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Arsenic impairs embryo development *via* down-regulating Dvr1 expression in zebrafish

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HIGHLIGHTS

- Dvr1 played an important role in the left-right asymmetry establishment of zebrafish embryo.
- Dvr1 was involved in arsenic-mediated embryo toxicity.
- It gives novel insight into molecular mechanism of arsenic-mediated embryo toxicity.

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ABSTRACT

Exposure to environmental inorganic arsenic compounds has serious health effects on humans, including cancer, cardiovascular disease, developmental and reproductive problems. Our previous study showed that arsenic exposure caused various signs of toxicity in early stages of zebrafish development, including cardiac and neural system, such as pericardium edema, circulation failure, heart malformation. However, how arsenic exerts these effects is little known. Here, real-time quantitative RT-PCR and whole-mount *in situ* hybridization data showed that zebrafish Dvr1, a mammalian homolog of GDF1 related to the formation of left–right axis, was significantly down-regulated in embryos exposed to arsenite. Embryos with Dvr1 knockdown by antisense morpholino displayed abnormal development, including pericardium edema and failed looping, which is similar to the defects phenotype with arsenic treatment. Furthermore, overexpression of GDF1 significantly rescued development anomalies caused by morpholino or arsenite. Taken together, our results indicated for the first time that Dvr1 played an important role in the left-right asymmetry establishment of zebrafish embryo, and Dvr1 was involved in arsenic-mediated embryo toxicity, which gives novel insight into molecular mechanism of arsenic-mediated embryo toxicity.

1. Introduction

The experimental virtues of the zebrafish such as small size, development outside of the mother, the transparency, cheap maintenance of the adult made the zebrafish an outstanding model for studying the effects that environmental contaminants or drugs have on developmental processes. The ability of zebrafish to survive several days without a functioning circulatory system is a distinctive advantage when studying the development of the cardiovascular system (Bakkers, 2011). Inorganic arsenic contamination of drinking water is a major worldwide public health problem. Chronic arsenic ingestion increases risk and incidence of cancer, cardiovascular disease, reproductive and developmental problems (Abernathy et al., 2003; Bernstam and Nriagu, 2000). Our previous study identified arsenic embryotoxicity in zebrafish development, including cardiac and neural system. We found that exposure of zebrafish larvae to arsenic causes cardiovascular toxicity such as pericardium edema, circulation failure and failing looping (Li et al., 2009).

Heart development is a complex process which begins with the induction of cardiogenic cells from mesoderm. Many signal molecules such as bone morphogenetic proteins 2 (BMP-2) (Yamada et al., 2000), fibroblast growth factors 8 (FGF-8) (Alsan and Schultheiss, 2002), and mesodermally-derived Wnt11 (Eisenberg and Eisenberg, 1999) induce mesodermal cells to become

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Table 1
Primers in quantitative realtime PCR analysis.

Gene-primer ID	Gene-Primer ID
β-Actin F	5′-GCCTTCCTTCCTGGGTATGG-3′
β-Actin R	5′-CAGACGGAGTATTTACGCTCAG-3′
BMP2a F	5′-CCAGCAGAGCCAACACTATC-3′
BMP2a R	5′-GGTCAGGTTGAAGAGGAACC-3′
BMP2b F	5′-AGCAGAGCAAACACGATACG-3′
BMP2b R	5′-ACTGGCATCTCCGAGAACTT-3′
tbx2b F	5′-TTTCCAGCCCACTTAGGTTT-3′
tbx2b R	5′-ATGGAGATGAAGGCTCTGGT-3′
Dvr1 F	5′-TCAGCCCTGTTGCGTTCC-3′
Dvr1 R	5′-ACTCATCCACCACCATGTCTTC-3′
FGF8a F	5′-CATTTGAGCTGGAAAACCTG-3′
FGF8a R	5′-CAAAACGCAAAGAGGTGAAG-3′
bmp4 F	5′-TGAAAGATCCACCAGTCGAG-3′
bmp4 R	5′-CTGGGATGCTGCTGAGATTA-3′

