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Ethanol Exposure Induces Upregulation of Specific MicroRNAs in Zebrafish Embryos

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Prenatal exposure to ethanol leads to a myriad of developmental disorders known as fetal alcohol spectrum disorder, often characterized by growth and mental retardation, central nervous system damage, and specific craniofacial dysmorphic features. The mechanisms of ethanol toxicity are not fully understood, but exposure during development affects the expression of several genes involved in cell cycle control, apoptosis, and transcriptional regulation. MicroRNAs (miRNAs) are implicated in some of these processes, however, it is not yet clear if they are involved in ethanol-induced toxicity. In order to clarify this question, we have exposed zebrafish embryos to ethanol and evaluated whether a miRNA deregulation signature could be obtained. Zebrafish embryos were exposed to 1 and 1.5% of ethanol from 4 h postfertilization (hpf) to 24 hpf. The miRNA expression profiles obtained reveal significant miRNA deregulation and show that both ethanol concentrations upregulate miR-153a, miR-725, miR-30d, let-7k, miR-100, miR-738, and miR-732. Putative gene targets of deregulated miRNAs are involved in cell cycle control, apoptosis, and transcription, which are the main processes affected by ethanol toxicity. The conservation of affected mechanisms among vertebrates leads us to postulate that similar miRNA deregulation occurs in humans, highlighting a relevant role of miRNAs in ethanol toxicology.

Key Words: **ethanol toxicity; microRNAs; zebrafish; apoptosis.**

MicroRNAs (miRNAs) are small (~21 nt) noncoding RNA molecules that regulate translation efficiency and stability of target genes (Ambros, 2004; Bartel, 2004; Filipowicz *et al*., 2008). Each miRNA can target multiple messenger RNAs and may regulate several pathways or gene networks. These molecules are highly conserved among vertebrates and play major roles in regulating a variety of biological processes, namely morphogenesis (Giraldez *et al*., 2005; Yin *et al*., 2008), cell cycle control (Adlakha and Saini, 2011), and differentiation (Tay *et al*., 2008), among others. Altered expression of miRNAs often results in gene deregulation and perturbation of cellular homeostasis. Indeed, miRNA misexpression is implicated in a variety of diseases, ranging from cancer (Huang *et al*., 2008; Silber *et al*., 2008) to heart disease (Care *et al*., 2007; Zhao *et al*., 2007), and major efforts are ongoing to obtain miRNA expression profiles and signatures of human diseases to improve disease diagnosis and prognosis.

Because miRNA expression is sensitive to cellular degeneration (Ikeda *et al*., 2007; Volinia *et al*., 2006), it is reasonable to hypothesize that environmental factors will also play a role in miRNA biology. Therefore, variation in miRNA expression may help understand drug toxicity mechanisms being useful in toxicological diagnosis. In fact, miRNAs are implicated in the effects of environmental cigarette smoke in the lung of rodents and humans, where most of the deregulated miRNAs are downregulated and are involved in the control of stress response, apoptosis, angiogenesis, and transcription (Izzotti *et al*., 2009; Schembri *et al*., 2009). Also, exposure to perfluorooctane sulfonate (PFOS), a widely distributed environmental organic compound that causes developmental toxicity, alters miRNA expression in zebrafish embryos (Zhang *et al*., 2011). In addition, exposure to heavy metals, namely arsenate, cadmium, and aluminum, has been linked to altered miRNA expression, with miR-146a being commonly upregulated upon exposure to cadmium and aluminum (reviewed in Hou *et al*., 2011).

Alcohol (EtOH) is a teratogen, which crosses the placenta during pregnancy, thus affecting many aspects of fetal development. It is well documented that its consumption during pregnancy is responsible for developmental disorders, known as fetal alcohol spectrum disorders (FASD) (Ismail *et al*., 2010). However, the underlying molecular mechanisms of EtOH toxicity and how it affects fetal development are not yet fully understood. Hence, the development of tools to unravel the impact of EtOH on gene expression is crucial to understand EtOH effects during embryonic development. Because miRNAs regulate multiple targets, alteration of their expression by a potent teratogen, such as EtOH, can induce relevant developmental alterations and explain some of the pleiotropic effects of EtOH.

Several studies have already shown that miRNAs are affected by EtOH exposure, suggesting that their deregulation may mediate its teratogenic effects. The first study was carried

out in fetal mouse cortical neurons *in vitro* and showed that EtOH, at a level attained by alcohol addicted people, suppressed the expression of miR-21, miR-335, miR-9, and miR-153a, suggesting that miRNA expression was sensitive to this teratogen (Sathyan *et al*., 2007). Another study showed that miR-10a and miR-10b are significantly upregulated in fetal brains of mice after 25 and 38% prenatal alcohol exposure. This study also showed that the HOXA1 protein, a miR-10a target, was downregulated in the brain of exposed mice. These findings established an association between miR-10 and EtOH-induced teratogenesis, suggesting a possible role for this miRNA in the pathogenesis of birth defects (Wang *et al*., 2009). Both studies show that miRNAs are sensitive to EtOH, however, the differential effects of EtOH on miRNA expression indicate that miRNA deregulation varies according to EtOH concentration, exposure period, and tissue/cell type.

In order to further understand how miRNAs mediate the teratogenic effects of EtOH during the most responsive stages of development to this stressor, we have studied the effects of different alcohol concentrations on miRNA expression in 24-h postfertilization (hpf) zebrafish embryos. DNA microarrays were used to analyze alterations in miRNA expression in zebrafish embryos exposed to 1 or 1.5% EtOH from 4 to 24 hpf. This model is excellent to study developmental toxicity because the external embryo development allows one to test EtOH effects by directly placing the embryos in toxic solutions. Zebrafish embryo transparency also allows for monitoring developmental alterations easily and its recent validation as a model of embryonic exposure to EtOH allows one to extrapolate results to other vertebrates (Bilotta *et al*., 2004). We found that seven miRNAs were commonly upregulated upon exposure to both 1 and 1.5% EtOH, which indicates that these miRNAs are potential key regulators of the EtOH response and constitute a signature for EtOH-induced toxicity in vertebrates.

MATERIALS AND METHODS

Zebrafish maintenance and chemicals. Wild-type zebrafish (AB strain) was maintained at 28°C on a 14/10-h light/dark cycle in a closed flow-through system. Animal husbandry followed the Portuguese law for animal experiments. Absolute EtOH was obtained from Sigma. Test solutions were prepared immediately prior to the addition to the embryos.

