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Differential Expression of MicroRNAs in Embryos and Larvae of Mahi-Mahi (*Coryphaena hippurus*) Exposed to *Deepwater Horizon* Oil

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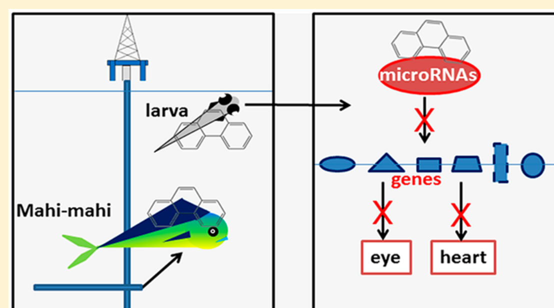
Supporting Information

ABSTRACT: *Deepwater Horizon* (DWH) oil causes developmental cardiotoxicity in fish species, but the molecular mechanisms are still not well understood. MicroRNAs (miRNAs) play key roles in a number of biological processes, including heart development. Therefore, the expression of miRNAs in mahi-mahi (*Coryphaena hippurus*) exposed to weathered slick oil and nonweathered source oil was analyzed. miRNAs were sequenced and annotated using the *Fugu rubripes* genome (termed the *Fugu*-guided approach) or aligned with known mature animal miRNAs using the Basic Local Alignment Search Tool (BLAST) method (termed the phylogenetically guided approach). Exposure of embryos to slick oil resulted in more differentially expressed miRNAs than exposure to source oil at all developmental stages. Gene ontology analysis on the target mRNAs was consistent with pathway analysis of miRNAs, predicting disruption of cardiovascular system development after oil exposure, and specific miRNA−mRNA interactions identified by ingenuity pathway analysis. Slick oil caused an overexpression of miR-133, which correlated with the decrease in the level of expression of genes related to the cardiovascular system such as potassium voltage-gated channel subfamily H member 2 (*kchn2*). This work is the first study linking miRNAs and mRNAs in crude oil-exposed fish, providing a new understanding of the molecular mechanism(s) of oil toxicity.



INTRODUCTION

In 2010, the largest oil disaster in U.S. history occurred with the explosion of the *Deepwater Horizon* (DWH) oil rig, releasing millions of barrels of oil into the Gulf of Mexico. The timing and location of the DWH oil spill overlapped with the spawning season of economically and ecologically relevant fish species, including mahi-mahi (*Coryphaena hippurus*).[1] Oil-derived polycyclic aromatic hydrocarbons (PAHs) from the DWH spill have been shown to negatively affect cardiac development of mahi as well as other pelagic fish like the bluefin tuna (*Thunnus maccoyii*), yellowfin tuna (*Thunnus albacares*), and yellowtail amberjack (*Seriola lalandi*).[2−4] In mahi, pericardial edema, impaired looping, atrial contractility, reduced stroke volume, and reduced cardiac output were observed along with altered expression of genes such as atrial myosin heavy chain (*amhc*) and ventricular myosin heavy chain (*vmhc*) after oil exposure.[5] Oil exposure can also affect Ca^{2+} and K^{+} currents in isolated fish cardiomyocytes.[6] Collectively, the effects induced by PAHs during development can translate into adverse effects on later life stages as previously reported for mahi, potentially altering the overall fitness of an organism.[3]

Oil-derived PAHs has been shown by many studies to negatively impact normal development. In the water column, because of natural weathering processes, the composition and structure of PAHs can be altered.[2−4] It has been shown that weathering can shift the composition of PAHs in oil by decreasing the number of lower-molecular weight PAHs and subsequently increasing the proportion of higher-molecular weight PAHs.[4] Interestingly, it is suggested that weathered slick oil can be more toxic than nonweathered oil.[4]

PAH-induced developmental toxicity can be mediated through the activation of the aryl hydrocarbon receptor (*ahr*).[7,8] However, PAHs that are poor AhR ligands can also cause developmental toxicity in fish.[7,9] Similarly, transcriptomic studies in fish have indicated a number of non-AhR pathways involved in oil toxicity; however, it is unclear how these transcripts are regulated.[10,11] Expression of mRNA can be post-transcriptionally controlled with microRNAs (miRNAs), which

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are small noncoding RNAs.[12] miRNAs play a key role during embryogenesis and in regulating other biological processes such as cell differentiation, apoptosis, and metabolism. In addition, recent studies have also shown that oil can regulate miRNA expression through methylation of miRNA promoters.[13,14] On the basis of the differential expression of genes observed after oil treatment, we sought to investigate the role of miRNA in the transcriptomic changes induced by oil in embryonic and larval fish exposed to two different types of DWH oil, weathered slick oil and nonweathered source oil.

