Unraveling the role of AHR gene duplication in PAH toxicity in zebrafish

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Outline

- A little history on zebrafish AHR genes
- PAH toxicity
- Classifying PAHs
- AHR Downstream events
- AHR regulated non-coding RNAs
AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes
The AHR and PAH pathways of toxicity

- AHR Binding
- HSP 90
- AIP
- CYP Induction
- Metabolites
- AHR Independent Toxicity
- No metabolism
- AHR
- ARNT
- Transcription
- Disruption of endogenous pathways
Predicting AHR Ligands
Modeling a “Target” Zebrafish AHRs

Zebrafish three AHRs

- AHR2 primary mediator of toxicity
- AHR1A deficient in TCDD binding and transactivation activity
- AHR1B functional but no known toxicological roles

AHR Homology Model

- AHR ligand binding domain models built using NMR structure of HIF2α (PAS domain)
- Mouse, rat, human, zebrafish
- Performed molecular docking of putative AHR ligands

TCDD Molecular Docking with the Zebrafish AHRs

AHR2  AHR1B  AHR1A

Unable to dock

-3.97  -4.86

Predicted binding energy (kcal/mole)

The $ahr2^{hu3335}$ Zebrafish Line

T → A mutation in residue 534 resulting in a premature stop

- Truncated protein is predicted to be non-functional
- Basal mRNA expression suggests mutant $ahr2^{hu3335}$ transcript is degraded

Ahr2hu3335 Mutants Are Resistant to TCDD-Induced Developmental Toxicity
Leflunomide Molecular Docking

AHR2  AHR1B  AHR1A

-2.13  -1.97  -2.19

Predicted binding energy (kcal/mole)

O’Donnell, E.F. et al. 2010, PLOS One
Leflunomide-induced CYP1A expression is partially AHR2 dependent
AHR1A Dependent CYP1A Expression

1a 1b 2

Control morpholino
ahr2hu3335 10 uM Lef

AHR1B morpholino
ahr2hu3335 10 uM Lef

AHR1B + AHR1A morpholino
ahr2hu3335 10 uM Lef

Control morpholino
ahr2hu3335 1% DMSO
ahr2 Mutants Are Resistant to TCDD-induced CYP Expression Changes
AHR2 importance confirmed in CRISPR/Cas9 line

A

-11 bp deletion in exon 1

ahr2

| ATG TCG GCG GGT ATC ACA TAT GCG GTC AAG AAA CGG AAG |

ahr2<sup>mut1</sup>

| ATG TCG GCG GGT ATC GGT CAA GAA ACG GAA GAA GCC GT TCA GAA (-11 bp) |

B

Translation of mutant sequence predicts premature stop codon

1. ATG TCG GCG GGT ATC GGT CAA GAA ACG GAA GAA GCC GT TCA GAA
   45 Met Ser Ala Gly Ile Gly Gin Glu Thr Glu Glu Ala Arg Ser Glu

2. AAT ACC CAA ACC ACC ACC ACC TGA
   46 Asn Thr Gln Thr Thr Thr Pro
   89 End

C

PAS domains

<table>
<thead>
<tr>
<th>ahr2&lt;sup&gt;+&lt;/sup&gt;</th>
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<tr>
<td>PAS</td>
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<th>ahr2&lt;sup&gt;mut1&lt;/sup&gt;</th>
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<tr>
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Garcia GR, Bugel SM, Truong L, Spagnoli S, Tanguay RL. AHR2 required for normal behavioral responses and proper development of the skeletal and reproductive systems in zebrafish. PloS one. 2018;13(3)
OSU/PNNL Superfund Research Program
Polycyclic Aromatic Hydrocarbon Mixtures: New Technologies and Emerging Health Risks
Toxicity Mechanisms for Most PAHs are Unknown

- Environmental samples can contain 100’s PAHs
- Environmentally Dynamic
- Parent, substituted compounds
- Toxicity data is scarce for substituted PAHs
- PAHs induce AHR-dependent and AHR-independent developmental toxicity, dependent on structure
- We lack the structural basis for developmental and neurotoxicity
High-Throughput Screening of PAHs

**Exposure**
- Dechorionated
- 6 hpf to 120 hpf
- 5 concentrations
  - 50-1 μM
  - 5-0.1 μM
- N=32

**24 hour evaluations**
- Behavior
- Morphology

**5 day evaluations**
- Morphology
- Behavior
- CYP1A Localization

**Timeline**
- 6 hpf
- 24 hpf
- 120 hpf

**Images:**
- Early developmental stages
- Behavioral and morphological assessments
- CYP1A localization

**Assessments:**
- 6 hpf to 120 hpf
- 5 concentrations
- N=32
HTS Platform for Chemical Screening

Embryo Collection

Chemical Exposure

10 min 6 hr 1 day 5 days


All dependent on a custom made LIM system “ZAAP”
What About The 24 hpf Photomotor Response (EPR)?

