Transcriptome kinetics of arsenic-induced adaptive response in zebrafish liver

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Lam, Siew Hong, Cecilia Lanny Winata, Yan Tong, Svetlana Korzh, Wen San Lim, Vladimir Korzh, Jan Spitsbergen, Sinnakarupan Mathavan, Lance D. Miller, Edison T. Liu, and Zhiyuan Gong. Transcriptome kinetics of arsenic-induced adaptive response in zebrafish liver. *Physiol Genomics* 27: 351–361, 2006. First published August 1, 2006; doi:10.1152/physiolgenomics.00201.2005.—Arsenic is a prominent environmental toxicant and carcinogen; however, its molecular mechanism of toxicity and carcinogenicity remains poorly understood. In this study, we performed microarray-based expression profiling on liver of zebrafish exposed to 15 parts/million (ppm) arsenic [As(V)] for 8–96 h to identify global transcriptional changes and biological networks involved in arsenic-induced adaptive responses in vivo. We found that there was an increase of transcriptional activity associated with metabolism, especially for biosyntheses, membrane transporter activities, cytoplasm, and endoplasmic reticulum in the 96 h of arsenic treatment, while transcriptional programs for proteins in catabolism, energy derivation, and stress response remained active throughout the arsenic treatment. Many differentially expressed genes encoding proteins involved in heat shock proteins, DNA damage/repair, antioxidant activity, hypoxia induction, iron homeostasis, arsenic metabolism, and ubiquitin-dependent protein degradation were identified, suggesting strongly that DNA and protein damage as a result of arsenic metabolism and oxidative stress caused major cellular injury. These findings were comparable with those reported in mammalian systems, suggesting that the zebrafish liver coupled with the available microarray technology present an excellent in vivo toxicogenomic model for investigating arsenic toxicity. We propose an in vivo, acute arsenic-induced adaptive response model of the zebrafish liver illustrating the relevance of many transcriptional activities that provide both global and specific information of a coordinated adaptive response to arsenic in the liver. microarray expression profiling; arsenic toxicity; oxidative stress; fish toxicogenomics

ARSENIC IS AN IMPORTANT and ubiquitous environmental toxicant, and the risk of arsenic poisoning in humans is a public health issue worldwide (1, 23, 27, 46, 54). In addition, arsenic is classified as a human carcinogen based on several epidemiological studies showing an association of arsenic exposure with cancers in lung, bladder, kidney, and liver (33, 54). Aside from cancers, arsenic ingestion is also associated with other human diseases such as blackfoot disease, atherosclerosis, hypertension, diabetes mellitus, skin lesions, and liver injury (19, 20, 37, 54, 62). Despite these findings and the fact that arsenic is the most extensively studied of the metals and metalloids in drinking water, the molecular mechanisms of arsenic toxicity and carcinogenicity are poorly understood (34, 46). This is partly due to the difficulty of inducing cancer with arsenic in animal models and the complex effects induced by arsenic through a variety of mechanisms that influence numerous signal transduction pathways, as these effects vary depending on cell type, dosage, and form of arsenic used (7, 60). Thus many different modes of action have been proposed, and these include both genotoxic and nongenotoxic effects (33, 34, 46). Some of the genotoxic effects include induction of chromosomal abnormalities, oxidative stress, gene amplification, and altered DNA repair, whereas nongenotoxic actions involved altered cellular signaling (growth factors and cell proliferation), altered DNA methylation patterns, and disruption of protein function. To add to the complexity, a genotoxic event can lead to nongenotoxic effects and vice versa.

The zebrafish has been used as a premier model not only for vertebrate development (28, 38, 42) but also for understanding human diseases (3, 35, 41, 49) and toxicology (50). Although there have been in vitro studies (13, 14), until recently there was no published report on in vivo exposure of zebrafish to arsenic. Since initiation of our analysis, this gap started to fill rather rapidly (30, 44, 61), indicating an increased interest to use zebrafish in toxicology studies of arsenic. The long history of zebrafish in carcinogen toxicology studies also provides good experimental and literature backgrounds (9, 51) for investigating arsenic carcinogenic and toxic mechanisms. Many physiological and molecular similarities in xenobiotic metabolism and adaptive response to toxicant insult were found between zebrafish and mammals, rendering zebrafish a suitable toxicology model (13, 50). Moreover, fish have long been used as sentinels for biomonitoring of aquatic environmental pollutants and are good indicators of arsenic toxicity (55). In addition, the amenability of the zebrafish system to various molecular techniques and the vast genomic resources, including the near-completed zebrafish genome project and available zebrafish microarrays, make it a highly versatile system for toxicogenomic studies.

