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Embryonic atrazine exposure alters zebrafish and human miRNAs associated with angiogenesis, cancer, and neurodevelopment

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Abstract

MicroRNAs (miRNAs) are short, single-stranded RNA that regulate post-transcriptional control of mRNA translation. Knowledge on the role of these critical regulators in toxicological responses in increasing, but is still limited. Atrazine is a herbicide used throughout the Midwestern US that is reported to frequently contaminate potable water supplies above the maximum contaminant level of 3 parts per billion. Atrazine is a suspected endocrine disrupting chemical and studies have begun to investigate the genetic mechanisms of toxicity; however, studies investigating epigenetic mechanisms are limited. In this study both zebrafish and human miRNAs were significantly altered in response to an embryonic atrazine exposure of 0.3, 3, or 30 ppb in zebrafish. Altered miRNAs are known to play a role in angiogenesis, cancer, or neuronal development, differentiation, and maturation. Targeted analysis of altered human miRNAs with genes previously identified to be altered by atrazine exposure revealed several targets linked to cell cycle and cell signaling. Further analysis of hsa-miRNA-126-3p, which had altered expression in all three atrazine treatments at 72 hpf, revealed alterations also occurred at 60 hpf in the 30 ppb treatment group. Results from this study indicate miRNA deregulation in zebrafish and human miRNAs following an embryonic atrazine exposure in zebrafish.

Keywords

atrazine; development; miRNA; targeting analysis; transcriptomics; zebrafish

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1. Introduction

Particular interest in recent years has shifted towards the study of the underlying epigenetic mechanisms of gene regulation and the role it plays in developmental reprogramming of the genome and disease susceptibility. Epigenetics can be defined as the study of the molecular mechanisms that regulate gene expression often leading to permanent, yet reversible, changes that can be stable throughout the lifespan of an organism and potentially be heritable (Goldberg et al., 2007; Portela and Esteller, 2010). The term was initially coined by Conrad Waddington in the 1940s while describing the outward appearance, or phenotype, that an organism displays based upon the genetic material and functions of the genes in an organism (Hanson and Gluckman, 2014). Three types of known molecular epigenetic mechanisms include DNA methylation, histone modifications, and small non-coding RNAs that regulate gene expression (Jirtle and Skinner, 2007). Of these, the alteration of DNA methylation patterns and subsequent reprogramming of developmental processes by changing transcriptional gene expression has received most attention, especially as it pertains to the heritability of these epigenetic alterations (Anway et al., 2005, 2006; Guerrero-Bosagna et al., 2010; Inawaka et al., 2009; Stouder et al., 2010; Vandegehuchte et al., 2009). More recently, studies have implicated the importance of post-transcriptional regulation by non-coding RNAs in regulating gene expression, namely through short sequences of RNA referred to as microRNAs (miRNAs).

miRNAs are short (~22 nucleotides in length), single-stranded RNA genes that regulate post-transcriptional gene expression by targeting messenger RNAs (mRNAs) through their complementary sequences in the 3’ untranslated region and repressing their translation. MicroRNAs are associated with a broad spectrum of cellular and developmental processes including responses to xenobiotic stresses. Thus, miRNAs are being widely studied for their mechanistic role in toxicological outcomes and have been implicated in cardiovascular, developmental, liver, and neurotoxicity pathways (Tal and Tanguay, 2012; Yokoi and Nakajima, 2001). miRNAs are found in diverse organisms suggesting evolutionary conservation of mechanisms related to miRNA regulation (Li et al., 2010; Lim et al., 2003). However, the majority of miRNA functions are unknown and different animal models are being used to identify miRNA functions and differences in miRNA expression in laboratory studies (Ason et al., 2006). Moreover, few studies have investigated the role of miRNAs in toxicological responses thereby limiting the knowledge of relevance of these critical regulators in mechanisms of toxicity.

Endocrine disrupting chemicals (EDCs) are exogenous agents that alter the endocrine system through multiple pathways. EDCs are diverse in structure and are present in many products such as plasticizers, pharmaceuticals, and pesticides (Roy et al., 2009; Swedenborg et al., 2009). Rapid increases in industrialization and in the production of these chemicals have increased the risk of human exposure, therefore, heightening public concern and the need for investigation into their mechanisms of action. Studies have reported that exposure to EDCs can cause irreversible changes in tissue formation, decreased reproductive potential, obesity, and cancer (Cooper et al., 2000; Hatch et al., 2011; Roy et al., 2009; Swedenborg et al., 2009; Wetzel et al., 1994). Moreover, evidence suggests that exposure to

