (Ertzer *et al.*, 2007; Shkumatava *et al.*, 2004). The transgene does not reflect, however, the reactivity of the endogenous *shh* gene but presumably represents rather the response of an unknown locus, into which the transgene has integrated in the *Tg(-2.4shh:gfpABC line #15)* (Hadzhiev *et al.*, 2007). In mutants impairing the activity of Shh signal transduction, both the gap in the melanophore streak as well as transgene expression are abolished (Hadzhiev *et al.*, 2007). The effect of MeHg on the integrity of the fin fold and the potential involvement of the Shh pathway prompted us to investigate the fin primordium in MeHg-treated embryos in detail.

MATERIALS AND METHODS

Fish maintenance. The *Tg(-2.4shh:gfp:ABC#15)* transgenic line contains the 2.4-kb *shh* promoter upstream of and the elements ar-A, ar-B, and ar-C of the *shh* introns 1 and 2 downstream of the *gfp* coding sequence as described (Ertzer *et al.*, 2007; Hadzhiev *et al.*, 2007; Müller *et al.*, 1999, 2000). The *Tg(-2.4shh:gfp:ABC#15)* line and wild-type zebrafish strains AB, ABO, and Tübingen were maintained on a 14 h/10 h light-dark cycle at 28.5°C in recirculization systems (Schwarz Ltd, Germany, and Müller and Pfleger Ltd, Germany) and fed commercial food and in-house hatched brine shrimp as described (Westerfield, 1993). Zebrafish embryos were obtained from spawnings of wild-type or identified *Tg(-2.4shh:gfp:ABC#15)* fish in spawning cages (Westerfield, 1993).

Chemical treatments. MeHg (methyl mercury chloride; CH_3ClHg), lead (II) chloride ($PbCl_2$), arsenic (III) oxide (As_2O_3), and cadmium chloride ($CdCl_2$) were purchased from Sigma-Aldrich (St Louis, MO). Stock solution of 100 mg/l MeHg was prepared in water and working solutions (6–120 µg/l) were obtained by diluting the stock solution in embryo medium (60 µg/ml; Instant Ocean, Red Sea, Houston, TX, pH 6.73, Ca 0.8 mg/l; K 0.6 mg/l; Mg 2 mg/l; Na 16 mg/l; S 2 mg/l [Westerfield, 1993]) to the desired concentrations. Working solutions of 20 mg/l $PbCl_2$, 79 mg/l As_2O_3, and 15 mg/l $CdCl_2$ in embryo medium were prepared in the same way from 5, 10, and 4 g/l stock solutions in fish water, respectively. As described previously (Yang *et al.*, 2007), toxicant concentrations were chosen so that more than 80% treated embryos showed malformation but that embryo mortality was minimal.

Unless otherwise stated, embryos were exposed to chemicals from 4 to 72 hours postfertilization (hpf). A control group under the same conditions as the treatment groups but with the embryos reared in embryo medium only was included in all experiments. Embryos were anesthetized with 0.02% tricaine (3-aminobenzoic acid ethyl ester) for documentation with a stereomicroscope (Leica MZ 16F) or a compound microscope (Leica DM 5000 B).

In situ hybridization and antibody labeling. The following genes were selected with specific expression in the tail primordium by an RNA *in situ* hybridization screen. The cDNA of the *sequestosome 1* (*sqtm1*) gene (accession number NM_213173) was obtained by screening a cDNA library, derived from a mix of mRNA from 16, 24, and 36 hpf zebrafish embryos, with a radioactive oligonucleotide probe (5′-GGATCAAGATGTCTCAACAAGTTTC-3′). The digoxigenin (DIG)-labeled antisense RNA of *sqtm1* was synthesized by digestion with *Xho*I followed by transcription with T3 polymerase using standard procedures (Oxtoby and Jowett, 1993; Strähle *et al.*, 1994). Genes for *NM_213361* (accession number NM_213361), *serum/glucocorticoid regulated kinase 1* (*sgk1*) (accession number BC052134), AW115990 (accession number AW115990), and BM101698 (accession number BM101698) were purchased as I.M.A.G.E. clones from ImaGenes (Germany). Antisense probes of *NM_213361*, *sgk1*, *AW115990*, and *BM101698* were generated by PCR using primers 5′-CCGGATCCGGTGGTGCAAATCAAAGAACTGCTCCT-CAGTG-3′ and 5′-CAGAGATGCATAATACGACTCACTATAGGGAGA-GACAAACCACAA CTAGAATG-3′ and DIG-labeled RNA was then transcribed by T7 polymerase from the PCR fragment. The antisense RNA probes of *mmp9* (NM_213123), *mmp13* (NM_201503), *l-plastin* (NM_131320), *myeloid-specific peroxidase* (*mpx*, NM_212779), and *apolipoprotein-E* (NM_131098) were transcribed with T7 and Sp6 RNA polymerase (Table 1). Further details are available upon request.

Embryos were grown in embryo medium or in chemical solution containing 0.003% phenyl-thiourea to prevent the formation of pigment at 28.5°C. Control and treated embryos were fixed in 4% paraformaldehyde overnight at 4°C and then stored in 100% methanol at −20°C. The *In situ* hybridization and antibody labeling of caspase3 (Research & Diagnostics Systems, Inc., USA) were performed according to the published protocols (Oxtoby and Jowett, 1993; Strähle *et al.*, 1994). For double RNA, labeling probes were labeled with either DIG (Roche) or DNP-11-UTP (PerkinElmer). These two probes were detected by anti-Dig POD (Roche) and anti-DNP-HRP (PerkinElmer) sequentially and the staining was developed following the manuals of the tyramide signal amplification (PerkinElmer) plus Cyanine 3 and Cyanine 5 kits (PerkinElmer). Once developed, the embryos were dissected from the yolk and mounted in 70% glycerol for whole-mount imaging or embedded in Aqua Polymount (Polyscience) followed by analysis with a Leica SP2 confocal microscope. For sectioning, stained embryos were embedded in 3% agarose, and 20-µm sections were cut with a vibratome.

For detection of apoptotic cells, 72 hpf embryos were incubated in 0.5 µl/ml of AO (acridine orange hydrochloride, Sigma) in embryo medium for 1 h. After staining, AO was removed by washing the embryos with embryo medium three times, 5 min each.

Real-time PCR. Three independent total RNAs were extracted from each of the 6, 30, 60 µg/l MeHg-treated, and control embryos according to the procedures provided by the RNeasy Mini Kit (Qiagen). The reverse transcription of cDNA was performed with 1 µg total RNA following standard procedures (Sambrook *et al.*, 1989). Real-time PCR of each sample including three biological repeats and two technical replications was carried out following the supplier's instructions (Applied Biosystem) by using SYBR Green and the comparative *Ct* method. The raw data were processed and analyzed with the Excel software. The mRNA levels were calculated using $2^{-\Delta\Delta Ct}$.

Morpholino knockdown. The p53 antisense morpholino oligonucleotide (GACCTCCTCTCCACTAAACTACGAT) synthesized by GeneTools Ltd (Ekker and Larson, 2001) was resuspended in water at 0.5mM in 0.1% phenol red. The morpholino was injected into the yolk of one- to two-cell–stage embryos using a gas-driven microinjector (Müller *et al.*, 2000).

Mmp inhibitor experiments. Zebrafish embryos were treated with 0.1mM GM6001 (general Mmp inhibitor; Calbiochem), 1% DMSO, 100 µg/l MeHg or 0.1mM Mmp Inhibitor II (Mmp9- and Mmp13-specific inhibitor; Calbiochem), 1% DMSO, and 100 µg/l MeHg in embryo medium. Controls were exposed to 100 µg/l MeHg and 1% DMSO in embryo medium without the Mmp inhibitors. All the treatments were conducted from 4 to 72 hpf. At 72 hpf, MeHg and the Mmp inhibitors were removed by washing the embryos with embryo medium three times, 5 min each. Photos of the tail region of the embryos were captured by a CCD camera connected to a stereomicroscope, and the area of the fin fold (Supplementary fig. 5) was calculated by the software ImageJ (Abramoff *et al.*, 2004). One-way ANOVA followed by Bonferroni's or Dunnett's multiple comparison tests have been performed by comparing the various treatment groups.

