DNA Methylation Profile of Estrogen Receptor Alpha in Fish Exposed to EE2

J Fetke[2,3]; R Flick[1]; J Martinson[1] ; W Huang[1]; M See[1]; E Pilgrim[1]; A Biales[1]

[1]US EPA, Office of Research and Development (ORD), [2]University of Cincinnati, [3]Oak Ridge Institute of Science & Education @US EPA ORD, Cincinnati, OH

Introduction

Exposure to exogenous estrogens such as 17α-ethynylestradiol (EE2), is associated with reproductive problems in fish
- Decreased fecundity, sperm production, and feminization of males (1, 2)

Estrogen receptors genes have important roles in reproduction
- Encode nuclear receptors which allow binding of estrogens, or estrogen mimics, for transport to the nucleus where estrogens interact with estrogen response elements (EREs) in the upstream region of genes important for egg production

Transcriptional regulation via epigenetic mechanisms is one method by which organisms adapt to changing conditions (3)
- DNA methylation can dynamically respond to external environmental stimuli including toxins and allow alteration of gene function without changing the underlying DNA sequence (4)

Estrogen receptor alpha (ERα) is upregulated in male fathead minnows when fish are exposed to EE2. Since DNA methylation in the promoter region of genes is known to be associated with active transcription, we hypothesized that an upregulation of ERα would be inversely correlated with decreases of DNA methylation.

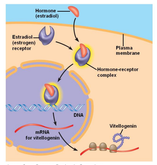


Image from Pearson Benjamin Cummings

DNA methylation studies in fish are limited, so it is unknown whether EE2 affects DNA methylation level and/or pattern in the upstream region of ERα in fish and how that might be associated with reproductive pathways.

Objectives

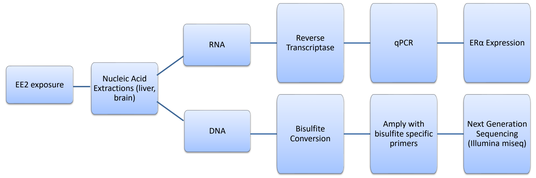
- Characterize the pattern and level of DNA methylation for ERα in liver and brain tissue of mature male fathead minnows (*Pimephales promelas)* exposed to EE2 for 48 hours
- Identify potential DNA methylation changes in brain and liver following a depuration period to determine whether potential changes persist post-exposure
- Determine whether DNA methylation changes are associated with gene expression

Methods and Materials

Water Chemistry
- Water samples were taken daily from all aquaria to quantify actual EE2 dose delivered to fish. Nominal doses of EE2 were 2.5 and 10 ng/L. Mean measured doses of EE2 for each treatment were 1.37 ± 0.31 and 3.47 ± 1.34 ng/L, respectively. Control group measured 0 ng/L EE2

Necropsies
- Following exposure and at each time point, fish were anesthetized in 400mg/l tricaine methanesulfonate (MS-222) and brains and livers were dissected from each fish and flash frozen using liquid nitrogen

Nucleic Acid Extractions and Gene Expression
- Total RNA and DNA were extracted using the Allprep DNA/RNA Mini Kit (Qiagen) for each tissue type. 100 ng of total RNA per reaction was used in 20 µl reverse transcription (RT) reactions carried out in duplicate. No-template controls and no amplification controls (RT enzyme omitted) were included with each set of samples being processed. Resultant cDNA was used in qPCR reactions to quantify ERα expression



Bisulfite Conversion and Sequencing
- Total DNA was bisulfite-converted using EZ-96 DNA Methylation-Lightning MagPrep Kit (Zymo Research) according to manufacturer's protocol. Following bisulfite treatment, CpG enriched regions spanning 3kb (5' upstream through the second exon) targeted were amplified with bisulfite specific primers

Bioinformatics
- QC of sequencing data: FastQC (v0.11.8)
- Remove adapter sequences andprimers: Cutadapt (v1.18)
- Read alignment: Bismark (v0.19.0), Bowtie2 (v2.3.1)

Statistics
- Bisulfite analysis: BiSeq (v1.20.0), beta regression (5, 6)
- Gene expression: ANOVA, Tukey multiple comparisons of means

