Transcriptional Activation of Estrogen Receptor-alpha and Estrogen Receptor-beta from Elephant Shark (*Callorhynchus milii*)

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Short title: Transcriptional activation of elephant shark estrogen receptors

Abstract

Humans and other terrestrial vertebrates contain two estrogen receptors (ERs), ER-alpha and ERbeta. Among cartilaginous fish (sharks, rays, skates), which are jawless vertebrates that evolved about 525 million years ago, only activation by steroids of ER-beta orthologs has been characterized. To remedy this gap in understanding estrogen signaling in jawless vertebrates, we studied estrogen activation of orthologs of human ER-alpha and ER-beta. Unexpectedly, we found that *C. milii* contained three estrogen-responsive ER-alpha genes: ER-alpha1 (596 amino acids), ER-alpha-2 (600 amino acids), and ER-alpha-3 (599 amino acids) with strong sequence similarity to each other. We also found an estrogen-unresponsive gene, ER-alpha4 (561 amino acids), with a 39 amino acid deletion in the DNA-binding domain. An estrogen-responsive ER-beta ortholog (580 amino acids) also was present in *C. milii*. The three active *C. milii* ER-alphas have a similar length to human ER-alpha (595 amino acids); however, *C. milii* ER-beta is longer than human ER-beta (530 amino acids). We determined the half-maximal response (EC50) and fold-activation to estradiol (E2), estrone (E1), and estriol (E3) of *C. milii* ER-alpha1, ER-alpha2, ER-alpha3, and ER-beta. Among these estrogens, E2 had the lowest EC50 for all four ERs. Fold-activation by E2 and E3 was similar for ER-alpha1, ER-alpha2, ER-alpha3, and ER-beta. Overall, estrogen activation of *C. milii* ER-alpha and ER-beta was similar to that for human ER-alpha and ER-beta indicating substantial conservation of the vertebrate ER in the 525 million years since the divergence of cartilaginous fish and humans from a common ancestor.

Keywords: elephant shark; estrogen receptor evolution; ER-alpha; ER-beta.

1. Introduction

Estrogens are a class of steroid hormones that have diverse physiological activities in females and males in humans and other vertebrates [1-5]. Estrogens act by binding to estrogen receptors (ERs) [5-6], which are transcription factors that belong to the nuclear receptor family, which also includes receptors for progestins, androgens, and corticosteroids [7-9]. To date, two distinct estrogen receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), respectively, have been isolated in mammals and other vertebrates [10-12]. The response of ERα and ERβ in humans and other vertebrates to 17β-estradiol (E2), the main physiological estrogen, as well as to estrone (E1) and estriol (E3) (Figure 1) has been studied extensively [5,6,10,13].

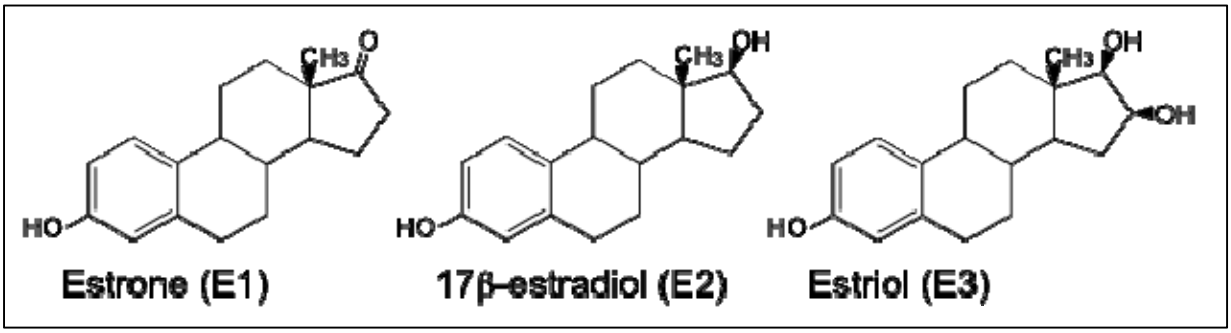

Figure 1. Structures of estrogen.
Estrone (E1), 17β-estradiol (E2), and estriol (E3) are the natural estrogens. E1 is a minor female sex hormone and serves mainly as a precursor of E2. E3 is produced in the placenta during pregnancy. E2 is a major female sex hormone mainly produced in the ovary [35, 36].