EDCs can cause adverse effects not only in organisms that come into contact with them, but also to future progeny of exposed individuals (Anway et al., 2005, 2006).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a pre-emergent herbicide used to prevent the growth of broadleaf and grassy weeds on crops such as corn, sorghum grass, sugar cane, and wheat and is reported to have endocrine disrupting effects (Barr et al., 2007; Eldridge et al., 1999; Ochoa-Acuña et al., 2009; Solomon et al., 2008). The United States Environmental Protection Agency (U.S. EPA) estimates approximately 76.5 million pounds of atrazine are applied annually in the United States, making it one of the most widely used herbicides (Rinsky et al., 2012). The U.S. EPA has set the maximum contaminant level (MCL) for atrazine at 3 parts per billion (ppb; μg/L) in drinking water supplies; however, during spring and summer months, this level is often exceeded (Barr et al., 2007; Ochoa-Acuña et al., 2009; Rohr and McCoy, 2010; U.S. EPA, 2002). Numerous studies have investigated the adverse health effects of atrazine on the neuroendocrine system. Results report that atrazine decreases gonadotropin releasing hormone (GnRH) release, the pre-ovulatory surge of luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin (PRL) (Cooper et al., 2000; Foradori et al., 2009, 2013). The genetic and cellular mechanisms behind the observed hormonal alterations are also under investigation both *in vitro* and *in vivo* (Fa et al., 2013; Kucka et al., 2012; Pogrmic-Majkic et al., 2010, 2014; Quignot et al., 2012). Furthermore, epidemiological studies highlight the need for understanding the developmental and reproductive effects of atrazine exposure and its impact on rural communities as exposure is evident in areas where this herbicide is used (Munger et al., 1997; Ochoa-Acuña et al., 2009; Winchester et al., 2009).

We have previously reported that developmental atrazine exposure results in morphological alterations and expression alterations in genes associated with reproductive system function and development, cell cycle regulation, and cancer in zebrafish larvae (Weber et al., 2013). The zebrafish provides a strong complementary vertebrate model when investigating epigenetic mechanisms of toxicity and developmental toxicity including *ex utero* fertilization and development, short developmental periods, a relatively short life span, and high genetic homology to humans. In addition, the zebrafish has structural and functional homology of the central nervous system (CNS) (de Esch et al., 2012; Howe et al., 2013).

In the present study we exposed zebrafish embryos to 0.3, 3 or 30 ppb atrazine from 1-72 hours post fertilization (hpf; the end of embryogenesis) and identified zebrafish and human miRNAs that were significantly altered in response to atrazine. Targeted analysis was then performed to determine the regulation of gene expression by miRNAs on altered gene targets from our previous transcriptome analysis (Weber et al., 2013). Furthermore, developmental characterization and atrazine toxicity of hsa-miR-126-3p, a miRNA reported to be altered in all atrazine treatments, was established.

2. Materials and Methods

2.1 Zebrafish husbandry and experimental design

Zebrafish (wild-type AB strain) were housed in a Z-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 hour light:dark cycle and maintained at 28°C (±1°C) with a pH of 7.0 - 7.2

and conductivity range of 470-520 μ S. Adult zebrafish were bred in cages and embryos were collected, staged, and rinsed with system water as described previously (Peterson et al., 2011). Embryos were dosed with 0, 0.3, 3, or 30 ppb atrazine (CAS #1912-24-9; Chem Service, 98% purity) from 1-72 hours post fertilization (hpf) as previously described (Weber et al., 2013; Wirbisky et al., 2015). Four biological replicates (n=4), each from a different clutch, were included in each of the experiments. All animal protocols were approved and performed in accordance with Purdue University's Institutional Animal Care and Use Committee guidelines.

2.2 miRNA microarray analysis of 72 hpf zebrafish larvae

Upon completion of the exposure period, 50 embryos from each treatment group of each replicate were collected, pooled, and homogenized in Trizol reagent and flash frozen in liquid nitrogen. Total RNA was isolated from the pooled embryo samples using a miRNeasy kit (Qiagen). The Agilent miRNA Complete Labeling and Hyb Kit was used to fluorescently label samples along with the MicroRNA Spike-In Kit to measure the efficiency of the labeling process. A total of 100 ng total RNA from each sample was used for labeling. Samples were dephosphorylated using Calf Intestinal Phosphatase and then denatured with dimethyl sulfoxide. Following denaturation, samples were cooled and subsequently labeled with Cyanine 3-pCp using a T4 RNA Ligase. Labeled samples were purified using MicroBioSpin 6 columns (Bio-Rad, Hercules, CA) and dried using a Savant Speed Vac on medium-high heat for 1 hour. Dried samples were resuspended in nuclease-free water and prepared for hybridization using GE Blocking Agent and Hi-RPM Hybridization Buffer. Samples were loaded onto a custom designed 8X60K Agilent miRNA array in which all human and zebrafish miRNAs were included (based on miRBase release 18.0). Arrays were loaded into SureHyb chambers and hybridized at 55°C for 20 hours in an Agilent Microarray Hybridization Oven. The following day, arrays were removed from the oven, washed with Agilent Gene Expression Wash Buffers and scanned on an Agilent SureScan Microarray scanner. Probe information was extracted from generated tif images using Feature Extraction image analysis software 9.5.3 using QC Metric Set to evaluate the quality of labeling and hybridization. A single replicate was removed from the 30 ppb treatment group as it did not meet QC standards. Data were background-subtracted, normalized to the 90^{th} percentile, and analyzed for differential expression in GeneSpring 12.5 software using a one-way analysis of variance (ANOVA) with a Tukey's post hoc test ($p<0.05$) to determine which atrazine treatment groups were different from the control.

