Functional Differences in Paralogous Aryl Hydrocarbon Receptors (AHRs) of *Xenopus laevis*

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Question

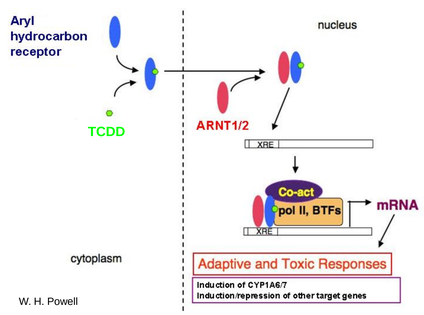
Do AHR1α and AHR1β display functional differences in *Xenopus laevis*?

Abstract

2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) is a ubiquitous environmental contaminant and potent toxicant in most vertebrates. The AHR, a ligand-activated transcription factor, mediates TCDD toxicity. The frog *Xenopus laevis* possesses two AHR paralogs, AHR1α and AHR1β; however, it is unknown if each plays a specific, non-redundant role in the toxicity of TCDD or in the frog's physiology. We sought to determine whether these AHRs exhibit distinct biological functions using XLK-WG, kidney epithelial cells, and antisense approaches to knock down expression of each paralog. We first used morpholino antisense oligonucleotides and Endo-Porter, a reagent that delivers morpholinos into cells. This approach proved inefficient. Fluorescence of control oligos was not observed in cells following transfection. Next, we transfected siRNAs against each AHR paralog using two transfection reagents. Using reverse transfection, siPORT *Amine* Transfection Agent with AHR1α siRNA demonstrated up to 85% reduction in AHR1α mRNA, but a 9-fold induction of AHR1β mRNA. With AHR1β siRNA, this reagent induced both AHR1α and AHR1β mRNA. However with pre-plated transfection, siPORT *Amine* Transfection Agent resulted in no effect on AHR knockdown. After reverse transfection, siPORT *NeoFX* Transfection Agent also did not lead to any AHR expression knockdown. These unexpected results may relate to problems involving transfection efficiency or siRNA sequences. Ultimately, these studies will contribute to the understanding of the role of multiple AHRs in the unusual insensitivity of *Xenopus laevis* to TCDD toxicity.

Background

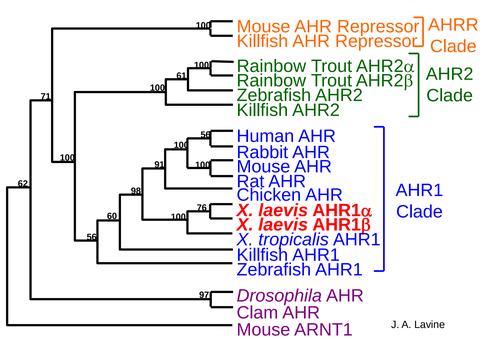
AHR Signaling Pathway



- The AHR, a ligand-activated transcription factor and member of the basic-helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) gene family, regulates the transcription of target genes [1].
- TCDD exhibits its wide variety of toxic effects via aryl hydrocarbon receptor (AHR)-mediated signaling pathways [2].
- After ligand binding, the AHR enters the cell's nucleus where it dimerizes with ARNT and binds DNA, altering and inducing the expression of numerous genes [1].
- TCDD-induced expression of the target gene CYP1A is used as a biomarker of AHR activity [3].

Figure 1. The AHR signaling pathway.

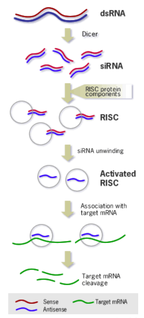
Evolution and AHR Gene Multiplicity



- Throughout evolution, several genome and gene duplication events have occurred, illustrating why the number of AHRs in each species can vary greatly.
- While mammals, including humans, only express one type of AHR (AHR1), non-mammalian vertebrates typically have multiple AHR genes.
- In *X. laevis*, a recent gene duplication resulted in the expression of two AHR1s, AHR1α and AHR1β. These two paralogs share 86% amino acid identity [3].
- At this point, the functional significance of multiple AHR genes is not well understood.

Figure 2. Phylogenetic analysis of *X. laevis* AHR sequences.

Methods



AHR Expression Knockdown

- XLK-WG growth conditions have been optimized using 20% fetal bovine serum.
- Antisense approaches to knock down expression of each AHR paralog.
- Used morpholino antisense oligonucleotides and Endo-Porter, a peptide reagent that delivers morpholinos into cells. This approach proved inefficient when fluorescence of control oligos was not observed in cells following transfection.
- siRNA sequences against each AHR paralog were transfected into *X. laevis* cells using two transfection reagents:
  1. siPORT *NeoFX*, a lipid-based reagent
  2. siPORT *Amine*, a proprietary blend of polyamines reagent
- After 24 hours, the success of AHR1α or AHR1β knockdown was determined using a quantitative real-time PCR.

Figure 3. The mechanism of siRNA. Double-stranded RNAs (dsRNAs) can silence the expression of target genes. First, the dsRNAs get processed into small interfering RNAs (siRNAs) by an enzyme called Dicer. Then, the siRNAs assemble into RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the associated RNA.

