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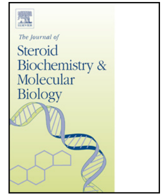

Evolution of estrogen receptors in ray-finned fish and their comparative responses to estrogenic substances

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In vertebrates, estrogens play fundamental roles in regulating reproductive activities through estrogen receptors (ESRs), and disruption of estrogen signaling is now of global concern for both wildlife and human health. To date, ESRs of only a limited number of species have been characterized. We investigated the functional diversity and molecular basis or ligand sensitivity of ESRs among ray-finned fish species (Actinopterygii), the most variable group within vertebrates. We cloned and characterized ESRs from several key species in the evolution of ray-finned fish including bichir (Polypteriformes, ESR1 and ESR2) at the basal lineage of ray-finned fish, and arowana (Osteoglossiformes, ESR1 and ESR2b) and eel (Anguilliformes, ESR1, ESR2a and ESR2b) both belonging to ancient early-branching lineages of teleosts, and suggest that ESR2a and ESR2b emerged through teleost-specific whole genome duplication, but an ESR1 paralogue has been lost in the early lineage of euteleost fish species. All cloned ESR isoforms showed similar responses to endogenous and synthetic steroidal estrogens, but they responded differently to non-steroidal estrogenic endocrine disrupting chemicals (EDCs) (*e.g.*, ESR2a exhibits a weaker reporter activity compared with ESR2b). We show that variation in ligand sensitivity of ESRs can be attributed to phylogeny among species of different taxonomic groups in ray-finned fish. The molecular information provided contributes both to understanding of the comparative role of ESRs in the reproductive biology of fish and their comparative responses to EDCs.

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1. Introduction

Estrogens play important roles in growth, development, reproduction and behavior in vertebrates and their effects are mediated principally through estrogen receptors (ESRs), members of the nuclear steroid hormone receptor superfamily which are activated by their cognate ligands [1,2]. Upon ligand binding, the complex of receptor and ligand binds to a specific DNA sequence (estrogen response element; ERE), located in the regulatory regions of their target genes and subsequently activating gene transcription. Like other nuclear receptors, ESRs contain six distinct domains labeled from A to F, as defined previously [3]. The C-domain (DNA-binding domain; DBD) and E-domain (ligand-binding domain; LBD) are responsible for DNA-binding and ligand-binding, respectively, and are highly conserved among species as key functional domains [4–6]. The standardized nomenclatures for human estrogen receptor subtypes are designated ESR1 and ESR2, respectively (HUGO Gene Nomenclature Committee [7]). The only existing approved nomenclature for fish is for zebrafish (*Danio rerio*) where the ERα and ERβ subtypes are designated Esr1, Esr2b and Esr2a (Official Zebrafish Nomenclature Guidelines; http://zfin.org). Although this nomenclature has been adopted widely for teleosts, some contradicting nomenclature exists for the ESR2 orthologues (*e.g.* zebrafish ERβ2 corresponds to medaka ERβ1). Based on this and to standardize the ESR subtype

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nomenclature across all species we have adopted the official ESR nomenclature for human here.

Estrogen-responsive ESRs have been identified in all classes of vertebrates. To date, early-branching vertebrates including lamprey (Agnatha, a stem lineage of vertebrate), shark (Chondrichthyes, a sister group of Osteichthyes or bony fish) and lungfish (Diplocercidae, an early branching lineage of tetrapods) have been shown to have two distinct types of ESR, ESR1 and ESR2, probably originating from a duplication of the ESR gene early in the vertebrate lineage [5] (see also Fig. S5). However, in some lineages one isoform has been subsequently lost [8–10] (see also Discussion). Intriguingly, the largest extant group within the Actinopterygii (ray-finned fish), teleosts, have three forms of ESRs: ESR1, ESR2a and ESR2b [11], where ESR2a and ESR2b appear to be closely related to each other. Only one type of ESR2 has been reported previously in gar (Lepisosteiformes, a sister group of teleosts). These observations suggest that the duplication of ESR2 seen in teleosts reflects a teleost-specific whole genome duplication (WGD) event [12–15]. Detailed information relating to the characterization of ESRs in ray-finned fish, however, is still limited. Cladistia (*e.g.*, bichir: Polypteriformes) are generally regarded as the most basal lineage of ray-finned fish leading to the teleost lineage [16], whereas Osteoglossomorpha (*e.g.*, arowana: Osteoglossiformes) and Elopomorpha (*e.g.*, eel: Anguilliformes) belong to an ancient family and early-branching lineages of teleost. As such, these organisms are highly relevant fish species for investigating the evolution and functional divergence of the ESRs as they diverged at times prior to (bichir) and after (arowana, eel) the teleost-specific WGD. To date, however, ESRs in these species have not been characterized.