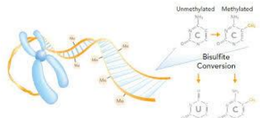


Image from Babraham Bioinformatics

Results

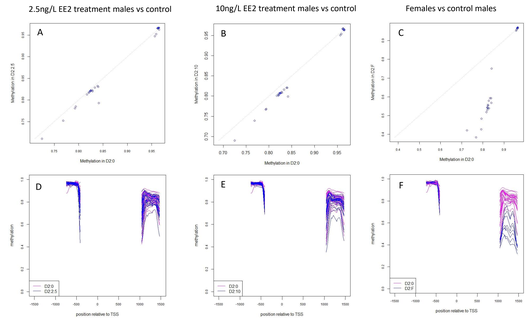


Figure 4. Scatter plot of 2d treatment group (2.5 ng/L and 10 ng/L of EE2) males compared to control (A, B) and females compared to control males (C). Figures D, E, and F show mean DNA methylation (y-axis) of each fish with respect to CpG location (x-axis) where the predicted transcriptional start site (TSS) is designated by "0".

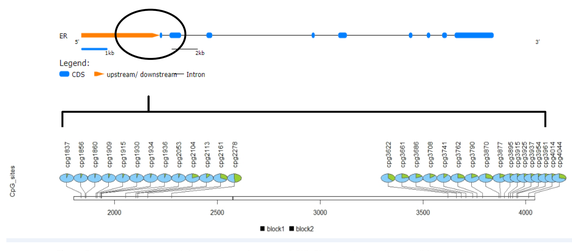


Figure 5. Putative gene model diagram (top) showing a 2.5Kb 5' region (orange), exons (blue), and intronic regions (black line). Along the bottom, CpG sites are shown with mean methylation level for 2d 10 ng/L EE2 treatment group males indicated by corresponding pie charts (graphics generated by (7, 8).

Study Design

Reproductively mature male fathead minnows were exposed to 2.5 (n=46) and 10 ng/L (n=45) of EE2 in a flow-through diluter system for 48h. Unexposed males served as a control group (n=44) as well as separately contained unexposed females (n=8). Following exposure, a subset of fish were depurated in water for 7 (n=8 to 9 fish/group) and 14 (n=8 to 10 fish/group) days (Figure 1, Figure 2).

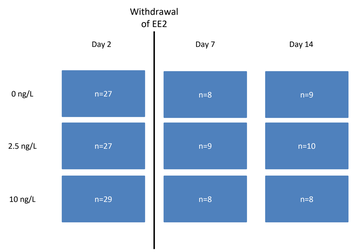


Figure 1. Diagram of study design.

Figure 2. Exposure tanks.

Results

- In liver, gene expression of treatment groups are significantly different from control at both doses of EE2 (Figure 3, p<0.001).
- DNA methylation of upstream and coding regions of ERα is not correlated with gene expression (Pearson's correlation).
- 40 CpG sites were targeted in this study, 29 had enough sequence coverage for analysis (13 in the upstream region of the gene, 17 sites in the coding region).
- In female fish liver, all 17 CpG sites located in the coding region of ERα display significantly different DNA methylation from males (BH, p < 0.001) (Figure 4C, 4F). No significant difference is found in brain.

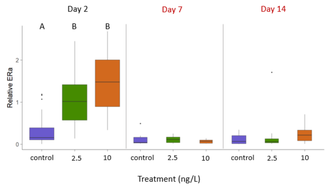


Figure 3. Relative expression of ERα in male fathead minnows at 2, 7, and 14 days in liver tissue. At Day 2, both treatment groups differed significantly from control (p<0.001)

- For liver, after the 2d EE2 exposure at CpG site 2161, DNA methylation is significantly different in females (p<0.005) relative to control males.
- After the 2d EE2 exposure, CpG sites 2113, 2161, and 2278 were found to be differentially methylated in the 10 ng/L EE2 treatment group (beta regression, p=0.03, 0.006, 0.05 respectively).