RESULTS

MeHg Impairs Formation of the Fin Fold and Abolishes the Tail Fin Primordium

During previous studies (Samson and Shenker, 2000; Yang *et al.*, 2007), it was noted that exposure of embryos to MeHg resulted in

TABLE 1
Information of the Probes for In Situ Hybridization

Gene name	Accession number	Primers	Vector	Restriction enzyme	Transcriptase
apolipoprotein-E (apoe)	NM_131098	F: tctggcagtatgtgtctgaactc R: aatagcctccaagacagacaag	pGEM-T Easy	*Sal*I	T7
E74-like factor 3 (elf3)	XM_684268	F: gagcgaatctgaacgtcacc R: gccggaaggagctctctgta	pGEM-T Easy	*Sac*II	SP6
l-plastin	NM_131320	F: gccaaggtggttcatgactt R: ccagtcgatgtcctggtttt	pGEM-T Easy	*Sac*I	T7
mmp13	NM_201503	F:ggcacgagaaacatgaagacc R:cacacacaaatgattttcaagga	pCS2+	*Eco*RI	T7
mmp9	NM_213123	F:cagcaacactgcttgcactt R:aggaaacccatcatgaccaa	pCS2+	*Bam*HI	T7
Mpx	NM_212779	F: ctgcccatggttagactggt R: aagacgttcgcaatggtagg	pGEM-T Easy	*Sac*II	SP6
peroxiredoxin 1 (prdx1)	NM_001013471	F: gtatcgcgagacttgagcac R: gcattaattcacatcacttctgtc	pGEM-T Easy	*Sal*I	T7
Sequestosome 1 (sqtm1)	NM_213173	ggatcaagatgtctcaacaagtttc	pBlueScript II SK(+)	*Xho*I	T3
serum/glucocorticoid regulated kinase 1 (sgk1)	BC052134	F:ccggatccggtggtgcaaatc aaagaactgctcctcagtg R:cagagatgcataatacgactca ctatagggagagacaaaccaca actagaatg	pME18S-FL3	Nil	T7
AW115990	AW115990	F:ccggatccggtggtgcaaatcaaa gaactgctcctcagtg R:cagagatgcataatacgactcacta tagggagagacaaaccacaact agaatg	pME18S-FL3	Nil	T7
BM101698	BM101698	F:ccggatccggtggtgcaaatcaa agaactgctcctcagtg R:cagagatgcataatacgactca ctatagggagagacaaaccaca actagaatg	pME18S-FL3	Nil	T7
NM_213361	NM_213361	F:ccggatccggtggtgcaaatcaa agaactgctcctcagtg R:cagagatgcataatacgactcac tatagggagagacaaaccaca actagaatg	pME18S-FL3	Nil	T7

a malformed tail fin fold (Figs. 1A and 1B). Instead of forming a clear, thin and fan-shaped membrane as in the controls (Fig. 1A), embryos treated with 60 μg/l MeHg from 4 to 72 hpf had a smaller and less well-structured fold surrounding the tail (Fig. 1B).

These defects in the fin fold were strikingly correlated with an altered pattern of pigment distribution. At 72 hpf (Fig. 1C), the melanophores form two rows above and below the tail at the boundary to the fin folds. The ventral row is characterized by a gap in the row of melanophores slightly anterior to the tip of the tail (Fig. 1A). This gap was previously shown to correlate with a region which functions as the primordium of the tail fin and which is dependent on Shh signaling (Hadzhiev *et al.*, 2007). This gap was missing in 72 hpf embryos exposed to 60 μg/l MeHg from 4 to 72 hpf (Figs. 1A–D) as in embryos with defects in the Shh signaling pathway (Hadzhiev *et al.*, 2007). MeHg (60 μg/l) caused, in addition, pericardial edema, and the head and eyes were smaller as previously reported (Samson and Shenker, 2000, 2001; Yang *et al.*, 2007).

Expression of the green fluorescent protein (GFP) from the transgene *Tg(-2.4shh:gfpABC)* marks the embryonic primordium of the tail fin that will grow out much later during larval stages (Hadzhiev *et al.*, 2007). We tested whether treatment with MeHg impairs expression of this early marker of the tail fin primordium. Transgenic embryos were exposed to 60 μg/l MeHg from 4 to 72 hpf. A high proportion of embryos (86%, $n = 5, 20$ embryos each) showed a complete loss of expression of GFP in the tail fin primordium (Figs. 1E and 1F). This correlated with loss or reduction of the gap in pigmentation (Figs. 1G and H). Expression of the transgene in the floor plate or in the pectoral fins was not affected by the MeHg treatment, indicating that MeHg specifically impaired GFP expression in the tail fin primordium.

The Effect on the Tail Fin Primordium Is Specific for MeHg

We next wished to test whether other chemicals such as $CdCl_2$, As_2O_3, or $PbCl_2$ with related toxicogenomic response profiles (Yang *et al.*, 2007) could also induce this malformation.