Ethanol exposure. Zebrafish embryos were obtained from spawning adults in groups of about 10 males and 10 females. Spawning was induced in the morning and zebrafish embryos were collected. Petri dishes with approximately 250 eggs in system water were incubated at 28°C to allow normal zebrafish development until 4 hpf, when blastula was reached. At this stage, embryos were examined under a dissecting microscope and those that had developed normally were selected for EtOH exposure (approximately 200 eggs). Briefly, embryos were randomly distributed into plastic petri dishes containing 20 ml of EtOH test solutions (0.5, 1, 1.5, 2, or 3% EtOH). All solutions were prepared by dilution of absolute EtOH in system water. Exposure was from 4 to 24 hpf. At this stage, solutions were changed with system water, and embryos were allowed to grow until 96 hpf. The control group was allowed to grow in plain system water. Morphological changes, mortality, and deformations were recorded at 24, 48, 72, and 96 hpf. Three biological replicates were performed for each experiment.

For the miRNA analysis, only 1 and 1.5% EtOH concentrations were used as higher concentrations had very high incidence of malformations and mortality. Zebrafish embryos were collected at 24 hpf for microarrays and quantitative PCR (qPCR) analysis. Two biological replicates were performed for each assay. Embryos were frozen in liquid nitrogen and were kept at −80°C until RNA extraction.

Cell death assay. Acridine orange (AO) was used to evaluate cell death in embryos exposed to EtOH. Twenty-four-h postfertilization control embryos and 24 hpf embryos exposed to the different EtOH concentrations tested were dechorionated with pronase and incubated with a 5 μg/ml AO solution in system water at 28°C for 30 min. Embryos were washed eight times with system water and anesthetized with tricaine and examined using a Zeiss Imager Z1 fluorescence microscope. Images were obtained with an AxioCam Hrm camera. The number of AO-stained cells was measured in the head area, between the front of the embryo head and the otolith using ImageJ.

RNA extraction and quantification. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Invitrogen). RNA quantity and quality were assessed using the NanoDrop and Agilent 2100 bioanalyzer systems, respectively. Samples with a RNA Integrity Number above seven were used.

miRNA microarray production. miRNA microarrays (miRNAChip_MS_V1) used in this study were printed in the Portuguese National DNA-Microarray Facility located at the University of Aveiro. The arrays contained a total of 1164 probes spotted in quadruplicate. Each array was printed with optimized probe sequences targeting all of the known mature miRNAs deposited in the Sanger miRBase Sequence Database, Release 9.0, for human, mouse, rat, Drosophila, *Caenorhabditis elegans*, and zebrafish (NCode Multi-Species miRNA probe set V2 from Invitrogen) and also mismatch controls for monitoring hybridization specificity and the NCode Positive Control for labeling quality control and easier spot finding. In addition, 24 probes corresponding to the novel zebrafish miRNAs identified by our previous study (Soares *et al*., 2009) were also included in the array. These unmodified oligonucleotides, which are 34–44 bases in length, were dissolved in 150mM phosphate buffer pH 8.5 at a concentration of 20μM and printed onto Nexterion E slides (SCHOTT) using a MicroGrid II compact spotter. Printed slides were further processed according to manufacturer's recommendations.

miRNA microarray hybridization. Total RNA from control and test samples was labeled using the ULS microRNA labeling kit (Kreatech) according to the manufacturer's instructions. Briefly, 2 μg of total RNA from control and test samples were incubated with Cy3-ULS for 15 min at 85°C. The labeled RNAs were purified to remove nonreacted Cy3-ULS to produce a fluorescently labeled RNA sample for microarray analysis. Dye incorporation was monitored by ultraviolet-visible spectroscopy. Hybridization was carried out in the miRNAChip_MS_V1 at 42°C for 16 h. Slides were washed following the manufacturer's recommendations and immediately scanned using an Agilent G2565AA microarray scanner.

Computational analysis of miRNA microarray data. Microarray images were analyzed using Quantarray v3.0 software (PerkinElmer). Cy3 median pixel intensity values were background subtracted, normalized, and subjected to further analysis. Data points were removed when intensity values were below 100. A global median normalization microarray data of the zebrafish 24-hpf larvae were applied using BRB-ArrayTools v3.4.0 software. Data were normalized for each miRNA with the correspondent NCode Positive Control probe intensity. Log transformed (base 2) of the ratio between miRNA and NCode Positive Control intensities of each sample was calculated. Differentially expressed miRNAs from each sample type were identified using the Multiple Experiment Viewer Mev software version 4.6.1. A paired *t*-test ($p < 0.01$) with standard Bonferroni correction was performed to identify miRNAs that demonstrated statistically significant difference in expression between untreated and 1 and 1.5% EtOH-treated 24-hpf zebrafish embryos.

Microarray data submission. The microarray raw data have been submitted in Gene Expression Omnibus database and have been given the following accession numbers: GSE32632, GPL14672, and GSM808869-GSM908874.

miRNA quantitative real-time PCR. Quantification of miRNA expression was carried out using NCode miRNA First-Strand cDNA module and Platinum SYBR Green qPCR Super Mix-UDG from Invitrogen. Briefly, miRNAs were polyadenylated using poly-A polymerase and adenosine triphosphate, and complementary DNA (cDNA) was prepared from 500 ng of total RNA with the SuperScript III Reverse Transcriptase (RT) and a universal RT primer, following the manufacturer's protocol. qPCR was performed with the synthesized cDNA using SYBR Green detection reagent, the universal qPCR primer provided in the kit, and a forward primer designed to target the desired miRNA. U6 small RNA (RNU6) was used as an endogenous control for normalization. Reverse transcriptions and no template controls were run at the same time following the RT step. Quantitative real-time PCR was carried out using the ABI Prism 7500 Real-Time PCR System (Applied Biosystems). Reactions were incubated in a 96-well optical plate, and the cycling began with template denaturation at 95°C for 2 min, followed by 50 cycles at 95°C for 15 s and 60°C for 35 s. The threshold cycle data (CT) and baselines were determined using auto settings. All assays including no template controls were carried out in triplicate. REST software (Pfaffl *et al.*, 2002) was used to calculate the relative quantification of miRNA expression. In our dataset, the control sample was the untreated 24-hpf sample.

Target genes prediction of the differentially expressed miRNAs. Analysis of predicted targets was carried out using different algorithms: Eimmo, miRanda, and TargetScan. Putative target genes were considered whenever they were predicted by at least two of the databases used.

