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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.

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 and Paoloni-Giacobino, 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

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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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 to 72 h 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 h light:dark cycle and maintained at 28 °C (±1 °C) with a pH of 7.0–7.2 and conductivity range of 470–520 μS. 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 to 72 h 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 h. 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 8 × 60 K 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 h 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

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