2.3 miRNA targeted analysis of gene expression microarrays

miRNAs found to be differentially expressed were uploaded in Ingenuity Pathway Analysis (IPA) software program for targeting analysis of previously obtained 72 hpf gene expression data (Weber et al., 2013). The microRNA Target Filter in IPA provides insights into the biological effects of microRNAs and filters microRNA-mRNA data relationships using experimentally validated interactions from TarBase, miRecords, and peer-reviewed biomedical literature. This software currently does not support this analysis for zebrafish miRNAs.

2.4 Deposition of data

Array data has been deposited at the gene expression data base GEO (GSE78805).

2.5 Quantitative polymerase chain reaction (qPCR) developmental characterization of mir-126-3p

Zebrafish embryos were exposed to control aquaria water for completion of the developmental characterization of hsa-miR-126-3p. Embryos were collected at 12, 24, 36, 48, 60, and 72 hpf (n=4). In addition, to determine the effects of atrazine on hsa-miR-126-3p throughout development, zebrafish embryos were exposed to control aquaria water or an atrazine treatment of 0.3, 3, or 30 ppb throughout embryogenesis and collected at 12, 24, 36, 48, and 60 hpf (n=3). Following the specified developmental period (for both developmental characterization and atrazine exposed embryos), 50 embryos from each treatment group in each replicate were collected, pooled, and homogenized in Trizol reagent and flash frozen in liquid nitrogen. miRNA was isolated from the pooled embryo samples using a miRNeasy kit (Qiagen). miRNA was synthesized to cDNA using the Universal RT cDNA synthesis kit from Exiqon following manufacturer's recommendations (Exiqon, Woburn, MA). The probe specific to hsa-miR-126-3p was designed using locked nucleic acid technology by Exiqon. qPCR was performed using Exiqon SYBR Green kit according to manufacturer's recommendation (Exiqon, Woburn, MA). qPCR was performed following similar methods as previously described (Weber et al., 2013; Wirbisky et al., 2015) following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). Several genes were assessed to determine the best reference to be used for this data set (data not shown). U6 was found to be most consistent and least variable for this analysis. Experimental samples were run in triplicate (technical replicates) and gene expression was normalized to U6. Efficiency and specificity were checked with melting and dilution curve analysis and no-template controls. The developmental time course and atrazine-treated samples were analyzed for statistical significance with an ANOVA and a post-hoc least significant difference test when a significant ANOVA was observed ($p<0.05$).

3. Results

3.1 Developmental atrazine exposure alters zebrafish and human miRNAs

Microarray analysis using a platform containing all known sequences for zebrafish and human miRNAs revealed only one human miRNA (hsa-miR-126-3p) with altered expression in the 0.3 ppb atrazine treatment group following an embryonic exposure. In the 3 ppb atrazine treatment group, expression of two zebrafish miRNAs and three human miRNAs were significantly decreased. The 30 ppb atrazine treatment group had 16 zebrafish miRNAs and 6 human miRNAs with a significant decrease in expression. Overall a dose response increase in the number of miRNAs affected was observed (Table 1).

3.2 Targeting analysis with altered miRNAs and past transcriptomic results

Targeting analysis between the microarray miRNA results with genes previously identified to be altered at the same atrazine exposures was completed for the human miRNAs using Ingenuity Pathway Analysis (IPA). This analysis revealed no targeted genes in the 0.3 ppb

treatment by hsa-miR-126-3p. The 3 ppb atrazine treatment showed three human miRNAs (hsa-126-3p, hsa-miR-3907, and hsa-miR-455-3p) targeting 5 genes (*ANTXR2*, *SIK2*, *KIF5A*, *PODNL1*, and *RSL24D1*) (Table 2; Figure 1A). The most robust response occurred in the 30 ppb treatment with 5 miRNAs (hsa-miR-101-3p; hsa-miR-124-3p, hsa-126-3p, hsa-miR-3907, and hsa-miR-455-3p) targeting 15 unique genes (Table 3; Figure 1B).

3.3 Quantitative polymerase chain reaction (qPCR) developmental characterization of hsa-miR-126-3p

As hsa-miR-126-3p was identified to be altered in all three atrazine treatment groups, analysis on the developmental time course expression was first conducted to further characterize this miRNA. Expression at 12 hpf was significantly different from all other time points with minimal expression observed. Expression at 24 and 48 hpf showed statistically similar medial expression, while 36, 60, and 72 hpf displayed higher expression levels ($p<0.0001$) (Figure 2).