Discussion

ERα is upregulated when fish are exposed to EE2 in liver but not in brain. Bisulfite analysis of the 5' upstream region of ERα did not support our hypothesis that gene expression is inversely correlated with decreases in methylation level (Figure 3, Pearson's correlation). However, at the end of the 2 day EE2 exposure three CpG sites were found to be differentially methylated in the 10 ng/L EE2 treatment group. Significant differences in DNA methylation were found at CpG sites 2113, 2161, and 2278 (beta regression, p=0.03, 0.006, 0.05 respectively). The 2.5 ng/L EE2 treatment group approaches significance at site 2113 (p=0.051). In female fish, CpG site 2161 is significantly different from untreated males (p<0.005). These data appear positively correlated for this specific cluster of CpG sites which are also near the transcription start site of the gene. These p-values reflect uncorrected values. Currently we are investigating an appropriate post-hoc multiple testing procedure for this dataset which accounts for spatial dependencies for which the Benjamini Hochberg approach may be too conservative (9, 10).

We found a significant difference in DNA methylation of females and control group males in the coding region of ERα (Figure 4C and 4D, p<0.001) in liver. No significant difference in DNA methylation was found in the coding region of ERα for females, or among any group, in brain. This indicates that ERα has a tissue-specific role in liver of female fish which is not present in males.

Future steps for this research include evaluating another estrogen receptor subtype, ERβ, for differential DNA methylation or other epigenetic modifications in order to better understand the mechanisms that underlie gene regulation and gain insight into potential cross-talk between estrogen receptor subtypes in fish (11).

Contact

Janine Fetke, M.S.
ORISE/US Environmental Protection Agency
University of Cincinnati, Department of Biological Sciences
Email: fetke.Janine@epa.gov, fetkeje@mail.uc.edu

References

1. Parrott, J. L., & Blunt, B. R. (2005). Life-cycle exposure of fathead minnows (Pimephales promelas) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environmental toxicology, 20*(2), 131-141
2. Kristensen, T., Baatrup, E., & Bayley, M. (2005). 17α-ethinylestradiol reduces the competitive reproductive fitness of the male guppy (Poecilia reticulata). *Biology of reproduction, 72*(1), 150-156
3. Mirbahai, L., & Chipman, J. K. (2014). Epigenetic memory of environmental organisms: a reflection of lifetime stressor exposures. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 764*, 10-17
4. Tost, J. (2010). DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Molecular biotechnology, 44*(1), 71-81
5. Hebestreit K, Klein H (2019). *BSeq: Processing and analysing bisulfite sequencing data*. R package version 1.24.0
6. R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/
7. Ou J, Zhu LJ (2019). "trackViewer: a Bioconductor package for interactive and integrative visualization of multi-omics data." *Nature Methods*, **16**, 453–454. doi: 10.1038/s41592-019-0430-y, https://doi.org/10.1038/s41592-019-0430-y
8. Bo Hu, Jinpu Jin, An-Yuan Guo, He Zhang, Jingchu Luo and Ge Gao. (2015). GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics*, 31(8):1296-1297
9. Ramdas, A. K., Barber, R. F., Wainwright, M. J., & Jordan, M. I. (2019). A unified treatment of multiple testing with prior knowledge using the p-filter. *The Annals of Statistics*, 47(5), 2790-2821
10. Zhou, Z., Lunetta, K. L., Smith, A. K., Wolf, E. J., Stone, A., Schichman, S. A., ... & Logue, M. W. (2019). Correction for multiple testing in candidate-gene methylation studies. *Epigenomics*, (0)
11. Nelson, E. R., & Habibi, H. R. (2013). Estrogen receptor function and regulation in fish and other vertebrates. *General and comparative endocrinology*, *192*, 15-24.

Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Any mention of trade names, products, or services does not imply an endorsement by the U.S. Government or the U.S. Environmental Protection Agency (EPA). The EPA does not endorse any commercial products, services, or enterprises.

This project was supported in part by an appointment to the Research Participation Program at the U.S. Environmental Protection Agency, ORD/CCTE/GLTED/MIB, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and EPA.