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Early life stage trimethyltin exposure induces ADP-ribosylation factor expression and perturbs the vascular system in zebrafish

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Abstract

Trimethyltin chloride (TMT) is an organotin contaminant, widely detected in aqueous environments, posing potential human and environmental risks. In this study, we utilized the zebrafish model to investigate the impact of transient TMT exposure on developmental progression, angiogenesis, and cardiovascular development. Embryos were waterborne exposed to a wide TMT concentration range from 8 to 96 hours post fertilization (hpf). The TMT concentration that led to mortality in 50% of the embryos (LC₅₀) at 96 hpf was 8.2 μ M; malformations in 50% of the embryos (EC₅₀) was 2.8 μ M. The predominant response observed in surviving embryos was pericardial edema. Additionally, using the Tg (*flil*a: EGFP) y1 transgenic zebrafish line to non-invasively monitor vascular development, TMT exposure led to distinct disarrangements in the vascular system. The most susceptible developmental stage to TMT exposure was between 48–72 hpf. High density whole genome microarrays were used to identify the early transcriptional changes following TMT exposure from 48 to 60 hpf or 72 hpf. In total, 459 transcripts were differentially expressed at least 2-fold ($P < 0.05$) by TMT compared to control. Using Ingenuity Pathway Analysis (IPA) tools, it was revealed that the transcripts misregulated by TMT exposure were clustered in numerous categories including metabolic and cardiovascular disease, cellular function, cell death, molecular transport, and physiological development. *In situ* localization of highly elevated transcripts revealed intense staining of ADP-ribosylation factors *arf3* and *arf5* in the head, trunk, and tail regions. When *arf5* expression was blocked by morpholinos, the zebrafish did not display the prototypical TMT-induced vascular deficits, indicating that the induction of *arf5* was necessary for TMT-induced vascular toxicity.

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Keywords

zebrafish; trimethyltin chloride; vascular; ADP-ribosylation factors (*arf*); gene expression

1. Introduction

As thermal- and photo-resistant stabilizers, organotin compounds have been widely used in polyvinyl chloride, pesticides, fungicides, and antifouling paints (Blunden et al. 1984). TMT is one of the most widely used organotins and a ubiquitous environmental contaminant (Hoch 2001). TMT is well recognized as a toxic chemical to humans, and incidences of TMT poisoning have been reported across the world. Though many e TMT poisoning cases are associated with occupational exposure, incidences occur through the ingestion of organotin polluted food or water (Hoch 2001; Tang et al. 2010; Tang et al. 2002). Furthermore, the presence of TMT in aquatic environments is well documented (Liu et al. 2003; Shawky and Emons 1998) and its potential risk to aquatic organisms was recently examined (Li et al. 2011). Upon adult TMT exposure, neurotoxicity is the major symptom of poisoning in both humans and experimental animals. Because of this, TMT has been widely used as a model toxicant for studying central nervous system toxicity (El-Fawal and O'Callaghan 2008; Zuo et al. 2009). More recently, TMT was also recognized as a cytotoxicant (Holden and Coleman 2007; Liu et al. 2004; Yoneyama et al. 2009), an immunotoxicant (Gomez et al. 2007; Holloway et al. 2008), and an apoptotic inducer in some systems (Cai et al. 2009; Mundy and Freudenrich 2006). These toxicities have led to the potential application of organotin compounds, as a class, in metal-based chemotherapy for treating human cancers (Alama et al. 2009). Therefore, a comprehensive understanding of the toxic effects of organotin compounds and their mode of action will not only help reveal the environmental health effects caused by these chemicals, but also provide the biological basis for their applications in anti-tumor activities. In particular, potential cardiovascular toxicity is a significant concern for the use of organotin compounds in human chemotherapy (Nath 2008 598-612.).

Earlier studies with mammalian model systems indicated that organotin compounds interfered with heme metabolism, decreased blood pressure resulting from a depression of the vascular smooth muscle, altered blood composition, and decreased organ-to-heart mass ratios in rats and mice (Nath 2008 598-612.). These results suggested that the cardiovascular system is a target organ of organotins. However, no studies have looked into the possible effect of TMT exposure on vascular development. In zebrafish, it is hypothesized that, during the early embryonic stage, the same molecular mechanisms specify differentiation and patterning of the nervous and vascular systems (Melani and Weinstein 2010). We hypothesized that TMT may elicit adverse effects on the developing vascular system. Our preliminary data in zebrafish showed that developmental exposure to TMT resulted in a high incidence of vascular deficits and decreased heart rate.

In this study, we used zebrafish as a model system to define the developmental toxicity of TMT, and to begin to dissect the toxicity mechanism. We report that TMT exposure induced pericardial edema and aberrant vascular development. Using the vascular effects as phenotypic anchors, global mRNA expression microarray studies were completed to identify the early gene expression changes that preceded these phenotypes. *In situ* localization of highly elevated transcripts in qRT-PCR and microarrays revealed intense staining of ADP-ribosylation factors *arf3* and *arf5* in the head, trunk, and tail regions. When *arf5* expression was blocked by morpholinos, the zebrafish did not display the prototypical TMT-induced vascular deficits, indicating that the induction of *arf5* was necessary for TMT-induced vascular toxicity.