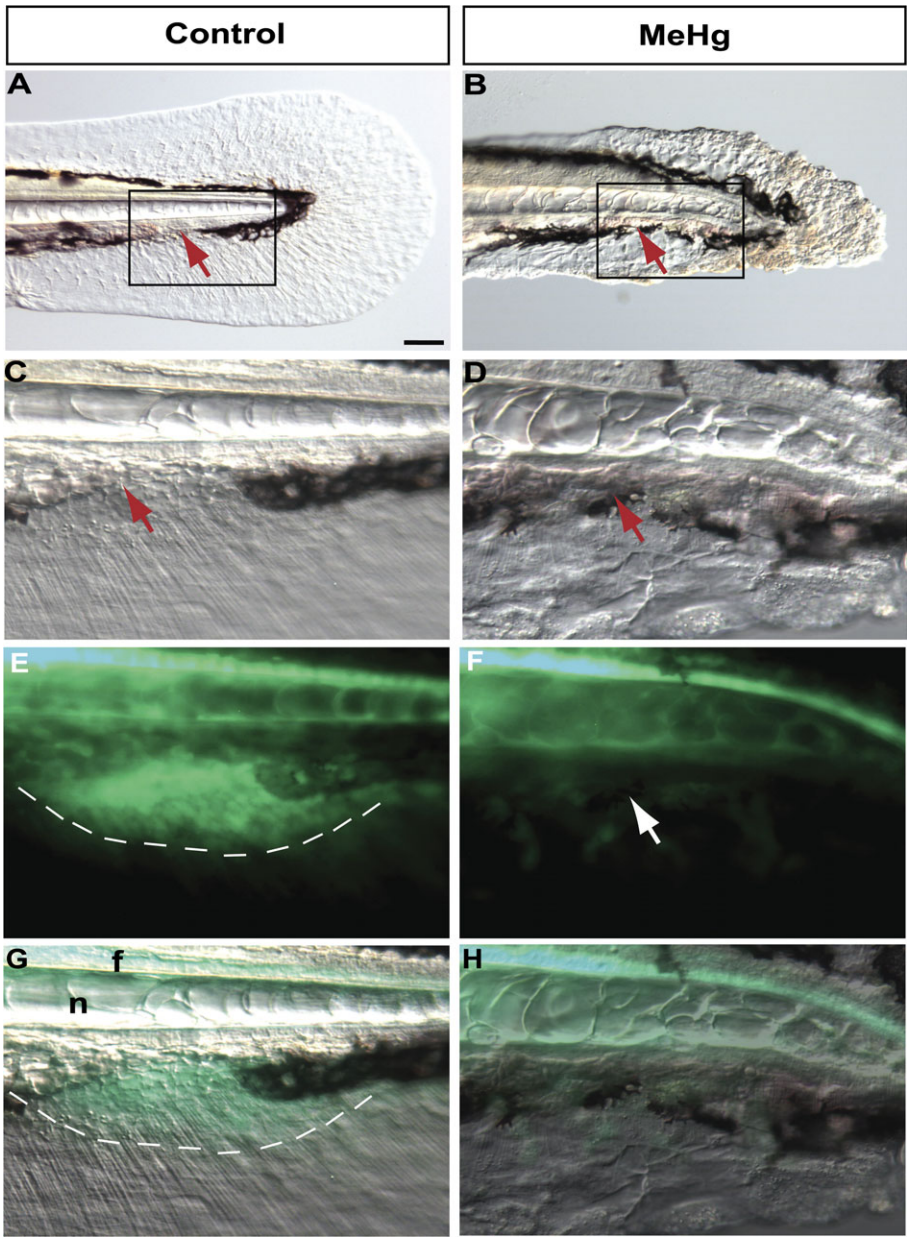

FIG. 1. MeHg treatment causes tail fin fold defects and abolishes the tail fin primordium. (A–D) Differential interference contrast optic (DIC) views of the tails of 72 hpf embryos treated with embryo medium (control) or with 60 µg/l MeHg (MeHg) from 4 to 72 hpf. In untreated embryos, the pigment cells form two streaks of melanophores with one above and one below the tail at the boundary to the fin folds. The ventral streak is characterized by a gap in the row of pigment cells (arrow) slightly anterior to the tip of the tail. This region represents the primordium of the tail fin that will develop during larval stages. The gap in pigmentation (arrow) was missing at high frequency in embryos treated with MeHg (B). These embryos have also malformed fin folds. Panels C and D show high magnification views of the regions boxed in panels (A) and (B). Anterior, left and, dorsal up. Scale bar: 25 µm (A, B); 50 µm (C–F). (E and F) Fluorescence images outline the tail fin primordium. This region is marked by GFP expression in the *Tg(-2.4shh:gfpABC #15)* transgenic line (E, dashed line). This domain of GFP expression is missing in MeHg-treated embryos (arrow, F). (G, H) Overlay of DIC views of panels (E) and (F), respectively. Anterior, left, and dorsal up. n, notochord; f, floor plate.

We exposed embryos to water (Fig. 2A, control), to 60 µg/l methyl mercury (Fig. 2B), to 20 mg/l $PbCl_2$ (Fig. 2C), to 79 mg/l As_2O_3 (Fig. 2D), or to 15 mg/l $CdCl_2$ (Fig. 2E) from 4 to 72 hpf. At the same or lower concentrations, all four compounds induced a profound toxicogenomic response (Yang *et al.*, 2007). Embryos were examined at 72 hpf for defects in fin fold formation and whether the gap in the ventral streak of pigment cells (indicative of the tail fin primordium) is present. Neither $PbCl_2$ nor $CdCl_2$ nor As_2O_3 treatment led to defects in the fin folds or abolished the gap in pigmentation (Figs. 2A–E). These data suggest that, within the substances tested, the defect in the tail primordium is specific to treatment with MeHg.

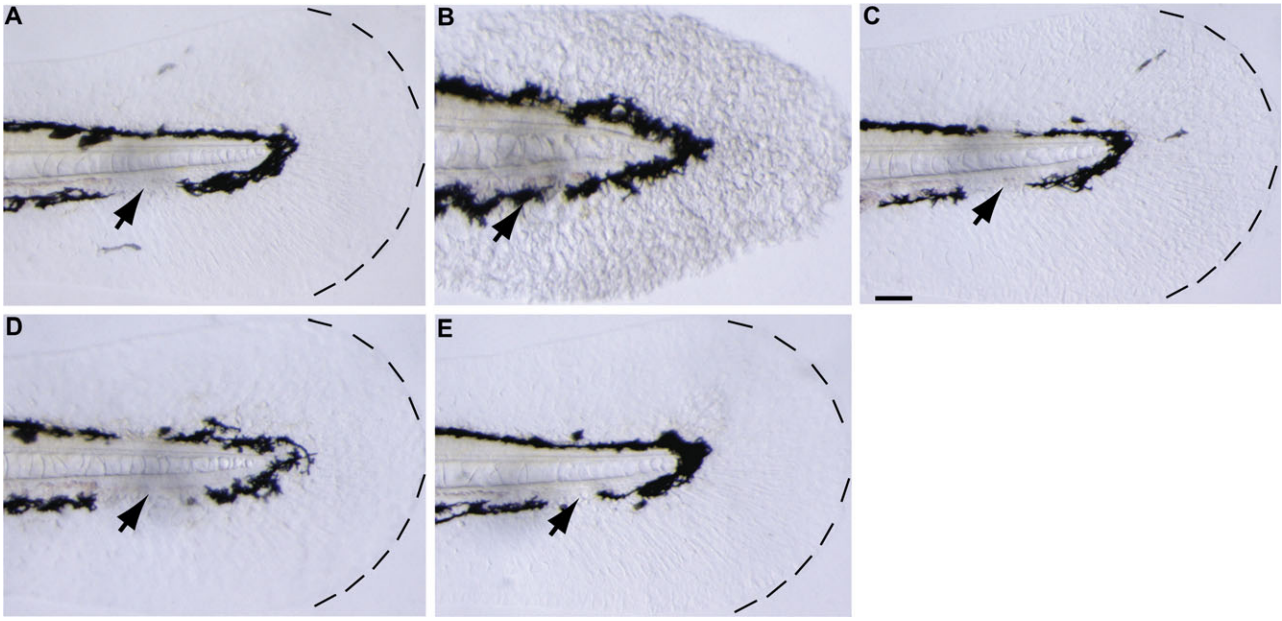

FIG. 2. Loss of the melanophore gap is specific to MeHg treatment. Embryo medium control (A) and 60 µg/l MeHg- (B), 20 mg/l $PbCl_2$- (C), 79 mg/l As_2O_3- (D), and 15 mg/l $CdCl_2$ (E)-treated 72 hpf embryos. Only MeHg-treated embryos lack the gap in the ventral melanophore streak (arrow), suggesting that impairment of the development of the tail fin primordium is specific to MeHg. Lateral views of the tails with dorsal up and anterior to the left. Scale bar: 50 µm.

Next, we asked whether MeHg treatment would induce apoptosis. Both acridine orange (Darzynkiewicz *et al*., 1992) and immunohistochemical staining of caspase3 (Cohen, 1997) were utilized to detect apoptotic cells. The number of cells undergoing programmed cell death was slightly increased in MeHg embryos in comparison to controls (Supplementary fig. 1A–D″). We also tested whether we could prevent the defects in the tail fin primordium by blocking apoptosis. To this end, we injected a morpholino that blocks translation of p53 (Robu *et al*., 2007). The concentration of injected Mo-p53 used blocks effectively the unspecific apoptotic effects of morpholinos in the brain (data not shown, Robu *et al*., 2007). This Mo-p53 concentration could, however, not abolish the defect in the fin fold (Supplementary fig. 1E) underscoring the notion that cell death is not the primary or only cause of the defects in the tail of MeHg-treated embryos.

Concentrations as Low as 6 µg/l MeHg Cause Defects in the Tail Fin Primordium

Next, we determined the effective dose of MeHg. Transgenic embryos were exposed and the size of the domain expressing GFP in the tail fin primordium was scored. Two classes of effects were observed (Fig. 3A–C): a reduction of the GFP expression domain (type I, Fig. 3B) and a complete loss of the GFP expression in the fin fold (type II, Fig. 3C). Reduced GFP expression was noted in 10% embryos exposed to 6 µg/l MeHg from 4 to 72 hpf (Fig. 3D). More than 80% of embryos showed a reduction of the GFP domain (type I), when 20 µg/l MeHg was administered (Fig. 3D). Bathing embryos in 30 µg/l caused defects in all exposed embryos and an increase to slightly more than 50% embryos with type II defects (Fig. 3D). More than 80% of embryos exposed to 60 µg/l MeHg showed the complete loss of transgene expression in the tail fin primordium (Fig. 3D).