■ MATERIALS AND METHODS

Oil Preparation and Embryo Treatment. Mahi embryos used for the study were collected and prepared using the standard UMEH method at the University of Miami Experimental Hatchery (UMEH).[10,15] After collection and assessment, embryos were concurrently exposed to diluted high-energy water accommodated fractions (HEWAF) of the source and weathered slick oil from the DWH spill as previously described.[10] Source oil was collected directly over the well from the subsea containment system (sample SO-20100815-Mass-001 A0075K). Weathered oil termed slick oil was collected from a surface skimming operation (sample OFS-20100719-Juniper-001 A0091G).[10]

Following exposure 6 hours post fertilization (hpf), 30 surviving embryos or larvae were pooled in RNAlater 24, 48, and 96 hpf, and each time point and treatment had three replicates. At the pharyngula stage (24 hpf), the embryo forms a heart tube, which starts to beat. By the yolk-sac larval stage (48 hpf), the ventricle and atrium are discernible and the size of the yolk sac decreases. At the last stage (96 hpf), the larvae have undergone morphogenesis and are free swimming.[16] During the treatment, the health of the embryos and larvae was monitored and dead animals were removed. Toxicity data along with pericardial area and heart rate measurements were recorded and analyzed and have been previously published.[10] Water quality was also monitored throughout the experiment, and PAH concentrations were measured.[10]

miRNA Isolation, Library Construction, and Sequencing. miRNAs of pooled samples were isolated using the miRNeasy mini kit from Qiagen (Valenica, CA). The quality and concentration of miRNA were determined using the Agilent (Santa Clara, CA) 2100 small RNA chip. miRNA libraries were made using the New England Biolabs (Ipswich, MA) NEBNext Multiplex Small RNA Sample Prep kit following the manufacturer's protocol. The size distribution and concentration of the libraries were determined using the Agilent High Sensitivity DNA Assay Chip. Single-Read 1 × 75 sequencing was performed on an Illumina NextSEQ v2 instrument at the Institute of Integrative Genome Biology of the University of California, Riverside. The read data were deposited in the NCBI database (GSE102966).

Phylogenetically Guided miRNA Annotation Method. Two methods were used to annotate the miRNAs from mahi. Because previous mRNA annotation experiments used exclusively *Fugu rubripes* with the "On-Ramp" pipeline,[10] annotation methods for miRNA were similarly performed solely using *Fugu*, comparing and supplementing the output with a broader phylogenetic method. For both methods, the quality of the raw sequences was evaluated by the FastQC toolkit (Cambridge, U.K.). Adaptor sequences were trimmed off using the FASTX toolkit (Cambridge, U.K.), to obtain a mean Phred score of ≥30. Only reads that are longer than 18 bp and shorter than 30 bp were kept for downstream alignment. The filtered sequencing data were aligned against known mature miRNA sequences from miRbase (release 21)[17] by using the Basic Local Alignment Search Tool (BLAST)[18] (Figure S1). Only sequencing that (1) had no gap in the alignment with known mature miRNA, (2) matched exactly with known miRNA nucleotides 2−17, and (3) was at maximum 3 bp longer than known miRNA was considered as a candidate miRNA. A customized perl script was then designed to count the sequencing read of each unique miRNA.[19] Differential expression analysis was conducted using DESeq2 with default independent filtering.[20] miRNAs were considered differentially expressed when the false discovery rate (FDR) was <0.01 (Benjamini−Hochberg correction). Identified differentially expressed miRNAs and mRNA-seq data from Xu et al.[10] were analyzed using the microRNA target filter in ingenuity pathway analysis (IPA; Qiagen, Valencia, CA) to identify experimentally demonstrated miRNA−mRNA relationships and predict the impact of changes in expression of miRNA and its target mRNA on biological processes and pathways. A miRNA was considered to be regulatory only if the expression levels of miRNA and its mRNA targets are reversely correlated. As previous studies showed that slick oil increased the pericardial area and altered the gene expression in the cardiovascular system and the nervous system, we further filtered the differentially expressed (DE) miRNA/mRNA data and focused on cardiovascular and ocular functions and diseases in IPA.[10] The target mRNAs identified by IPA were then used for gene ontology (GO) analysis by using the Database for Annotation, Visualization and Integrated Discovery (DAVID).[21,22]