2. Materials and methods

2.1. Fish husbandry and exposure protocols

The Tg (*flil1a*: EGFP) y1 line used for vascular patterning assessments was obtained from Dr. Brant Weinstein at the National Institute for Child Health and Human Development. The Tropical 5D and AB wild type lines were used to conduct all other experiments. All fish were raised and kept at the Sinnhuber Aquatic Research Laboratory at Oregon State University under standard laboratory conditions of temperature 28 ($\pm 0.5^{\circ}\text{C}$), pH 7.2 (± 0.2) on a 14:10 dark/light photoperiod according to standard zebrafish breeding protocols (Westerfield 1995). Trimethyltin chloride (PN#146498; purity >97%) was purchased from Sigma-Aldrich. A TMT stock solution of 1mM in water was stored at 4°C until further diluted with embryo medium (van Eeden et al. 1999; Westerfield 1995).

Embryos were individually waterborne-exposed to TMT at various concentrations in plastic 96-well plates (200 μL /well). Developmental progression was monitored daily and mortality was determined by heartbeat cessation. Malformations such as yolk sac edema, pericardial edema, body axial/tail curves, swim bladder inflation, and pigmentation were tabulated. TMT concentrations from 0.01 to 100 μM were first used to estimate LC_{50} and to determine the most sensitive toxicological response. Once the dose-dependent response range was defined, a narrower final concentration range between 1 and 15 μM was used for three separate trials with 12 embryos in each concentration to calculate the LC_{50} and EC_{50} values.

2.2. Vascular patterning in transgenic larvae after TMT exposure

Tg (*flil1a*: EGFP) y1 embryos were exposed to 0, 2, 5, 8, 10 and 13 μM TMT from 8 to 96 hpf in 6 well plates with 20 embryos in 5 ml of solution. At 96 hpf, live larvae were euthanized with 0.02% MS-222 for 5 min and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) over night. After fixation, they were washed three times with PBS and stored in PBS at 4°C prior to imaging with a fluorescence microscope (Nikon ECLIPSE TE2000-U). 5–7 intersegmental vessels in each field of view were imaged utilizing a 20 $\times$ objective, and 3–4 segments at yolk sac and tail regions were imaged utilizing a 40 $\times$ objective. We used a scoring metric where abnormal vasculature was scored if more than four abnormal vessels (including missing vessels, inappropriate crossing or hyper-proliferation) were present in the zebrafish, regardless of the position or distribution of the abnormal structures. Vasculature abnormality was thus expressed as the percent larvae with abnormal vessels. Total vascular number and distance between vessels (including the yolk and the tail region) were calculated using Image-Pro Plus7.0 (IPP, Media Cybernetics).

2.3. Sensitive exposure period screening

To determine which phase of the embryogenesis was most TMT sensitive, embryos/larvae were waterborne-exposed to TMT (5, 10, 15 and 20 μM) in 6-well plates during the following developmental windows: 8–24 hpf, 24–48 hpf, 48–72 hpf, or 72–96 hpf. At the end of each exposure period, the zebrafish were rinsed three times with embryo media (EM) and transferred to 96-well plates for continuous development until 96 hpf where the incidence of pericardial edema was scored.

2.4. Microarray Analysis

The most susceptible developmental stage to TMT exposure was between 48–72 hpf; therefore, 48 hpf embryos were exposed to 10 μM TMT or vehicle EM, and at 60 and 72 hpf, total RNA was collected from pooled embryos. There were three replicates and each replicate pool consisted of 40 individual zebrafish. Total RNA was isolated with TRIzol Reagent (cat#15596-026, Life Technologies) according to the manufacturer's instructions. The quantity and quality of RNA was determined using the Nanodrop-1000

Spectrophotometer and the Agilent Bioanalyzer. All RNA samples passed concentration, and quality requirements (A260/A280 = 1.8, and A260/A230 = 1.8). For microarray processing, 10 µg of total RNA was reverse transcribed using SuperScript III and oligo (dT) primer (Invitrogen, Carlsbad, CA), and double stranded cDNA was synthesized and purified using Qiagen Minelute PCR Purification spin columns. Double strand cDNAs were labeled with Cy5 dNTP, and hybridized to 12 × 135K zebrafish gene expression arrays (Roche Nimblegen, Madison, WI) and scanned using the Axon GenePix 4200A Pro scanner (Molecular Devices, Sunnyvale, CA) with a green laser (532 nm) and a hardware setting of 450 pmt, laser power of 100, and a pixel size of 5. Histogram analysis was performed to assure that the normalized counts were between 1e-4 and 1e-5 at the signal intensity of saturation (65,000).

2.5. Gene expression data processing and pathway analysis

Raw data were extracted, background subtracted and quantile normalized (Bolstad et al. 2003) using NimbleScan v2.5 software. Gene calls were generated using the Robust Multichip Average algorithm (Irizarry et al. 2003). Statistical analysis was performed by one-way ANOVA for unequal variance with Tukey's posthoc test ($P < 0.05$) in GeneSpring GX v11.0 (Agilent Technologies, Santa Clara, CA) to generate significant gene lists and a Venn diagram identifying unique and common expressions at 60, and 72 hpf. Importing the statistically significant gene list into Multi-Experiment Viewer produced a bi-hierarchical clustering heat map. Individual clusters were analyzed further with the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatic package (<http://david.abcc.ncifcrf.gov/home.jsp>) to determine common and unique functional pathways (Dennis et al. 2003; Huang et al. 2009). A zebrafish Nimblegen background, and individual cluster gene lists were uploaded into DAVID using entrez gene identifiers. Functional annotation of clustering using only biological process levels 3, 4, and 5 was applied to each gene list. Only biological processes that received an enrichment score greater than one were noted on the bi-hierarchical clustering heat map. Zebrafish mRNA sequences on the microarray were blasted at the NCBI website to find the human orthologs with the highest blast score, then subjected to IPA analysis (IPA, Ingenuity® Systems, www.ingenuity.com). The identified genes were mapped to corresponding gene objects in the Ingenuity Pathways Knowledge Base to generate networks, bio-functions and canonical pathways. Additionally, a comparison analysis among the three gene lists (60 hpf, 72 hpf and the common genes) was used to show the relationship of different development period gene changes involved in the certain pathway.

