Does Exposure to BPA Change the Epigenome of Zebrafish?

A systems level analysis of the miRNome

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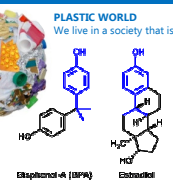
MUSC DEPARTMENT of MEDICINE

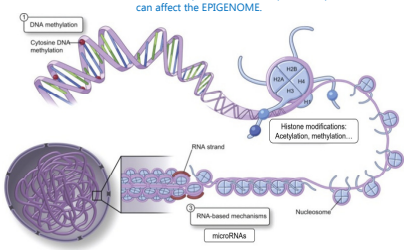
INTRODUCTION

PLASTIC WORLD
We live in a society that is addicted to plastic!



PLASTICIZERS MIMIC ESTROGEN!
Bisphenol-A (BPA) is a widely used PLASTICIZER.
Because of its chemical similarity to estradiol (estrogen hormone), BPA mimics this hormone, by interfering with several nuclear hormone receptors, disrupting the ENDOCRINE SYSTEM IN OUR BODY.

BEYOND DNA, THERE IS EPIGENETICS
The EPIGENOME includes all factors that regulate gene expression without changing the DNA sequence (histone modifications, DNA methylation, miRNAs). Environmental influences, such as a person's diet or exposure to pollutants, can affect the EPIGENOME.



WHAT ARE THE CONSEQUENCES
of this constant exposure to a man-made contaminant like BPA that mimics a natural hormone?

ECOSYSTEM

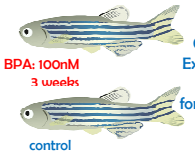
MANKIND

HYPOTHESIS

SYSTEMS TOXICOLOGY MODEL: ZEBRAFISH
Our laboratory exploits the unique advantages of **Zebrafish** (*Danio rerio*) as a systems toxicology model to protect and improve human and environmental health.

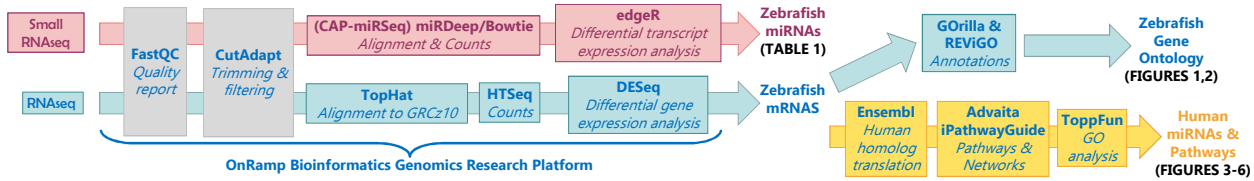
HYPOTHESIS
Exposure to BPA during adulthood changes the epigenome by affecting the expression of many miRNAs and their targeted genes, causing a cascade of dysregulated pathways and adverse health outcomes.

EXPERIMENTAL DESIGN



Collect the liver. Extract small RNA and total RNA for high throughput Sequencing.

BIOINFORMATICS: WORKFLOW & PLATFORMS



RESULTS OF STEP1: ZEBRAFISH DATA

TABLE 1: List of miRNAs differentially expressed identified with miRDeep.

miR-1	miR-204
miR-16c	miR-205
miR-19a	miR-214
miR-19b	miR-375
miR-122	miR-430a
miR-129	miR-430c
miR-130c	miR-451
miR-133a	miR-457a
miR-144	miR-458
miR-148	miR-499
miR 153a	miR 724
miR 184	miR 725
miR-187	miR-2185
miR 193a	miR 2189
miR 202	

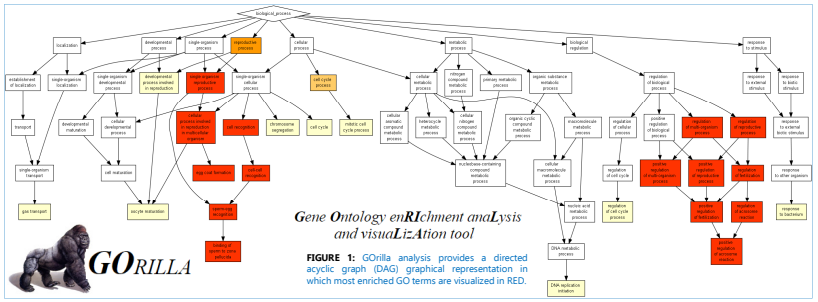


FIGURE 1: GOrilla analysis provides a directed acyclic graph (DAG) graphical representation in which most enriched GO terms are visualized in RED.

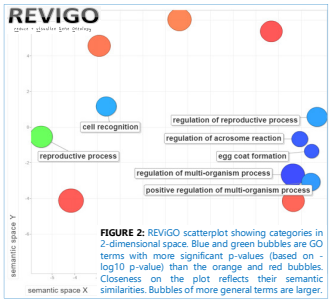


FIGURE 2: REViGO scatterplot showing categories in 2-dimensional space. Blue and green bubbles are GO terms with more significant p-values (based on -log10 p-value) than the orange and red bubbles. Closeness on the plot reflects their semantic similarities. Bubbles of more general terms are larger.