Statistical analysis. Data for edema and hatching rate, embryo length, eye diameter, and cell death assay, expressed as mean ± SD were analyzed by one-way ANOVA. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Phenotypic Effects of EtOH Exposure

Zebrafish embryos were initially exposed to 0 (control), 0.5, 1, 1.5, 2, and 3% EtOH at 4 hpf, and the exposure was kept up to 24 hpf when zebrafish embryos showed a basic body plan with all major organs formed, following previous protocols (Bilotta *et al.*, 2004; Marrs *et al.*, 2010; Sylvain *et al.*, 2010). The exposure period occurred between gastrulation and somitogenesis, which are the developmental stages that are more responsive to EtOH treatment (Bilotta *et al.*, 2004; Reimers *et al.*, 2004). Mortality, morphological changes, and deformations were recorded at 24, 48, 72, and 96 hpf. EtOH increased the incidence of malformations (including axial malformations and edema) and mortality. At 72 hpf, 2% EtOH induced 50% of embryo mortality while 3% EtOH resulted in 100% of mortality already at 24 hpf (Fig. 1A), confirming previous studies (Bilotta *et al.*, 2004; Sylvain *et al.*, 2010). Although embryos exposed to 0.5 and 1% EtOH showed no significant increase in malformations or mortality, 1% EtOH produced slight physical differences in growth, retinal diameter, and significant edema formation (Figs. 1B–D). Measurements of larvae length and eye diameter

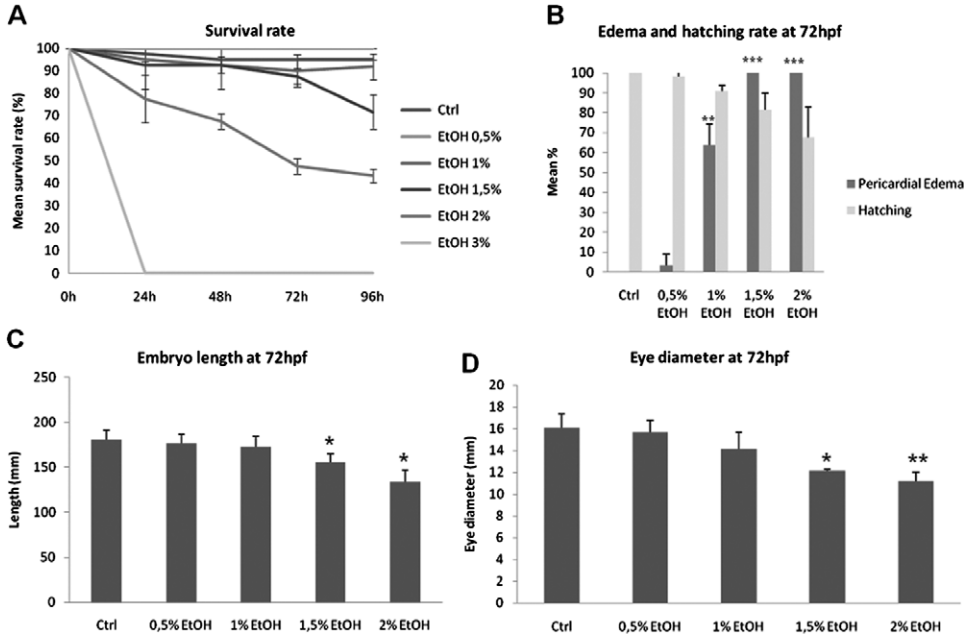


FIG. 1. Effects of EtOH on embryos development. Zebrafish embryos were exposed to different EtOH concentrations (0 [control], 0.5, 1, 1.5, 2, and 3%) from 4 to 24 hpf. Mortality was recorded every 24 h until 96 hpf. All other measurements were carried out at 72 hpf. (A) There was a decrease in survival with increasing EtOH concentrations already observable at 24 hpf. Highest concentration of EtOH (3%) was lethal for zebrafish embryos at 24 hpf. Two percentage EtOH produced 50% mortality at 72 hpf. (B) Hatching rate decreased with increasing EtOH concentrations. At 72 hpf, control embryos were outside the chorion. After 2% EtOH exposure, there was a 30% decrease in hatching rate. Increasing EtOH concentrations induced edema formation. One percentage EtOH induced 70% edema formation at 72 hpf (**$p < 0.01$) and increased with increasing EtOH concentrations (1.5 and 2%, ***$p < 0.001$). (C) Embryo length decreased with increasing EtOH concentrations. This difference was statistically significant after 1.5 and 2% EtOH exposure (*$p < 0.05$). (D) Increasing EtOH concentration also decreased the eye diameter. This difference was statistically significant for 1.5% (*$p < 0.05$) and 2% EtOH exposure (**$p < 0.01$).

were performed at 72 hpf for control, 0.5, 1, 1.5, and 2% EtOH-exposed embryos. Both embryo length and eye diameter decreased with increasing concentrations of EtOH. These differences were statistically significant for embryos exposed to 1.5 and 2% EtOH (Figs. 1C and D).

EtOH of 0.5% had little or no effect in zebrafish development, whereas 1 and 1.5% EtOH showed small effects on brain development at 24 hpf. Embryos exposed to 2% EtOH had severe brain malformations, whereas 3% EtOH-exposed embryos arrested development and were not viable (Fig. 2).

Previous studies showed that EtOH reduces cell proliferation and increases cell death during various stages of development (Anthony *et al.*, 2008). In zebrafish embryos, EtOH also induced cell death and apoptosis along the dorsal axis of the embryo, particularly in the brain region (Carvan *et al.*, 2004; Reimers *et al.*, 2006), and it is likely that cell death is correlated with the craniofacial malformations observed after EtOH exposure (Reimers *et al.*, 2006). In order to confirm these results in our model system, embryos were stained with AO for 30 min. EtOH increased apoptotic cell death, especially in the central nervous system region with increasing EtOH concentrations (Fig. 3). Embryos exposed to 0.5% EtOH did not show a significant difference in relative cell death, however, cell death increased 1.5× in 1% EtOH-exposed embryos and up to 2.5× in 1.5% EtOH-exposed embryos (Fig. 3).

miRNA Deregulation by EtOH

In order to detect miRNA changes derived from EtOH exposure, the miRNA analysis was restricted to 24-hpf embryos, which allowed for assessing early differences in miRNA expression at the most critical developmental period. miRNA profiling of 24-hpf zebrafish embryos exposed to 1 and 1.5% EtOH was performed as both concentrations induced different levels of alterations in embryo development, but were not lethal. We were able to detect differentially expressed miRNAs at both EtOH concentrations. One hundred and eighteen miRNAs were differentially expressed in response to 1% EtOH, whereas 108 miRNAs were differentially expressed in response to 1.5% EtOH. From these, only miRNAs with threshold fold change values above 1.5 and below −1.5 were considered as upregulated and downregulated, respectively (Table 1).