2.6. qRT-PCR analysis

Total RNA was isolated in triplicate from control embryos or embryos exposed to 10 µM TMT from 48 to 72 hpf ($n = 40$ per group). cDNA was prepared from 5 µg of total RNA per group using SuperScript III and oligo (dT) primers in a 20 µl volume. Quantitative PCR using gene specific primers (Table 1) was conducted on an Applied Biosystems Step One Real-Time PCR System. Briefly, 25 ng of cDNA was used for each qRT-PCR reaction using the Power SYBR Green PCR Master Mix (PN# 4367659, Applied Biosystems) according to the manufacturer's instructions. β -actin was selected as the internal reference due to its steady expression in both control and TMT treated larvae. The Comparative C_T ($\Delta\Delta C_T$) was used to measure gene expression compared to the control. Gradient annealing temperature studies were initially completed to determine the optimal annealing temperature for each primer set. Gel electrophoresis and thermal denaturation (melt curve analysis) were used to confirm product specificity. To compare the results from microarray and qRT-PCR, the fold changes of the array expression profiles relative to vehicle control group were compared to the qRT-PCR data. All oligonucleotide primers were synthesized by Eurofins MWG Operon (<http://www.operon.com>).

2.7. Whole mount in situ hybridization

To localize *arf3*, *arf5*, and *atf3* transcripts, *in situ* hybridization (Thisse and Thisse 2008) was performed using 72 hpf embryos from control and 10 μ M TMT exposed groups (exposed from 48 to 72 hpf). The *arf3*, *arf5*, *atf3*, and a reference control *sonic hedgehog* (*shha*) probes were prepared by RT-PCR with cDNA template derived from the RNA isolated from the 72 hpf vehicle larvae. The T3 RNA polymerase site primer 5' CATTAACCCTCACTAAAGGGAA 3' was added to the 5' end of the antisense primer to produce templates for the *in vitro* transcription of antisense probes (Table 2). The embryos were exposed to phenylthiourea (Sigma) at a final concentration of 0.0045% at 24 hpf to inhibit the formation of pigmentation.

2.8. Morpholino microinjection

The sequence of the *arf5* morpholino was 5'-GACCAACTGTGAACATACACGTTTA-3', and the sequence for the standard control morpholino was 5'-CTCTTACCTCAGTTACAATT-3' (Gene Tools, Philomath, OR). Morpholinos were diluted to 1mM in UltraPure distilled water. Approximately 2 nl of 0.5 mM *arf5* morpholino solution was microinjected in the embryos at the 1–2 cell stage. The Tg (*fli1a*: EGFP) y1 embryos of control and *arf5* morphants were allowed to develop until 96 hpf to evaluate the trunk vasculature formation phenotype as described in section 2.2. Primers spanning *arf5* exon 1 (forward primer: 5'-TCGCACTCCGAGCATTCTTTCTGC-3') and exon 4 (reverse primer: 5'-TCTGTCTGTCAGCTCATGGACCGG-3') were used for RT-PCR analysis to confirm the effectiveness of gene knock down. The predicted size of the amplified cDNA fragment containing the *arf5* intron 1 was 642 bp, whereas a product generated from cDNA without intron 1 would be 478 bp.

2.9. Statistical analysis

Nonlinear regression was used to generate the dose response curves for LC₅₀ and EC₅₀ calculations (GraphPad Prism5). A one-way ANOVA was performed to determine statistical significance followed by a Dunnett's post hoc test to independently compare each exposure group to the control group (SPSS, Chicago, IL, USA). All the data were reported as means $\pm$ standard error (SEM) unless otherwise stated.

3. Results

3.1. TMT-mediated malformations

Wild type 5D tropical embryos were exposed to 10 fold TMT serial dilutions (0.01–100 μ M) from 8 to 96 hpf. No mortality was observed at concentrations lower than 1 μ M, and all larvae died at 100 μ M TMT. At 10 μ M, TMT produced mortality in $80 \pm 4.5\%$ of embryos. The surviving embryos displayed malformations including pericardial edema and yolk sac edema. Based on this preliminary trial, embryos were exposed to a narrower concentration range of TMT (1–15 μ M) from 8 hpf and monitored daily until 96 hpf. The calculated LC₅₀ at 96 hpf was 8.25 μ M and the EC₅₀ at 96 hpf was 2.78 μ M (Fig. 1). Similar concentration responses were obtained with the wild type AB line and Tg (*fli1a*: EGFP) y1 embryos (data not shown). TMT exposure induced a variety of deformities including pericardial edema, yolk sac edema, uninflated swim bladder, and curved body axis. The most prominent TMT-dependent endpoint was pericardial edema (Fig. 2); 100% of embryos exposed to 4 μ M of TMT displayed this response.