The liver is a major target organ of arsenic toxicity in both mice (59, 63) and humans (17, 18, 37, 39, 54). Arsenic-induced liver injury in humans is a common phenomenon, typically manifesting initially as degenerative lesions with jaundice, progressing to noncirrhotic portal hypertension, fibrosis, cirrhosis, and neoplasia such as hepatocellular carcinoma (15, 37, 39). Previous microarray studies on arsenic were mainly performed in vitro using human or murine cell lines (4, 6, 21, 22, 45, 64, 66, 67) and yeast (29), while in vivo studies typically involved chronic exposure to arsenic (37, 62, 63). A recent
study investigated subacute in vivo effects of dimethylnarsinic acid on gene expression profiles of rat urothelium (48). Although many arsenic-induced, differentially expressed genes have been identified, there are no data on in vivo kinetics evaluated by the microarray approach on the adaptive response of a specific targeted organ, such as liver; undoubtedly, these data from microarray analyses should aid further understanding of arsenic mechanisms of toxicity resulting in pathology.

In this study, we used a zebrafish oligoarray with 16,416 gene probes (representing ~14,900 unique genes or about one-third of the genes in the zebrafish genome) to investigate the arsenic-liver molecular interaction in zebrafish subjected to acute arsenic exposure. Arsenate [As(V)] was used in our experiment, as it can be rapidly metabolically reduced to arsenite [As(III)] and biotransformed to other methylated arsenicals (33, 34), and this has been shown to occur in fish as well (10, 52). In addition, we believe that As(V) is found abundantly in natural aquatic systems, the predominant arsenic form under aerobic conditions (46), and it has also been reported in a study in Lake Washington that about two-thirds of arsenic in rain and lake water was arsenate (24). Our main objective was to investigate whether the in vivo zebrafish liver system would show adaptive responses to arsenic toxicity at the molecular level similar to those reported in mammalian systems based on the arsenic-inducible genes, especially those involved in the many proposed genotoxic and nongenotoxic modes of action (33, 34, 46). This would provide molecular evidence of the relevance of using zebrafish liver as a toxicogenomic model for investigating arsenic toxicityology. In addition, by using available computational tools, we were able to capture the transcriptome changes that indicate important physiological events that could lead to possible pathological conditions in zebrafish liver exposed to arsenic. Our findings provide further insights into the underlying mechanisms of arsenic toxicity and indicate that the zebrafish liver coupled with the available microarray technology present an excellent in vivo model for investigating arsenic toxicity at the transcriptome level.

**MATERIALS AND METHODS**

**Arsenic Exposure**

To estimate the toxicity and a suitable concentration of arsenic for the exposure experiment, adult zebrafish were treated with different concentrations of arsenic [As(V)] acid (Na$_2$HAsO$_4$·7H$_2$O; Sigma) ranging from 20 to 100 mg/l (~5–25 parts/million (ppm) As(V)) for 96 h at a density of 1 fish/150 ml at 27 ± 2°C in a static condition. Control fish were kept in water under similar conditions, and a total of 12 fish were used for each group. Test solutions and water were changed daily. Fish were fed once a day with commercial frozen bloodworms (Hikari). Arsenic from a stock solution was added to the exposure experiment, as it can be rapidly metabolically reduced to arsenite before clearing in Histoclear and embedding in paraffin. The paraffin-embedded tissues were sectioned serially at 6-μm thickness and stained with hematoxylin and eosin or used for immunohistochemical study.

Staining for glycogen was performed using periodic acid Schiff staining (PAS) with or without diastase according to the manufacturer’s protocol (Sigma-Aldrich). For immunohistochemistry of paraffin sections, antigen retrieval was performed by heating before treating sections with 6% hydrogen peroxide in methanol for peroxidase blocking and in blocking buffer (10% bovine serum-PBS) for 20 min to block nonspecific binding. Primary antibodies including mouse monoclonal anti-human cytokeratin-18 (Ks18.04, Maine Biotechnology Services; 1:500 dilution) and mouse anti-human E-cadherin (BD Biosciences, 1:300 dilution) were incubated at room temperature for 4 and 2 h, respectively. Rabbit anti-mouse antibody conjugated with horseradish peroxidase (Zymax grade, DAKO) for detection by 3,3’-diaminobenzidine tetrahydrochloride (DAB) substrate-chromagen (DAKO) was used as a secondary antibody. Negative controls were set up by omitting primary antibodies. Positive reactivity was revealed by the formation of brown-colored precipitate at the site of the target antigen. Sections were lightly counterstained with hematoxylin.

Images of the stained liver sections were captured using either an Axioptot 2 or Axiovert microscope (Zeiss) equipped with an imaging system and were analyzed with a computer-assisted image analyzer program (Axiovision, Zeiss). Density of nucleated cells (no. of nucleated cells/9,600 μm$^2$) and the size of nucleus were determined with an image analyzer in both the arsenic-treated group and the control group using hematoxylin- and eosin-stained sections. Three images of liver sections (630 × magnification) for each liver from five control and five arsenic-treated fish samples of the 48-h group were used to determine the density of nucleated cells. Five images of liver sections (1,000 × magnification) from three control and three arsenic-treated fish (n = 3 liver samples) of the 72-h group were used to measure the size of the nuclei for 50–60 hepatocytes/image. A two-tailed heteroscedastic t-test was used to determine the statistical significance (P < 0.05).