cardiogenic cells, probably by leading the expression of cardiogenic transcription factor genes, such as Tbx2, 3, and 5, Tal 1, Nkx2.5 and Gata4 (Laverriere et al., 1994; Moses et al., 2001; Yamada et al., 2000). Then, the cardiac disc which migrated from cardiogenic cell transforms into a cardiac tube with a rightward looping which is critical to normal heart formation. The left–right (L/R) asymmetry is initiated by asymmetric expression of activinβB, which inhibits Shh expression in the right portion but allows its expression in the left lateral plate mesoderm (LPM), where it induces Nodal expression. Nodal can upregulate Pitx2 gene transcription, which establishes a leftward identity in the LPM (Wagner and Siddiqui, 2007). Accumulating evidences demonstrated that Vg1 (Vg1 in *Xenopus* and chicken; GDF1 in mouse; Dvr1 in zebrafish) acts upstream of these asymmetric genes, such as Nodal, Pitx2, and plays a role in establishing the L/R axis (Dohrmann et al., 1996; Schier and Shen, 2000; Whitman, 2001).

Our previous study mainly focused on the toxicity effect of arsenite (Li et al., 2009). However, little is known about the molecular mechanism underlying developmental defects caused by arsenic exposure. In this study, we used zebrafish as a model to investigate the possible mechanism of arsenic toxicities. We found that arsenic treatment caused serious cardiac and neural development defects of zebrafish embryo. Taking advantage of morpholino technique and rescue experiments, we determined that the abnormalities induced by arsenite were rescued mostly by mouse GDF1, implying that arsenic-induced defects were mainly caused by the inhibition of Dvr1 activity.

2. Materials and methods

2.1. Maintenance of animals and embryo toxicity test

Wild type AB strain and *Tg* (cmlc2: GFP) zebrafish were raised and embryo collection was performed using standard protocols as described previously (Li et al., 2009). Briefly, adults were maintained at 28 °C in a 14:10 light:dark (L:D) cycle in a closed flow-through system supplied with filtered water and Instant Ocean salt. Eggs were collected during the first hour after fertilization and incubated in egg water at 28.3 °C (Westerfield, 2000) and staged in hours or days post fertilization (hpf or dpf) according to standard criteria (Kimmel et al., 1995). At 4 hpf, part of normal embryos at blastula stage were selected under a stereomicroscope (Zeiss Lumar, V12, Germany), and then randomly distributed in 90-mm diameter Petri dishes and treated with egg water or 2 mM sodium arsenite (Sigma–Aldrich, St. Louis, MO). The embryos were examined under a stereomicroscope at 48 hpf.

2.2. Isolation of RNA and real-time quantitative RT-PCR analysis

Real-time RT-PCR was used to detect the expression of candidate genes. Total RNA was extracted from zebrafish embryos, using Trizol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed to cDNA for PCR (TAKARA, Otsu, Japan). Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/primer3/) was used for designing specific forward and reverse primers. The primer pairs were designed to span introns to prevent amplification of any contaminating genomic DNA. The details of these fragments were described (Table 1). Real-time PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) with SYBR green fluorescent label. Samples (20 μl final vol.) contained the following: 1× SYBR green master mix (Roche, San Francisco, CA, USA), 5 pmol of each primer. Cycling parameters were as follows: an initial step of 10 min at 95 °C, followed by 15 s at 95 °C and 1 min at 60 °C for 40 cycles. Each sample in each group was measured in triplicate and experiment was repeated at least three times. The quantification was normalized to endogenous control β-actin.