Hierarchical clustering of the miRNA expression data (Fig. 4) identified miR-153a, miR-725, miR-30d, let-7k, miR-100, miR-738, and miR-732 as commonly upregulated after exposure of

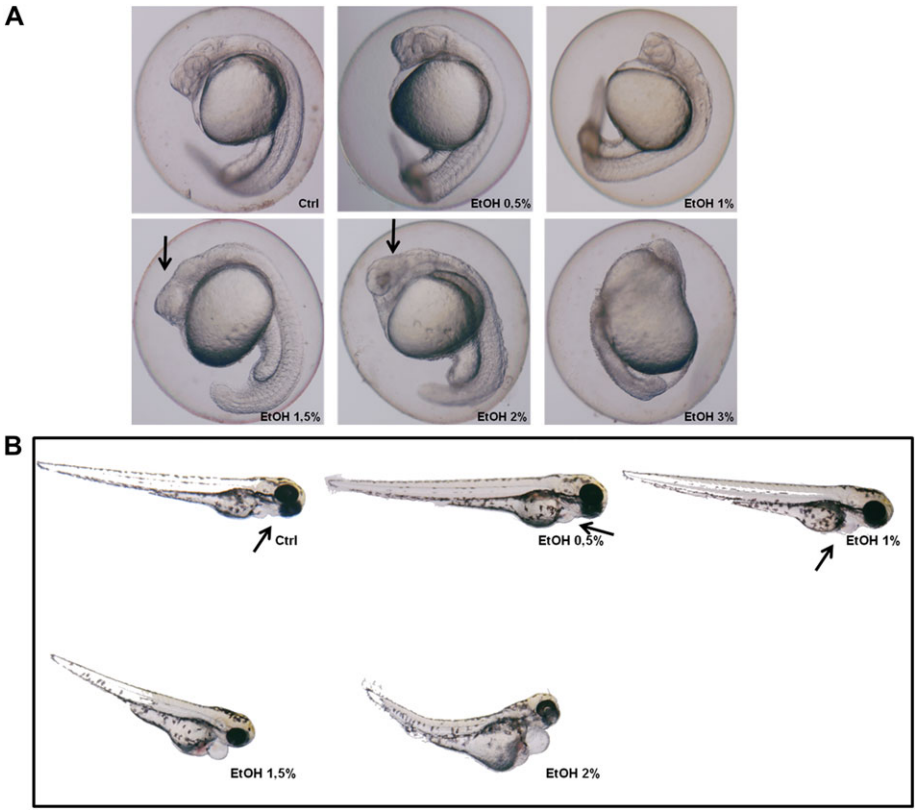


FIG. 2. Embryos exposed to EtOH show increased incidence of malformations. (A) Twenty-four h postfertilization embryo photos were taken with a Nikon camera attached to a Leica magnifier. Embryos exposed to 0.5 and 1% EtOH exhibited no visible differences compared with control embryos. Concentrations above 1.5% resulted in visible malformations, namely smaller heads, smaller tails, and underdeveloped eyes. These malformations were more extreme after 2% EtOH exposure. Three percent EtOH exposure was lethal. (B) Seventy-two h postfertilization embryos exposed to different EtOH concentrations. At 72 hpf, embryos exposed to 1% EtOH showed a slight edema (arrow). However, 1.5 and 2% EtOH resulted in smaller embryos with increased pericardial edema incidence (arrows). Two percentage EtOH-exposed embryos showed severe axial malformations.

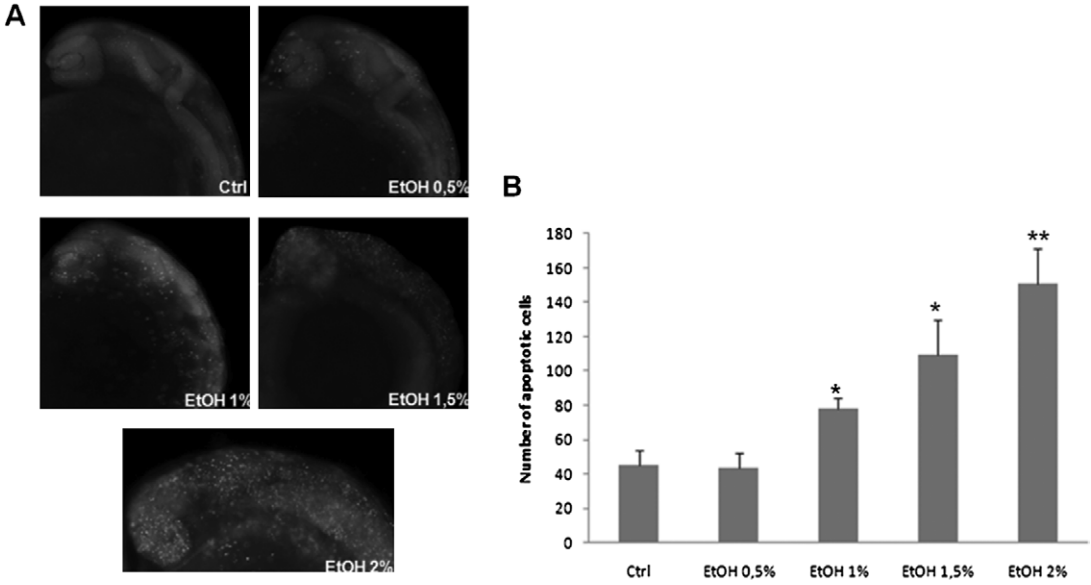


FIG. 3. EtOH increases cell death. (A) Embryos were stained with AO and were visualized by fluorescence microscopy. AO-stained cells in the head area between the front of the embryo head and the otolith were quantified using ImageJ. (B) AO-stained cells were quantified in three independent batches of embryos (Ctrl and EtOH-treated embryos). Mean and SDs were calculated for each condition and one-way ANOVA was carried out (*$p < 0.05$, **$p < 0.01$). There is a statistically significant increase in AO-staining cells with EtOH concentration above 1%.

zebrafish embryos to both 1 and 1.5% EtOH (Table 1). Interestingly, the fold change of these miRNAs was similar at both EtOH concentrations, except for miR-725 whose upregulation was higher at 1% EtOH than at 1.5% EtOH, and let-7k whose upregulation was more considerable at 1.5% EtOH than 1% EtOH (Table 1). Therefore, it is likely that this group of commonly upregulated miRNAs represents a specific signature for EtOH exposure. However, some miRNAs were exclusively overexpressed by one of the EtOH concentrations tested. For example, miR-216a, miR-142b-5p, let-7j, miR-735, miR-199*, miR-461, miR-133a, miR-731, miR-1, miR-130a, miR-146b, and miR-132* were upregulated by 1% EtOH, whereas no change was induced by 1.5% EtOH. On the other hand, miR-736, miR-2189, miR-183, miR-18b*, miR-727, miR-10d*, miR-210*, miR-130b, miR-2188, and miR-107 were exclusively upregulated by 1.5% EtOH (Table 1). This suggests that small changes in alcohol concentration may trigger different cellular responses and affect miRNA expression differentially. Surprisingly, we were not able to identify miRNAs downregulated in embryos exposed to 1.5% EtOH, but miR-23a, miR-203, let-7c, miR-128a, and miR-193b were downregulated by 1% EtOH treatment.