Although ERs with sequence similarity to ERα [14,15] and ERβ [14-16] have been isolated from elasmobranchs (sharks, rays, and skates), only the response to steroids by ERβ from the cloudy catshark (*Scyliorhinus torazame*) and whale shark (*Rhincodon typus*) has been studied [16]. Estrogen activation of a gnathostome ERα has not been reported. Elasmobranchs, along with Holocephali, comprise the two extant subclasses of Chondrichthyes, jawed fishes with skeletons composed of cartilage rather than calcified bone [17]. Chondrichthyes occupy a key position relative to fish and terrestrial vertebrates, having diverged from bony vertebrates about 450 million years ago [14,18]. As the oldest group of extant jawed vertebrates (gnathostomes), the properties of ERα and ERβ in cartilaginous fishes can provide important insights into the early evolution of ERα and ERβ from an ancestral ER in lamprey [19]. Moreover, elephant shark has the slowest evolving genome of all known vertebrates, including the slowly evolving coelacanth [14], which makes elephant shark genes windows into the past, and, thus, a good system to study the evolution of the ER, including selectivity of the ER in a basal vertebrate for steroids and the subsequent divergence in steroid selectivity in human ER, in other terrestrial vertebrate ERs and in ray-finned fish ERs [20]. Another point of interest for the ER in elephant shark is that elephant sharks contain three full-length ERαs [14], raising the question of each how these three ERαs compare with each other and with mammalian ERαs in their response to estrogens.

To remedy this gap in our understanding of ERα activation in a basal jawless vertebrate, we have studied the response to estradiol, estrone, and estriol of three full-length *Callorhinchus milii* ERαs, as well as *C. milii* ERβ. Here we report that despite the strong sequence similarity among *C. milii* ERα1, ERα2, ERα3, and ERβ, there are some differences in

their half-maximal response (EC50) and fold-activation to these three physiological estrogens. We find that E2 had the lowest EC50 for all four ERs. E2 and E3 had a similar fold activation for ERα1, ERα2, ERα3, and ERβ. Overall, estrogen activation of *C. milii* ERα and ERβ was similar to that for human ERα and ERβ [5,7], indicating substantial conservation of the vertebrate ER in the 525 million years since the divergence of cartilaginous fish and humans from a common ancestor.

2. Materials and methods

2.1. Animals

C. milii elephant sharks of both sexes were collected in Western Port Bay, Victoria, Australia, using recreational fishing equipment, and transported to Primary Industries Research Victoria, Queenscliff, using a fish transporter. The animals were kept in a 10-t round tank with running seawater (SW) under a natural photoperiod for at least several days before sampling. Animals were anesthetized in 0.1% (w/v) 3-amino benzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO, USA). After the decapitation of the animal, tissues were dissected out, quickly frozen in liquid nitrogen, and kept at −80°C. All animal experiments were conducted according to the Guideline for Care and Use of Animals approved by the committees of University of Tokyo [21].

2.2. Chemical reagents

Estrone (E1), 17β-estradiol (E2), and estriol (E3) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). All chemicals were dissolved in dimethyl-sulfoxide (DMSO). The concentration of DMSO in the culture medium did not exceed 0.1%.

2.3. Cloning of estrogen receptors

When we began this project, the genome of *C. milii* had not yet been sequenced [14]. Thus, we needed to sequence an ER in *C. milii* as a first step in determining the number of ERs and their sequences in *C. milii*. For this goal, we used two conserved amino acid regions in the DNA-binding domain (GYHYGVW) and the ligand-binding domain (NKGM/IEH) of vertebrate ERs to design the degenerate oligonucleotides to clone *C. milii* ERs. The second PCR used the first PCR amplicon, and nested primers that were selected in the DNA binding domain (CEGCKAF) and the ligand binding domain (NKGM/IEH). As a template for PCR, the

first-strand cDNA was synthesized using total RNA isolated from the ovary. The amplified DNA fragments were subcloned with TA-cloning plasmids pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The 5' and 3' ends of the ER cDNAs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech., Palo Alto, CA). To amplify the isoforms of elephant shark ER, we applied a PCR-based cDNA amplification technique using the primers et (5'-TGAAGTGTGATCGTCCAGGCGACAG-3' and 5'-CAAGCTGGAGGATAAGACATCGAC -3'). Sequencing was performed using a BigDye Terminator Cycle Sequencing kit and analyzed on the Applied Biosystems 3730 DNA Analyzer.