Transfection Methods

Reverse Transfection

Plate and Transfect cells → 24 hrs → Harvest mRNA

Pre-plated Transfection

Plate cells → 24 hrs → Transfect cells → 24 hrs → Harvest mRNA

Results: siPORT *Amine* Transfection Reagent

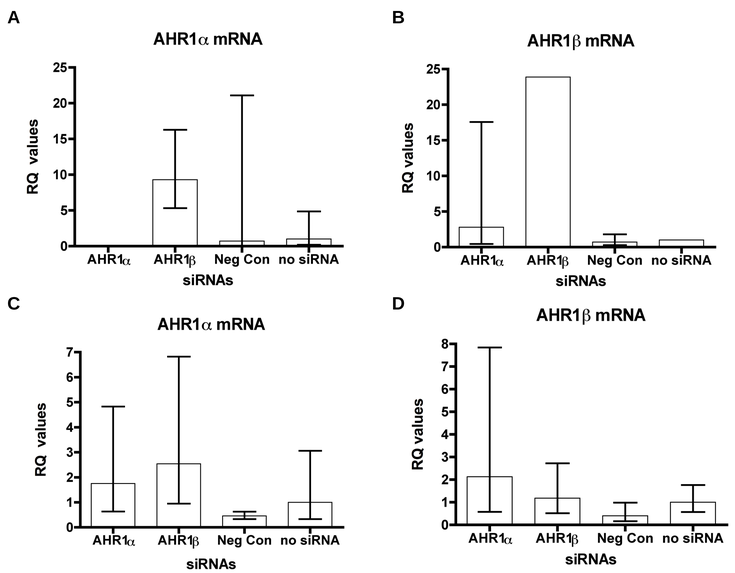


Figure 4. AHR expression knockdown using siRNAs and siPORT *Amine* Transfection Reagent. *X. laevis* XLK-WG cells were transfected for 24 hours before RNA extraction and subsequent quantification by a real-time PCR. A: Quantification of AHR1α mRNA after reverse transfection. B: Quantification of AHR1β mRNA after reverse transfection. C: Quantification of AHR1α mRNA after pre-plated transfection. D: Quantification of AHR1β mRNA after pre-plated transfection. AHR1α siRNA sequence, sense: GGAGUAGGAUGAACACGAtt and antisense: UUCGUGUUCAUCCUACUCCtt; AHR1β siRNA sequence, sense: GGAGCAGAAUGAACACGAAtt and antisense: UUCGUGUUCAUUCUGCUCCtt. siRNA sequences were designed by Ambion, Inc. (Austin, TX). Y-axis values represent Relative Quantification (RQ), error bars = possible RQ values based on the standard deviation of ΔΔCt.

Results: siPORT *NeoFX* Transfection Reagent

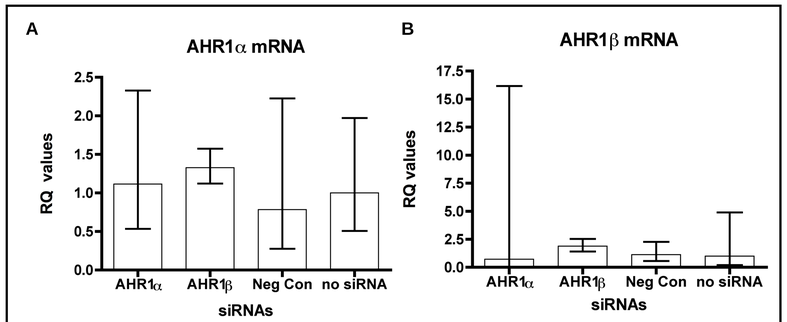


Figure 5. AHR expression knockdown using siRNAs and siPORT *NeoFX* Transfection Reagent. *X. laevis* XLK-WG cells were transfected for 24 hours before RNA extraction and subsequent quantification by a real-time PCR. A: Quantification of AHR1α mRNA after reverse transfection. B: Quantification of AHR1β mRNA after reverse transfection. AHR1α siRNA sequence, sense: GGAGUAGGAUGAACACGAtt and antisense: UUCGUGUUCAUCC UACUCCtt; AHR1β siRNA sequence, sense: GGAGCAGAAUGAACACGAAtt and antisense: UUCGUGUUCAUUCUGCUCCtt. siRNA sequences were designed by Ambion, Inc. (Austin, TX). Y-axis values represent Relative Quantification (RQ), error bars = possible RQ values based on the standard deviation of ΔΔCt.

Conclusions and Future Direction

- Using reverse transfection, siPORT *Amine* Transfection Agent with AHR1α siRNA demonstrated an 85% reduction in AHR1α mRNA, but a 9-fold induction of AHR1β mRNA. With AHR1β siRNA, this reagent induced both AHR1α and AHR1β mRNA (Figure 4a, b).
- After pre-plated transfection, siPORT *Amine* Transfection Agent and siRNAs did not lead to AHR expression knockdown (Figure 4c, d).
- With reverse transfection, siPORT *NeoFX* Transfection Agent and siRNAs resulted in no effect on AHR knockdown (Figure 5).
- These results may relate to problems involving transfection efficiency or siRNA sequences.
- If expression knockdown of individual AHR paralogs in *X. laevis* cells is accomplished with other transfection reagents, such as NIMT® FeOfection|PURPLE, assessing how these cells respond to TCDD treatment will determine whether functional differences do actually exist for AHR1α and AHR1β.

Literature Cited

1. Hahn, M.E., *Mechanisms of innate and acquired resistance to dioxin-like compounds*. Reviews in Toxicology 2, 1998: p. 395-443.
2. Mandal, P.K., *Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology*. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 2005. **175**(4): p. 221-230.
3. Lavine, J.A., et al., *Aryl Hydrocarbon Receptors in the Frog Xenopus laevis: Two AhR1 Paralogs Exhibit Low Affinity for 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD)*. Toxicological Sciences, 2005. **88**(1): p. 60-72.

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