Microarray Data Validation by Quantitative Real-Time PCR

In order to validate the above microarray data, miRNA deregulation was confirmed by qPCR. For this, we have tested five miRNAs whose expression was deregulated by EtOH, namely miR-23a, miR-133, miR-153, miR-183, and miR-736. We also tested miR-218a whose expression did not change by exposure to 1 or 1.5% EtOH (Fig. 5). RNU6 was used as an internal control for data normalization. In all cases, the qPCR and microarray data showed high correlation. miR-153a was commonly upregulated by both 1 and 1.5% EtOH treatments (2.19- and 5.18-fold change, respectively). However, the level of miR-153a upregulation in 1.5% EtOH-exposed embryos was higher (5.18-fold change) than that determined by the DNA microarrays (1.94-fold change), indicating that miR-153a deregulation increases with increasing EtOH concentrations (Fig. 5). miR-23a downregulation by 1% EtOH and miR-736 upregulation by 1.5% EtOH were confirmed by qPCR (−2.94- and 4.18-fold change, respectively) (Figs. 5A and B). Interestingly, although miR-736 was not considered significantly deregulated in the microarray analysis of samples treated with 1% EtOH, qPCR analysis revealed that this miRNA was upregulated in this condition (2.19-fold change), but at lower levels than in 1.5% EtOH-exposed embryos (4.18-fold change) (Fig. 5A). To further confirm the overall microarray data, we have validated miR-133 and miR-183, which were exclusively upregulated by 1 and 1.5% EtOH, respectively, after microarray analysis (Figs. 5A and B). qPCR confirmed that miR-183 was upregulated by 1.5% EtOH only (1.18-fold change in 1% EtOH and 1.66-fold change in 1.5% EtOH). miR-133a fold change was 1.40 in 1% EtOH samples, as determined by qPCR, which was below the upregulation threshold (fold change 1.5). Nevertheless, qPCR data indicated that miR-133a fold change by 1% EtOH was higher (fold change 1.40) than by 1.5% EtOH (fold change 1.20), which was in agreement with the microarray data (Figs. 5A and B). Validation of miR-218a expression in embryos exposed to 1 and 1.5% EtOH confirmed that EtOH does not deregulate significantly this miRNA (1.44- and 1.28-fold change, respectively) (Figs. 5A and B).

TABLE 1
miRNAs Deregulated by EtOH

	Fold change	
	1% EtOH	1.5% EtOH
miR-153	**2.22**	**1.95**
miR-725	**2.14**	**1.54**
miR-30d	**1.74**	**1.85**
let-7k	**1.69**	**2.30**
miR-100	**1.65**	**1.63**
miR-738	**1.59**	**1.61**
miR-732	**1.5**	**1.71**
miR-216a	**1.76**	*1.40*
miR-142b-5p	**1.69**	*1.35*
let-7j	**1.66**	*1.26*
miR-735	**1.61**	*1.30*
miR-199*	**1.60**	*1.22*
miR-461	**1.59**	*1.40*
miR-133a	**1.58**	*1.41*
miR-731	**1.55**	*1.43*
miR-1	**1.54**	*1.22*
miR-130a	**1.54**	*1.26*
miR-146b	**1.54**	*1.10*
miR-132*	**1.50**	*1.46*
miR-736	NS	**5.43**
miR-2189	NS	**2.06**
miR-183	*1.21*	**1.83**
miR-18b*	*1.13*	**1.66**
miR-727	*1.38*	**1.64**
miR-10d*	*1.25*	**1.62**
miR-210*	*−1.21*	**1.58**
miR-130b	*1.18*	**1.54**
miR-2188	NS	**1.53**
miR-107	*1.26*	**1.52**
miR-23a	**−1.59**	NS
miR-203	**−2.18**	NS
let-7c	**−2.48**	NS
miR-128a	**−3.01**	*−1.31*
miR-193b	**−8.91**	NS

Notes. List of deregulated miRNAs by both 1 and 1.5% EtOH (bold). miR-153, miR-725, miR-30d, let-7k, miR-100, miR-738, and miR-732 were commonly upregulated by both EtOH concentrations tested, showing similar fold variation. The exceptions were miR-725 that showed higher upregulation by 1% EtOH (fold change 2.14) than by 1.5% EtOH (fold change 1.54) and let-7 whose upregulation by 1.5% EtOH (fold change 2.30) was stronger than by 1% EtOH (fold change 1.69). miR-216a, miR-142b-5p, let-7j, miR-735, miR-199a*, miR-461, miR-133a, miR-731, miR-1, miR-130a, miR-146b, and miR-132* were exclusively upregulated by 1% EtOH, whereas miR-23a, miR-203, let-7c, miR-128a, and miR-193b were exclusively downregulated by 1% EtOH. miR-736, miR-2189, miR-183, miR-18b*, miR-727, miR-10d*, miR-210*, miR-130b, miR-2188, and miR-107 were upregulated by 1.5% EtOH only. In general, fold change of 1.5% EtOH upregulated miRNAs was higher than that observed for 1% EtOH upregulated miRNAs. Downregulated miRNAs were not detected in embryos exposed to 1.5% EtOH. Upregulation was considered whenever fold change was higher than 1.5, and downregulation was considered whenever fold change was lower than −1.5. Only statistically significant miRNAs were considered ($p < 0.01$). NS, not significant. Values in italics represent unaltered miRNA expression.

Prediction of Differentially Expressed miRNA Targets

Because miRNAs control gene expression at the post-transcriptional level, we have identified *in silico* the putative targets of five miRNAs deregulated by EtOH exposure, namely miR-153a, miR-30d and miR-736 (upregulated by both EtOH concentrations), miR-183 (upregulated by 1.5% EtOH only), and miR-23a (downregulated by 1% EtOH). For this, we have used the Eimmo, Miranda, and TargetScan algorithms, which contain zebrafish miRNA target predictions. Potential targets were considered whenever they were identified by at least two of these algorithms (Table 2).