3.4 qPCR analysis of hsa-miR-126-3p expression following embryonic atrazine exposure at additional developmental time points

To determine if atrazine exposure would alter expression of hsa-miR-126-3p at additional developmental time points, qPCR analysis was conducted at 12, 24, 36, 48, and 60 hpf. Expression at 12 hpf was minimal and did not meet expression threshold for analysis. No significant alterations in hsa-miR-126-3p was found at 24, 36, or 48 hpf in any of the atrazine concentrations ($p=0.1497$; $p=0.2557$; $p=0.0998$, respectively) (Figure 3A-C). However, a significant increase was observed at 60 hpf in the 30 ppb atrazine treatment ($p=0.0281$) (Figure 3D).

4. Discussion

A limited number of studies to date have investigated miRNA expression alterations in response to EDC exposure in zebrafish and humans (Avissar-Whiting et al., 2010; Hsu et al., 2009; Jenny et al., 2012; Tilghman et al., 2012; Veiga-Lopez et al., 2013). Furthermore, there are limited to no published reports on the disruption of epigenetic regulators in response to atrazine exposure in human or zebrafish studies. The investigation into the adverse effects of an embryonic atrazine exposure on miRNAs is important as miRNAs are essential for embryogenesis, tissue morphogenesis, and cellular processes. In addition, miRNAs are thought to contribute to disease progression and genetic disorders (Bhattacharya et al., 2016; Esteller 2011; Tal and Tanguay, 2012). Thus, our goal in this study was to identify miRNAs that were altered in response to atrazine exposure using miRNA expression arrays interrogating both human and zebrafish miRNAs. This approach was chosen based on conservation of miRNA sequences and the fact that zebrafish miRNAs are still being identified. We identified a total of 17 zebrafish and 6 human unique miRNAs significantly altered in response to atrazine exposure, albeit, robustness in change was not high. A dose response in the number of miRNAs altered was also observed with additional miRNAs changed as treatment concentration increased.

An interesting finding was the alteration of several members of the zebrafish miR-10 family in the 3 and 30 ppb atrazine treatments. The miR-10 family is located in an evolutionary conserved region of the genome which contains the *Hox* cluster of genes. The *Hox* genes are critical regulators of the anterior-posterior body axis and the miR-10 family regulates the expression of *HoxB1a* and *HoxB3a* and seemingly works in conjunction with the *HoxB4* gene (Woltering and Durston, 2008). Alterations of the miR-10 family could be a contributing factor in the previously reported increase in head length following embryonic atrazine exposure (Weber et al., 2013). Furthermore, as epigenetic alterations can be inheritable, we have also previously shown offspring of adult zebrafish that were only exposed to 0.3 or 3 ppb atrazine during embryogenesis displayed a significant increase in their head width to total body length ratio, while offspring of adult zebrafish exposed to 30 ppb atrazine during embryogenesis had a significant decrease in their head length to total body (Wirbisky et al., 2016). In addition, a recent study employing both the zebrafish model and human umbilical vein endothelial cells, found that miR-10 members regulate angiogenic signaling, making it a potential marker for modulation of angiogenesis (Hassel et al., 2012). Other significantly altered miRNAs involved in the regulation of angiogenesis include miR-23a and miR-24. These miRNAs are members of the miR-23/27/24 cluster which is highly conserved through vertebrate animals (Bang et al., 2011).

miRNA dysregulation has also been reported as a key regulator in the development of various cancers (Heneghan et al., 2010; Hu et al., 2010; Lawrie et al., 2008). In this study we observed alterations within the miR-16 family (miR-16a and miR-16b). miRNAs of the miR-16 family have been evaluated as a tool in the identification of prostate, gastric, and breast cancer (Mobarra et al., 2015). The miR-16 family has also been identified to inhibit cell proliferation, regulate cell cycle, and promote apoptosis, further supporting its role in tumorigenesis (Aluru et al., 2013; Mobarra et al., 2015). Embryonic atrazine exposure also elicited a decrease in miR-18a and miR-18b-5p. These miRNAs are members of the miR-17-92 cluster, and are highly expressed in several cancers (Komatsu et al., 2014). Other miRNAs significantly altered include miR-143, miR-216b, and miR-217. These miRNAs are also primary tools in cancer development as they function as tumor suppressors (Faraji et al., 2015; Shen et al., 2014).

miRNAs are also implicated to play a role in brain development and neurogenesis with dysregulation contributing to various neurological diseases (Sun and Shi, 2015). We observed zebrafish miR-26a altered in the 30 ppb atrazine treatment group. miR-26 functions in neuronal development and is shown to target the *ctdsp2* gene in zebrafish which is important in the differentiation process of neural stem cells to neurons. Intriguingly, this miRNA is encoded in an intron of the *ctdsp2* gene and is expressed along with the gene rather than independently (Dill et al., 2012).