***Fugu* Genome miRNA Annotation.** The methods of quality assessment and filtration of the raw reads were the same as described above. In contrast to a total phylogenetic screen, for the *Fugu* annotation method, the processed reads were mapped solely to the *F. rubripes* genome, and both known and novel miRNAs were identified using miRDeep2, a probabilistic algorithm based on the miRNA biogenesis model and designed to detect miRNAs from deep sequencing reads.[23,24] Read counts generated from miRDeep2 were used for differential expression analysis using DESeq2. The same downstream analysis using IPA and DAVID was conducted as described above (Figure S1). All analysis was performed on a local server running under the Institute for Integrative Genome Biology (IIGB)'s Linux cluster, Biocluster, environment (http://manuals.bioinformatics.ucr.edu/home/hpc).

Quantitative Reverse Transcription Real-Time Polymerase Chain Reaction (qPCR). Differentially expressed miRNAs identified in the miRNaseq analysis were validated by qPCR. miRNA primers were designed using the Qiagen custom miRNA primer assay. Melt curve analysis and 1% agarose gel electrophoresis were performed to assess the specificity of the qPCR products. cDNA was generated using the Qiagen miScript II RT Kit. Relative qPCR expression was determined by the 2ΔΔCt method with the Qiagen miScript SYBR Green PCR Kit using RNU6 as the normalizing gene and run on the CFX Connect Real-Time PCR Detection System.

■ RESULTS AND DISCUSSION

Global Expression Profiles of miRNAs after Source and Slick Oil Exposure. miRNAs play an important role during development and are potentially involved in PAH-induced toxicity. To assess the role of miRNAs in oil toxicity

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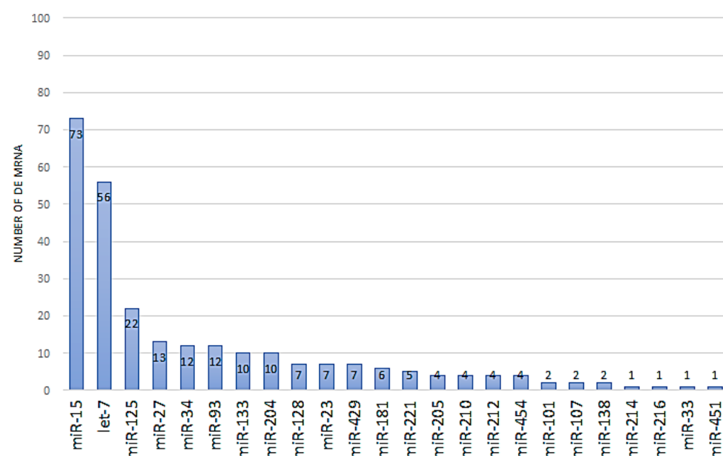
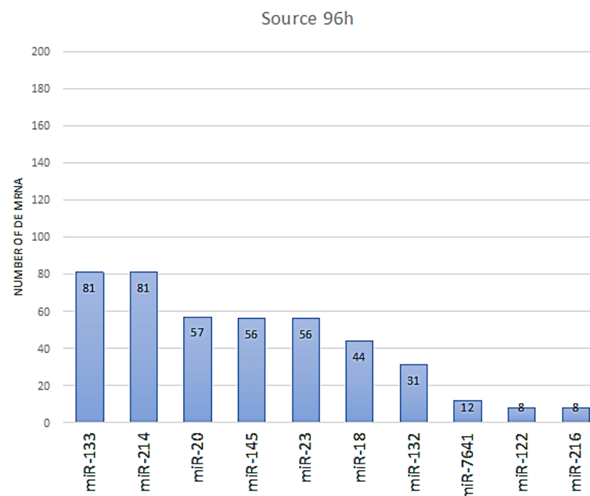
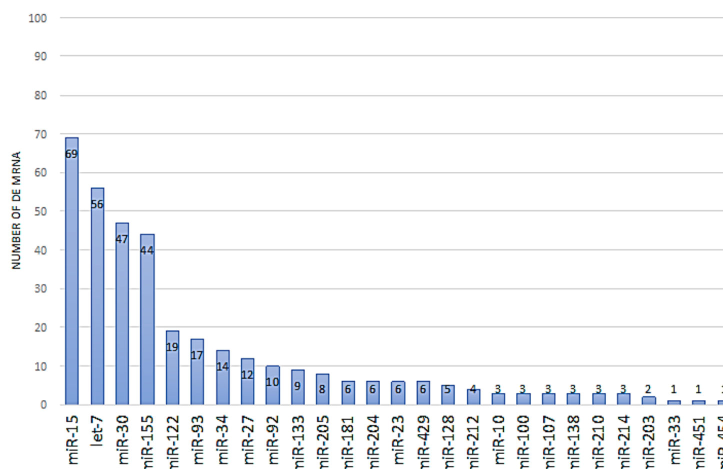
A. Source oil - Phylogenetic-guided approach



