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Cloning of multiple ERα mRNA variants in killifish (*Fundulus heteroclitus*), and differential expression by tissue type, stage of reproduction, and estrogen exposure in fish from polluted and unpolluted environments

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ABSTRACT

To test the hypothesis that alternative splicing could be an adaptive mechanism for populations subject to multi-generational estrogenic exposures, we compared estrogen receptor alpha (ERα) splicing variants in two populations of killifish (*Fundulus heteroclitus*): one resident in an estrogenic polluted environment (New Bedford Harbor, NBH, MA, USA) and one from a relatively uncontaminated reference site (Scorton Creek, SC, MA, USA). In total we identified 19 ERα variants, each with deletions of one or more coding exons. Four of the variants with potential functional relevance were analyzed by qPCR to test for population differences in expression by tissue type, site, sex, seasonal reproductive status and estrogen treatment. Significantly, a 5′-truncated short form variant ($ER\alpha_S$) was highly expressed in liver and ovary, and was associated with seasonal reproductive activity in SC but not NBH fish. Both $ER\alpha_S$ and the full-length long variant ($ER\alpha_L$) were estrogen-inducible ($ER\alpha_S > ER\alpha_L$) but the induction response was lower in NBH than in SC fish. In contrast, NBH killifish were hyper-responsive to estrogen as measured by expression of two other estrogen responsive genes: vitellogenin (Vtg) and aromatase B (AroB). Most strikingly, two ERα deletion variants (Δ6 and Δ6–8), lacking ligand binding and activation function domains, were identified in a subset of NBH fish, where they were associated with reduced responsiveness to estrogen treatment. Together, these results support the hypothesis that alternative splicing of the *esr1* gene of killifish could be an autoregulatory mechanism by which estrogen modulates the differential expression of ERα, and suggests a novel and adaptive mechanistic response to xenoestrogenic exposure.

1. Introduction

Estrogen-like endocrine disrupting chemicals (EDCs) or xenoestrogens interact with the ligand-binding site of estrogen receptors (ER) to alter transcription (Diamanti-Kandarakis et al., 2009). However, the extent to which EDCs could alter estrogen signaling though mechanisms other than direct transcriptional regulation remains largely unexplored in both human and wildlife populations. Because such mechanisms could provide adaptive opportunities for populations subject to multi-generational EDC exposures, we chose to study two populations of killifish (*Fundulus heteroclitus*): one resident in a polluted EPA Superfund site, New Bedford Harbor (NBH), MA, USA, and one resident in a clean reference site, Scorton Creek (SC), MA, USA. The NBH site is highly contaminated with polychlorinated biphenyls (PCBs) whose metabolites are weak ER agonists (DeCastro et al., 2006). Additionally, outflow from wastewater is the presumed source of several natural and synthetic estrogens that have been identified in NBH (Zuo et al., 2006), and other contaminants present in urban estuaries are also estrogenic (Benotti and Brownawell, 2007). The NBH killifish have demonstrated resistance to polycyclic aromatic hydrocarbons (PAHs), dioxins, and dioxin-like PCBs as measured

Abbreviations: AhR, aryl hydrocarbon receptor; AroB, brain aromatase or cytochrome P450 19b; Cyp1a, cytochrome P450 1a; *elfa*, gene encoding elongation factor alpha; EDC, endocrine disrupting chemical; ER, estrogen receptor; *esr1*/*ESR1*, gene encoding ER alpha in teleosts/mammals; NBH, New Bedford Harbor, MA; PAH, polycyclic aromatic hydrocarbons; PCB, polychlorinated biphenyl; qPCR, quantitative PCR; SC, Scorton Creek, MA; SNP, single nucleotide polymorphism; UTR, untranslated region; Vtg, vitellogenin.

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by reduced toxicity and decreased cytochrome P450 1a1 (Cyp1a) expression and activity (Bello et al., 2001; Nacci et al., 1999). This trait is heritable (Nacci et al., 2010), and has been ascribed to a genome-wide disruption of aryl hydrocarbon receptor (AhR) signaling (Oleksiak et al., 2011; Whitehead et al., 2010); however the exact mechanism remains to be determined (Aluru et al., 2011; Hahn et al., 2004; Reitzel et al., 2014).

ERα/β are members of the steroid receptor family of ligand-activated nuclear receptors and interact as homo- or heterodimers on estrogen responsive elements (ERE) of target gene promoters to enhance or repress transcription (Heldring et al., 2007). In killifish, like other teleost fishes, estrogenic responses are mediated by three ERs (ERα, -βa, -βb) but only ERα is estrogen responsive (Greytak and Callard, 2007). As measured by increased expression of estrogen responsive genes in reproductively inactive adults (vitellogenin, Vtg; and the predominant brain form of cytochrome P450 aromatase, AroB) (Greytak et al., 2005), the NBH environment is highly estrogenic, but ERα mRNA (itself a marker of estrogen exposure) remains unchanged, nor are there changes in the non-estrogen-regulated ERβa and ERβb (Greytak and Callard, 2007). Moreover, seasonal increases in ERα mRNA seen in SC are dampened in NBH fish; for example, in female liver ~3.5-fold increases are reduced to less than 2-fold in NBH (Greytak and Callard, 2007; Greytak et al., 2005). Although this suggests attenuation of the ERα auto-regulatory feedback loop that amplifies estrogenic responses in teleosts, induction of Vtg and AroB mRNAs was the same or greater in NBH males as compared to SC male fish (Greytak et al., 2010). Paradoxically, NBH embryos/larvae overexpress ERα ~5-fold, yet they are hypo-responsive to estrogen treatment as measured by ERα and AroB induction (Greytak and Callard, 2007; Greytak et al., 2010). Together these findings suggest that the mechanism by which NBH killifish may be resistant to estrogenic exposures is gene- and life stage-specific.