In addition to the altered zebrafish miRNAs, we examined the effects of atrazine on human miRNAs and report 6 unique significantly altered miRNAs. The human miRNA miR-126-3p was found to be significantly and robustly down-regulated in all three atrazine treatments with just over a 7-fold decrease in expression. miR-126 has been investigated in a number of cancer-based studies with several reports pointing towards a potential tumor-suppressive role in non-small cell lung cancer, breast cancer, pancreatic and gastric cancers (Feng et al.,

2010; Hamada et al., 2012; Yang et al., 2012; Zhang et al., 2013). In addition, a significantly reduced human miRNA in the 30 ppb atrazine treatment belongs to the miR-124 family (miR-124-3p). This miRNA is specifically expressed in the central nervous system in post-mitotic neurons and promotes neuronal differentiation and maturation (Sun and Shi, 2015). The role of miR-124 in development and neurogenesis has also been studied in rodent and zebrafish models (Aluru et al., 2013; Cheng et al., 2009). Results also showed human miR-10b to be significantly altered in the 30 ppb atrazine treatment which affirms the high homology between the zebrafish and human miR-10 family.

Human miRNAs that were found to be significantly altered were further analyzed using IPA to determine if these miRNAs targeted genes previously altered by a developmental atrazine exposure (Weber et al., 2013). In our previous study, alterations in larval morphology, mRNA, and corresponding protein levels of selected mRNA targets were altered with mRNA and proteins linked to neuroendocrine function, cell cycle, and carcinogenesis (Weber et al., 2013). Only human miRNAs could be included in this analysis, as the software doesn't currently allow zebrafish miRNAs. Targeting analysis of the previously identified 23 altered genes revealed no altered targeted mRNAs in the 0.3 ppb treatment. Targeting analysis of the 62 previously altered genes in the 3 ppb treatment group revealed three miRNAs (hsa-miR126-3p, hsa-miR-3907, and hsa-miR-455-3p) which targeted 5 genes (*ANTXR2*, *SIK2*, *KIF5A*, *PODNL1*, and *RSL24D1*). Of key interest is the targeted regulation of *SIK2*. *SIK2* is a kinase of the AMPK family that plays a role in CREB1 mediated gene transcription and numerous cell signaling processes including insulin signaling and gluconeogenesis (Henriksson et al., 2012; Walkinshaw et al., 2013). In addition, as we have previously discussed, numerous miRNAs altered by atrazine play a role in tumorigenesis; therefore, alterations in *SIK2*, which is currently under investigation as a target in cancer therapy further supports miRNA regulation of cancer targets (Ahmed et al., 2010; Bon et al., 2015).

Targeting analysis of the 30 ppb gene set showed the highest number of genes targeted by miRNAs with 5 miRNAs targeting 15 unique genes. Alterations in cell cycle progression are reported to occur following atrazine exposure resulting in an accumulation of cells in S-phase *in vitro* (Freeman et al., 2006; Powell et al., 2011). Our previous gene expression analysis revealed alterations in numerous genes associated with cell cycle progression and proliferation (*AVP*, *BRCA2*, *MAD2L2*, *MCM7*, *PIM1*, and *TPD52L1*) (Weber et al., 2013). Here, we report that targeting analysis revealed 2 miRNAs which targeted *PIM1*; miR-101-3p and miR-124-3p. *PIMI* has a known function of regulating cell cycle progression from late G1 through S-phase. In addition, *STAG2*, *TOP1*, and *MAD2L2* were also miRNA targets that are involved in cell cycle processes including formation of the cohesion complex which is necessary for the separation of sister chromatids, alterations of topologic states, and accurate mitosis (Li et al., 2015; Listovsky et al., 2013; Xu et al., 2015). One limitation of the targeting analysis is that the samples analyzed in the current study and in the past transcriptomic analysis was from different biological samples. Although both sets of samples were analyzed at the same developmental time point (72 hpf) the rate of development is known to differ slightly among clutches with data providing a snapshot of expression. As such, this may create some difficulties in a direct comparison of the direction of change between the miRNA and mRNA, but observations in this study were

in agreement with expected results in that decreases in miRNA expression were associated with increased mRNA expression signifying that there would have been less miRNA to repress mRNA expression.

As previously stated, we reported a significant and robust alteration in the human miRNA hsa-126-3p in all three atrazine concentrations. miR-126 is conserved among species and is found within intron 7 of the EGF-like domain-containing protein 7 (*EGFL7*) gene in vertebrates (Meister and Schmidt, 2010). miR-126 is found in highly vascularized tissues such as the heart, liver, and lung, and is the only miRNA known to be specifically expressed in the endothelial cell lineage, hematopoietic progenitor cells, and endothelial cell lines (Meister and Schmidt, 2010). Studies indicate that miR-126 is involved in regulating multiple cellular processes including blood cell development, inflammation, and angiogenesis (Meister and Schmidt, 2010).