B. Source oil- Fugu-guided approach



C. Slick oil - Phylogenetic-guided approach



D. Slick oil- Fugu-guided approach

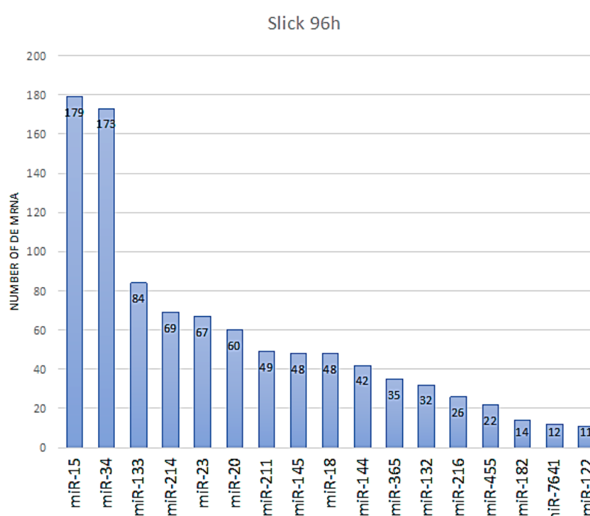


Figure 1. Distribution of the number of differentially expressed (DE) mRNAs correlated to differentially expressed miRNA identified using IPA at 96 hpf. The bar graphs show the numbers of correlated mRNAs and miRNAs at 96 hpf after source (A and B) and slick (C and D) oil exposure.

during development, miRNA expression was assessed in mahi embryos treated with slick and source oil from the DWH oil spill. The number of DE miRNA increased as development progressed, and slick oil induced more DE miRNAs than source oil as determined by both methods (Figure S2). Using the phylogenetic miRNA annotation method, the numbers of DE miRNAs were 46, 101, and 178 at 24, 48, and 96 hpf after source oil exposure, respectively (Table S1). Exposure to slick oil resulted in 139, 213, and 213 altered miRNAs at 24, 48, and 96 hpf, respectively (Table S1). As predicted, the *Fugu*-guided annotation method identified fewer DE miRNAs than the phylogenetic method did. At 24, 48, and 96 hpf, exposure to source oil resulted in 25, 24, and 165 DE miRNAs, respectively (Table S1). Similar to the phylogenetic method, more DE miRNAs were identified after slick oil exposure than after source oil exposure using the *Fugu* approach. There was a total of 42 DE miRNA at 24 hpf after slick oil exposure. At 48 hpf, there were 82 DE miRNAs, and at 96 hpf, there were 254 DE miRNAs (Table S1). Common DE miRNAs were also identified between the two methods (Figure S3). Some miRNAs were consistently upregulated (e.g., miR-7641) or downregulated (e.g., miR-21b) at all three developmental stages (Tables S2−S4). The smaller number of DE miRNAs with the *Fugu* method is consistent with the smaller number of DE mRNAs observed using a *Fugu*-selective annotation platform for mRNA relative to *de novo* methods that incorporated additional phylogenetic screening.[25] The expression of a subset of miRNAs was confirmed by qRT-PCR at 96 hpf of mahi exposed to slick oil (Figure S4). Previous studies have indicated that weathered slick oil caused more severe developmental defects than source oil did probably because of a larger number of three-ring PAHs are present in the slick oil.[10] Similarly, more DE miRNAs were observed after slick oil exposure than after source oil exposure of the mahi embryos at all stages.