Alternative splicing of the *esr1* gene encoding ERα, is a plausible candidate mechanism to explain the complexity and diversity of estrogenic responses in NBH killifish. Like other nuclear receptors, ERα is a modular protein with five distinct functional domains (A–F) encoded by eight exons; the DNA- (DBD, D) and ligand-binding (LBD, E) domains; two activation functions (AF), the constitutively active AF-1 (AB) at the N-terminus and the ligand dependent AF-2 (EF) in the LBD (Heldring et al., 2007). In humans and mice, differential splicing of the eight coding exons of the *ESR1* gene generates an exceptional number of structurally and functionally different ERα splice variants, some of which are associated with disease phenotypes (for a review see, Taylor et al., 2010). Moreover, transcription and splicing are mechanistically coupled and co-regulated (Moore and Proudfoot, 2009; Auboeuf et al., 2004), suggesting that xenoestrogens that regulate transcription of a gene target also have the potential to regulate its splicing. Initial cloning efforts demonstrated no significant site differences in single nucleotide polymorphisms (SNPs) in the coding region of the killifish *esr1* gene (Greytak and Callard, 2007), nor were any identified in a more comprehensive candidate gene scan SNP analysis which included ERα (Proestou et al., 2014). However, one alternatively spliced ERα was cloned from killifish (ERαx), which was present in both SC and NBH populations (Greytak and Callard, 2007). Alternatively spliced ERα variants have been identified in many fish species (Cotter et al., 2013; Pakdel et al., 2000; Patino et al., 2000; Pinto et al., 2012; Seo et al., 2006; Tan et al., 1996; Xia et al., 1999). In particular, the expression of alternative long ($ER\alpha_L$, full-length) and short ($ER\alpha_S$, 5′-truncated) mRNA variants appears to be a conserved feature of teleosts (Cotter et al., 2013). In rainbow trout, where the processing of the *esr1* gene and production of alternative $ER\alpha_S$ and $ER\alpha_L$ isoforms was first described, the two variants have different estrogen binding and transactivation functions (Menuet et al., 2001; Pakdel et al., 2000). Also, in zebrafish, alternatively spliced $ER\alpha_L$ and $ER\alpha_S$ transcripts are differentially expressed by tissue type, during development and in response to estrogens and xenoestrogens (Cotter et al., 2013).

Here we investigated the hypothesis that the *esr1* gene is differentially processed in NBH and SC killifish, and that resultant expression patterns of alternatively spliced ERα mRNAs could account for observed gene-, tissue-, and site-related differences in estrogen responses. We applied a targeted PCR cloning approach to characterize the killifish *esr1* gene and to comprehensively survey the spectrum of ERα splice variants in fish from both sites. Four of the most abundant and functionally relevant variants were then analyzed by quantitative (q) and semi-quantitative PCR to document SC and NBH population differences in expression patterns by tissue type, reproductive status, and estrogen treatment. A total of 19 alternatively spliced ERα transcripts were identified, some with more than one alternative splicing event. $ER\alpha_L$ and $ER\alpha_S$ transcripts were differentially expressed by site, tissue type, reproductive status, and estrogen treatment. Strikingly, two deletion variants lacking exons that encode both the ligand binding and second activation domains were specific to a subset of fish from NBH and, in these individuals, were associated with reduced estrogen responsiveness.

2. Materials and methods

2.1. Collection and treatment of adult killifish

To obtain tissues for PCR cloning and determine the tissue distribution of identified ERα mRNA variants (Section 3.3), adult killifish (six of each sex) were collected monthly from SC and NBH between May (reproductively active) and October (reproductively inactive), as described previously (Greytak and Callard, 2007; Greytak et al., 2005). Size, body weight and gonadosomatic index (GSI) of these fish were also reported in these earlier publications. Previously collected tissues and RNA extracts as an ethanolic precipitate used for cloning and variant analysis, respectively, were stored at −80 °C.

To determine effects of estrogen on expression of ERα, AroB and Vtg mRNAs, (Section 3.4) additional reproductively active male and female killifish were collected from the two sites in June 2013 and kept until August 2013 in separate flow-through tanks with ambient temperature and light cycles at the National Health and Environmental Research Laboratory (Aquatic Ecology Division, US EPA), as previously described (Nacci et al., 1999), at which time they were past the period of seasonal reproductive activity at these sites (Greytak et al., 2005). Fish were then injected intraperitoneally with 5 mg/kg 17β-estradiol (E2; Sigma, St Louis, MO) in sesame oil or vehicle alone 5 days before decapitation. This concentration was chosen based on previous studies (Greytak et al., 2010; Pait and Nelson, 2003; Urushitani et al., 2003), which demonstrated significant induction of Vtg, AroB, and ERα mRNAs with this dosing protocol. Average body weights of these groups were SC males 8.13 g ± 0.48 g, SC females 10.05 g ± 0.85 g, NBH males 6.52 g ± 0.34 g, NBH females 7.39 g ± 0.48 g, and reproductive regression was verified by visual inspection of the gonads. All fish were anesthetized in 0.06% MS-222 (Sigma) before decapitation. Tissues were immediately quick-frozen on dry ice, and stored at −80 °C.

2.2. RNA extraction and reverse transcription

Frozen tissues were homogenized in Tri Reagent (Sigma) and total RNA was extracted according to the manufacturer's instructions. Extracts were treated with DNase I (Promega, Madison, WI) to minimize gDNA contamination. Concentration and quality of RNA was determined spectrophotometrically and confirmed by