Due to the consistent and robust alteration in miR-126 across all atrazine treatments, we sought to characterize the basal levels of miR-126 expression throughout zebrafish development. Results indicated that the expression of miR-126 is developmental time point specific. At 12 hpf, miR-126 had the lowest expression level with rapid increases at 36, 60, and 72 hpf. Due to miR-126's known involvement with angiogenesis and vascular integrity, the variation in expression observed in this study is hypothesized to be linked to the rapid vascular development occurring during zebrafish embryogenesis. In early zebrafish development, angiogenesis occurs in two surges to form the intersegmental vessels (ISVs). The first wave begins at 20– 24 hpf, with the formation of the first ISVs from the dorsal aorta (Ellertsdóttir et al., 2010). Around 30 hpf, each new sprout formed from the dorsal aorta is made up of 3 to 4 cells with distinct positional fates (Baldessari et al., 2008). The differentiated functions of these cells allow each ISV to grow dorsally to form segmental arteries and eventually connect with neighboring ISVs to form the dorsal longitudinal anastomotic vessel (Ellertsdóttir et al., 2010). In the current study, miR-126 was found to have minimal expression at 12 hpf and then increase in expression at 24 hpf corresponding to the first wave of angiogenesis. At 32–34 hpf, the second wave of angiogenesis begins with the formation of small branches from the posterior cardinal vein. These new vessels merge with existing segmental arteries to form segmental veins or grow separately to contribute to the lymphatic vasculature (Ellertsdóttir et al., 2010). Our observed increase of miR-126 at 36 hpf, suggests a correlation to the second wave of angiogenesis. Furthermore, a study by Nicoli and colleagues (Nicoli et al., 2010) analyzed angiogenesis of the accessory fifth aortic arch (AA5x), which occurs around 60 hpf, and its dependence on blood flow. Results revealed that the flow-dependent transcription factor, krueppel-like factor 2 (*klf2a*), was responsible for activating miR-126 to induce *vegf* signaling and allow for the formation of a patent circulatory connection (Nicoli et al., 2010). The increase in miR-126 expression later in zebrafish development at 60 and 72 hpf suggests a relationship to this mechanism.

A previous study conducted by Zou and colleagues (2011) utilized the Tg(fli1:EGFP)^{y1} transgenic zebrafish line and also analyzed miR-126 expression throughout zebrafish embryogenesis (Zou et al., 2011). This study examined the expression of miR-126 at similar developmental time points (12, 24, 36, 48, and 72 hpf) and revealed increasing expression as development progressed; however, statistical analysis was not completed in order to

determine statistical significance between developmental time points. While findings from Zou and colleagues (2011) are in agreement that miR-126 expression increases after 12 hpf, they differ in their analysis at 48 hpf. This difference may be attributed to the use of differing zebrafish lines. The current study used wild type AB zebrafish, while Zou and colleagues (2011) used the Tg(fli1:EGFP)^{y1} transgenic line (Zou et al., 2011). Thus, genetic profiles of the zebrafish are different and may also result in a slightly different maturation progression.

Investigating the effects of EDCs on epigenetics and in particular, miRNAs, is beginning to come into view in order to define mechanisms of toxic action (Casati et al., 2015; Hsu et al., 2009; Tilghman et al., 2012). To date, no studies have been completed examining the effects of atrazine on miRNA expression. Therefore, in the present study, we also demonstrated that miR-126 deregulation was not observed in the majority of the developmental time points following atrazine exposure. However, zebrafish embryos treated with 30 ppb atrazine through 60 hpf also showed a significant increase in expression. While atrazine does not appear to have an effect on ISV formation due to the lack of deregulation at 24 and 36 hpf, the up regulation at 60 hpf may have an effect on the mechanism governing angiogenesis in the aortic arches, specifically AA5x. As mentioned above, angiogenesis during AA5x development is induced by blood flow, which activates *klf2a* to induce miR-126, which results in enhanced *vegf* expression (Nicoli et al., 2010).. Although vascular integrity was not addressed in this study, results warrant further investigation.

While the objectives of this study surround the developmental toxicity of atrazine exposure, previous studies from our laboratory have focused on defining the later-in-life effects of an embryonic atrazine exposure (Wirbisky et al., 2015, 2016). Our previous studies show that an embryonic atrazine exposure alters 5-hydroxyindoleacetic acid (5-HIAA) and serotonin turnover in adult female brain tissue in addition to numerous gene alterations throughout the serotonergic system (Wirbisky et al., 2015). In addition, we have identified reproductive dysfunction through a decrease in spawning events, increases in follicular atresia and ovarian progesterone, and alterations in gene expression throughout the hypothalamus-pituitary-gonadal (HPG) axis in adult female zebrafish (Wirbisky et al., 2016). Studies investigating epigenetic alterations in various adult tissues following an embryonic atrazine exposure are needed as epigenetics are thought to contribute to the developmental origins hypothesis of disease onset.

5. Conclusions

In this study, we identified alterations in miRNA expression associated with a developmental atrazine exposure. We assessed both zebrafish and human miRNAs in this approach and found changes in miRNA expression for both species which further corroborates the high homology of miRNAs across species. Members of the miR-10 family of miRNAs were found to be altered in both zebrafish and human targets analyzed suggesting that this family of miRNAs may be a specific target of atrazine toxicity. Multiple zebrafish miRNAs associated with cancer were also identified, further supporting previous transcriptome analysis identifying numerous genes associated with carcinogenesis following an embryonic atrazine exposure. Targeting analysis of human miRNAs with previously identified altered

gene sets from a developmental exposure to atrazine was carried out to identify downstream functional pathways that are affected from the exposure. These results suggest epigenetic regulators of cell cycle and cell signaling may be targeted by atrazine. Lastly, our characterization and atrazine profile of miR-126 shows support that atrazine could affect cellular processes associated with angiogenesis and warrants future studies into the potential lifespan effects of these alterations