ZEBRAFISH miRNAs:
Small RNA sequencing revealed **29 differently expressed miRNAs** in the liver of BPA-exposed zebrafish (cutoff adjusted p-value: 0.4).

GENE ONTOLOGY:
Based on total RNA sequencing, Gene Ontology Enrichment analysis indicates that BPA disrupts the **REPRODUCTIVE PROCESSES** in zebrafish, including binding of sperm to zona pellucida and positive regulation of acrosome reaction.

WHY DO WE ANALYZE HUMAN HOMOLOGS:
- Zebrafish genome is not as well characterized as human genome.
- Gene ontology (GO) terms are poor in zebrafish, better defined in human.
- 70% of protein-coding human genes are related to genes found in the zebrafish (Howe 2013).
- 84% of genes known to be associated with human disease have a ZF counterpart.

RESULTS OF STEP2: HUMANIZED DATA

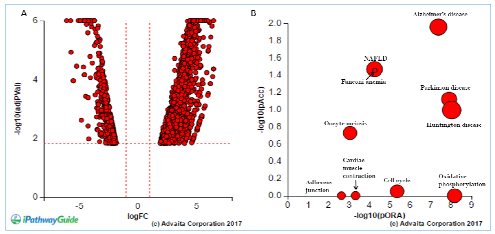


FIGURE 3: iPathwayGuide (Advaita) report. **A)** Volcano plot: All 2,220 significantly differentially expressed (DE) genes are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis). **B)** Pathways perturbation vs. over-representation plot. The top 10 biological pathways are plotted in terms of the two types of evidence computed by iPathwayGuide: over-representation on the x-axis (pORA) and the total pathway accumulation on the y-axis (pAcc). Significance is -log10 of the p-value.

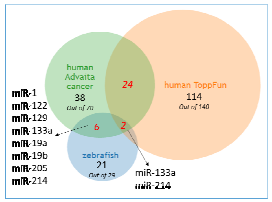


FIGURE 7: Venn diagram for DE miRNAs identified **1)** from small RNA sequencing analysis (zebrafish), and from data obtained from total RNA sequencing projected on the corresponding human homologs and analyzed with **2)** iPathwayGuide (human Advaita cancer) and **3)** ToppGene suite online tool (human Toppfun). This proportional Venn diagram was generated using VENNY 2.1 online tool (Oliveros, 2007).

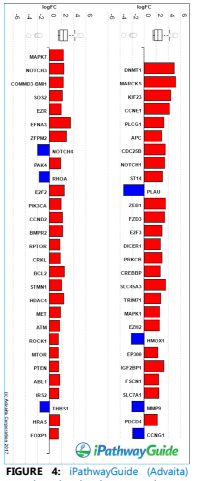


FIGURE 4: iPathwayGuide (Advaita) gene bar plot showing genes that are important in cancer.

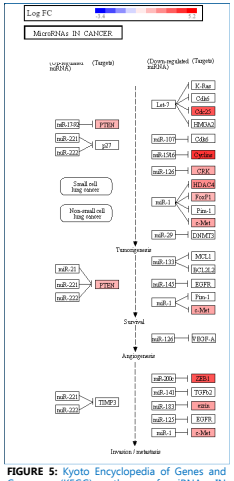


FIGURE 5: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of miRNAs IN CANCER generated by iPathwayGuide (Advaita).

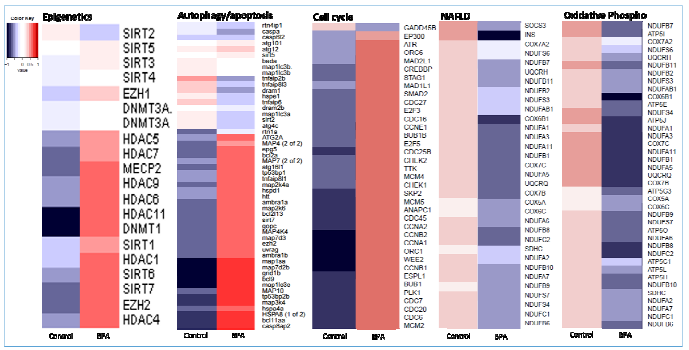


FIGURE 6: Heatmaps for different pathways generated with Ward clustering and Euclidean distance via the heatmap.2 package. In BPA-exposed specimen, many genes related to Epigenetics, Autophagy/apoptosis and Cell cycle pathways were upregulated as compared to control. Genes belonging to the Non-alcoholic fatty liver disease (NAFLD) and Oxidative phosphorylation pathways were downregulated in BPA-exposed zebrafish.