We used the *C. milii* extracted RNA to clone ERα1, ERα2, ERα3, ERα4, and ERβ, which had sequences corresponding to the sequences of the *C. milii* genome deposited by Venkatesh et al., 2014 [14].

2.4. Database and sequence analysis

All sequences generated were searched for similarity using BLASTn and BLASTp at the web servers of the National Center of Biotechnology Information (NCBI). We found four ERα genes and one ERβ gene [14], which were aligned using ClustalW [22].

2.5. Reporter gene assay

Full-length estrogen receptors were amplified using specific forward and reverse primers designed at start and stop codons and cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). In addition, ERs with a FLAG-tag added to the N-terminus were also prepared by PCR. A reporter construct, pGL4.23-4xERE was produced by subcloning of oligonucleotides having 4xERE into the *Kpn*I-*Hind*III site of pGL4.23 vector (Promega). All cloned DNA sequences were verified by sequencing.

Reporter gene assays using full-length ERs were performed in Human Embryonic Kidney 293 cells (HEK293 cells). HEK293 cells were seeded in 24-well plates at 5 x 10^4 cells/well in phenol-red-free Dulbecco's modified Eagle's medium with 10% charcoal/dextran-treated fetal bovine serum. After 24 h, the cells were transfected with 400 ng of reporter construct, 25 ng of pRL-TK (as an internal control to normalize the variation in transfection efficiency; contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter), and 200 ng of pcDNA3.1-estrogen receptor using polyethylenimine (PEI). After 5 h of incubation, ligands were applied to the medium at various concentrations. After an additional 43 h, the

cells were collected, and the luciferase activity of the cells was measured with the Dual-Luciferase Reporter Assay System. Promoter activity was calculated as firefly (*P. pyralis*)-luciferase activity/sea pansy (*R. reniformis*)-luciferase activity. The values shown are mean ± SEM from three separate experiments, and dose-response data, which were used to calculate the half maximal response (EC50) for each steroid, were analyzed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA). All experiments were performed in triplicate.

3. Results

3.1. Cloning and sequence analysis of cDNAs for four elephant shark ERα genes and one ERβ gene.

Using standard RT-PCR and RACE techniques, we successfully cloned four full-length ERs, designated as ERα1, ERα2, ERα3 (Figure 2A, B), and ERβ (Figure 2C), from elephant shark ovary RNA. The cDNA for elephant shark ERα1 predicted a 596 amino acid protein with a calculated molecular mass of 66.6 kDa (GenBank accession no. LC068847), and the cDNA for elephant shark ERβ predicted a 580 amino acid protein with a calculated molecular mass of 64.9 kDa (GenBank accession no. LC068848), Figure 2A, B. In addition, we cloned the other two full-length elephant shark ERs: ERα2 and ERα3, which are found in GenBank (GenBank accession no. XM_007894403 for ERα2, XM_007894404 for ERα3**)**. ERα4 (GenBank accession no. XM_007894406) with a 39 amino acid deletion in the DNA-binding domain was also cloned (Figure 2A, B).

The elephant shark ERα and ERβ sequences contain the five recognizable steroid hormone receptor sub-domains in the expected order: the N-terminal region (A/B domain), DNA binding domain, DBD (C domain), hinge region (D domain), ligand binding domain, LBD (E domain), and C-terminal extension (F domain) (Figure 2A). In Figure 2A we compare the functional domains of ERα1, ERα2, ERα3 and ERα4. Figure 2A shows that there is excellent conservation among these ERs of the A/B, C, D, E and F domains. This strong sequence conservation also is seen in the amino acid alignment of ERα1, ERα2, ERα3 and ERα4 shown in Figure 2B. Figure 2B shows the deletion in the DBD of ERα4, which probably explains the lack of binding of estrogens by *C. milii* ERα4. The amino acid sequence of ERα4 is closest to ERα3. Overall, Figures 2A and 2B show the strong sequence conservation in the four *C. milii* ERα genes.