Predicted targets were mainly involved in developmental, cellular, and cell cycle processes. Interestingly, some of the targets, namely Cyclin E (*Ccne*), *Neurod*, transforming growth beta 3 (*Tgfb3*), and ribosomal protein S7 (*Rps7*) are involved in cell cycle and p53 signaling (Cheah *et al*., 2010; Chen *et al*., 2007; Duan *et al*., 2011; Lindahl *et al*., 2004).

Other putative target genes are involved in CNS and neuron development, namely *Neurod* (miR-153a), *Foxa2*, *Zic1*, *Zic5* (miR-23a), *Bdnf*, and *Sox3* (miR-30d) (Table 2) (Caldwell *et al*., 2008; Dee *et al*., 2008; Elsen *et al*., 2008; Ferri *et al*., 2007; Lee, 1997; Nyholm *et al*., 2009). Interestingly, the *Nog3* gene, which is involved in pattern specification and determination of left/right symmetry was identified in our analysis as a putative miR-183 target. This miRNA was only upregulated by 1.5% EtOH, where embryos showed visible axial malformations.

Some of the putative miRNA target genes identified are transcriptional factors for which the function is not well determined but are mainly expressed in the developing nervous system and most probably play a role in neuronal differentiation, namely *Pou4f1* (miR-153a and miR-30d), *Lhx1a* and *Lhx1b* (miR-30d), *Foxg1*, and *Sox21b* (miR-23a).

DISCUSSION

Heavy EtOH consumption during pregnancy can induce gene expression alterations and alter various signaling pathways in the developing embryo (Aroor and Shukla, 2004; Hill *et al*., 1989). However, the exact mechanisms of EtOH toxicity are still poorly understood. It is possible that miRNAs are targeted by EtOH and play important roles in the cellular responses to its toxicity. Because miRNAs can regulate multiple targets, alteration of expression of the former by EtOH can induce developmental alterations and explain some of the EtOH pleiotropic effects. In fact, a correlation between EtOH exposure and miRNA deregulation is observed in an *in vitro* model of the second trimester of gestation of cerebral cortical neuroepithelium. This corresponds to an important period in brain development when the germinative neuroepithelial zones of the neural tube give rise to neurons that populate the brain cerebral cortical plate. For this reason, EtOH exposure during this period will alter brain development (Camarillo and Miranda, 2008). High levels of