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
CNS	central nervous system
EDCs	endocrine disrupting chemicals
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
hpf	hours post fertilization
IPA	Ingenuity Pathway Analysis
ISVs	intersegmental vessels
LH	luteininzing hormone
miRNA	microRNA
ppb	parts per billion
PRL	prolactin
US EPA	United States Environmental Protection Agency

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Highlights

- Developmental atrazine exposure alters zebrafish and human miRNAs
- Targeted analysis links miRNAs to mRNA involved in cell signaling and cell cycle
- hsa-miR-126-3p was characterized through development and following developmental atrazine exposure

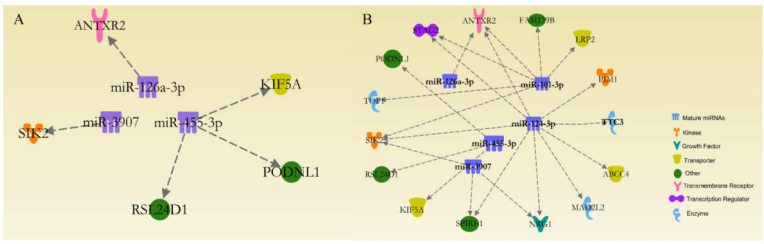

Figure 1. Schematic diagram of the targeted analysis of the 3 and 30 ppb atrazine treatments

Targeted analysis of the altered miRNAs in the 3 ppb atrazine treatment to the previously altered mRNAs identified 3 miRNAs targeting 5 mRNAs through the Ingenuity Pathway Analysis (IPA) program (A). Targeted analysis of the altered miRNAs in the 30 ppb atrazine treatment to the previously altered mRNAs identified 5 miRNAs targeting 15 mRNAs through the IPA program (B). Overall a dose response was observed with similar miRNAs and more miRNAs changed as you increase in dose. In addition, as expected the down regulation of miRNA expression resulted in an increase in mRNA expression for all targets.

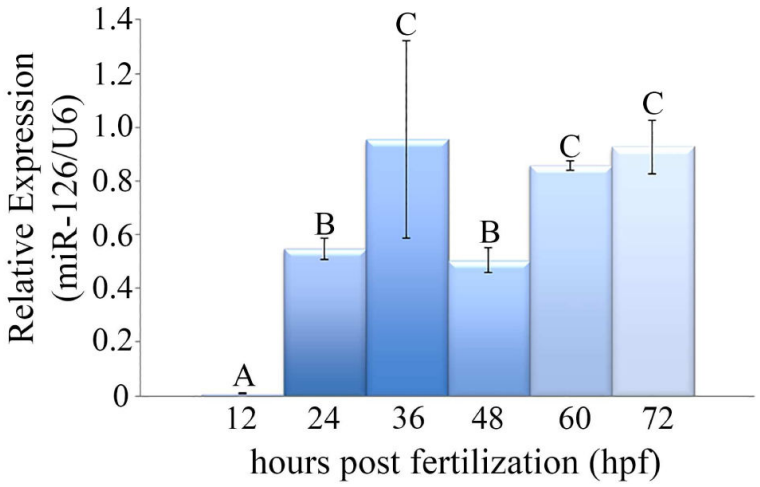

Figure 2. Developmental expression characterization of hsa-miR-126-3p in the zebrafish

Zebrafish embryos were exposed to control aquaria water from 1-72 hpf and collected at 12, 24, 36, 48, 60, and 72 hpf for developmental characterization of miR-126. Expression of miR-126 was time point specific ($p<0.0001$). A dramatic increase in expression occurred after 12 hpf with peak expression at 36 hpf. After a decrease in expression at 48 hpf, expression increased again at 60 and 72 hpf. **(**n=4; different letters indicate that time points are significantly different from each other; miR-126 expression was normalized to U6 as a reference. Error bars indicate standard deviation).

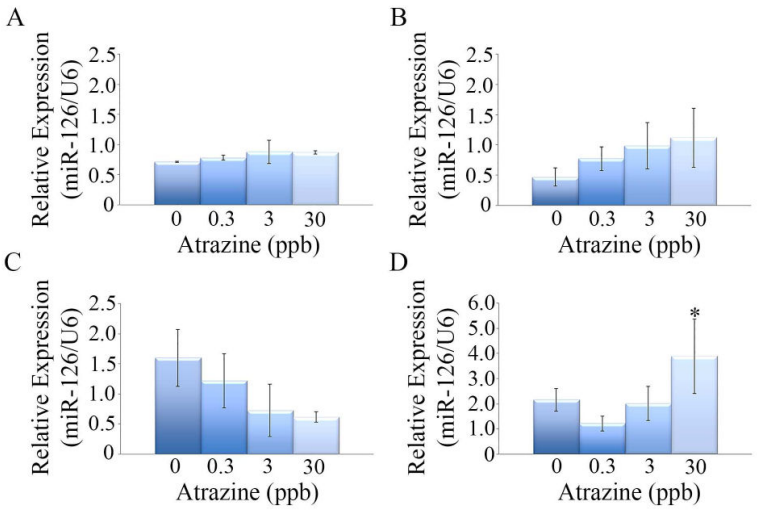

Figure 3. Expression analysis of hsa-miR-126-3p at multiple developmental time points following atrazine exposure in zebrafish