In Figure 2C, we show a sequence alignment of *C. milii* ERα1 and ERβ. The DNA response element recognition motif (P-box, CEGCKA) in the DBD and the AF-2 motif (core LLLEML region) in the LBD, which is required for interaction with transcriptional co-activators, are conserved in all four -elephant shark ERα sequences and in ERβ (Figure 2B and 2C).

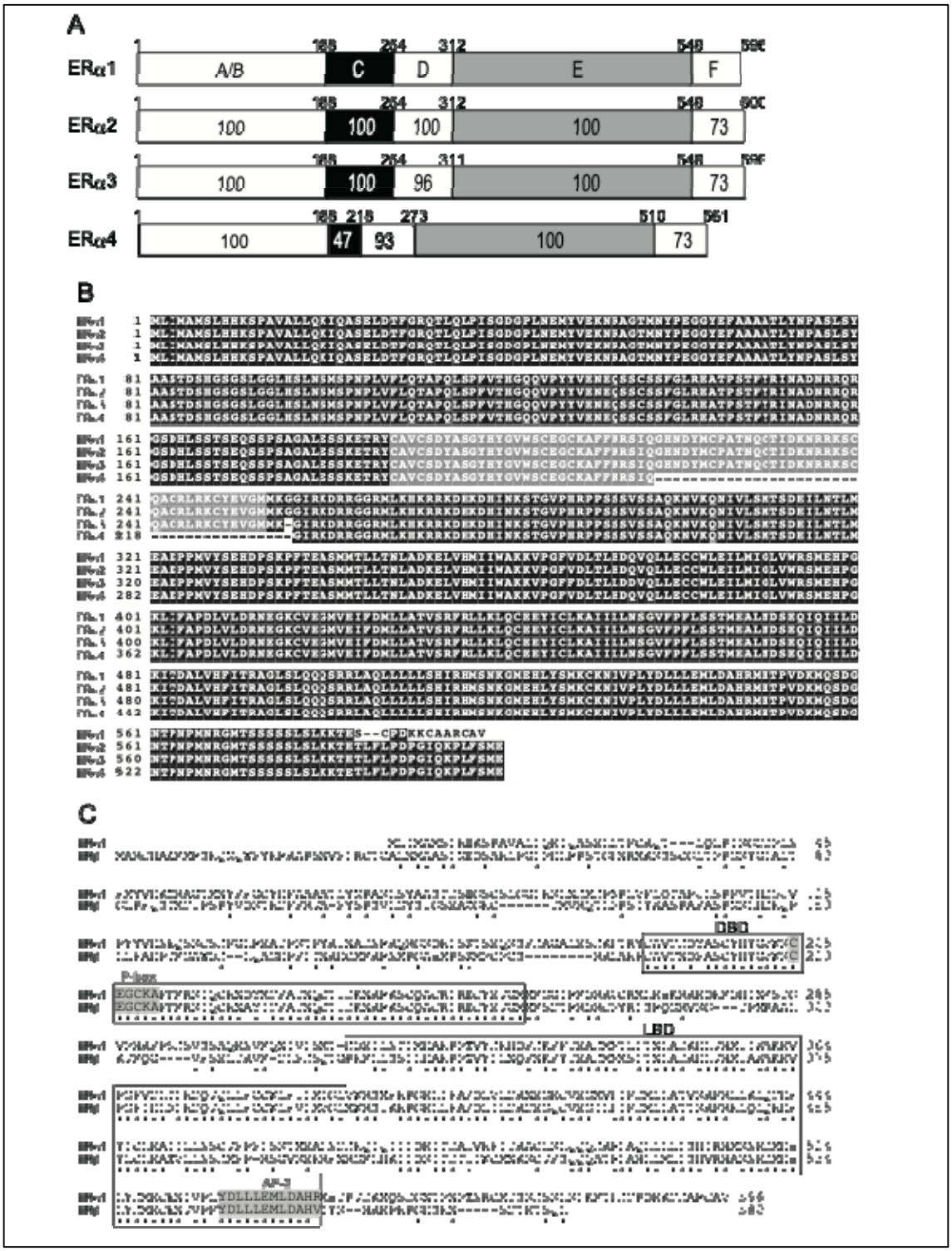

Figure 2. Amino acid sequences of the elephant shark ERs.
(A) Comparison of functional domains of four elephant shark ERα genes. Elephant Shark ERα1, ERα2, and ERα3 show strong sequence conservation of their A/B, C, D, and E domains. ERα4 has a deletion in the DNA-binding domain [C domain], which explains its lack of transcriptional activation by estradiol. (B) Sequence alignment of Elephant Shark ERα1, ERα2, ERα3, and ERα4. There is excellent conservation of the four-elephant shark ERα genes. (C) Aligned sequences of the elephant shark ERα1 and ERβ. The DBD and LBD are indicated by open boxes. Residues are important for DNA response element recognition (the P-box), and the AF-2 region, which mediates contact of the LBD with transcriptional coactivators, is shaded gray. Numbers to the right indicate amino acid position. Asterisks indicate residues conserved in both ERα1 and ERβ.

3.2 Comparison of functional domains on human ERα and ERβ with functional domains in elephant shark ERα and ERβ.

To gain additional insights into the evolution of the *C. milii* ERs, we compared the functional domains of elephant shark ERα1to elephant shark ERβ (Figure 3A), of elephant shark ERα1 to human ERα and human ERβ (Figure 3C), and of elephant shark ERβ to human ERα and human ERβ (Figure 3D). We also compared human ERα and ERβ to each other (Figure 3B).

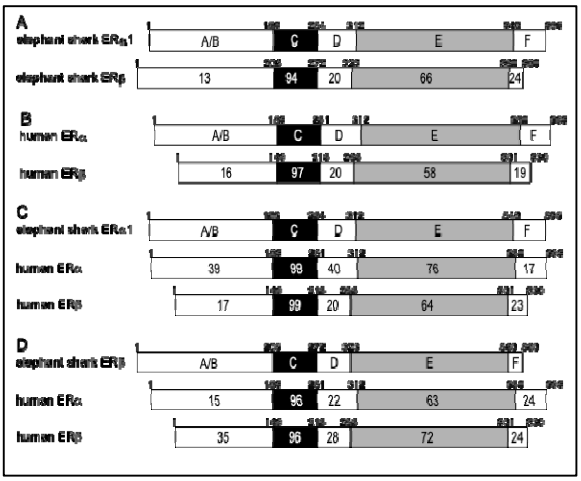

Figure 3. Comparison of the functional domains of elephant shark ERs with human ERs.