A 6-h Exposure Window Any Time between 12 and 48 h Is Sufficient to Induce the Tail Defects

We next asked when MeHg exposure during development will affect the formation of the melanophore gap. Embryos were exposed to 60 µg/l MeHg for 6- or 12-h periods in the first 2 days of development. Treatment from 4 to 12 h of development during blastula, gastrula, and early segmentation stages did not significantly effect the formation of the melanophore gap in comparison to untreated controls (Fig. 4). In contrast, all intervals of treatment in the next 1.5 days lead to loss of the gap in melanophore distribution (Fig. 4). Moreover, a 6-h period of exposure at any time during this phase is sufficient to induce the defect. Exposure of embryos after 48 hpf did not elicit any significant effect anymore, thus limiting the sensitive phase to 48 hpf. This suggests that the most sensitive phase coincides with a period, when tail fin development has been initiated by emergence of the tail fin primordium, but prior to the apparent outgrowth of the tail fin.

MeHg Treatment Causes Ectopic Expression of Matrix Metalloproteases mmp9 and mmp13 in the Embryonic Tail

In previous studies assessing the toxicogenomic responses to MeHg (Yang *et al*., 2007), we noted that the mRNAs of the metalloproteases *mmp9* and *mmp13* were strongly upregulated in response to MeHg treatment in whole-embryo extracts. This prompted us to assess whether the expression of *mmp9* (NM_213123) and *mmp13* (NM_201503) mRNA could be

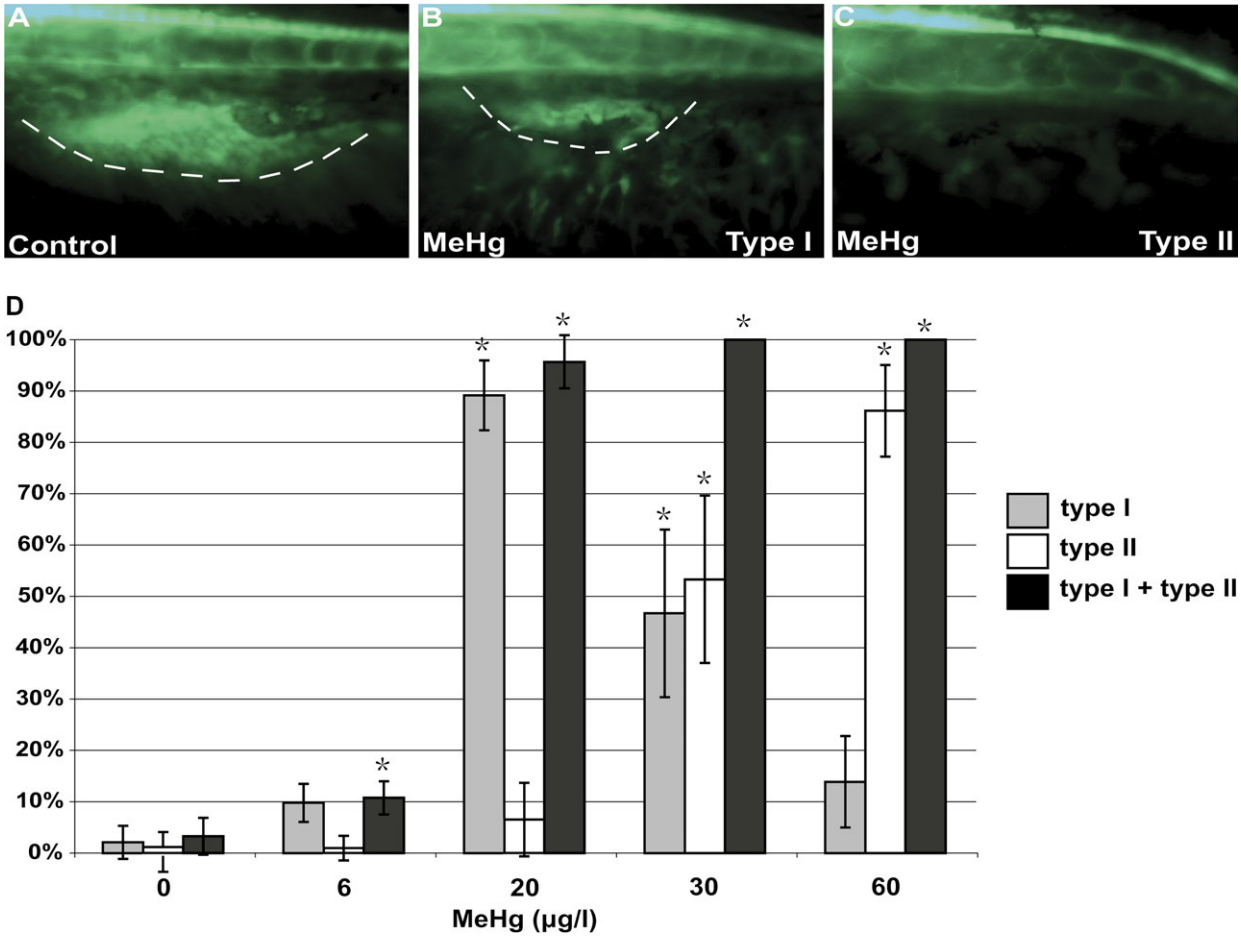

FIG. 3. Concentration dependence of reduction of GFP expression at the primordium of the tail fin. (A–C) Medium control (A) and MeHg-treated embryos (B and C). Depending on the degree of reduction of GFP expression in the growth zones, embryos were categorized either as type I when they had a reduced GFP domain (B) in comparison to controls (A) or as type II when they lacked GFP expression completely (C). Anterior to the left and dorsal up. 72 hpf embryos are shown. (D) Frequency of type I (gray bar) and type II (white bar) or all misformed embryos (black bar). Embryos were exposed to 0, 6, 20, 30, and 60 µg/l of MeHg. Already 6 µg/l showed a significant increase in embryos with reduced expression of GFP. At 60 µg/l, almost all treated embryos fell into the type II class. Mean values ± SEM from five sets of experiments are depicted. Type I, ANOVA: $F = 94.51$ and Dunnett: $*p < .01$; type II, ANOVA: $F = 108.1$ and Dunnett: $*p < .01$; and type I + type II, ANOVA: $F = 1601$ and Dunnett: $*p < .01$). Y-axis means percentage of embryos showing reduction or loss of GFP expression.

correlated with the observed defects in the tail region. We exposed embryos to 60 µg/l MeHg from 4 to 72 hpf and assessed the pattern of expression at 72 hpf by whole-mount *in situ* hybridization with antisense probes complementary to *mmp9* and *mmp13* mRNA. Both mRNAs were ectopically expressed in the fin folds and in individual cells scattered over the tail region in MeHg-treated embryos (Figs. 5A, 5B, 5D, and 5E). This upregulation of *mmp9* and *mmp13* transcripts was quantitatively confirmed by real-time PCR with reverse-transcribed cDNA prepared from total RNA of embryos exposed to 6, 30, and 60 µg/l MeHg. The results demonstrate that exposure to 30 and 60 µg/l MeHg increase the levels of *mmp9* and *mmp13* transcripts dramatically (Supplementary fig. 2). However, only background levels of *mmp9* and *mmp13* mRNAs were detected upon exposure to 6 µg/l MeHg (Supplementary fig. 2) as for solvent controls.

Double *in situ* hybridization, with both probes simultaneously showed that increased expression could already be noted at 24 hpf in embryos treated with MeHg from 4 hpf onward (Supplementary figs. 3A–F). We found cells that expressed only *mmp9* but not *mmp13* mRNA (Supplementary fig. 3). Sectioning of embryos hybridized to *mmp9* and *mmp13* antisense RNA showed a strong enrichment of the expressing cells in the epidermis (data not shown). We wondered whether the scattered *mmp9*- and *mmp13*-expressing cells are immune cells involved in tissue clearance. MeHg-treated embryos were hybridized to the *l-plastin* antisense probe, which is specific for macrophages and monocytic cells (Bennett *et al.*, 2001). Neutrophils were detected with the *mpx* antisense probe (Bennett *et al.*, 2001). Neither gene was significantly upregulated by MeHg or was expressed in a pattern resembling that of the *mmp9*- and *mmp13*-expressing cells (Supplementary fig. 4).