3.2. Vascular patterning

TMT exposure altered vascular patterning in the yolk sac and tail extension regions (Fig. 3). Vessel disarray such as crossing (arrow point) and proliferation (arrowhead) were only

present in TMT exposed larvae. Quantitative analysis revealed significant increases in the percent of larvae with abnormal vessels with increasing TMT concentrations (Table 3). Though the number of vessel segments from the yolk sac extension to the caudal fin did not change, TMT exposure reduced the distance between intersegmental vessels (ISVs) in both yolk sac and tail extension regions with more pronounced reductions present in the tail region (Table 3). The TMT-induced reduction in the distance between ISVs was generally proportional to the reduction in total body length (Table 1), suggesting there was a secondary effect from delayed development or changes in body curvature in the treatment group. There was a lower incidence of vessel abnormalities in zebrafish exposed to TMT from 48 to 72 hpf than those exposed from 8 to 96 hpf (data not shown). We also found vascular defects in the brain region (Fig. S1), however, it was difficult to quantify the defects in this region due to complicated vessel networks and resultant high GFP intensity.

3.3. Developmental window of TMT sensitivity

5D strain embryos exposed to TMT from 48 to 72 hpf recapitulated the pericardial edema observed when continuously exposed from 8 to 96 hpf (Table 4). The higher 15 μ M TMT exposure induced malformations in all four developmental windows and high mortalities between 24–72 hpf. But 10 μ M TMT induced 100% pericardial edema and no mortality when administered between 48 to 72 hpf. Identical trials using either the wild type AB or the Tg (*flia*: EGFP) y1 lines revealed the sensitivity window was the same for each line. All malformations were registered at 96 hpf. At 72 hpf, larvae in all TMT-exposed groups appeared morphologically normal.

3.4. TMT induced gene expression alternation

To identify the TMT-mediated transcriptional responses that preceded the display of a visual phenotype, whole animal global microarray analysis was completed using RNA isolated from zebrafish exposed to 10 μ M TMT for 12 h (from 48 to 60 hpf) or 24 h (48–72) 72 hpf. To reiterate, TMT exposure from 48 to 60 or 48 to 72 hpf did not register reliably detectable abnormalities until approximately 96 hpf. Analysis of the differentially expressed transcripts resulted in a total of 1657 transcripts that were significantly misregulated ($P < 0.05$) by TMT exposure at 60 and 72 hpf compared to the control group (Fig. 4A). There were fewer differentially expressed transcripts at 60 hpf compared to 72 hpf. Further pathway analysis using DAVID was applied to elucidate the statistical enrichment for functional perturbations upon TMT exposure. The elevated expression categories included disease mutation, lysosome, response to stimulus, regulation of locomotion and protein transport. The lower expressed categories included neurogenesis, learning and memory, central nervous system, cell proliferation, chemotaxis, and locomotor behavior (Fig. 4B).

A Venn diagram was created to compare these transcript sets (Fig. 5). A total of 84 and 1,515 transcripts were changed relative to the vehicle control group at 60 and 72 hpf, respectively, while the misregulation of 58 transcripts was common to both time points. A >2 fold filter was applied, which resulted in a total of 475 transcripts (Fig. 5B), including 8 and 459 transcripts misregulated at 60 and 72 hpf, respectively, while the misregulation of 8 transcripts was common to both time points. For example, *arf1* and *arg2* were elevated, and *bcl6* was reduced at 60 hpf. The genes at 72 hpf are involved in cellular, skeletal and muscular system development and function, tissue development, molecular transport, RNA trafficking, cardiovascular and neurological disease.

3.5. qRT-PCR validation of gene expression profiles

Transcripts were selected for qRT-PCR expression validation. Comparison of mRNA abundance determined by microarray and qRT-PCR revealed similar trends for all transcripts validated (Table 5). Among these, thirteen transcripts were identified by DAVID

pathway analysis: two elevated transcripts were involved in angiogenesis (*lepr*, *zgc:162630*), and the rest were involved in neurological processes (*ned11b*, *arg2*, *casp9*, *galca*, *mmp9*, *fam63a*, *grck7a*, *opnsin1*, *gnat2*, *nnara6* and *fen1*). It was noteworthy that the ADP-ribosylation factor related family members, *arf3* and *arf5*, were elevated 30 and 15 fold, respectively using microarray analysis; however when validated by qRT-PCR, the fold changes were consistently, 1,900- and 1,500-fold elevated compared to the vehicle control group, respectively. The qRT-PCR results generally displayed a higher magnitude of change (Table 5).

3.6. In situ hybridization analysis

To localize *arf3*, *arf5*, *atf3* and *shha* expression, embryos were exposed to 10 μ M TMT or vehicle from 48 to 72 hpf followed by *in situ* hybridization (Fig. 6). *Shha* was used as a reference gene control and stained mainly the notochord, posterior and ventral diencephalon, and the expression was only modestly impacted by TMT exposure (Figs. 6A, B). The expression of *arf3* was greatly increased by TMT exposure. *Arf3* expression in control larvae was detected weakly in the eye and fore and midbrain, but intensely in the head, trunk and tail regions of TMT-exposed larvae (Figs. 6C, D). Similarly, *arf5* expression in the TMT-exposed group was increased and localized to the head, trunk and tail regions compared to control larvae, but there was diminished stain intensity in the eye region (Figs. 6E, F). The expression of *atf3* was present in the center of the eye of control group larvae, but in TMT treated larvae, *atf3* expression extended to the eye periphery and to the midbrain and hindbrain regions (Figs. 6G, H).