**Fish**

**Total RNA Extraction**

Total RNA was extracted from livers using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Reference RNA was obtained by pooling equal amounts of male and female total RNA extracted from liver tissues of wildtype zebrafish. The integrity of RNA samples was verified by gel electrophoresis, and the concentrations were determined by UV spectrophotometer.

**Histology Procedures and Image Analysis**

Fish were slit open ventrally from the heart to the anus to expose the digestive organs. The gut, together with surrounding tissues including the liver, was removed and fixed in a freshly prepared Bouin’s solution. Fixed gut samples were washed several times with 70% ethanol, followed by dehydration in a graded series of ethanol before clearing in Histoclear and embedding in paraffin. The paraffin-embedded tissues were sectioned serially at 6-μm thickness and stained with hematoxylin and eosin or used for immunohistochemical study.

Staining for glycogen was performed using periodic acid Schiff staining (PAS) with or without diastase according to the manufacturer’s protocol (Sigma-Aldrich). For immunohistochemistry of paraffin sections, antigen retrieval was performed by heating before treating sections with 6% hydrogen peroxide in methanol for peroxidase blocking and in blocking buffer (10% bovine serum-PBS) for 20 min to block nonspecific binding. Primary antibodies including mouse monoclonal anti-human cytokeratin-18 (Ks18.04, Maine Biotechnology Services; 1:500 dilution) and mouse anti-human E-cadherin (BD Biosciences, 1:300 dilution) were incubated at room temperature for 4 and 2 h, respectively. Rabbit anti-mouse antibody conjugated with horseradish peroxidase (Zymax grade, DAKO) for detection by 3,3’-diaminobenzidine tetrahydrochloride (DAB) substrate-chromagen (DAKO) was used as a secondary antibody. Negative controls were set up by omitting primary antibodies. Positive reactivity was revealed by the formation of brown-colored precipitate at the site of the target antigen. Sections were lightly counterstained with hematoxylin.

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**Zebrafish Oligonucleotide Microarray Construction and Hybridization**

Zebrafish oligonucleotide probes for this array were designed by CompuGene and synthesized by Sigma Genosis. For each gene feature in the array, one 65-mer oligonucleotide probe was designed from the 3’-region sequences. Each probe was selected from a sequence segment that is common to a maximum number of splice variants predicted for each gene. The arrays contained 16,416 oligonucleotide probes (more information can be obtained from http://www.labonweb.com/chips/libraries.html). The array also contains 172 spots representing the same β-actin probe as controls. Oligonucleotide probes were resuspended in 3× SSC at 20 μM concentration and spotted onto poly-L-lysine-coated microscope slides using a custom-built DNA microarrayer in the Genome Institute of Singapore (GIS). The arrays
were spotted and quality controlled essentially as described by Eisen and Brown (26).

For fluorescence labeling of cDNAs, 20 μg of total RNA from the reference and sample RNAs were reverse transcribed in the presence of Cy3-dUTP and Cy5-dUTP (Amersham), respectively. Labeled cDNA were pooled, concentrated, and resuspended in DIG EasyHyb (Roche Applied Science) buffer for hybridization at 42°C for 16 h in a hybridization chamber (Gene Machines). After hybridization, the slides were washed in a series of washing solutions (2 × SSC with 0.1% SDS, 1 × SSC with 0.1% SDS, 0.2 × SSC and 0.05 × SSC; 30 s each), dried using low-speed centrifugation, and scanned for fluorescence detection.

Data Acquisition, Processing, and Analysis

The arrays were scanned using the GenePix 4000B microarray scanner (Axon Instruments), and the generated images with their fluorescence signal intensities were analyzed using GenePix Pro 4.0 image analysis software (Axon Instruments). All the data were uploaded into the GIST Microarray Database where normalization (median-centered normalization), statistical filtering, and analyses were carried out.

The microarray raw data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; accession no. GSE3048). They are compliant with the minimum information about a microarray experiment (MIAME) standard.

All the arrays had >80% of the gene features that gave a measurable signal. Only gene features that were not flagged and those with signal-to-background ratio >1.5 were extracted for analyses. Statistical comparison of genes between arsenic and control samples for different time point groups was performed using heteroscedastic t-test, and the resulting P values were adjusted for Benjamini and Hochberg false discovery rate (FDR) (11). The FDR adjustment is introduced to minimize the number of false-positive genes that could be identified by chance because of multiple hypothesis testing of large data sets (57).