The functional domains A/B through F are shown schematically with the number of amino acid residues indicated. The percentage of amino acid identity is shown. (A) Comparison of elephant shark ERα1 with elephant shark ERβ. The N-terminal (A/B) domains of elephant shark ERα1 and ERβ show strong divergence. There is also some divergence between the ligand (E) binding domain on elephant shark ERα1 and ERβ. (B) Comparison of human ERα (accession no. NM000125) with human ERβ (accession no. AB006590). The domains of human ERα with human ERβ have similar sequence similarity to each other as elephant shark ERα1 and ERβ. (C) Comparison of elephant shark ERα1 with human ERα and human ERβ. Elephant shark ERα1 is closer to human ERα than to human ERβ. (D) Comparison of elephant shark ERβ with human ERα and human ERβ. Elephant shark ERβ is closer to human ERβ than to human ERα.

Figure 3A shows the excellent conservation of the DNA-binding domains [C domains] (94% identity) and ligand-binding domains [E domains] (66% identity) in elephant shark ERα1

and ERβ, in contrast to the weak conservation of the A/B domains (13% identity) at their N-terminus. Comparison of Figures 3A and B reveals that the DBD in human ERα and *C. milii* ERα1 and ERβ have similar sequence conservation, while the LBD sequence in *C. milii* ERα1 and *C. milii* ERβ are more conserved (66% identity) than the LBD in human ERα and ERβ (58% identity).

Figure 3C shows the strong sequence conservation of the DBD in elephant shark ERα1 and human ERα and ERβ. Figure 3C also shows that there is stronger conservation (76%) of the LBD in elephant shark ERα1 and human ERα, compared to the similarity (64%) of the corresponding LBD in elephant shark ERα1 to human ERβ. Figure 3D shows the strong sequence conservation of the DBD in elephant shark ERα1 and human ERα and ERβ. Figure 3D also shows the stronger conservation (72%) of the LBD in elephant shark ERβ and human ERβ, compared to the similarity (64%) to the corresponding LBD in human ERα.