3.7. Pathway analysis

The TMT-misregulated transcripts were examined in the IPA software package to identify putative TMT targets. 24 of the 60 hpf TMT genes are involved in vasculature development and disease, with 11 transcripts elevated (e.g., *fgf7*, *atp1a1*, *stat4*, *slc1a4*, *dpysl5*, and *ptk2*) and 13 transcripts reduced (e.g., *antnap1*, *arid4b*, *fhl1*, *bcl6*, *nrob2*, *erbb4*, *rnhl1*, and *adarb1*). 44 of the 72 hpf TMT genes are involved in vasculature development and disease, with 36 transcripts elevated (e.g., *mapt*, *scd*, *rrad*, *lepr*, *mmp9*, *jun*, *vegfa*, *casp9*, and *bcl2l1*) and 8 transcripts reduced (e.g., *angptl4*, *ca2*, *slc1a2*, and *map2k6*). These transcripts and the highly misregulated *arf3*, *arf5*, and *atf3* genes were used to construct a putative TMT targeted vasculature pathway network (Fig. S2). In this network, a total of 26 transcripts were over expressed (e.g., *lepr*, *stat3*, *vegfa*, *jun*, *bcl2l1*, and *mmp9*, red labels in Fig. S2), and 2 transcripts were under expressed (e.g., *gch1*, and *angpt1*, green labels in Fig. S2).

3.8. Arf5 morphants protect TMT-induced vascular defects

To identify functional TMT target genes, we focused on *arf3* and *arf5* because of their strong induction by (Figs. 6D, F). *arf3* and *arf5* share 99% sequence similarity thus, we focused on morpholino knock down of *arf5*. The *arf5* morpholino significantly reduced the parent *arf5* transcript, and produced the predicted splice variant in 24 hpf embryos (Fig. 7A). Injection of *arf5* morpholino at higher concentrations (e.g., 0.5 mM) was lethal. In the absence of TMT, vascular development was not affected by partial *arf5* knock down indicating *arf5* is not essential for vascular development. On the other hand, when *arf5* morphants were exposed to 5 μ M or 10 μ M TMT, the TMT-elicited vascular phenotype was rescued in a significant number of larvae (Fig. 7B), suggesting that *arf5* is a functional TMT target gene. Representative images of control morpholinos injected and *arf5* morphants is illustrated in figure 7C.

4. Discussion

In this study, we reported that TMT exposure produced pronounced developmental toxicity, including pericardial edema and aberrant blood vessel formation in larval zebrafish. The critical developmental window for TMT toxicity causing pericardial edema is between 48–72 hpf. Blood vessel deformities were vascular endothelial cell expansion and aberrant intersegmental vessel patterning. Whole genomic microarray analysis revealed that TMT misregulated numerous transcripts in pathways critical for normal vascular system development. In particular, *arf3* and *arf5* were significantly upregulated by TMT exposure and *arf5* morphants were vascularly unaffected by TMT, suggesting that *arf5* and its associated signaling pathway are TMT targets.

In the present study, the total vessel number was not altered under TMT exposure implying that TMT did not affect extension of the intersegmental arteries and veins. The ISVs are fully formed by 48 hpf at the trunk region under normal conditions, while sprouts for the vertebral artery (associated with the spinal cord) and the parachordal vessel (adjacent to the notochord, at the level of the horizontal myoseptum) do not begin to appear until 84 – 96 hpf (Isogai et al. 2001). Our studies pinpoint that these vessels are the ones affected most by TMT exposure during the 48–72hpf exposure window. It is well established that angiogenesis of small blood vessels occurs via sprouting of endothelial cells from the major axial artery and vein (Zhong et al. 2006). TMT exposure appears to lead to uncontrolled angiogenesis of small blood vessels. Mutations of angiogenic genes such as *ephrinB2* and its receptors *EphB4*, *obd*, and *plxnD1* affect blood vessel pathfinding in the zebrafish (Childs et al. 2002; Helbling et al. 2000; Torres-Vázquez et al. 2004). The knockdown of caldesmon (*cal*) leads to serious defects in vasculogenesis and angiogenesis in zebrafish morphants (Zheng et al. 2009). Vascular endothelial growth factor (*vegf*) is a key regulator of physiological angiogenesis during embryogenesis and signaled through receptor tyrosine kinases *flk1* and *flt1*, and neuropilin (NP)1 and NP2 (Ng et al. 2006; Swift and Weinstein 2009). In the present unbiased microarray study, early life stage zebrafish exposed to TMT exhibited misexpression of many of these and additional angiogenic transcripts. These include *vegfa*, angiopoietin 1 (*angpt1*), *angptl4*, angiogenin inhibitor 1 (*rnhi*), calcium and calmodulin dependent protein kinase II delta (*camk2d*), Ras-related genes associated with diabetes (*rhoa* and *rrad*). Previous studies with cell lines indicated that TMT induced toxicity was mediated through oxidative species which stimulated induction of iNOS followed by increased Bax expression and caspase-executed cell death (Zhang et al. 2006). However, these gene regulations along with others such as stannin, nuclear factor-kappa B (NF-κB), presenilin-1, apolipoprotein E, and pituitary adenylylcyclase-activating polypeptide (Florea and Busselberg 2009; Reese et al. 2005; Toggas et al. 1992) were likely related to TMT induced neuronal degeneration. Their roles in TMT induced vascular deformity are unknown.

At the 60 hpf time point, *stat4* was elevated and it is an upstream regulator for *stat3*, *atf4*, *vegfa*, and *bcl2l1* that were also elevated at 72 hpf in our study. At the 72 hpf time point 459 transcripts were altered >2 fold, with the majority elevated 80% relative to controls. Only four transcripts were reduced suggesting that TMT functioned as signal stimulator rather than inhibitor. Among the 339 transcripts at 72 hpf with greater than 2-fold increase expression in response to TMT, 38 genes were transcription regulators and 74 genes were nuclear. Many of these transcription regulators are activated by phosphorylation, and involved in cascade signaling transduction. We hypothesize that TMT-mediated misexpression of these transcriptional regulators lead to secondary changes in gene expression.