Gene Ontology (GO) categories were analyzed using a web-based program, GOTree Machine (GOTM) (65). Genes were classified according to their annotated role in biological processes, molecular functions, and cellular components from GO (the Gene Ontology Consortium). The GOTM program identifies genes belonging to different GO categories based on available standard biological identifiers, and in this study we used the zebrafish UniGene identity number (build 79) as the identifier for the genes. The GOTM program calculates the statistical significance (P value) of nonrandom representation, that is, enrichment, of a GO category in the gene set of interest that is under investigation by comparison with a reference gene set (in this study, we used the entire list of gene probes on the array as the reference gene set) using a hypergeometric test. Therefore, the calculation of the statistical significance for a GO category takes into account the size of the gene set of interest compared with the entire reference gene set on the array. An “expected value” for the number of gene probes called significant, which increases with the size of the gene set of interest as a result of random chance selection, has been calculated for each GO category based on the entire gene set on the array. A GO category is considered significantly enriched when the ratio of the observed number of gene probes over the expected number of gene probes for the GO category surpasses a threshold value determined at P < 0.01 by the hypergeometric test in GOTM. It is therefore important to note that a GO category that is termed enriched here is relative, with a default statistical cutoff at P value < 0.01. A GO category that is significantly enriched in one time point group (P < 0.01) but not in another group (P > 0.01) does not necessarily mean that the biological significance associated with that particular GO has completely ceased to exert its role in the liver of the later group, but rather that its role has been relatively reduced.

Quantitative Real-Time PCR

Equal amounts of total RNA samples from control and arsenic-treated liver tissues and reference were reverse transcribed to cDNA. The cDNA samples were used for quantitative real-time PCR analysis, performed using the Lightcycler system (Roche Applied Science) with Lightcycler-FastStart DNA Master SYBR Green 1 (Roche Applied Science) according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

Arsenic Acute Toxicity Test

On the basis of our arsenic acute toxicity test results (Fig. 1), the 50% lethal concentration (LC50) at 96 h of arsenic treatment for adult zebrafish was estimated to be within 20–25 ppm As(V) (256–320 μM). Zebrafish treated with 5–15 ppm As(V) usually died within the first 48 h of treatment, and thereafter surviving fish appeared to be able to tolerate subsequent arsenic exposure with minimal mortality in the next 48 h. These observations suggest that the arsenic concentrations used in this study produced toxicity, and fish would either succumb to or recover from it. Fish that recovered from initial arsenic exposure appeared to show better tolerance to subsequent exposure. Increased tolerance to arsenic following initial exposure has been reported at both cellular and organism levels and is attributed to activated adaptive responses such as detoxification and expulsion mechanisms (36, 53). However, fish treated with 20–25 ppm As(V) showed a continuous decline in survival throughout the 96 h of treatment, suggesting that these concentrations were too high for tolerance to be achieved in many of the fishes. The arsenic acid concentration of 15 ppm As(V) (192 μM) was chosen for treatment of fish in the subsequent microarray experiment, as this concentration would likely result in acute hepatotoxicity leading to liver injury, with a sufficient number of surviving fish for the microarray experiment. Doses of 5–400 ppm of inorganic arsenic have been used in mouse models to generate desirable pathological conditions, with treatment duration ranging from 16 wk to 24 mo (60). Therefore, we reasoned that use of an acute arsenic exposure of 15 ppm As(V) over 96 h would be suitable for producing a spectrum of responses to arsenic toxicity, ranging from early to late/advanced toxic effects, to capture the transcriptome changes that recapitulate an acute arsenic-induced adaptive response in the zebrafish liver.

![Fig. 1. Percentage of fish survival throughout 96 h of 5–25 parts/million (ppm) arsenic [As(V)] treatment.](https://physiolgenomics.physiology.org/doi/10.220.33.1.on.April.9.2017)
Histopathology of the Liver in Arsenic-Treated Zebrafish

In our experiments, no gross morphological changes and histopathological alterations in the liver could be convincingly detected by the naked eyes or under light microscopy in fish treated with 15 ppm As(V) for 8 and 24 h compared with the control group. However, after 48 h of arsenic treatment, we found that the liver tissues became more delicate (soft and fragile) over time compared with control samples, indicating a rapid change in liver tissue consistency of arsenic-treated fish. In addition, green coloration was observed in some livers of arsenic-treated fish sampled at 72 and 96 h, indicating cholestasis (not shown). The presence of cholestasis has also been suggested in humans exposed to arsenic (31), further implying similar arsenic toxicopathology in both human and fish liver.

At lower magnification (200×), the hepatic parenchyma of arsenic-treated fish sampled from 48 h onward (Fig. 2A) was less homogenous compared with the liver of control fish (Fig. 2B). In addition, the uneven staining intensity of eosin, with an increase in cytoplasmic eosinophilia, observed in the liver of arsenic-treated fish compared with the more uniform staining in the liver of control fish clearly indicates cytopathological alterations of hepatocytes. Higher magnification (1,000×) revealed changes in hepatocyte morphology of arsenic-treated fish (Fig. 2C). Hepatocytes were more dissociated and irregular in shape with swelling compared with the more compact polygonal-shaped hepatocytes in control fish (Fig. 2D). The measured nucleus size of hepatocytes from fish treated with arsenic for 72 h was 7.62 ± 0.77 (SD) µm, significantly (P < 0.001)
larger than hepatocytes from control fish (6.28 ± 0.63 μm).
Density of nucleated cells in livers of arsenic-treated fish
sampled at 48 h was 88 ± 10 cells/9,600 μm², significantly
(P < 0.05) lower than that in livers of control fish (106 ± 6
cells/9,600 μm²). Nuclear anomalies such as karyomegaly,
karyorhexis, and karyolysis were observed more frequently in
livers of arsenic-treated fish than in livers of control fish (Fig.
2, C and D). Collectively, these data suggest that the nucleus,
cytoplasm, and surface cell membranes are targets of arsenic.
Genotoxic damage from arsenic is reported in both humans and
zebrafish (7, 44).