Biological Processes Affected by Slick and Source Oil Exposure. IPA identified miRNA−mRNA networks in both oil types at the three stages based on the reverse expression of miRNAs and their target mRNAs (Figure 1, Figure S5, and Figure S6). To determine the biological impact of miRNA−mRNA expression, a GO term analysis (molecular function,

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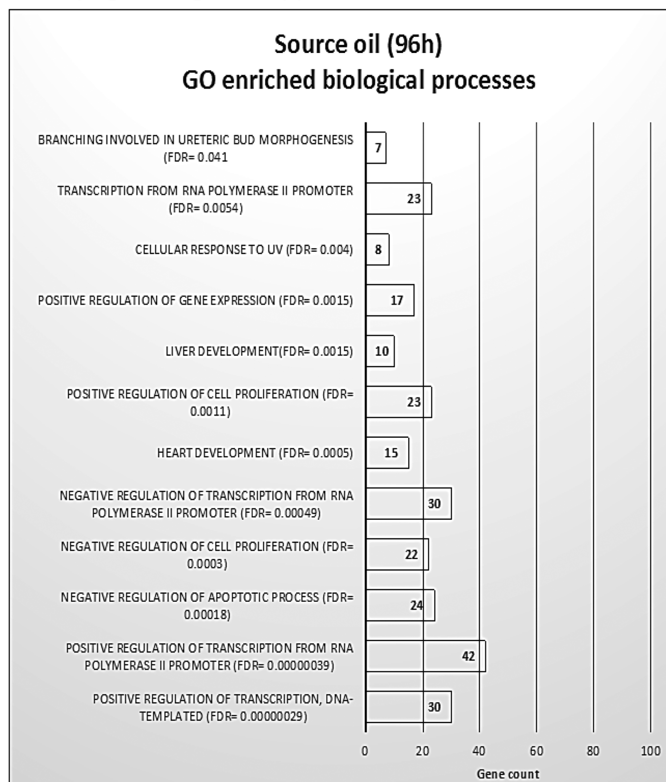