In situ hybridization revealed TMT-induced *arf3* and *arf5* expression in the head, trunk, and tail regions. Expression of *arf3* and *arf5* in the trunk region co-aligned with the ISV region, suggesting that *arf3* and *arf5* may play a significant role in TMT induced vascular hyperproliferation. The strong staining of *arf3* and *arf5* in the heavily vascularized head region might be expected to affect development of the zebrafish brain. However, we cannot preclude the potential effects of *arf3* and *arf5* on the heavily innervated brain region. It is known that the nervous and vascular systems often overlap with each other in anatomy and share similar molecular machinery (Melani and Weinstein 2010). Thus, *arf3* and *arf5* may target the developmental nervous system. Future studies are needed to identify the function of *arf3* and *arf5* in TMT induced neurotoxicity. It is noteworthy that developmental TMT exposure also results in behavioral deficits in larval zebrafish (Chen et al. 2011). The expression of *arf3* was mainly restricted to the eye region, consistent with other studies where *arf3* expression was elevated in the ganglion cell layer of the retina, the nerve fiber layer, and the optic nerve of the injured eye (Saul et al. 2010). The function of *arf3* in TMT induced zebrafish developmental toxicity is unknown.

When *arf5* expression was reduced by morpholino knockdown, the TMT-induced vascular deficits were significantly less pronounced than control morphants exposed to the same concentrations of TMT. These findings suggested that *arf5* plays a significant role in TMT-mediated vascular toxicity. Arfs are a family of highly conserved Ras-related GTP binding proteins that function in the regulation of vesicular traffic and actin remodeling (McGonnell and Fowkes 2006; Schrick et al. 2006). More recently, membrane or vesicular trafficking has been suspected to be involved in multiple steps of angiogenic processes such as vessel sprouting (Wu et al. 2011) and luminal expansion (Herbert and Stainier 2011; Zovein et al. 2010). Further exploration is necessary to fully unravel the underlying mechanism for *arf* mediated TMT-induced vascular toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Concentration-responses of TMT exposure in 5D line zebrafish embryos. Percent mortality (A) and malformation (B) of embryos after waterborne exposure to various concentrations of TMT from 8 to 96 hpf. This experiment was replicated three times with 12 embryos per treatment in each replicate.

Figure 2.

Typical morphology of zebrafish larvae at 96 hpf with and without TMT exposure: (A) control larva without TMT exposure showing normal appearance and inflated swim bladder (SB); embryos exposed to TMT at 2 μ M (B), 5 μ M (C), and 8 μ M (D) from 8 to 96 hpf exhibited different degrees of pericardial edema (PE), yolk sac edema (YSE), and uninflated swim bladder (USB). Scale bars = 1.0 mm.

Figure 3.

TMT induced blood vessel malformation in larval Tg (*fli1a*: EGFP) y1 zebrafish at 96 hpf. A control larva showing normal blood vessel patterning in yolk sac region (A) and tail region (B). TMT treated larvae display moderate vessel defects at 5 μ M (C, D), and severe malformation at 10 μ M (E, F). Arrows point to intersegmental vessel (ISV) crossing; the arrowheads point to abnormal vessel sprouts from the ISVs. Distance shown indicates the length between ISVs.

Figure 4.

Hierarchical clustering with time-matched control and DAVID pathway. (A) The hierarchical clustering ($P < 0.05$, 1627 genes) with the four treatment groups each with three replicates; (B) Further analysis of data from A (controls were removed) by database for annotation, visualization and integrated discovery (DAVID). From the DAVID pathway analysis, TMT induced the statistical enrichment for functional terms including upregulation for chemotaxis, locomotor behavior, cell proliferation, central nervous system, neurogenesis learning or memory, and downregulation for protein transport, disease mutation, lysosome, response to stimulus, regulation of locomotion. Values are Log₂ fold-changes; $P = 0.05$ by one way ANOVA.

Figure 5.

Venn diagram depicting the transcripts that were significantly differentially regulated after TMT exposure. Total RNA from vehicle control or TMT exposed larvae were isolated at 60 and 72 hpf. One-way ANOVA analysis assuming unequal variance and employing Tukey's post-hoc ($n = 3$, $P < 0.05$) showing genes changed versus time-matched control (**A**) and those with at least 2-fold changes (**B**).

Figure 6.

Representative images of *in situ* localization of *shha*, *arf3*, *arf5* and *atf3* expression in the control (A, C, E, G) and 10 μ M TMT (B, D, F, H) exposed larvae at 72hpf. Inserted images show dorsal view of the head and tail region from the same larvae. Embryos were exposed to TMT from 48 to 72 hpf, fixed, permeabilized, and stained for imaging.

Figure 7.

Arf5 morpholino (*arf5*-MO) rescues TMT-induced blood vessel malformation in Tg (*fli1a*:EGFP) y1 zebrafish larva. **(A)** *arf5* mRNA showing increased PCR band with the intron 1 addition (1-1*-2-3-4) from *arf5* morpholino injected group compared to the control morpholino (control-MO) injected group (exon 1-2-3-4) at 24 hpf. **(B)** The percent larvae with abnormal vessels are significantly reduced with *arf5* morphants exposed to TMT from 8 to 96 hpf compared to control larvae ($n = 10 \times 3$). **(C)** Representative images of at 96 hpf Tg (*fli1a*:EGFP) y1 zebrafish injected with control or *arf5* morpholinos exposed to vehicle or TMT. TMT exposed larvae display vessel defects. Arrows point to the intersegmental vessel (ISV) crossing part; the arrowheads point to abnormal vessel sprouts from the ISVs. The *arf5* morphants have normal vascular patterns.