miR-126 expression was examined at five developmental time points (12, 24, 36, 48, and 60 hpf) following an atrazine exposure of 0.3, 3, or 30 ppb or a control treatment. Expression at 12 hpf was minimal and below the threshold for analysis. No significant differences were observed at 24 (A), 36 (B), or 48 hpf (C). Expression levels were significantly increased in the 30 ppb atrazine treatment at 60 hpf (D). (n=3; *p<0.05; miR-126 expression was normalized to U6 as a reference. Error bars indicate standard deviation.)

Table 1

List of significantly altered zebrafish and human miRNAs following embryonic atrazine exposure.

Atrazine Treatment	Systematic Name	Log_2	p-value
0.3 ppb	hsa-miR-126-3p	−2.9067	0.0028
3 ppb	dre-miR-10a-5p	−0.1808	0.0087
	dre-miR-10c	−0.2451	0.0139
	hsa-miR-126-3p	−2.9201	0.0028
	hsa-miR-3907	−0.3769	0.0021
	hsa-miR-455-3p	−0.1223	0.0037
30 ppb	dre-miR-10a-3p	−0.2872	0.0277
	dre-miR-10a-5p	−0.1961	0.0087
	dre-miR-10d-5p	−0.1709	0.0161
	dre-miR-143	−0.2389	0.0168
	dre-miR-153c	−0.1718	0.0260
	dre-miR-16a	−0.2870	0.0188
	dre-miR-16b	−0.1513	0.0290
	dre-miR-18a	−0.1510	0.0046
	dre-miR-18b-5p	−0.1335	0.0426
	dre-miR-216b	−0.1909	0.0301
	dre-miR-217	−0.2326	0.0295
	dre-miR-218b	−0.2144	0.0162
	dre-miR-23a	−0.1612	0.0489
	dre-miR-24	−0.1186	0.0242
	dre-miR-26a	−0.2229	0.0213
	dre-miR-30e-5p	−0.1302	0.0353
	hsa-miR-101-3p	−0.4652	0.0432
	hsa-miR-10b-3p	−4.0142	0.0011
	hsa-miR-124-3p	−0.1533	0.0220
	hsa-miR-126-3p	−2.9791	0.0028
	hsa-miR-3907	−0.4577	0.0021
	hsa-miR-455-3p	−0.1079	0.0037

Table 2

Targeting analysis of human miRNAs and genes altered at 72 hpf in the 3 ppb atrazine treatment[a].

Systematic Name	miRNA p-value	Gene Symbol	mRNA Log_2	Biological Processes
hsa-miR-126-3p	0.00285	*ANTXR2*	0.9949	Unknown
hsa-miR-3907	0.00213	SIK2	1.5266	Cellular Processes
hsa-miR-455-3p	0.00374	KIF5A	1.2455	Cellular Processes
		PODNL1	1.5216	Unknown
		RSL24D1	0.5926	Translation

[a]Gene data is previously published in Weber et al. [35].

Table 3

Targeting analysis of human miRNAs and genes altered at 72 hpf in the 30 ppb atrazine treatment[a]

Systematic Name	miRNA p-value	Gene Symbol	mRNA Log_2	Biological Processes
hsa-miR-101-3p	0.0432	*ANTXR2*	1.2624	Unknown
		FAM179B	0.8655	Unknown
		LRP2	0.9030	Cellular Signaling
		PIM1	0.8883	Cellular Processes
		SIK2	1.6906	Cellular Processes
		STAG2	0.6248	Cell Division
		TOP1	0.6645	Cell Cycle
hsa-miR-124-3p	0.0220	*ABCC4*	0.6933	Metabolic Processes
		ANTXR2	1.2624	Unknown
		MAD2L2	1.4547	Cell Cycle
		NRG1	0.7991	Cell Signaling
		PIM1	0.8883	Cellular Processes
		SIK2	1.6906	Cellular Processes
		SPIRE1	0.7190	Cellular Processes
		STAG2	0.6248	Cell Division
		TTC3	1.0902	Differentiation
hsa-miR-126-3p	0.0029	*ANTXR2*	1.2624	Unknown
hsa-miR-3907	0.0021	*NRG1*	0.7991	Cell Signaling
		SIK2	1.6906	Cellular Processes
		SPIRE1	0.7190	Cellular Organization
hsa-miR-455-3p	0.0037	*KIF5A*	0.8041	Cellular Processes
		PODNL1	1.6064	Unknown
		RSL24D1	0.6708	Translation

[a]Gene data is previously published in Weber et al. [35].