3.3 Comparison of functional domains on elephant shark ERα and ERβ with functional domains in zebrafish, and whale shark.

To gain an additional insight into the evolution of elephant shark ERα and ERβ, we compared their sequences to ERα and ERβ sequences in zebrafish and whale shark (Figure 4).

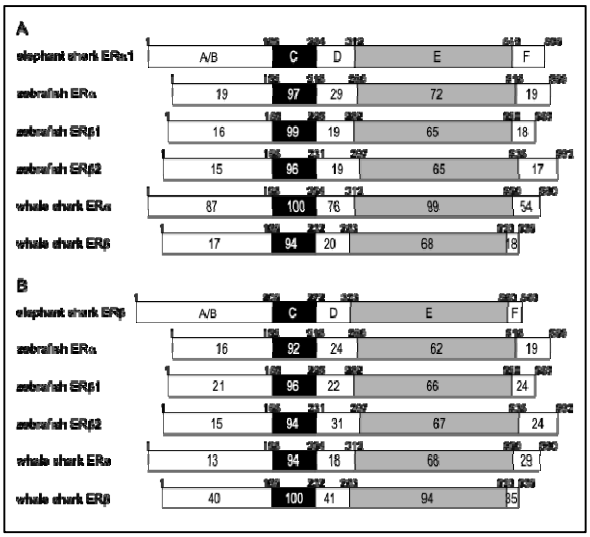

Figure 4. Comparison of the functional domains of elephant shark ERs with ERs from zebrafish and whale shark.
The functional domains A/B through F are shown schematically with the number of amino acid residues indicated. The percentage of amino acid identity is shown. (A) Comparison of elephant shark ERα1 with zebrafish and whale shark ERs. (B) Comparison of elephant shark ERβ with zebrafish and whale shark ERs. Genbank accession no. NM_152959 (zebrafish ERα), AF516874 (zebrafish ERβ1), AF349413 (zebrafish ERβ2), XM_048600891 (whale shark ERα), and AB551716 (whale shark ERβ).

The sequence comparisons between elephant shark ERα and ERβ domains with ERα and ERβ domains in whale shark revealed an unexpected sequence similarity (87% identity) between the A/B domain on elephant shark ERα and whale shark ERα. The A/B domain in whale shark ERβ and elephant shark ERβ has 40% identity. Interestingly, the A/B domains on elephant shark ERα and ERβ domains have 39% and 35% sequence identity, respectively, with the A/B domains on human ERα and human ERβ, respectively.

Figure 3C shows the stronger conservation of the LBD in elephant shark ERβ and human ERβ (72% identity), compared to the similarity to the corresponding LBD in human ERα (63% identity). The LBD of elephant shark ERα1 is closer to the LBD in human ERα than the LBD in human ERβ, and the LBD of elephant shark ERβ is closer to the LBD in human ERβ than the LBD in human ERα.