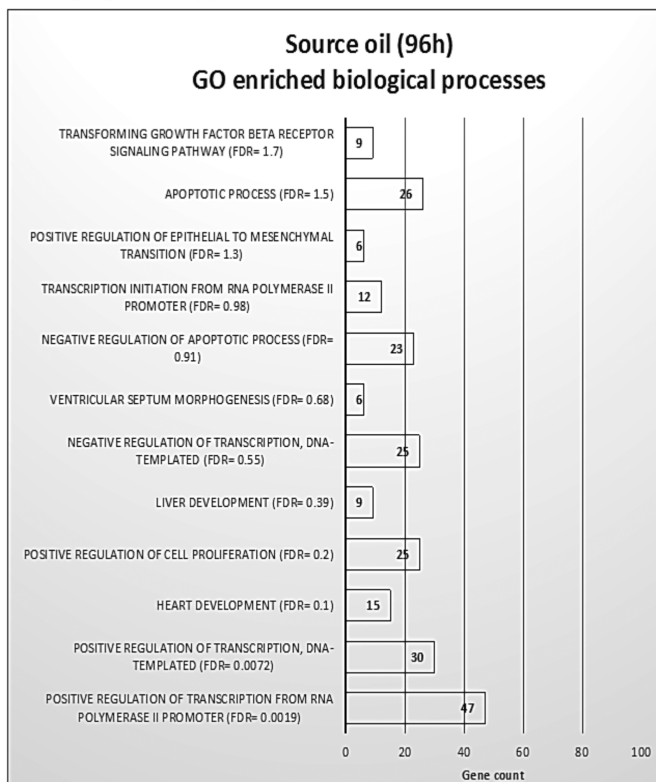

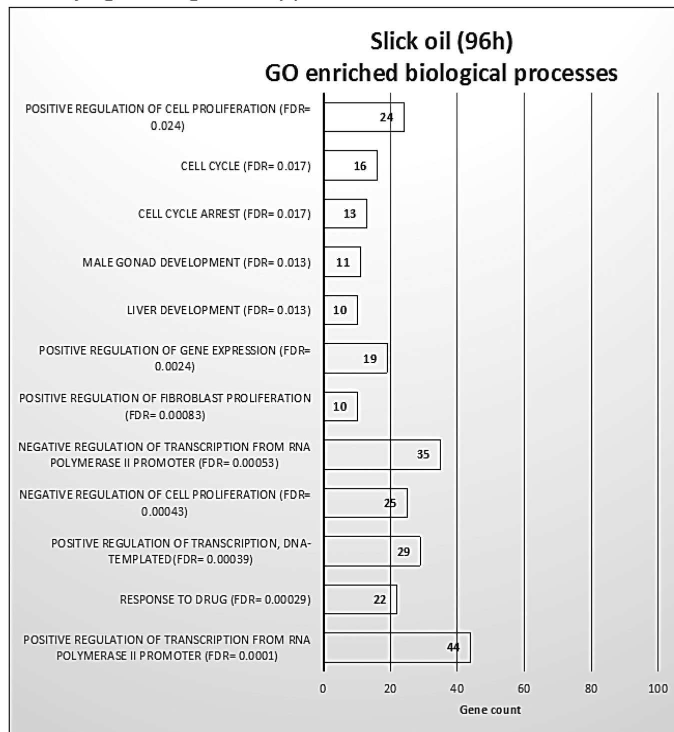

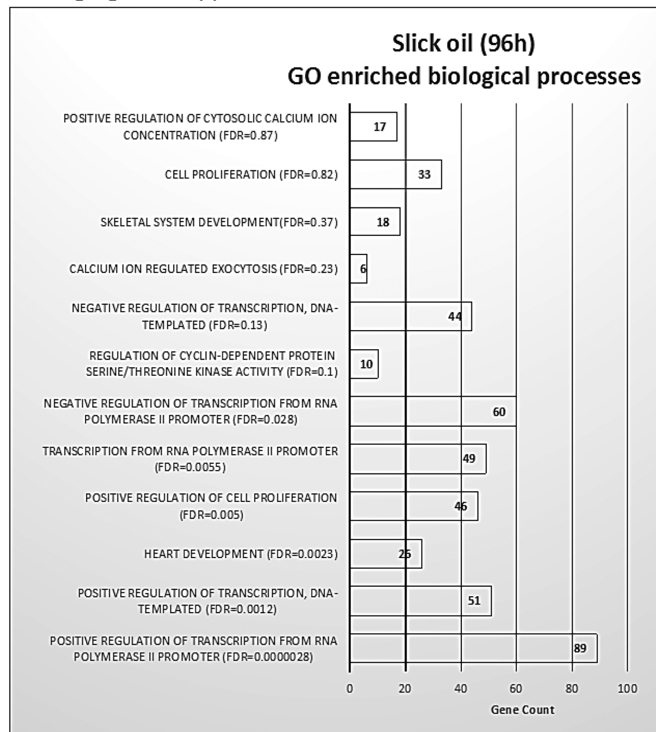

Figure 2. Gene ontology (GO) analysis of enriched biological processes of the correlated differentially expressed mRNA at 96 hpf after source and slick oil exposure. The top 12 biological terms identified after source oil exposure using (A) the phylogenetically guided approach and (B) the *Fugu*-guided approach. Top 12 biological terms identified after slick oil exposure using (C) the phylogenetically guided approach and (D) the *Fugu*-guided approach. The gene count and FDR *p* value associated with the GO category from DAVID are presented in the bar graph.

cellular component, and biological process) was conducted by analyzing the target mRNAs using DAVID (Figure 2 and Table S8). Using the phylogenetic method, the top enriched biological processes were transcription and cellular proliferation terms. Several cardiac-related terms were also identified for slick oil, e.g., cardiac muscle hypertrophy in response to stress