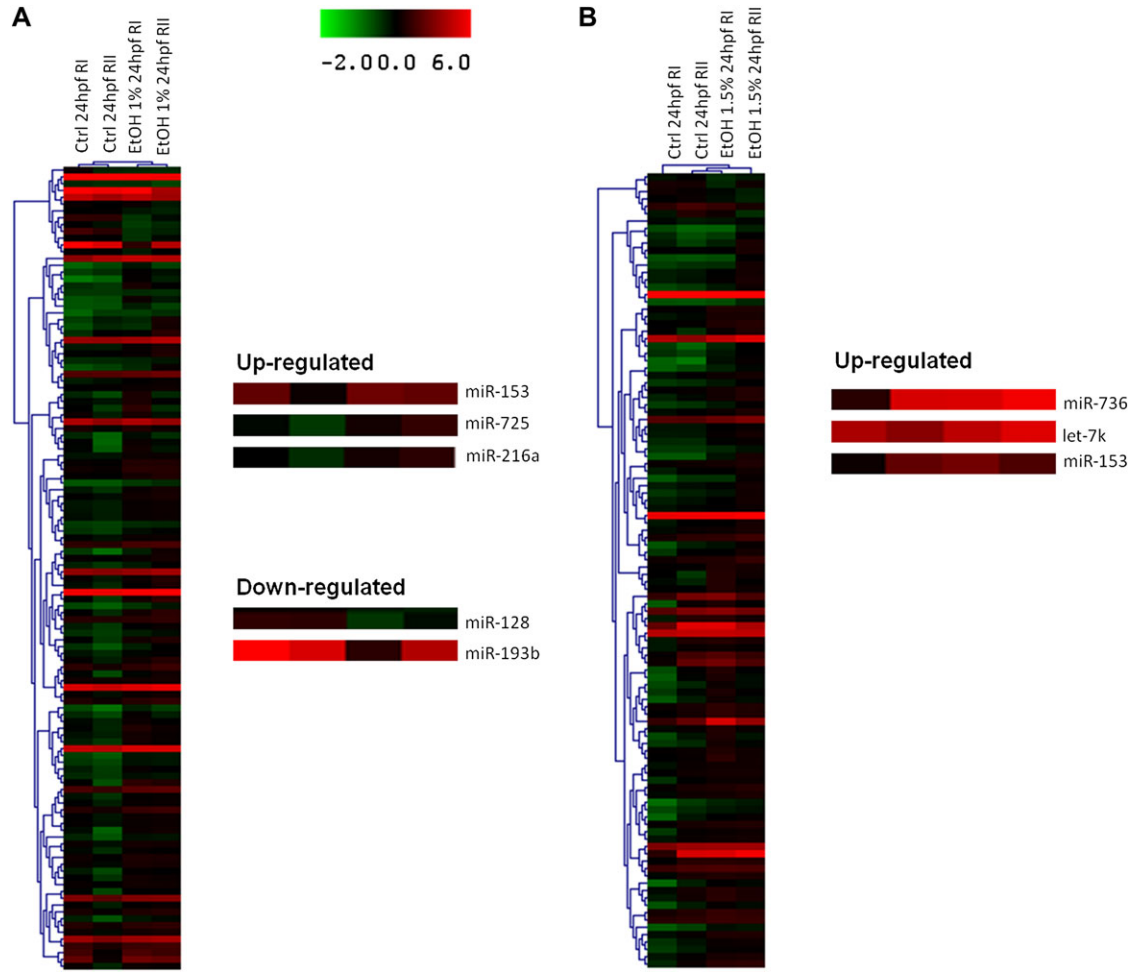


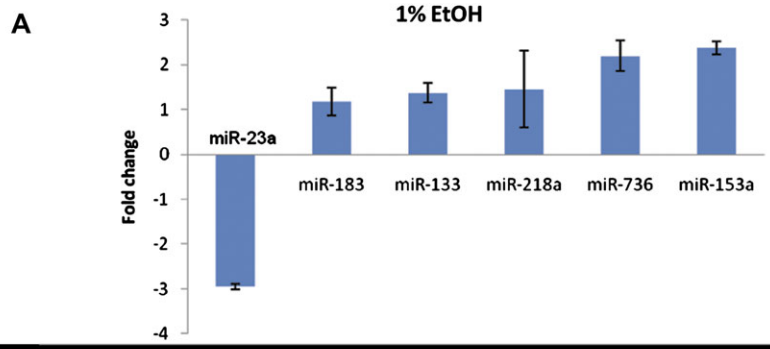
FIG. 4. Hierarchical clustering of deregulated miRNAs. Microarray data were clustered to compare miRNA deregulation between control and 1% EtOH (A) and 1.5% EtOH (B). The highlighted miRNAs are examples of those that showed the highest deregulation by EtOH. One percentage EtOH upregulated miR-153a, miR-725, and miR-216a by ~twofold. miR-128 and miR-193b were the most downregulated miRNAs by this EtOH concentration. EtOH of 1.5% upregulated miR-736 by 5.43-fold. let-7k and miR-153a were upregulated by approximately twofold. miR-153a was upregulated by both 1 and 1.5% EtOH, by approximately the same fold change.

EtOH (70mM), which are achieved in alcohol addiction, suppress miR-21, miR-335, miR-9, and miR-153, whereas low levels (13mM) upregulate miR-335 (Sathyan *et al*., 2007). On the other hand, miR-9 is upregulated by 20mM EtOH in adult neurons (Pietrzykowski *et al*., 2008), indicating that miRNAs are affected differently by EtOH depending on the concentration and tissue type. However, alcohol effects on miRNA expression during development are mostly unexplored and how different EtOH concentrations affect the miRnome during fetal development is still largely unknown.

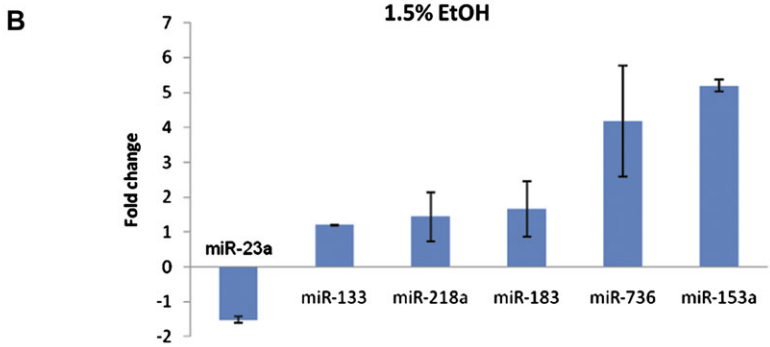
In this study, we have analyzed the effects of sublethal EtOH concentrations on miRNA regulation during vertebrate development using microarray profiling of miRNAs of 24-hpf zebrafish embryos exposed to 1 and 1.5% EtOH, which induce developmental malformations and gene expression alterations (Bilotta *et al*., 2004; Fan *et al*., 2010). EtOH exposure during the first 24 h of zebrafish development (gastrulation stages) is equivalent to the third week in human pregnancy, when EtOH effects are more deleterious to the embryo (Bilotta *et al*., 2004). Analysis of 24-hpf embryos permits identification of immediate effects of EtOH on miRNA expression during the most critical phase of development instead of focusing in a particular organ or tissue.

Our study revealed that seven miRNAs are commonly upregulated by both 1 and 1.5% EtOH (miR-153a, miR-725, miR-30d, let-7k, miR-100, miR-738, and miR-732), indicating that these miRNAs represent a signature of sublethal embryonic EtOH exposure. However, some miRNAs were deregulated by only one of the EtOH concentrations, confirming that different EtOH concentrations exert different effects on embryo development, inducing different cellular responses and different levels of miRNA deregulation.

Interestingly, expression of miR-153a, which was upregulated in our study, is suppressed by alcohol in neural stem cells (NSCs)/neural progenitor cells (Sathyan *et al*., 2007). These cells are resistant to apoptosis upon EtOH exposure, whereas EtOH



1% EtOH	miR-23a	miR-183	miR-133	miR-218a	miR-736	miR-153
microarray	-1.59	1.22	1.58	0.70	-	2.22
qPCR	-2.94	1.18	1.40	1.44	2.19	2.38



1.5% EtOH	miR-23a	miR-133	miR-218a	miR-183	miR-736	miR-153
microarray	-	1.41	1.28	1.83	5.65	1.94
qPCR	-1.51	1.20	1.44	1.66	4.18	5.18

FIG. 5. Validation of microarray data using qPCR. The qPCR results were normalized using U6 small RNA (RNU6) as an endogenous control. qPCR fold change was calculated using REST software, and microarray fold change was calculated using the ratio between signal intensities of control and EtOH-treated samples after application of between-subject *t*-test with standard Bonferroni correction. (A) qPCR validation of miRNAs deregulated by 1% EtOH. miR-153a showed ~twofold upregulation as demonstrated by both microarrays and qPCR. miR-183 and miR-218a expression did not in the presence of 1% EtOH in both the qPCR and microarray data sets. (B) qPCR validation of deregulated miRNAs after embryo exposure to 1.5% EtOH. qPCR confirmed that miR-183, miR-153a, and miR-736 were upregulated, whereas miR-133 and miR-218a did not change significantly in the presence of 1.5% EtOH.

increased apoptotic events in our zebrafish embryos. Nevertheless, deregulation of this miRNA correlates with apoptosis levels and with the known role of miR-153 in apoptosis in both cases (Liu *et al*., 2012; Xu *et al*., 2010), suggesting that it is a key regulator of EtOH toxicity. The same study shows that miRNAs of the let-7 family are highly expressed in neurons, but no significant deregulation of these miRNAs was observed after EtOH treatment of NSCs/NpCs cultures (Sathyan *et al*., 2007). Conversely, we have observed that let-7 family members, namely let-7k, let-7j, and let-7c, are deregulated by at least one of the EtOH concentrations tested in zebrafish embryos. Our data also showed that the EtOH concentrations used do not induce miR-9 deregulation, contrary to what was observed in *in vitro* models (Pietrzykowski *et al*., 2008; Sathyan *et al*., 2007), supporting the hypothesis that EtOH-induced miRNA deregulation depends on tissue type and on the EtOH concentration used. These factors affect miRNA signatures and help us understand in deeper detail the global effects of EtOH toxicity.