PAS staining was used to assess liver glycogen content. As
shown in Fig. 2E, the glycogen content in livers of arsenic-
treated fish (48 h onward) was much lower than that in livers
of control fish (Fig. 2F). Hepatic glycogen is rapidly mobilized
during stress situations in fish (32), and, in this case, hepatic
glycogen was rapidly depleted as a result of the arsenic-
induced toxic stress. Immunohistochemical staining of cyto-
keratin for bile canaliculi and ducts revealed short, shriveled
canalicular staining in livers of arsenic-treated fish (72 h
onward; Fig. 2G) compared with the stronger and longer
canalicular staining in livers of control fish (Fig. 2H), suggest-
ing defects in the hepatobiliary system of arsenic-treated fish
that could lead to cholestasis, as observed in some of the livers.
Immunohistochemical staining for E-cadherin, a transmem-
brane glycoprotein that mediates epithelial cell-to-cell adhe-
sion, revealed weak signals on the surface membrane of hepa-
tocytes in arsenic-treated fish (72 h onward; Fig. 2I) compared
with the stronger staining observed in controls (Fig. 2J). This
suggests that arsenic affected intercellular adhesion, compro-
mising tissue integrity in the liver.

Similar effects of arsenic on cell morphology, cytoskeleton,
and surface membranes, although poorly understood, have
been reported for the liver, brain, bladder, endothelial cells, and
skin of mammals (12). Taken together, these findings are
consistent with acute arsenic exposure at 15 ppm As(V) within
96 h causing liver injury in zebrafish.

General Assessment and Identification of Temporal
Differentially Expressed Gene Sets in the Liver of
Arsenic-Treated Zebrafish

The gene expression profiles of 12 pooled liver samples
from 48 arsenic-treated fish (each pooled liver sample was
taken from 4 individual fish) and 12 similarly pooled liver
samples from 48 control fish sampled from four time points
(each time point consisting of 3 pooled liver samples) were
characterized. To have a better assessment of the liver tran-
scriptome changes in response to arsenic, we systematically
explored how the number of significant gene probes changes
with individual and combined time point groups based on
duration of arsenic exposure (Fig. 3A). As early as 8 h of
exposure to arsenic, a substantial number (516) of gene probes
called significant (FDR <10%) were differentially expressed,
but it was not until 48 h of exposure to arsenic that a peak response occurred at the transcription level. With FDR <10%, the number (1,584) of gene probes called significant at 48 h was about threefold of that at 8 or 24 h and less than twofold of that in 96 h, indicating marked changes in the transcriptome between 24 and 48 h.

Combining two or more time points together for analysis allowed us to check for the behavior of the expression kinetics between the time points. Increase of sample size improved statistical power and resulted in a greater number of gene probes called significant to be identified (as observed in the combined time point groups compared with the individual time point groups). However, it is interesting to note that the number of gene probes called significant in the combined 48- and 96-h group \((n = 6)\) is similar to the overall 8- to 96-h group despite the later group having double the sample size \((n = 12)\), suggesting existing differences in expression kinetics between the time points \((8, 24, 48, 96)\). The findings again highlight the transcriptome changes between the earlier 8- and 24-h time points compared with the later 48- and 96-h time points. Thus we termed the combined 8- and 24-h group as the “Early” group and the combined 48- and 96-h group as the “Late” group to facilitate assessment of the expression kinetics between the early and late time points in subsequent analyses.

Comparison of overlapping genes called significant at a high stringency of FDR <1% between the Early and Late groups revealed 285 overlapping gene probes, representing 38 and 16.7% of the genes called significant in the respective group (Fig. 3B). These 285 gene probes, regarded as the common Early-Late arsenic-responsive gene set \((I)\), would be most suitable to serve as a liver biomarker gene set for acute arsenic exposure, as they were responsive in both Early and Late groups. The remaining nonoverlapping genes between the Early and Late groups indicate that these genes are significantly differentially expressed in a temporal manner, in that they were either only early responsive \((II; 465 \text{ gene probes regarded as unique Early arsenic-responsive gene set})\) or late responsive \((III; 1,417 \text{ gene probes regarded as unique Late arsenic-responsive gene set})\) to arsenic exposure (see Supplemental Data 1 for each complete gene set; Supplemental Materials are available in the online version of this article). These data also confirmed the marked transcriptome changes between 24 and 48 h of exposure to arsenic. These gene sets can be used as a reference for comparative purposes in future arsenic studies involving different concentrations, forms, and durations of exposure.