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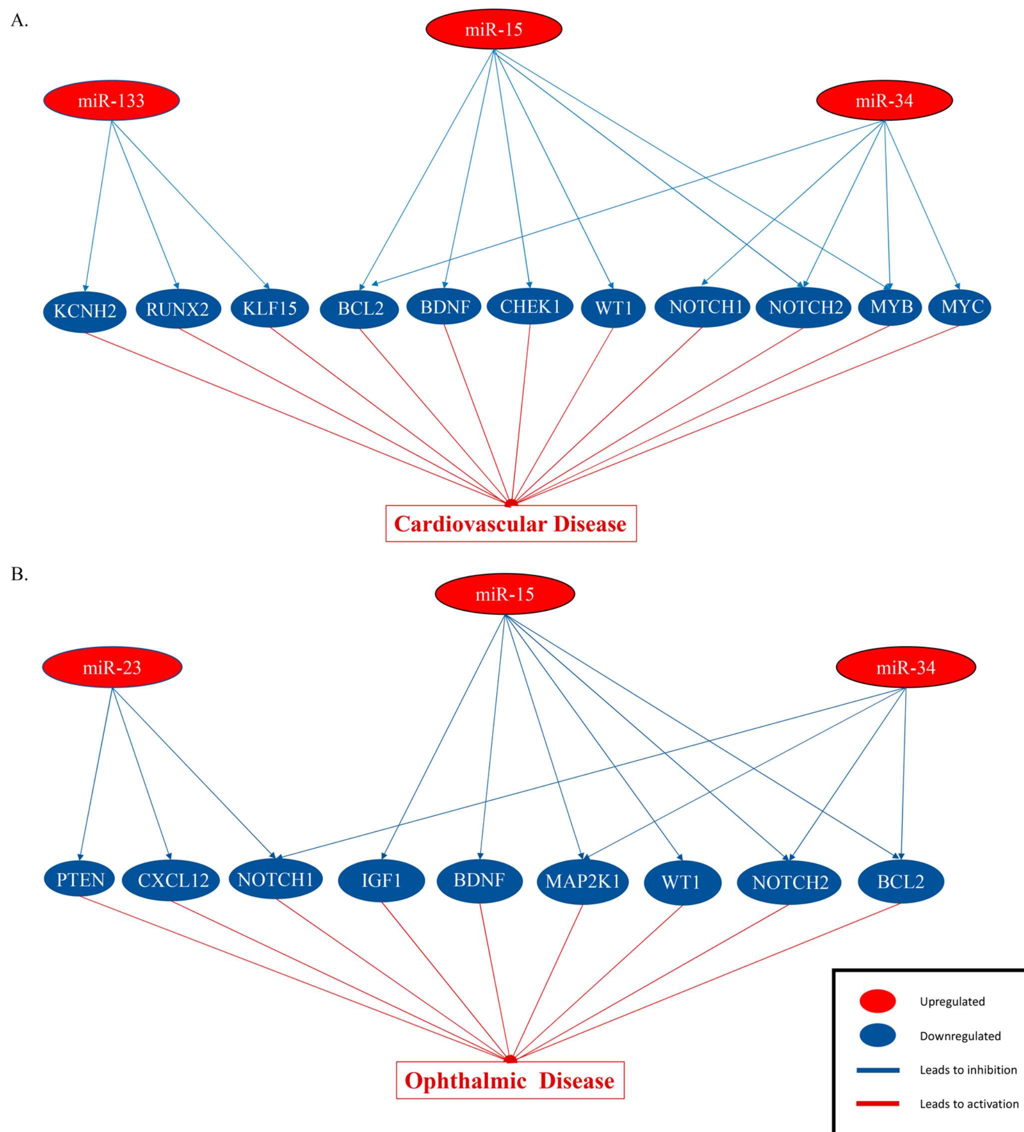

Figure 3. Ingenuity pathway analysis of correlated miRNA and mRNA networks related to (A) cardiovascular disease and (B) ophthalmic disease 96 hpf after slick oil exposure.

(ranked 18) and heart development (rank 28) (Figure 2C). For source oil, DAVID identified terms related to regulation of transcription as well as heart development as the top-ranked terms (Figure 2A). Similar to the phylogenetic method, annotation based on *Fugu* also predicted regulation of transcription, heart development, and cell proliferation as the top enriched terms for both slick and source oil at 96 hpf (Figure 2B,D). In addition, calcium regulation, liver development, and ventricular septum morphogenesis were also significantly enriched (Figure 2). The impacts of oil exposure on fish development have been well documented, and the cardiovascular system has been reported to be a primary target associated with oil exposure, which is consistent with the results of this study.[2−4,10,26] Recent studies have also shown that ion gradients that are important for normal excitation−contraction coupling are altered because of a disruption in Ca^{2+} and K^{+} currents after exposure to oil and phenanthrene.[6,27] Down-regulation of ion transporters *kcnh2* and *ncx1* have been observed following PAH and oil treatment, but it is unclear how the transporters are regulated by the toxicants.[11]