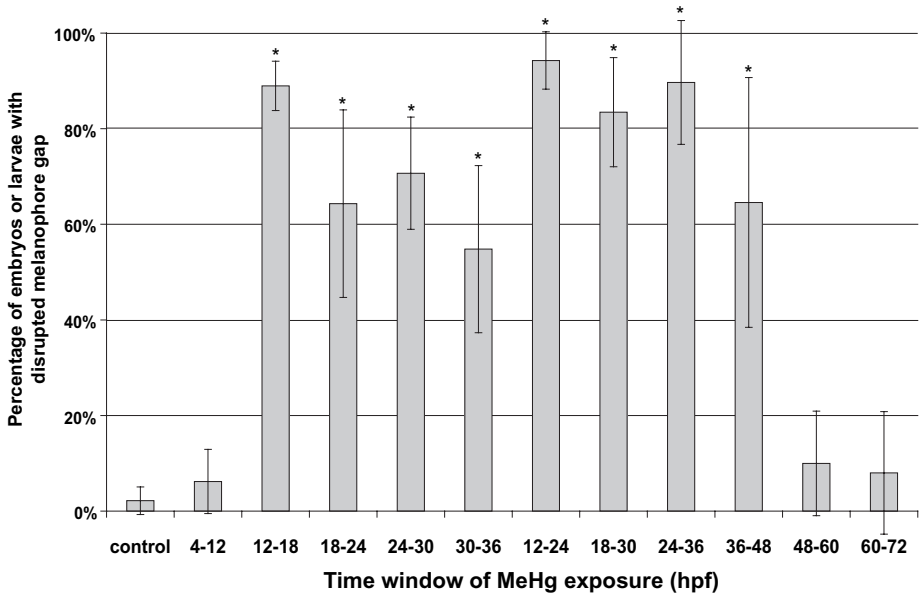

FIG. 4. Frequency of occurrence of disruption of tail fin primordium under different time windows of MeHg exposure. Embryos were treated with 60 µg/l MeHg from 4 to 12, 12 to 18, 18 to 24, 24 to 30, 30 to 36, 12 to 24, 18 to 30, 24 to 36, 36 to 48, 48 to 60, and 60 to 72 hpf ($n = 5$, 20 embryos each). Significant differences were noted in all the time windows exposed to MeHg between 12 and 48 hpf. The mean values ± SEM are reported. ANOVA, $F = 30.11$; Dunnett * $p < 0.01$.

Thus, the scattered *mmp9* and *mmp13* mRNA-positive cells are distinct from neutrophils, monocytic cells, and macrophages.

We next tested whether $PbCl_2$, As_2O_3, and $CdCl_2$ would also induce an increase in expression of *mmp9* and *mmp13* mRNA in the tail. We exposed embryos to either water or to 20 mg/l $PbCl_2$ or to 79 mg/l As_2O_3 or to 15 mg/l $CdCl_2$ from 4 to 72 hpf. *In situ* hybridization of $PbCl_2$-treated embryos revealed only a few scattered cells expressing *mmp13* mRNA in the tail ectopically, while *mmp9* mRNA was not induced (Figs. 5C and 5F). The other two chemicals did not elicit ectopic expression of *mmp9* and *mmp13* mRNA at all (data not shown). Thus, the induction of *mmp9* and *mmp13* expression in the tail is highly specific to treatment with MeHg.

MeHg may cause defects by the ectopic activation of Mmp9 and Mmp13 enzymatic activities in the fin fold. To test this notion, we applied the Mmp inhibitors GM6001 or MMP-9/MMP-13 Inhibitor II (Yoshinari *et al.*, 2009) to MeHg-treated embryos (100 µg/l). To quantitatively assess the effect of inhibitors, we calculated the area of the fin fold by drawing a vertical line through the tip of the notochord (Supplementary fig. 5) and calculating the area of the fin fold posterior to this line using the ImageJ software (Abramoff *et al.*, 2004). MeHg treatment from 4 to 72 hpf reduced the area of the posterior fin fold by two-fold (Fig. 5G). Cotreatment of embryos with 0.1mM GM6001 or MMP-9/MMP-13 Inhibitor II led to a small but significant increase in the area of the fin fold with $p < 0.05$ (one-way ANOVA test) (Fig. 5G).

Multiple Genes Are Upregulated in the Tail Fin in Response to MeHg

In order to identify further genes that could be linked to the defects in tail fin morphogenesis, we screened a set of 43 genes previously noted to be regulated in response to MeHg exposure in microarray experiments (Yang *et al.*, 2007). We focused the *in situ* hybridization analysis on 72 hpf embryos that were exposed to 60 µg/l MeHg from 4 to 72 hpf.

In addition to the *mmp9* (32 out of 33 embryos showed ectopic expression, 32/33) and *mmp13* (40/46) genes, we identified eight further genes that are ectopically expressed in the tail region of embryos treated with MeHg. The expression of three genes *(NM_213361,* 24/36; *AW115990,* 17/44; *BM101698*, 30/44*)* with unknown function was strongly activated by MeHg treatment in a pattern that resembled that of *mmp9* and *mmp13* activation (Figs. 6A–F). The mRNA of *sgk1*, (NM_199212, 32/40) was prominently expressed in response to MeHg in individual cells in the fin fold in a pattern reminiscent of *mmp9* and *mmp13* mRNA expression (Figs. 6G and H). In addition, we scored ectopic expression of the *peroxiredoxin 1* (NM_001013471, 53/56), *E74-like factor 3* (XM_684268, 8/23), and the *sqtm1* (NM_213173, 34/34) gene. The latter three genes were expressed only in very few cells of the tail in response to MeHg (Figs. 6I–N). Strong expression in response to MeHg was noted for the *apolipoprotein-E* mRNA (NM_131098, 49/51) (Figs. 6O and 6P).

DISCUSSION

We show here that MeHg impairs development of the fin fold and specification of the tail fin primordium in the zebrafish embryo. The effective window is restricted to the period between 12 and 48 hpf and the effect appears to be specific to MeHg at least within the limited range of substances tested. Moreover, the tail fin primoridum is most sensitive to MeHg