Identification of Major Transcriptional Changes and Biological Networks in the Liver Affected by Arsenic

To identify major transcriptional changes and biological networks associated with the effects of arsenic on the liver transcriptome, we analyzed the GO category for the differentially expressed genes called significant (FDR <1%) for the Early and Late groups, using a web-based program, GOTM (65). Using the GOTM program, we were able to integrate the various enriched GO categories in the form of a directed acyclic graph (DAG) to aid visualization of GO networks and identification of important global biological networks in the liver affected by arsenic, as shown in Fig. 4, A and B, for the Early and Late groups, respectively. A summary of the number of genes observed in each of the GO categories and the corresponding \(P\) value is provided in Supplemental Table 1. In general, GO categories affected by arsenic exposure were biological processes associated broadly with various metabolisms including both catabolism and biosynthesis and response to stimulus and localization; molecular functions associated broadly with binding, translation, catalytic, and transporter activities; and cellular components associated broadly with the membrane and cytoplasm.

We found that 58 and 104 GO categories were significantly \((P < 0.01)\) enriched for the Early and Late groups, respectively. The almost twofold increase in the number of enriched GO categories in the Late group was caused by increases in GO categories associated with biological processes such as various metabolisms and biosyntheses, molecular functions such as transcription and transporter activities, and various cellular components associated broadly with membrane- and non-membrane-bound organelles. This revealed the major biological program changes in the liver between the Early and Late stages. This change is more evident when we examined the number of significant genes and their corresponding \(P\) values in the enriched GO categories (see Supplemental Table 1). Although in general, the number of genes increased in most of the enriched GO categories in the Late group compared with the Early group, a marked nonrandom increase was observed in GO categories associated with various metabolisms (especially biosynthesis), transporter activities, and cellular components (such as membrane, cytoplasm, and endoplasmic reticulum), as evidenced by the 1,000- to over 10,000-fold difference in \(P\) values corresponding to some of the GO categories between the Early and Late groups. This showed that the increased numbers of gene probes called significant observed in these enriched GO categories were not merely by chance, due to more significant gene probes being identified in the Late group, but were the result of a biologically coordinated global program responding to the arsenic load in the liver. This marked nonrandom increase in the number of genes accounts for the prominent changes and emphasizes the importance of these biological components and their roles in the liver between 24 and 48 h of exposure to arsenic. The continuous need to meet the cellular demands of the adaptive response to the arsenical insults may also explain the dramatic transcriptome changes between 24 and 48 h. Within the first 24 h following the initial arsenic exposure, the liver had adjusted to the early effects of arsenic toxicity; however, as the accumulation of arsenicals in the liver increased with prolonged exposures, more energy and precursor metabolites were needed to meet the demands of the arsenic-induced adaptive response, hence the marked increase of differentially expressed genes associated with metabolisms.

On the other hand, there also appeared to be some GO categories that remained significantly enriched but changed relatively little (in terms of statistical significance, i.e., <100-fold change in \(P\) values) between the Early and Late groups, such as organic acid metabolism, energy derivation by oxidation of organic compounds, electron transport, catabolism, response to stress, ATP binding, and catalytic activities. The findings suggest that these biological processes played crucial roles in the liver adaptive response to acute arsenic toxicity. Taken together with these findings, the GOTM analysis indicated that, during the 96 h of arsenic treatment, there was an increase in transcriptional activities for proteins associated
with metabolism, especially biosyntheses and transporter activities localizing in membrane, cytoplasm, and endoplasmic reticulum, while transcriptional programs for proteins associated with catabolism, energy derivation, and response to stress/stimulus remained significant, with relatively few changes throughout the arsenic treatment.

**Identification of Biologically Relevant Genes Important for Adaptive Responses in the Liver of Arsenic-Treated Zebrafish**

To identify genes that are important for adaptive responses in the liver exposed to arsenic, we examined the gene probes called significant that were enriched in various GO categories. However, because most of the gene probes are assigned with multiple GOs, we have selected representative genes and grouped them into a general functional category in relation to adaptive responses that span the Early, Late, and “Overall” (combining 8 to 96 h) groups to facilitate presentation and discussion of the findings (Supplemental Table 3). Using real-time PCR, we have validated 39 genes and confirmed that they are significantly \((P < 0.05)\) differentially expressed (Supplemental Table 2). On the basis of our findings, we proposed an acute arsenic-induced adaptive response model of the zebrafish liver (Fig. 5).

**Arsenic metabolism.** The liver is the organ where most of the biotransformation of inorganic arsenic takes place (25, 46). Uptregulation of several genes coding for glutathione transferases, S-adenosylmethionine-dependent methyltransferases, thioredoxins, and ArsA arsenite transporter, suggests that active arsenic metabolism and generation of arsenic radicals occurred in the zebrafish liver. It has been reported that a small proportion \((3–7\%)\) of accumulated arsenic in tissue can be biomethylated in the medaka fish, *Oryzias latipes*, when it is exposed to 5–20 mg/l inorganic arsenic \([\text{iAs(V)}]\) (52). There is a proposal that these reactive species are responsible for the arsenic-induced stress response (25). In yeast, it has been shown that the depletion of glutathione pools in the cell as a result of arsenic metabolism can lead to oxidative stress and arsenation of sulfhydryl groups on proteins which can alter protein turnover (29). Consequently, expression of genes associated with stress proteins that are involved in the protection and repair of vulnerable proteins and DNA was rapidly upregulated.