Table 1

Primers used for qRT-PCR. The genes named were identified in NCBI.

Target gene	Forward sequence 5' to 3'	Reverse sequence 5' to 3'
<i>arf3</i>	ACACCAAACACGGCGAACA	CTGCTGCATCCAGACCAACC
<i>arf5</i>	AAAACATCTCGTTCACTGTGTGGG	TCTTCAGTCTCTCTGCTGCC
<i>atf3</i>	TGCGTCAGCTTTGGTTCCCTGT	AGGCTCTGTCAGTGGTCAGGGT
<i>zgc:1105152</i>	GATGTCTCATTGGCTGCTT	TCTGTCACTCACCCGCTCC
<i>rpf582</i>	GCACCACAAACAGTCTACTGGC	TCGGGCACATGAGGGCTGTT
<i>stmn4l</i>	CTAAAGCACTTGGCTGAAA	TTCTCCTGAAGACGCTCC
<i>Novel</i>	TGTGGATCGACCCCATGGAGGT	TGAGACCGCTGAGCCACAGTCA
<i>fosl1</i>	AGCCCTCAACCTCGTCACGTCT	TCCCTCACAGCAGCCAATCGA
<i>NID12*</i>	TGCAGCCCACAGGTACCGTCA	AAGCGCTGACTGCGAGCCTTG
<i>NID 14*</i>	TGTGTGAGAGAAACAGTCGTGGT	AGTTGTGTTGCATGTGAGCCGA
<i>NID 13*</i>	ACAGCTCATCTTTCCCTGGTTCT	AGTGCGTTAGTCCAGTGTTATCA
<i>bbc3</i>	CGTCGGCTTCTGTAGTGTGGT	CGCATCAGCGAACAGTTTGGTTG
<i>NID 19*</i>	TGAGTGAAGTGCAGTCAGCCACA	TGGGGTGTGTCGAGAACGCTT
<i>atf5</i>	AGACATGCCCCCTACAGCTGCA	ACCAGGCGCAACAGCTGAGT
<i>Lepr</i>	TGCCAACTGCTAAATGCTA	CTGCTGCTCTACGGGTCTT
<i>mmp9</i>	GGCGGTGCTCTCCAATAC	GGTCTCCGTCGAATGTCTT
<i>zgc:162630</i>	GGGCTCTTGTAAGTTTGGC	GAGTGGGAGCGTGTCTGAT
<i>fam63a</i>	GCCGACCATGCCGGCGTATTAT	TCACCGAGGTGAGCCATCAGCT
<i>gcrk7a</i>	CTGCGTGTGGGCCTGTCTGT	AGTGCGTAGTCAGAAAATCCCTGC
<i>Ndellb</i>	CGAACTGGACGAGAAGGA	CACAGGTGTAGCTGGGAGA
<i>opsin1</i>	GGAAAGGTGGCTGGTCAT	GCCTAAGAGTGGAGGGAGT
<i>gnat2</i>	AAAAGGCTATGGAAGGAT	GTGGGAAGGTAGTCAGGT
<i>arg2</i>	AACGGCGGACTGACCTAC	CCAGAGCGGATGCAACTA
<i>Galca</i>	CAAGGTAGAAATAGGAGGCG	AGGGAAGACCGATCAGCT
<i>nnara6</i>	TTTTGGAAAGGGAGACGG	GAATAAACGACGAAAGAGC
<i>fen1</i>	AACCAACGGCACCAGCAA	GTCCCAGAAATACAATCAGCAT
<i>casp9</i>	GTGACCAAGCCAGGCAACT	AATGACAGGAGGGCGATG
<i>β-actin</i>	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG

* NID represents transcripts not identified, but differentially expressed by TMT exposure

Table 2

Oligonucleotides used to generate *in situ* hybridization probes.

Target gene	Sequence 5' to 3'
<i>Shha</i>	Forward: GGGCCAGCGGCAGATACGA
	Reverse: GCCAAATGCGCAAGCCCCTG
<i>arf3</i>	Forward: ACACCAAACACGGCGAACA
	Reverse: GTGAGCCAGTCGAGTCCTTCGTA
<i>arf5</i>	Forward: CTATGCCTCGCACTCCAAAATGGG
	Reverse: CCCAGTCTGTCTGTCAGCTCATGGA
<i>atf3</i>	Forward: TGCGTCAGCTTTGGTTCCCTGT
	Reverse: GCTGGTTGGTATGGCGGTGTGT

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Quantitative analysis of vascular structure in Tg (*fli1a*: EGFP) y1 zebrafish larvae at 96 hpf (n = 15). Values in parentheses are mean percentage reduction in length when compared to control larvae.

Table 3

TMT (μM)	% Larvae with abnormal vessel	Vessel number	Distance between two vessels (μm)		
			Yolk sac region	Tail region	Body length (mm)
0	0±0	28.6±0.7	64±4	51±3	3.15±0.07
2	16±8	28.9±0.6	61±3 (5%)	49±3 (4%)	2.95±0.15 (6%)
5	41±10*	28.6±0.6	59±3* (8%)	44±4** (14%)	2.82±0.07 (10%)
8	76±14*	28.8±1.1	58±4** (9%)	41±3*** (20%)	2.73±0.11* (13%)
10	70±11**	28.7±1.0	55±3** (14%)	39±3** (24%)	2.60±0.07* (17%)

Asterisks indicate a significant difference at P < 0.05(*) or P < 0.001(**). Larva with more than four trunk segments showing malformed vessels is considered as abnormal.