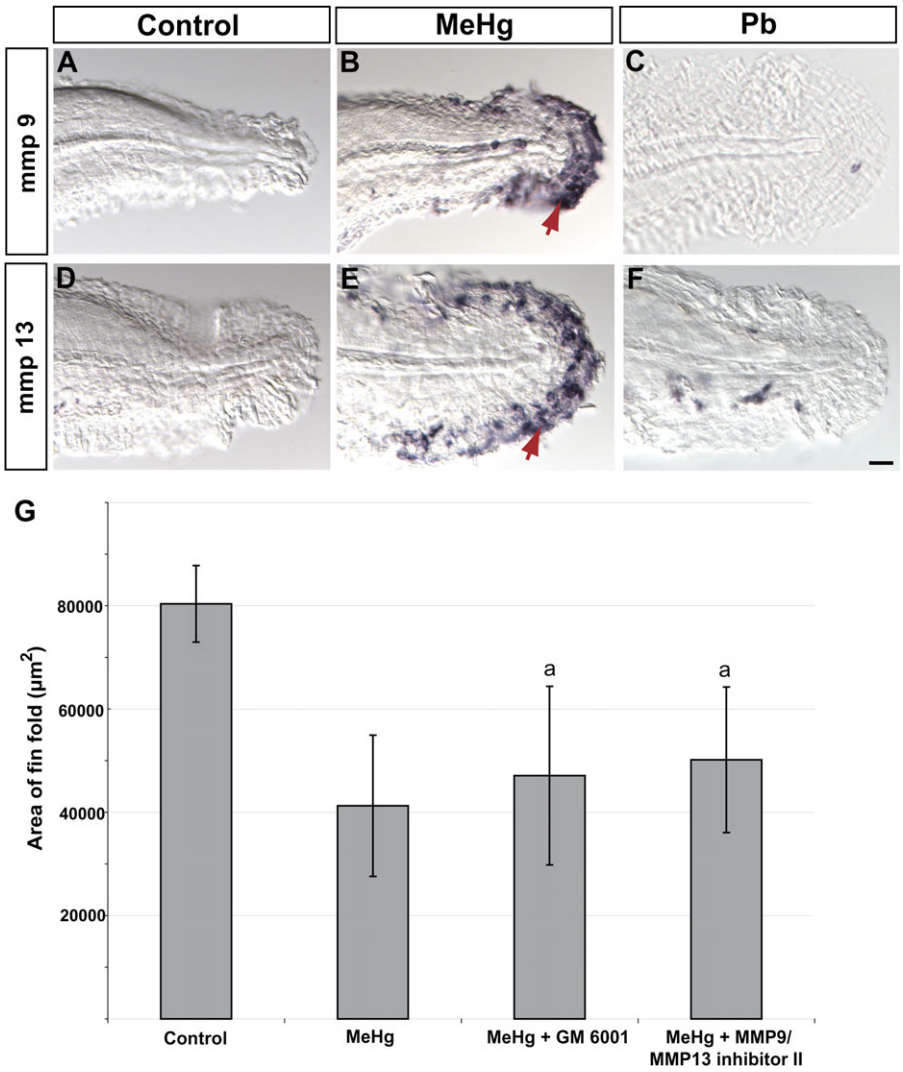

FIG. 5. *mmp9* and *mmp13* are ectopically expressed in the tail region of MeHg-treated embryos. (A–C) Tails of 72 hpf embryos exposed to embryo medium (A) or to 60 µg/l MeHg (B) or to 20 mg/l $PbCl_2$ (C) and hybridized to *mmp9* antisense probe. (D–F) Tails of 72 hpf embryos exposed to embryo medium (D) or to 60 µg/l MeHg (E) or to 20 mg/l $PbCl_2$ (F) and hybridized to *mmp13* antisense probe. The arrows indicate the ectopic expression of *mmp9* (B) and *mmp13* (E) in MeHg-treated embryos. (G) Partial rescue of MeHg-induced fin fold reduction by cotreatment with MMP inhibitors. A vertical line was drawn through the tip of the notochord (Supplementary Fig. 5), and the fin fold area posterior to this line was evaluated in embryos treated with embryo medium (''embryo culture'') or in 100 µg/l MeHg (''MeHg without inhibitor'') or with 100 µg/l MeHg and 0.1mM general MMP inhibitor, GM6001 (''MeHg + GM 6001'') or with 100 µg/l MeHg and 0.1mM MMP-9/MMP-13 Inhibitor II (''MeHg + MMP-9/MMP-13 inhibitor II''). The presence of MMP inhibitors increased the fin fold area by 18 and 25% relative to embryos treated with MeHg. The mean values and SEM of the area of the fin fold from 86 to 92 measurements in four independent experiments are presented. Bars marked with the letter ''a'' are not significantly different from each other. ANOVA, $F = 150.9$; Bonferroni, $p < 0.05$. Scale bar: 20 µm.

exposure; adverse effects of concentrations as low as 6 µg/l MeHg are detectable by a transgenic line expressing GFP in the caudal tail fin primordium. MeHg exposure leads to induction of a number of genes in the fin fold and tail including the metalloproteases *mmp9* and *mmp13*, suggesting that tissue remodeling plays a role in MeHg toxicity.

Interference with the Development of the Tail Fin Primordium

The tail fin develops as an asymmetric structure that grows out from a restricted zone of the ventral fin fold of the embryonic tail. The location of this zone correlates with a gap in the melanophore streak that runs along the flank of the embryo at 72 hpf (Hadzhiev *et al.*, 2007). MeHg has the same effect as loss-of-function mutations in the Shh signaling pathway, that is, loss of the pigment gap and loss of *Tg(-2.4shh:gfpABC #15)* transgene expression in the caudal fin primordium. However, MeHg treatment did not interfere with Shh-dependent processes at known other sites of Shh action (Barresi *et al.*, 2000; Schauerte *et al.*, 1998) in the embryo such as motor neuron specification or somite development (Yang, Ho, and Strähle, unpublished data). Moreover, Shh signaling appears to be

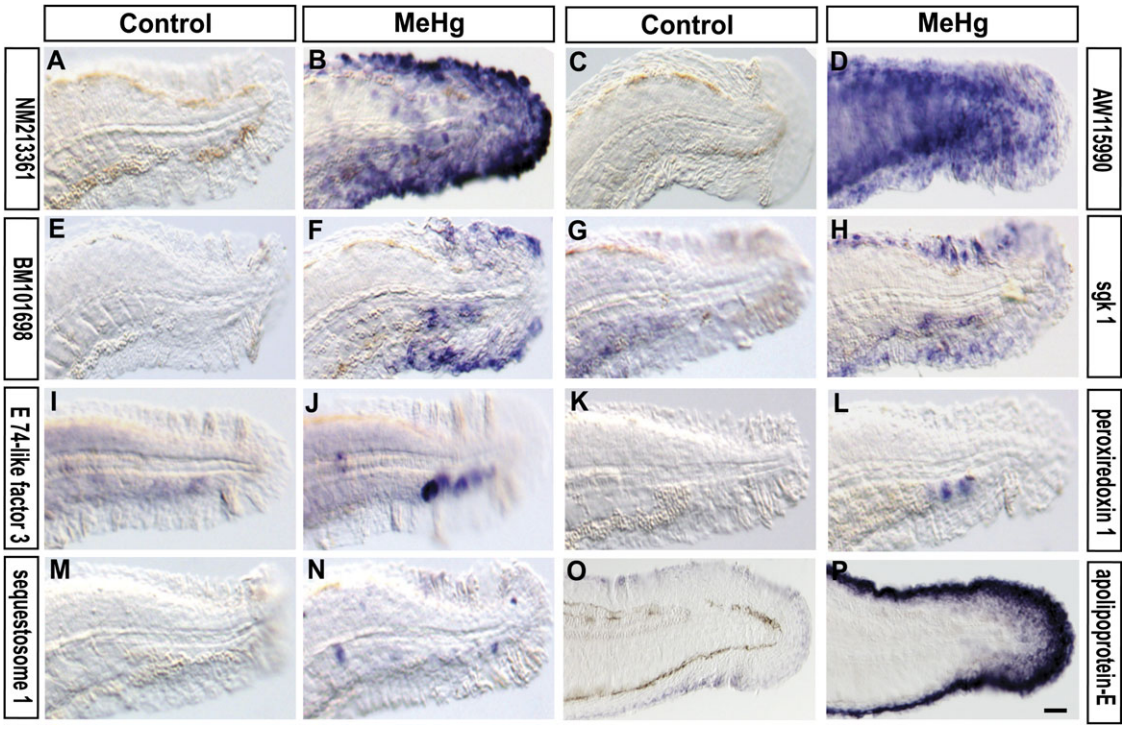

FIG. 6. *sgk1* and other genes are upregulated in the tail of MeHg-treated embryos. The tail region of 72 hpf zebrafish embryos stained by *in situ* hybridization with antisense probes to three unknown genes *NM_213361* (A and B), *AW115990* (C and D), and *BM101698* (E and F); *sgk1* (G and H); *E74-like factor 3* (I and J); *peroxiredoxin 1* (K and L); *sequestosome 1* (M and N); and the *apolipoprotein-E* gene (O and P). Each of these genes was specifically upregulated in the tail region and median fin fold of zebrafish embryos treated with 60 μg/l MeHg from 4 to 72 hpf. Anterior is to the left and dorsal up. Scale bar: 20 μm.

continuously required for tail fin development also at stages older than 48 hpf (Hadzhiev *et al.*, 2007). In contrast, exposure to MeHg does not impair transgene expression in the caudal fin primordium after 48 hpf. These observations make it rather unlikely that MeHg acts directly on Shh signaling. It has been proposed that neural crest cells migrate to the caudal fin primordium (Wood and Thorogood, 1984; van Eeden *et al.*, 1996). The time frame of MeHg effect (12–48 hpf) would fit with an interference by MeHg in this process. However, we did not find impairment of neural crest differentiation and migration as inferred from the patterns of crestin and sox10 expression in 24-h old embryos (Yang, Ho, Strähle, unpublished data).