- Control fish (in the absence of chemical) will respond after the 1st light pulse (Excitatory interval) but not after the 2nd light pulse (Refractory interval).
The LPR assay is 18 mins, with a 6 min acclimation period. Statistical significant was determined using a K-S test with a $p<0.01$, and a minimum of 30% AUC change (relative to control).
Larval Photomotor Response (LPR) Behavioral Testing (5 day Larvae)

96-well plate

Distance moved over time
Larval Photomotor Response (LPR)

Example Output (5 day Larvae)

NTP DNT 91–A8:2,2',4,4'-Tetrabromodiphenyl_ether

Start Time (min)

Total Moment (mm)

Conc 0 2 9 34
Conc 1 4.5 18 67
Comparative PAH Screening Effort
Developed a Library of PAHs for Comparative Analysis
CYP1A Expression Patterns as a Biomarker of AHR Activation

120 hpf
Summary of Results
Example Parent and Derivatives
16 EPA Priority PAHs Do Not Reflect Full Range of Effects
127 PAHs Clustered by Cyp1a Expression Patterns
RNA-Seq Analysis (Embryonic Expression)
Case Study – two OPAHs

Benz[a]anthracene-7,12-dione (7,12-Ba[A]Q)

1,9-Benz-10-anthrone (BEZO)
Differential Downstream AHR Activation (Measured CYP1A Expression)
Overt Toxicity for both - AHR2 Dependent

zfCyp1A Promoter → Green Fluorescent Protein
Complexity of Defining Molecular Responses – Even if MIE is known
Looking Downstream of AHR2 activation
Novel Transcript identified as long non-coding RNA (lncRNA) and mapped adjacent to Sox9b gene

Top significantly elevated transcripts identified from RNA-Seq in whole 48 hpf embryo exposure to 10 µM B[a]AQ

<table>
<thead>
<tr>
<th>Gene</th>
<th>log2 (fold change)</th>
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<tr>
<td>cyp1a</td>
<td>7.9</td>
</tr>
<tr>
<td>cyp1c1</td>
<td>4.8</td>
</tr>
<tr>
<td>cyp1c2</td>
<td>4.6</td>
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<tr>
<td>cyp1b1</td>
<td>3.6</td>
</tr>
<tr>
<td>novel transcript</td>
<td>3.2</td>
</tr>
</tbody>
</table>

- Spatial arrangement conserved
- Adjacency of AHREs in promoter region of lncRNA conserved
- Expect lncRNA-target regulation to be similar between fish and mammals
Sox9b and “SlincR” Expression in Mammalian Tissues

RNA-seq expression data from NCBI BioProjects PRJ NA66167 (Mouse) and PRJ EB4337 (Human)
**Sox9 Importance for Toxicology and Human Health**

Sox9 in humans:

- Dysregulation of Sox9 has been implicated in skeletal deformities, fibrosis, and cancer (Pritchett 2011)

Upon developmental exposure to TCDD, zebrafish Sox9b:

- Is one of the most reduced transcripts in several target organ toxicities
- Is hypothesized to have a **causal** role in TCDD toxicity

- The mechanism of Sox9b repression was unknown
Predicted s1nc R Structure

Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE)
slinc R Expression is Elevated by Other AHR Ligands

![Graph showing slinc R Expression over time]

- **Fold change (compared to 12 hpf control)**
- **Hours post fertilization**

- 0.1% DMSO
- 10 uM BaQ
- 0.25 ng/mL TCDD
Induction of slincR Expression Requires AHR2

- Induction of slincR (48hpf)
  - Fold change compared to ahr2^+ control
  - ahr2^+ Control
  - ahr2^+ TCDD
  - ahr2^+hu3335 Control
  - ahr2^+hu3335 TCDD

- slincR (48hpf) fold change compared to c-MO control
  - c-MO Control
  - ahr2-MO Control
  - c-MO B[a]AQ
  - ahr2-MO B[a]AQ
Expression of Sox9b and SlicnR Expression

48 hpf
Knockdown of slincR Results
In a Relief in Repression of Sox9b

**Confirmation of Knockdown:**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>slincR (48hpf)</th>
<th>Sox9b (48 hpf)</th>
</tr>
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<tbody>
<tr>
<td>c-MO Control</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sox9b-lnc-MO</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>c-MO TCDD</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Sox9b-lnc-MO</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.0</td>
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Fold change (compared to c-MO control)
What is the transcriptional impact of knocking slincR down?

**RNA-Seq Experimental Objectives:**

Identify the transcripts, pathways, and biological processes altered or regulated by slincR expression during normal development and during AHR2 activation

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**slincR splice blocking morpholino design:**

- E1
- E2
- E3

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**Graph:**

- 0.1% DMSO
- 1 ng/mL TCDD

* 98% 81%
Slinc R Transcriptional regulation

Garcia et al. Signaling events downstream of AHR activation that contribute to toxic responses: The functional role of an AHR-dependent long non-coding RNA (slincR) using the zebrafish Model In press EHP
Proposed mechanism of AHR-dependent control of gene expression during normal development

1. slincR is a direct AHR target gene
2. slincR is elevated in response to the activation of the AHR signaling pathway
3. slincR represses Sox9b & regulates other unknown targets
4. Altered biological processes
   - Translation (↓)
   - Inflammation (↓)
   - Cell adhesion (↑)
   - Notch signaling (↑)
To Summarize

- AHR gene duplication – zebrafish have three functional AHRs
- Increasingly possible to predict ligand/AHR binding
- Platform for structure-based PAH screening
- Complex downstream AHR targets
- AHR/SlincR/Sox9b relationship
- Highly amenable for mixture assessments
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