**Stress response.** “Response to stimulus” resulting in the upregulation of genes coding for stress proteins was one of earliest GO-categorized biological process to be significantly enriched and appeared to be sustained throughout the study. As shown in Supplemental Table 3, upregulation of heat shock protein genes, oxidative stress-inducible genes, and genes coding for proteins associated with antioxidant activity suggests increased oxidative stress and reactive oxygen species (ROS) in liver of arsenic-treated fish. Moreover, upregulation of genes associated with DNA damage/repair suggests occurrence of DNA damage. This was further supported by the histological observation of karyomegaly, indicative of DNA damage or damage to the mitotic spindle apparatus. Arsenic and its metabolites can cause toxicity directly, by attacking thiol or phosphate groups, resulting in impaired proteins, or indirectly, through the generation of ROS and free radicals, causing oxidative stress damage to both proteins and DNA (12, 25, 33). Heat shock proteins and several other chaperonins can help to stabilize and repair partly denatured or misfolded proteins. The upregulation of *aminolevulinate synthetase 1*, *ferritin heavy polypeptide 1*, and *heme oxygenase 1* suggests an increase in heme synthesis and deregulation of iron homeostasis. The induction of *heme oxygenase 1* detected in this study has been suggested as a response biomarker for arsenite exposure (40) and an indicator for oxidative stress (5, 47). Heme oxygenase 1 is responsible for the aerobic breakdown of heme to biliverdin, carbon monoxide, and iron, which has important effects on the liver (25). Biliverdin and its product bilirubin are free radical scavengers with an important role of protecting the cells from oxidative damage. Carbon monoxide, a potent vasodilator, may play a key role in the modulation of vascular dilation to cope with the physiological needs in the liver under stressful conditions. Free iron, on the other hand, increases oxidative stress through iron-dependent generation of ROS, and therefore upregulation of *ferritin* (as detected in this study), which encodes for an intracellular iron storage protein, could serve to lock up free iron. However, ferritin may act as another molecular target of arsenic, whereby interaction with methylated arsenic species can cause substantial release of iron from ferritin (2), resulting in a vicious cycle of oxidative damage. The upregulated *metallothionein 2* may also have a protective role in capturing ROS or chelating metal ions (25).

**Metabolism, biosyntheses, and catabolism.** The continuous exposure of the zebrafish liver to arsenic would impose on the hepatocytes a continuous requirement of the stress proteins to protect against the arsenical insults. In addition, arsenic also exerts its toxic effect through the impairment of cellular respiration by inhibition of various mitochondrial enzymes and the uncoupling of oxidative phosphorylation (54), further compounding the energy demand of the hepatocytes. This may account for the increased number of genes associated with metabolism, such as the continuous production of precursor metabolites and energy activities which in turn are used to support the increase of protein biosynthesis, such as the many stress proteins and other proteins required for homeostasis. Most of the upregulated genes associated with biosynthesis were involved with protein biosynthesis, for example various aminoacyl-tRNA synthetases, translation factors, and ribosomal proteins, while several others were associated with glycoprotein biosynthesis and carbohydrate biosynthesis (Supplemental Table 3). Chaperonins and heat shock proteins are again needed to help with proper protein folding, assembly, and distribution of newly synthesized proteins. On the other hand, improperly folded and damaged proteins by arsenicals or “short-lived” proteins are broken down via the ubiquitin-dependent protein degradation pathway. These may further account for the continuous expression of genes associated with heat shock proteins, protein transport, and ubiquitin-dependent protein degradation activities, as observed in this study. Increased expression of genes associated with ubiquitin-dependent protein degradation induced by arsenic has been reported (25, 29). Aside from protein catabolism, upregulation of genes associated with carbohydrate catabolism such as glycolysis and downstream genes associated with generation of precursor metabolites and energy clearly indicate a continuous need for production of precursor metabolites and energy to sustain various homeostatic and adaptive responses in the liver of arsenic-treated fish compared with the liver of control (non-
treated) fish. This is supported by the rapid depletion of glycogen content observed in the liver of arsenic-treated fish (Fig. 2, E and F). Liver metabolism is a known potential target for the toxic action of chemicals (32).

Transportation and cytoskeletal activities. The synthesis of many proteins would require active transportation to targeted sites in the cells or across cell membranes. It is therefore not surprising to detect the upregulation of genes associated with protein localization and ion transporters (Supplemental Table 3). Some of these gene products are important for homeostasis, and many are involved in ATPase activity coupled to transmembrane movement of substances or large movement within the cell including cytoskeletal reorganization. Genes coding for cell adhesion and cytoskeletal proteins such as tubulins, actins, and intermediate filament proteins were deregulated, suggesting large-scale reshaping and restructuring of cells. This corroborated with histopathological observations of more dissociated and irregular-shaped hepatocytes in arsenic-treated fish compared with control fish (Fig. 2, C and D), the altered staining for E-cadherin (Fig. 2, I and J), and the change in liver tissue consistency of fish after 48 h of arsenic treatment. Increased expression of hyaluronan synthase 2, which is involved in hyaluronan biosynthesis, a constituent of the extracellular matrix, suggested that wound healing and tissue repair were occurring in the arsenic-exposed liver. Hyaluronan is thought to have a role as a space filler (56) and in this case may occupy space left behind by degenerated hepatocytes and aid in resisting compressive forces in the injured liver tissues. However, if chronic arsenical insult persists, this could lead to scarring and fibrosis in the liver as observed in chronic arsenic-induced liver injury (15, 37).