The effect on the caudal fin primordium does not appear to be a reflection of overall tissue degradation in the exposed embryos. Although MeHg, As_2O_3, $PbCl_2$, and $CdCl_2$ induce related toxicogenomic responses (Yang *et al.*, 2007), only MeHg induced loss of the pigment gap and robust ectopic *mmp9* and *mmp13* mRNA expression in the embryonic tail. The effects on transgene expression in the tail fin primordium were observed at very low concentrations (6 μg/l), at which tissue degeneration in other embryonic regions is not evident. We do not know the absolute MeHg concentration in the embryo. Previous studies did not detect accumulation of MeHg in the fin fold. These studies reported MeHg accumulation in the lens and the brain (Korbas *et al.*, 2008; Santra *et al.*, 2009). Locomotor deficits of embryos treated with concentrations of 15 μg/l MeHg were attributed to neurotoxic effects (Samson *et al.*, 2001). Our results suggest that these low concentrations can also impair tail fin development, thereby contributing to the behavioral deficits observed previously (Samson *et al.*, 2001). In previous studies, concentrations as low as 27 μg/l and 266 μg/l MeHg triggered luciferase reporter activity in zebrafish embryos and in a zebrafish-derived cell line, respectively (Carvan *et al.*, 2000; Kusik *et al.*, 2008). Reduction of the shh:gfp transgene expression in the caudal fin primordium is thus a highly sensitive readout of MeHg exposure.

MeHg Induces Expression of Metalloproteases mmp9 and mmp13 in the Fin Fold of Exposed Embryos

Besides impaired specification of the tail fin primordium, administration of MeHg induced defects in the fin fold such as reduced size and increased apoptosis. Most strikingly, MeHg exposure triggered the expression of a set of 10 genes in the tail region, among which the metalloprotease genes *mmp9* and *mmp13* together with the lipid transporter *apolipoprotein-E* and three uncharacterized genes *(NM_ 213361, AW115990, BM101698)* were abundantly induced.

The *mmp9* and *mmp13* expression overlap in some cells. We found, however, also cells that expressed *mmp9* mRNA but not *mmp13* mRNA, suggesting that the *mmp9*- and *mmp13*-expressing cell populations are not identical or that the induction of the RNAs follows a stochastic process leading to expression in some but not all cells. We did not detect upregulation of markers such as *l-plastin* and *mpx* that are expressed by macrophages/monocytic cells and neutrophils, respectively (Bennett *et al*., 2001), suggesting that the *mmp9* and *mmp13* expression are not the reflection of an inflammatory response mediated by these cells invading the MeHg-damaged tissue. *Mmp*-expressing cells maybe dendritic, antigen-presenting cells, especially as expressing cells appear to be strongly enriched in the epidermis of MeHg-treated embryos (Yang, Ho, Strähle, unpublished data). However, very little is so far known about dendritic or Langerhans cells in the zebrafish embryo.

The *mmp9, mmp13*, and *sgk1* genes as well as the unknown genes *NM_213361*, *AW115990*, and *BM101698* are expressed in cells scattered throughout the fin fold. These genes could have a role in repair of the MeHg-inflicted tissue damage (Bai *et al*., 2005) or could be directly involved in the toxicological mechanism. It is tempting to speculate that misregulated *mmp9* and *mmp13* expression leads to tissue damage and thus to the observed effects seen in the fin folds. $CdCl_2$, As_2O_3, and $PbCl_2$ did not elicit a reduction of the tail fin primordium and induced ectopic expression of *mmp9* and *mmp13* mRNA only in a few cells or not at all. Upregulation of *mmp9* and *mmp13* mRNA expression was noted already at 24 h in MeHg-treated embryos, suggesting that an increased transcription of the *mmp9* and *mmp13* genes is an early consequence of MeHg exposure. In addition, blockage of Mmp9 and Mmp13 enzymatic activity with specific small molecule inhibitors reduced the observed defect in fin fold development. This argues in favor of Mmp9 and Mmp13 being at least part of the toxic mechanisms leading to impaired fin fold development. We noted a moderate increase in apoptotic cells in the fin fold. In addition, the literature reports a wide variety of molecular mechanisms, how MeHg can disturb cell homeostasis (Cambier *et al*., 2009; Castoldi *et al*., 2001; Hoffmeyer *et al*., 2006; QU *et al*., 2003; Senger *et al*., 2006; Vicente *et al*., 2004). The effect on *mmp9* and *mmp13* is one novel further aspect in the pleiotropic toxicological response to MeHg that may have, however, a very strong bearing on the developmental toxicity of MeHg.

Whether misregulated *mmp9* and *mmp13* could also cause the defects in the tail fin primordium remains to be addressed. We could not detect an elevation of *mmp9* and *mmp13* mRNA by RT-PCR relative to controls in embryos treated with 6 μg/l MeHg. This concentration caused impairment of the tail fin primordium as inferred from a reduction of the transgene expression domain in 10% of treated embryos. It is questionable whether a 10% increase in *mmp9*/*mmp13* mRNA levels above those of controls would have been detected by *in situ* hybridization or the RT-PCR assay. This underscores the sensitivity of the "transgene-readout" relative to these assays.

The transcriptional profiling of MeHg-treated embryos suggested that exposed embryos suffer from oxidative stress as they upregulated expression of oxidative stress genes (Kusik *et al*., 2008; Yang *et al*., 2007). With the exception of the *peroxiredoxin* 1 gene that was upregulated in the lateral line neuromasts located in the gap of the melanophore, expression of oxidative stress response genes was not significantly increased in the fin fold.

Altogether, we have identified 10 genes whose expression is specifically upregulated in the tail of MeHg-treated embryos and that have—to our best knowledge—not been linked to MeHg toxicity in the past. The *gfp* transgenic line, whose expression is abolished in the caudal fin primordium, represents a highly sensitive readout of MeHg-inflicted tail defects. Our experiments underscore the usefulness of the zebrafish as model to assess developmental toxicity of MeHg and expand its capabilities to obtain mechanistic insights by the identification of a number of novel genes that can serve as tools for further studies. These genes could serve as biosensors to monitor the effect of toxicants in living zebrafish embryos. Especially, the fluorescent readout of toxic impact in transgenic responder lines will not only allow the study of live animals but is probably the only way how, together with automation of embryo handling and image acquisition (Gehrig *et al*., 2009; Lam *et al*., 2009; Yang *et al*., 2009), to achieve the throughput that is necessary for the systematic assessment of the toxic effect of the large numbers of chemicals demanded by regulators (Hartung and Rovida, 2009).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics Int.* **11,** 36–42.

Bai, S., Thummel, R., Godwin, A. R., Nagase, H., Itoh, Y., Li, L., Evans, R., McDermott, J., Seiki, M., and Sarras, M. P., Jr. (2005). Matrix metalloproteinase expression and function during fin regeneration in zebrafish: analysis of MT1-MMP, MMP2 and TIMP2. *Matrix Biol.* **24,** 247–260.

Barresi, M. J. F., Stickney, H. L., and Devoto, S. H. (2000). The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. *Development* **127,** 2189–2199.

Bennett, C. M., Kanki, J. P., Rhodes, J., Liu, T. X., Paw, B. H., Kieran, M. W., Langenau, D. M., Delahaye-Brown, A., Zon, L., Fleming, M. D., *et al.* (2001). Myelopoiesis in the zebrafish, Danio rerio. *Blood* **98,** 643–651.