Putative target genes of the deregulated miRNAs, namely miR-153a, miR-30d, miR-183, miR-736, and miR-23a, are involved in the regulation of transcription, cell cycle, and brain development. The *Ccne* and *Tgfb3* genes are targeted by miR-153 and miR-30d, respectively, and are involved in p53 signaling, cell cycle, and apoptosis, suggesting that miRNAs play significant roles in cell cycle and cell growth control. In our zebrafish embryos, increasing concentrations of EtOH also

TABLE 2
Target Prediction for miR-153, miR-30d, miR-183, miR-23a, and miR-736

miRNA	Target	Go term (Process)
dre-miR-153a	*Thbs1*	Cell adhesion
	Ybx1	Regulation of transcription, DNA dependent
	Pou4f1	Regulation of transcription; regulation of transcription, DNA dependent
	Snap25a	Cytokinesis
	Ccne	Cell cycle; cell division
	Chrnd	Ion transport; myofibril assembly; transport
	Cpla2	Lipid catabolic process; metabolic process; phospholipid catabolic process
	Gucy2f	Cyclic guanosine monophosphate biosynthetic process; cyclic nucleotide biosynthetic process; intracellular signaling pathway; protein phosphorylation
	Neurod	Cell differentiation; multicellular organismal development; nervous system development
dre-miR-30d	*Nkx2.2a*	Schwann cell development; endocrine pancreas development; floor plate development; floor plate formation; glial cell migration; motor axon guidance; multicellular organismal development; regulation of transcription; regulation of transcription, DNA dependent; ventral spinal cord interneuron differentiation
	Rap2b	Signal transduction; small GTPase-mediated signal transduction
	Sox3	CNS development; neuron differentiation; positive regulation of transcription factor activity; regulation of transcription; transcription; multicellular organismal development
	Tgfb3	Cell growth; growth; transforming growth factor beta receptor–signaling pathway
	Znf395	Biological process
	Bdnf	Brain development
	Lhx1a	Regulation of transcription; regulation of transcription, DNA dependent; multicellular organismal development
	Wasl	Actin polymerization or depolymerization; protein complex assembly
	Pou4f1	Regulation of transcription; regulation of transcription, DNA dependent
	Rps7	Regulation of cell cycle; translation
	Prox1	Forebrain neuron development; lymphangiogenesis; multicellular organismal development; myofibril assembly; negative regulation of transcription factor activity; regulation of transcription
	Hoxa2b	Multicellular organismal development; regulation of transcription; regulation of transcription, DNA dependent; transcription
	Tpi1b	Fatty acid biosynthetic process; gluconeogenesis; glycolysis; lipid biosynthetic process; metabolic process; pentose-phosphate shunt
	Hoxb1b	Hindbrain development; multicellular organismal development; regulation of transcription; regulation of transcription DNA dependent; transcription
	Lhx1b	Regulation of transcription; regulation of transcription DNA dependent
	Ube2i2	Cell cycle; cell division; chromosome segregation; mitosis; multicellular organismal development; posttranslational protein modification; regulation of protein metabolic process
dre-miR-183	*Snx3*	Cell communication
	Egr1	Camera-type eye development; regulation of transcription; transcription
	Hspe1	Protein folding; response to stress
	Kif23	Cytokinesis; embryonic cleavage; microtubule-based movement
	Nog3	Cartilage development; cell differentiation; determination of right/left symmetry; dorsal/ventral pattern formation; multicellular organismal development; negative regulation of bone morphogenic protein-signaling pathway; negative regulation of cell differentiation
	Eya1	Adenohypophysis development; ear morphogenesis; inner ear morphogenesis; metabolic process; multicellular organismal development; otolith development
dre-miR-23a	*Mknk2*	Protein phosphorylation; regulation of translation; response to stress
	Acat2	Metabolic process
	Tef	Cellular response to light stimulus; positive regulation of transcription; regulation of transcription, DNA dependent
	Hoxd10a	Multicellular organismal development; regulation of transcription; regulation of transcription, DNA dependent; transcription
	Slc25a5	Transmembrane transport; transport
	Foxa2	Central nervous system myelin formation; endoderm development; multicellular organismal development; oligodendrocyte cell fate commitment; regulation of transcription, DNA dependent; ventral midline development
	Zic5	Canonical wnt receptor–signaling pathway; cell proliferation in midbrain; midbrain development; neural tube formation; regulation of cell proliferation
	Zic1	Brain development; cell proliferation in hindbrain; embryonic retinal morphogenesis in camera-type eye; forebrain morphogenesis; fourth ventricle development; hindbrain development
	Fgfr4	Fibroblast growth factor receptor–signaling pathway; positive regulation of cell proliferation; protein phosphorylation
	Foxg1	Neuron differentiation; nose development; regulation of transcription; regulation of transcription, DNA dependent; transcription
	Sox21b	Regulation of transcription; transcription
	Pik3r3	Phosphoinositide 3-kinase complex
	Metrnl	Extracellular region
dre-miR-736	*Eya1*	Adenohypophysis development; ear morphogenesis; inner ear morphogenesis; metabolic process; multicellular organismal development; otolith development

Note. Targets were predicted using Eimmo, miRanda, and TargetScan, which allow zebrafish miRNA target prediction. Putative genes were considered whenever they were predicted by at least two of the databases used.

induced cell death and apoptosis along the dorsal axis of the embryo, particularly in the brain region (Carvan *et al*., 2004; Reimers *et al*., 2006). Thus, it is possible that the activation of these processes is correlated with deregulation of the miRNAs mentioned above.

Besides targeting genes involved in cell cycle control and apoptosis, the majority of the deregulated miRNAs are highly expressed in the brain and some of their putative targets play major roles in CNS development. In zebrafish embryos, EtOH induces a number of morphological defects throughout the CNS, namely at the level of motor neuron and muscle fiber morphology (Sylvain *et al*., 2010). Additionally, EtOH also alters the fate of fetal human brain–derived stem cells and progenitor cells, which may contribute to the abnormal brain development observed in FASD (Vangipuram and Lyman, 2010). Therefore, the fact that most of the zebrafish miRNAs deregulated by EtOH in our study are brain specific or brain enriched is not surprising and it is likely that these miRNAs play a role in brain and CNS development. Future studies should focus on the *in vivo* validation of the targets of the deregulated miRNAs to experimentally validate this hypothesis.

In conclusion, our study shows that EtOH alters miRNA expression and that EtOH-induced miRNA deregulation may underlie the developmental defects observed in FASD, providing clues to the molecular mechanisms of EtOH toxicity. This is, to our knowledge, the first study that investigates miRNA expression deregulation in vertebrate developing embryos exposed to EtOH. The data also show that miRNAs can be used to assess the level of alcohol toxicity in vertebrate embryonic development, and the commonly upregulated miRNAs can be used as a signature of early embryonic exposure to EtOH.

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