Cardiovascular Disease-Related miRNA−mRNA Networks Identified Using IPA. The GO results of this study showed that slick oil can disrupt the cardiovascular system pathways at later developmental stages, which was consistent with the observed physiological responses (i.e., significantly decreased heart rate, higher variable heart rate, and increased pericardial area) at later developmental stages.[10] miRNA−mRNA networks were, therefore, filtered for cardiovascular system-associated functions and pathways 48 and 96 hpf after slick oil treatment using the IPA software (Tables S5 and S6). At 96 hpf, for the phylogenetic method, the top three miRNAs for slick oil were miR-15b, let-7e, and miR-30c (Table S6). For the *Fugu* method, miR-34a-5p, miR-15b, and miR-133b were the top three (Table S6). miRNA−mRNA network analysis showed that the DE of *kcnh2* was correlated with the DE of miR-133 (Figure 3A). *kcnh2* plays an important role in ion mobilization in cardiomyocyte cells, and alterations in kcnh2 function can disrupt action potentials.[28] The relationship between miR-133 and *kcnh2* is significant as it may explain how transporter expression is altered by PAHs and oil. Similarly, a study in mice has shown that miR-133 is also

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Environ. Sci. Technol. Lett. 2017, 4, 523−529

involved in the cardiac hypertrophy induced by phenanthrene.[13]

Slick oil exposure also upregulated miR-15b, which regulated the largest number of DE mRNAs (Figure 3A and Table S6). Members of the miR-15 family have been shown to play an important role in normal heart development by repressing cell proliferation through the regulation of cell cycle genes, such as checkpoint kinase 1 (*chek1*).[29,30] In mahi, *chek1* mRNA was downregulated, suggesting proliferation was impaired within embryos (Figure 3A and Table S6). Whether this occurred specifically in the heart or other targets deserves further study with *in situ* hybridization. The role of miR-15b and miR-133 in cardiovascular defects induced by oil also warrants further investigation.

Ophthalmic Disease-Related miRNA−mRNA Networks Identified Using IPA. In addition, the miRNA−mRNA networks were also filtered for ocular functions and ophthalmic diseases because vision-related pathways and diseases were identified in previous RNA-seq analysis in mahi,[10] and these vision-related pathways were linked to ocular toxicity in red drum fish.[31] The phylogenetic method identified let-7e, miR-15b, and miR-30c as the top three miRNAs correlated with the most DE mRNAs (Table S7). For the *Fugu* method, miR-34a-5p, miR-15b, and miR-20b were identified as the highest-ranking miRNAs (Table S7). Although eye-related processes were not observed in the GO term analysis using DE miRNA, some miRNA−mRNA networks were linked to ophthalmic disease. IPA showed that eye-related genes such as the downregulated mRNA of phosphatase and tensin homologue (*pten*) and neurogenic locus notch homologue protein 2 (*notch2*) genes were inversely correlated with several upregulated miRNAs (miR-34a, miR-15, and miR-23) (Figure 3B). PTEN is a phosphatase that is involved in tumor suppression and eye development.[32−34] NOTCH2 encodes a transmembrane receptor involved in cell differentiation.[35,36] In mice, a functional mutation of NOTCH2 caused abnormal eye vasculature, which can lead to several eye diseases such as cataracts and eye degeneration.[37] Cataract formation was one of the top diseases identified in the slick oil RNA-seq analysis.[10] With the growing body of evidence that the eye is negatively affected by PAHs, the need to further evaluate the role of miRNAs in ocular development is apparent.[38,39]

Overall, this study shows that exposure to both source and slick oil can alter the global expression of miRNAs during mahi embryonic and larval development. Consistent with toxicological effects and mRNA regulation observed in previous studies, a more pronounced change in miRNA expression was observed after slick oil exposure than after source oil exposure. GO term analysis also indicated heart development and ion regulation terms were affected by oil exposure. IPA identified miRNA−mRNA networks involved in cardiovascular and ocular functions and related diseases. To the best of our knowledge, this is the first study linking miRNAs and mRNA in fish responsive to crude oil exposure, providing a new opportunity for understanding mechanisms of oil-induced toxicity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00484.

Schematic of the bioinformatics pipeline (Figure S1), transcript expression profiles and Venn diagrams of miRNAs (Figures S2 and S3), a comparison of miRNaseq and qPCR methods (Figure S4), distribution of differentially expressed mRNA correlated to miRNA (Figures S5 and S6), the number of altered miRNAs (Table S1), lists of differentially expressed miRNAs (Tables S2−S4), IPA-enriched cardiovascular and ophthalmic diseases of miRNA and inversely correlated genes (Tables S5−S7), and top enriched cellular component and molecular function terms (Tables S8 and S9) (PDF)

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Notes

The authors declare no competing financial interest.

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