Cambier, S., Benard, G., Mesmer-Dudons, N., Gonzalez, P., Rossignol, R., Brethes, D., and Bourdineaud, J. P. (2009). At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (Danio rerio). *Int. J. Biochem. Cell Biol.* **41,** 791–799.

Carvan, M. J., III., Solis, W. A., Gedamu, L., and Nebert, D. W. (2000). Activation of transcription factors in zebrafish cell cultures by environmental pollutants. *Arch. Biochem. Biophys.* **376,** 320–327.

Castoldi, A. F., Coccini, T., Ceccatelli, S., and Manzo1, L. (2001). Neurotoxicity and molecular effects of methylmercury. *Brain Res. Bull.* **55,** 197–203.

Cohen, G. M. (1997). Caspases: the executioners of apoptosis. *Biochem. J.* **326,** 1–16.

Darzynkiewicz, Z., Bruno, S., Del, B. G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992). Features of apoptotic cells measured by flow cytometry. *Cytometry* **13,** 795–808.

Davidson, P. W., Myers, G. J., Cox, C., Wilding, G. E., Shamlaye, C. F., Huang, L. S., Cernichiari, E., Sloane-Reeves, J., Palumbo, D., and Clarkson, T. W. (2006). Methylmercury and neurodevelopment: longitudinal analysis of the Seychelles child development cohort. *Neurotoxicol. Teratol.* **28,** 529–535.

Ekino, S., Susa, M., Ninomiya, T., Imamura, K., and Kitamura, T. (2007). Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning. *J. Neurol. Sci.* **262,** 131–144.

Ekker, S. C., and Larson, J. D. (2001). Morphant technology in model developmental system. *Genesis* **30,** 89–93.

Ertzer, R., Muller, F., Hadzhiev, Y., Rathnam, S., Fischer, N., Rastegar, S., and Strähle, U. (2007). Cooperation of sonic hedgehog enhancers in midline expression. *Dev. Biol.* **301,** 578–589.

Gehrig, J., Reischl, M., Kalmar, E., Ferg, M., Hadzhiev, Y., Zaucker, A., Song, C., Schindler, S., Liebel, U., and Müller, F. (2009). Automated high-throughput mapping of promoter-enhancer interactions in zebrafish embryos. *Nat. Methods* **6,** 911–916.

Grandjean, P., and Landrigan, P. J. (2006). Developmental neurotoxicity of industrial chemicals. *Lancet* **16,** 2167–2178.

Hadzhiev, Y., Lele, Z., Schindler, S., Wilson, S. W., Ahlberg, P., Strähle, U., and Müller, F. (2007). Hedgehog signaling patterns the outgrowth of unpaired skeletal appendages in zebrafish. *BMC Dev. Biol.* **7,** 75.

Hartung, T., and Rovida, C. (2009). Chemical regulators have overreached. *Nature* **460,** 1080–1081.

Hoffmeyer, R. E., Singh, S. P., Doonan, C. J., Ross, A. R., Hughes, R. J., Pickering, I. J., and George, G. N. (2006). Molecular mimicry in mercury toxicology. *Chem. Res. Toxicol.* **19,** 1117–1118.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203,** 253–310.

Korbas, M., Blechinger, S. R., Krone, P. H., Pickering, I. J., and George, G. N. (2008). Localizing organomercury uptake and accumulation in zebrafish larvae at the tissue and cellular level. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 12108–12112.

Kusik, B. W., Carvan, M. J., III., and Udvadia, A. J. (2008). Detection of mercury in aquatic environments using EPRE reporter zebrafish. *Mar. Biotechnol.* **10,** 750–757.

Lam, C. S., März, M., and Strähle, U. (2009). gfap and nestin reporter lines reveal characteristics of neural progenitors in the adult zebrafish brain. *Dev. Dyn.* **238,** 475–486.

Müller, F., Albert, S., Blader, P., Fischer, N., Hallonet, M., and Strähle, U. (2000). Direct action of the nodal-related signal cyclops in induction of sonic hedgehog in the ventral midline of the CNS. *Development* **127,** 3889–3897.

Müller, F., Chang, B., Albert, S., Fischer, N., Tora, L., and Strähle, U. (1999). Intronic enhancers control expression of zebrafsih sonic hedgehog in floor plate and notochord. *Development* **126,** 2103–2116.

Myers, G. J., Davidson, P. W., and Strain, J. J. (2007). Nutrient and methyl mercury exposure from consuming fish. *J. Nutr.* **137,** 2805–2808.

Oxtoby, E., and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res.* **21,** 1087–1095.

Qu, H., Syversen, T., Aschner, M., and Sonnewald, U. (2003). Effect of methylmercury on glutamate metabolism in cerebellar astrocytes in culture. *Neurochem. Int.* **43,** 411–416.

Robu, M. E., Larson, J. D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S. A., and Ekker, S. C. (2007). p53 activation by knockdown technologies. *PLoS Genet.* **3,** e78.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Samson, J. C., Goodridge, R., Olobatuyi, F., and Weis, J. S. (2001). Delayed effects of embryonic exposure of zebrafish (Danio rerio) to methylmercury (MeHg). *Aquat. Toxicol.* **51,** 369–376.

Samson, J. C., and Shenker, J. (2000). The teratogenic effects of methylmercury on early development of the zebrafish, Danio rerio. *Aquat. Toxicol.* **48,** 343–354.

Santra, M., Ryu, D., Chatterjee, A., Ko, S. K., Shin, I., and Ahn, K. H. (2009). A chemodosimeter approach to fluorescent sensing and imaging of inorganic and methylmercury species. *Chem. Commun.* **16,** 2115–2117.

Schauerte, H. E., van Eeden, F. J. M., Fricke, C., Odenthal, J., Strähle, U., and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125,** 2983–2993.

Senger, M. R., Rico, E. P., de Bem Arizi, M., Frazzon, A. P., Dias, R. D., Bogo, M. R., and Bonan, E. D. (2006). Exposure to Hg^{2+} and Pb^{2+} changes NTPDase and ecto-5′-nucleotidase activities in central nervous system of zebrafish (Danio rerio). *Toxicology* **21,** 229–237.

Shkumatava, A., Fischer, S., Müller, F., Strähle, U., and Neumann, C. J. (2004). Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina. *Development* **131,** 3849–3858.

Strähle, U., Blader, P., Adam, J., and Ingham, P. W. (1994). A simple and efficient procedure for non-isotopic in situ hybridization to sectioned material. *Trends Genet.* **10,** 75–76.

Van Eeden, F. J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A., *et al.* (1996). Genetic analysis of fin formation in the zebrafish, Danio rerio. *Development* **123,** 255–262.

Vicente, E., Boer, M., Leite, M., Silva, M., Tramontina, F., Porciúncula, L., Dalmaz, C., and Goncalves, C. A. (2004). Cerebrospinal fluid S100B increases reversibly in neonates of methyl mercury-intoxicated pregnant rats. *Neurotoxicology* **25,** 771–777.

Westerfield, M. (1993). *The Zebrafish Book*. The University of Oregon Press, Eugene, OR.

Wood, A., and Thorogood, P. (1984). An analysis of in vivo cell migration during teleost fin morphogenesis. *J. Cell Sci.* **66,** 205–222.

Yang, L., Ho, N. Y., Alshut, R., Legradi, J., Weiss, C., Reisch, M., Mikut, R., Liebel, U., Müller, F., and Strähle, U. (2009). Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. *Reprod. Toxicol.* **28,** 245–253.

Yang, L., Kemadjou, J. R., Zinsmeister, C., Bauer, M., Legradi, J., Müller, F., Pankratz, M., Jäkel, J., and Strähle, U. (2007). Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo. *Genome Biol.* **8,** R227.

Yoshinari, N., Ishida, T., Kudo, A., and Kawakami, A. (2009). Gene expression and functional analysis of zebrafish larval fin fold regeneration. *Dev. Biol.* **325,** 71–81.