2.3. Whole-mount in situ hybridization (WISH)

The plasmid containing the second exon of *Dvr1* was obtained as a kind gift from Dr. Lehmann (Department of Ophthalmology and Medical Genetics, University of Alberta, Canada). Dvr1 RNA probe, which was generated from the Dvr1 plasmid after linearization was synthesized and labeled with digoxigenin UTP-NTP mix (Roche) by T7 RNA polymerase (Promega, Madison, WI, USA). The whole-mount *in situ* hybridization on embryos was performed as described (Moens, 2008). Embryos used for *in situ* hybridization were anesthetized in tricaine and fixed with 4% paraformaldehyde. The expression of Dvr1 in zebrafish embryos were detected using alkaline phosphatase-conjugated antibodies and visualized by 4-nitro blue tetrazolium (Promega) and 5-bromo-4-chloro-3-indolyl-phosphate (Promega) staining, then embryos were mounted in glycerol and photographed with a dissecting microscope (Zeiss Lumar, V12) and CCD camera (Axioncan MRc5, Germany).

2.4. Morpholino inhibition and microinjection

These plasmids (pCS2-GDF1, pCS2-BMP2-GDF1 and pCS2-BMP2-Vg1) were obtained as a kind gift from Daniel S. Kessler (Department of Ophthalmology and Medical Genetics, University of Alberta, Canada). pCS2-GDF1 encoded native GDF1. In an attempt to generate mature GDF1 or Vg1 protein, the signal sequence, prodomain, and cleavage site of BMP2 were fused to the mature domain of GDF1 (BMP2-GDF1) or Vg1 (BMP2-Vg1) as described before (Wall et al., 2000). Plasmids were purified with DNA purification kit (Promega, Heidelberg, Germany). Antisense morpholinos (MOs) were obtained from GeneTools (Philomath, OR, USA). The Dvr1 MO was designed targeting the translation start site of Dvr1. The MO sequence was GCTCTGAGGAGGACCAAGAACATTA. Embryos at 1–2 cell stage were injected with MO (10 ng) alone or in combination of these plasmids (100 ng), respectively, with or without arsenite treatment at 4 hpf. The embryos were examined under a stereomicroscope at 48 hpf.

2.5. Assessment of cardiovascular phenotype

After injection following the protocol above, wild-type, Mo and rescued embryos were removed from incubation and observed with a stereomicroscope (Zeiss Lumar, V12) at 48 hpf in order to quantify the cardiovascular phenotype. Three separate heart phenotypic categories including cardiac looping, pericardial edema and the presence of pooled erythrocytes were counted. Cardiac looping was rated as normal or abnormal using *Tg* (cmlc2:GFP) transgenic embryos, pericardial edema was either rated as present or absent through histology sections and impaired circulation was defined by the presence of pooled erythrocytes, low numbers of circulating erythrocytes. All phenotypes were judged objectively with comparison to normal phenotype. The counts at each time point were made separately by two independent observers and averaged (Mitchell et al., 2010). Staining of hemoglobin by *o*-dianisidine was performed as previously described (Detrich et al., 1995). To prepare embryos for hematoxylin and eosin (H&E) staining, embryos at 48 hpf were anesthetized in tricaine and fixed in 4% paraformaldehyde, dehydrated, paraffin-embedded and sectioned at 5 μm intervals.

2.6. Whole-mount immunochemistry

Whole-mount immunochemistry of acetylated α-tubulin (α-AT) was performed using the standard method as described previously. Briefly, embryos of 48 hpf were dechorionated, fixed with 4% paraformaldehyde at room temperature (RT) for 1 h and washed with PBST (PBS with 0.1% Tween-20), then blocked with blocking buffer containing 10% normal goat serum (Beijing Zhongshan Golden Bridge Co. Ltd.) and 2 mg/ml bovine serum albumin (BSA; Sigma) for 2 h at RT. The blocking buffer was then removed, and the embryos were incubated with primary antibody (α-AT antibody, Sigma), at 4 °C overnight. They were then washed and incubated with IgG-FITC (Beijing Zhongshan Golden Bridge Co.) rabbit anti-mouse secondary antibody for 2 h at RT. Then pictures were taken with a stereomicroscope (Zeiss Lumar, V12).

2.7. Statistical analysis

All data were performed using the SPSS 16.0 statistical software package. The incidence of embryo malformation at 48 hpf was analyzed using chi-square test and chi-square partition was further used to compare the differences between the treated groups. Differences between groups were considered to be significant with a P value of less than α or α'.