Upregulated genes under miscellaneous category. There are many upregulated genes that do not belong to any enriched GO category but still may have a role in the arsenic-induced adaptive response. Interestingly, several genes associated with cell cycle (ccng1, cdnk1b) and important intracellular signaling pathways such as jak-stat and ras-MAPK were also upregulated (Supplemental Table 3). Expression of ccng1 is known to be inducible by DNA damage (8) and together with cdnk1b may play a role in delaying or arresting cell cycle to provide time for damaged DNA to be repaired. Upregulation of jak-1 by arsenic has been reported (67). While it has been demonstrated that Rac1 and Mapkapk2 proteins are activated by arsenic (58) and activation of Stat-1 is enhanced by arsenic (16), it is not clear as to how their gene expressions are affected by arsenic. Nevertheless, deregulation of these genes may be associated with nongenotoxic effects and tumorigenic potentials of arsenic.

Downregulated genes associated with liver-specific functions and miscellaneous category. Several genes that were associated with liver-specific functions were also observed to be downregulated, suggesting disruption of certain liver-specific functions. Many of these genes were observed to be more significantly downregulated in the Late group compared with the Early group, likely because of arsenic toxicity rather than direct regulation by arsenic (Supplemental Table 3). These included genes associated with blood factors, hormone activity, and lipid metabolism. Lesions in the hepatobiliary system as indicated by the bile stasis observed in some livers and altered bile duct staining (Fig. 2, G and H) in arsenic-treated fish will certainly cause disruption to some of these liver-specific functions known to be associated with biliary excretion (32). Several genes under the “miscellaneous” category associated with growth factors, cytokeskeleton, and transcription factors were also downregulated. In addition, downregulation of DNA (cytosine-5-)-methyltransferase 1, which is involved in DNA methylation, may be an early indication of potential DNA hypomethylation, as has been reported (43, 63), and may have implications in carcinogenesis.

Fig. 4. Directed acyclic graph (DAG) of Gene Ontology (GO) networks revealing important global transcriptional programs in the liver of zebrafish exposed to arsenic [As(V)] acid. A: Early 8- and 24-h group. B: Late 48- and 96-h group. GO categories that are significantly (P < 0.01) enriched are in black, while nonenriched (P > 0.01) are in gray.

Fig. 5. Arsenic-induced adaptive response model in zebrafish liver. Major transcriptional changes associated with important biological processes and physiological events that could lead to possible pathological conditions in liver of zebrafish exposed to arsenic.
In summary, we examined liver transcriptome changes that occur following arsenic exposure at selected time points with the aim of understanding the transcriptome kinetics of arsenic-induced adaptive responses in vivo in the zebrafish liver. Our analysis demonstrates an increase of transcriptional activity associated with metabolism, especially biosyntheses and transporter activities localizing in membrane, cytoplasm, and endoplasmic reticulum, while transcriptional activity associated with catabolism, energy derivation, and response to stress remained significant throughout the study. Some of these findings were further supported by histopathological evidence, which may be used as a reference for phenotype-anchoring points for certain classes of genes in future studies. On the basis of the findings that many differentially expressed genes were associated with heat shock proteins, DNA damage/repair, antioxidant activity, hypoxia-inducible proteins, iron homeostasis, arsenic metabolism, and ubiquitin-dependent protein degradation, we have strong evidence suggesting that DNA and protein damage as a result of arsenic metabolism and oxidative stress are causing major cellular and tissue injury (Fig. 5). This injury was evident from the histopathology of arsenic-exposed liver and corroborated well with changes in the altered expression of genes associated with cytoskeletal organization and cell adhesion observed in this study. As the liver focused on adapting to the continual arsenical insults, increased transcripts associated with metabolism and decreased transcripts associated with certain liver-specific functions were observed. Although arsenic-induced oxidative stress is a known genotoxic event, this is the first description of the relevance of major transcriptional changes associated with biological networks that provides both global and specific information on a coordinated adaptive response to acute arsenic toxicity in the liver. Given these findings, it is not surprising that chronic exposure to arsenic, with prolonged damage to DNA and protein, could result in neoplasia, especially in tissues with regenerative capability such as liver. Our findings may be used to infer possible transcriptome changes associated with liver damages that could occur in individuals exposed to arsenic in an endemic area where high incidence of liver diseases occur in these populations (17, 18, 37) and where drinking water can contain >0.6 ppm and as high as 3.2 ppm arsenic (39, 44, 54).

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