Table 4

Percent normal morphology, pericardial edema, and mortality of 5D line zebrafish larvae at 96 hpf after TMT exposure at various developmental window (n = 16 embryos/larvae per group).

TMT (μ M)	Exposure window (hpf)	% Normal larvae	% Pericardial edema	% Mortality
0	8 to 96	100	0	0
5	8 to 24	94	6	0
	24 to 48	94	0	0
	48 to 72	63	37	0
	72 to 96	88	12	0
10	8 to 24	81	19	0
	24 to 48	19	81	0
	48 to 72	0	100	0
	72 to 96	88	13	0
15	8 to 24	92	8	0
	24 to 48	0	100	50
	48 to 72	0	100	87
	72 to 96	88	12	0

Table 5
Comparisons of TMT-regulated transcripts in larvae at 72 hpf between microarray and qRT-PCR analysis

Gene	Microarray			qRT-PCR			Gene targeting effect		SEQ_ID
	Fold	SEM	P-value	Fold	SEM	P-value			
<i>arf3</i>	30.2	4.4	0.02	1938.5	405.2	0.04	ADP-ribosylation factor related family member		TC252426 ^a
<i>arf5</i>	15.1	5.5	0.12	1523.4	174.6	0.01	ADP-ribosylation factor related family member		ENSDART00000056047 ^b
<i>atf3</i>	14.4	1.6	0.01	65.3	6.1	0.01	activating transcription factor 3 (<i>atf3</i>)		OTTDART00000006631 ^c
<i>zgc:1105152</i>	11.9	0.6	0.00	32.8	6.3	0.04	-		NM_001017853 ^d
<i>rfp582</i>	10.7	2.5	0.06	42.0	5.3	0.02	-		ZV700S00001968 ^e
<i>stmn4l</i>	9.9	1.2	0.02	14.3	1.4	0.01	stathmin-like 4; differentiation and proliferation		OTTDART000000025126
<i>Novel</i>	9.4	3.5	0.14	43.9	6.2	0.02	-		ENSDART00000058949
<i>fosl1</i>	9.3	1.8	0.05	81.4	8.0	0.01	protein similar to vertebrate FOS-like antigen 1		OTTDART000000023370
<i>NID 12</i>	8.6	1.9	0.06	14.6	2.0	0.02	-		ZV700S000006532
<i>NID 14</i>	8.6	3.0	0.12	45.6	8.8	0.04	-		ZV700S000006639
<i>NID 13</i>	8.1	1.4	0.04	12.4	1.9	0.03	-		TC254817
<i>bbc3</i>	7.8	1.9	0.07	12.8	2.1	0.03	control permeable of the outer mitochondrial membrane		ZV700S000004671
<i>NID 19</i>	7.4	1.5	0.05	16.3	2.6	0.03	-		ZV700S000002527
<i>atf5</i>	7.3	1.1	0.03	15.2	0.9	0.00	activating transcription factor 5		OTTDART000000021442
<i>Lepr</i>	4.9	1.1	0.07	5.5	0.5	0.01	leptin receptor; type 2 diabetes mellitus		ENSDART000000104616
<i>mmp9</i>	4.6	1.3	0.11	14.8	2.2	0.02	matrix metalloproteinase 9		OTTDART000000025258
<i>zgc:162630</i>	4.5	0.5	0.02	41.2	2.8	0.00	-		NM_001082825
<i>fam63a</i>	3.7	0.6	0.04	11.1	0.3	0.00	-		OTTDART000000017116
<i>gcrk7a</i>	-3.4	0.6	0.06	-5.7	0.9	0.04	G-protein-coupled receptor kinase 7a		OTTDART000000024144
<i>Ndel1b</i>	3.4	0.5	0.04	4.4	0.1	0.00	NudE-like protein 1b; neurological disorders		OTTDART000000024237
<i>opsin1</i>	-3.3	0.6	0.07	-7.1	0.8	0.02	opsin 1 (cone pigments), short-wave-sensitive 2		OTTDART000000002229
<i>gnat2</i>	-3.0	0.8	0.13	-6.9	1.1	0.04	G-protein alpha transducing activity polypeptide		ZV700S000006718
<i>arg2</i>	2.8	2.1	0.49	13.8	0.3	0.00	arginase, type II; smooth muscle disorder		OTTDART000000026242
<i>Galca</i>	2.8	0.9	0.18	2.7	0.6	0.09	galactosylceramidase; cellular surface cancers		OTTDART000000004460
<i>mnar6</i>	-2.7	0.4	0.06	-4.9	1.2	0.09	neuronal nicotinic acetylcholine receptor alpha 6		NM_001042684
<i>fen1</i>	-2.6	0.3	0.03	-3.8	0.2	0.01	elongation of very long chain fatty acids (<i>fen1</i>)		BC060897.1 ^f

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Gene	Microarray		qRT-PCR		Gene targeting effect		SEQ_ID
	Fold	SEM	P-value	Fold	SEM	P-value	
<i>casp9</i>	2.5	0.1	0.00	4.5	0.5	0.02	OTTDART00000028081

Note: fold changes with minus signs indicate down regulation. The sequence IDs were identified using designated online resources:

- ^a<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=zfsh;>
- ^b<http://www.ensembl.org/index.html;>
- ^c<http://vega.sanger.ac.uk/index.html;>
- ^d<http://www.ncbi.nlm.nih.gov/nuccore;>
- ^e<http://www.ncbi.nlm.nih.gov/nucest;>
- ^f<http://www.ncbi.nlm.nih.gov/gene>