Disruption of the endocrine system is of concern globally both for humans and wildlife. Endocrine disrupting chemicals (EDCs) enter the aquatic environment where fish species are especially at risk of exposure from discharges emanating from wastewater treatment works and agricultural run-off. EDCs are extremely wide ranging in their derived sources and they include alkylphenols, pesticides, plasticizers, bisphenols [*e.g.*, bisphenol A (BPA), 4-nonylphenol (NP)], dichlorodiphenyltrichloroethane (DDT) and its metabolites such as *o,p*'-DDT, natural and pharmaceutical estrogens [17β-estradiol (E2), estrone (E1), estriol (E3) and 17α-ethinylestradiol (EE2)]. Exposure to these EDCs, especially steroidal estrogens are known to induce a range of effects on fish including reproductive and developmental disorders such as skewed sex ratios and increased prevalence of intersex [17–20]. Most of these investigations, however, are limited to a few model fish species. Teleosts show a lot of divergence within vertebrates and have experienced WGD. This has resulted in a high complexity of the genetic structure of ESRs and this needs due consideration in unraveling species responsiveness to environmental estrogens.

We have developed and applied *in vitro* reporter gene assays to evaluate comparative estrogenic potency of EDCs for a variety of fish species [21,22]. Through these analyses, we clarified species-dependent transactivation of ESR1 where medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), bluegill (*Lepomis macrochirus*) and guppy (*Poecilia reticulata*) ESR1s show higher sensitivities to BPA, NP and *o,p*'-DDT compared with those of cyprinids [carp (*Cyprinus carpio*), roach (*Rutilus rutilus*)] [21]. In addition, we have found subtype-specific responses of ESR2 to EDCs with weaker reporter activity of ESR2a compared with ESR2b and these responses were comparable among medaka, stickleback, carp, roach and zebrafish (*Danio rerio*) [11]. To investigate whether such response patterns of ESR subtypes are common throughout ray-finned fish, and to understand the functional diversity of ESRs, we cloned ESRs from several key fish species of particular significance in terms of the evolution of ray-finned fish, including bichir, arowana and eel, and characterized the phylogenetic relationships and transactivities elicited by steroidal estrogens and estrogenic EDCs.

2. Materials and methods

2.1. Chemical reagents

E1 (purity >99.0%), E2 (purity >98.0%), E3 (purity >99.0%) and EE2 (purity >98.0%) were purchased from Sigma-Aldrich (St. Louis, MO), and 4-NP (purity >97.0%), BPA (purity >99.0%) and *o,p*'-DDT (purity >99.5%) were from Kanto-Kagaku (Tokyo, Japan). All compounds tested in the reporter gene assay were dissolved in dimethylsulfoxide (DMSO, Nacalai, Kyoto, Japan) and the concentration of DMSO in the culture medium did not exceed 0.1%.

2.2. Source of fish

Silver arowana (*Osteoglossum bicirrhosum*) and gray bichir (*Polypterus senegalus*) were purchased from a commercial supplier (Meito Suien, Nagoya, Japan). Japanese eel (*Anguilla japonica*) was kindly provided from Freshwater Resources Research Center, Aichi Fisheries Research Institute (Nishio, Japan). All animal handling procedures and protocols were approved by the institutional animal care and use committee at the National Institute for Basic Biology, Okazaki, Japan.

2.3. Cloning and construction of ESRs

For cloning bichir, arowana and eel ESRs, RNA was isolated from liver, brain and ovary, and reverse transcribed into cDNA which served as template for PCR using degenerate oligonucleotides designed at conserved amino acid region in the DBD and LBD [22]. The 5′- and 3′- ends of the ESRs were amplified by rapid amplification of the cDNA end (RACE) using the GeneRacer Kit (Life Technologies, Carlsbad, CA). A full-length transcript of the open reading frame was amplified using PrimeStar GXL polymerase (Takara, Ohtsu, Japan). The PCR reaction was performed independently using the cDNA templates from liver, brain and gonads. The resulting amplification products were subcloned into pCR-Blunt II-TOPO vector (Life Technologies) and at least 6 clones were sequenced for each PCR reaction. The PCR products were then subcloned into the pcDNA3.1 vector (Life Technologies). The full-coding region of eel ESR2b (AB003356) was amplified by a standard RT-PCR and subcloned into the pcDNA3.1 vector. The eel ESR2b cDNA isolated in the present study was identical to the previous sequence in the Genbank, with an amino acid substitution (glycine to serine at position 136). All primer information is listed in Supplemental Table 1.

2.4. Phylogenetic tree of ESRs

The deduced amino acid sequences of DBD and LBD, including hinge regions, were aligned using the Clustal X program. Alignments with questionable gaps were removed. A maximum likelihood tree based on the JTT-matrix-based model [23] was constructed from this alignment using a 1000 replicate bootstrap analysis using MEGA6 software [24]. Amphioxus (*Branchiostoma belcheri*) ESR was used as an outgroup of the phylogeny. The accession numbers of the sequences used in the phylogenetic analyses are listed in Supplemental Table 2.

2.5. Transactivation assay and data analysis

To examine the ligand-sensitivities with the ESRs, transactivation assays using pGL3-4xERE were performed as previously reported [21]. HEK293 cells (DS Pharma Biomedical, Osaka Japan)

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