HUMAN miRNAs & PATHWAYS: Several miRNAs and pathways are perturbed after BPA-exposure, as shown in the humanized data. By analyzing the humanized data with iPathwayGuide (Advaita) in STEP2, we identified several pathways that are perturbed, including Cell Cycle, NAFLD, Oxidative Phosphorylation (**FIGURE 3**) and Cancer (**FIGURE 4**). In BPA-exposed specimens, many genes related to Epigenetics, Autophagy/Apoptosis and Cell Cycle were upregulated as compared to control, and many genes important for NAFLD and Oxidative Phosphorylation were downregulated (**FIGURE 6**). Additionally, several miRNAs have been identified in the humanized data (zebrafish gene symbols translated to human homolog) by Advaita (**FIGURE 5**) and Toppfun analysis (data not shown), including some that are directly associated with cancer genes (**FIGURE 5**). When comparing these miRNAs to the list of 29 miRNAs identified directly from zebrafish samples (**FIGURE 7**), we identified 8 miRNAs that were listed in all analyses.

CONCLUSIONS & DISCUSSION

- According to a paper published in Nature by Howe et al. (2013), 70% of protein-coding human genes are related to genes found in the zebrafish (*Danio rerio*, ZF), and 84% of genes known to be associated with human disease have a ZF counterpart. In order to take a glimpse at the potential effects that BPA could have on human heath, we determined the human homologs of the ZF genes discovered in STEP1 and analyzed them using human databases such as iPathwayGuide and ToppFun.
- Our data suggest that a 3 week exposure to BPA deregulates several miRNAs in adult ZF, including some that are also expressed in humans, warranting further direct investigation in humans. Our study also indicates that BPA affect ZF reproductive system markers, as well as pathways related to non-alcoholic fatty liver disease (NAFLD), cell cycle, autophagy/apoptosis, oxidative phosphorylation and cancers. We also determined that several epigenetic factors are upregulated by BPA, including EZH2, a histone methyltransferase that links 2 epigenetic systems of gene silencing, specifically histone methylation and DNA methylation (Doherty et al. 2010). Overexpression of EZH2 has been described in a number of human cancers.
- Our "epigenetics" heatmap (**FIGURE 6**) reveals that BPA increased the expression of EZH2, as well as DNMT1, a DNA methyltransferase. This is consistent with Doherty et al. 2010 and Santangeli et al. (2016) respectively. Together these data suggest that "short term" exposure to BPA can modify the EPIGENOME, including the miRNome, in adult ZF.
- The zebrafish is a great toxicology system model that offers many advantages such as high fecundity, short generation cycles, low cost of colony maintenance, genome easily modified, transparency of the embryos and adults, embryos develop externally, high permeability, nearly the entire genetic code is expressed/active during early life stages and spontaneous development of cancers. The idea of using zebrafish as cancer model emerged 10 years ago and now starts to yield results (White et al. 2013). In unison with the cancer biology community which uses human and mouse systems, the zebrafish model could offer a unique set of tools that could help cancer research efforts.
- The same is true for other research fields, including NAFLD, a highly prevalent form of severe chronic liver disease that affects 1/3rd of all Americans. A zebrafish model of NAFLD exists based on the mutation of the gene *foie gras* (*foigr*) that leads to fatty liver disease resembling human NAFLD, characterized by large lipid filled hepatocytes and cellular apoptosis in larvae as young as 5 dpf (Goldsmith & Jobin, 2012).
- Given that changes in epigenetic profile, including miRNAs, has been shown to drive the progression of many diseases in both animal and human models, it is important to clearly define how BPA affects the epigenome and perturbs downstream pathways. To our knowledge, this is the first study that examined the effect of BPA on the zebrafish miRNome.

LITERATURE CITED:
Howe, K., et al. (2013). "The zebrafish reference genome sequence and its relationship to the human genome." Nature. **Doherty, L. F., et al. (2010).** "In Utero Exposure to Diethylstilbestrol (DES) or Bisphenol-A (BPA) Increases EZH2 Expression in the Mammary Gland: An Epigenetic Mechanism Linking Endocrine Disruptors to Breast Cancer." Hormones and Cancer.
White, R., et al. (2013). "Zebrafish cancer: the state of the art and the path forward." Nat Rev Cancer. **Goldsmith, J. R. and C. Jobin (2012).** "Think small: zebrafish as a model system of human pathology." J Biomed Biotechnol. **Santangeli, S., et al. (2016).** "BPA-Induced Deregulation Of Epigenetic Patterns: Effects On Female Zebrafish Reproduction." Scientific Reports

FUTURE DIRECTIONS

- Investigate how BPA regulates the expression of miRNAs of interest; identify which receptors are involved; examine miRNA promoter regions by chromatin immunoprecipitation (ChIP) to identify alterations in transcriptional machinery after BPA exposure.
- Using human primary hepatocyte cells treated with BPA (different concentrations), examine the miRNome by systems levels analysis.
- Hypothesis: miRNome alterations precede the increased risk of CANCER and adult onset disease (e.g. NAFLD) after *in utero* exposure to endocrine disruptors.
- Examine "epigenetic transgenerational inheritance of adult onset disease" in F3 generation after exposure to BPA in F0.