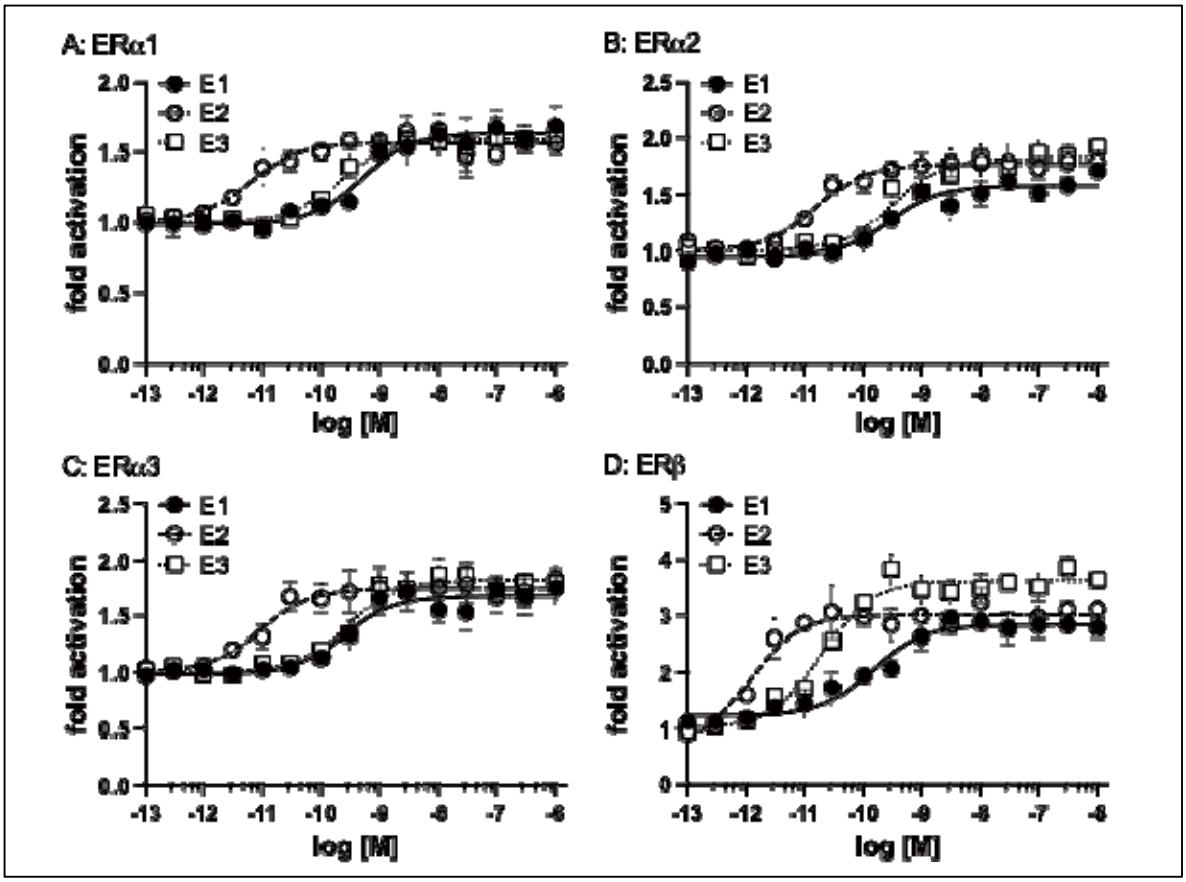

Figure 5. Transcriptional activation of elephant shark ERs.
Elephant shark ERs were transfected into HEK293 cells with an ERE-driven reporter gene. Concentration-response profile for ERα1 (A), ERα2 (B), ERα3 (C), and ERβ (D) for E1, E2, and E3 (10^{-13}M to 10^{-6}M). Data are expressed as a ratio of a vehicle (DMSO) to other test chemicals. Each point represents the mean of triplicate determinations, and vertical bars represent the mean ± SEM.

3.4. Transcriptional activities of elephant shark ERα and ERβ.

A transactivation assay was used to examine the response to the physiological estrogens E1, E2, and E3 of the three active isoforms of elephant shark ERα. The three isoforms, ERα showed ligand-dependent transactivation (Figure 5A, B, C). The EC50s for transcriptional activation of ERα1, ERα2, and ERα3 were 0.49 nM, 0.29 nM, and 0.25 nM (E1), 0.0059 nM, 0.016 nM, and 0.0093 nM (E2), and 0.2 nM, 0.31 nM, and 0.29 nM (E3), respectively (Table 1).

We also examined the induction of elephant shark ERβ transcriptional activity by different concentrations of estrogens (E1, E2, and E3). All three estrogens activated transcription of elephant shark ERβ in a dose-dependent manner (Figure 5D). The half-maximal response (EC50) for transcriptional activation of elephant shark ERβ was 0.14 nM for E1, 0.001 nM for E2, and 0.02 nM for E3 (Table 1). Although there was no significant difference in EC50 values for activation by an estrogen of ERα1 and ERβ, the fold activation values for E2 were higher than those for E1 and E2 (Table 1). Thus, the elephant shark estrogen receptors exhibited a ligand responsiveness observed human estrogen receptors [1-10].

Table 1. Gene transcriptional activities of elephant shark ERs by estrogens in HEK293

		E1	E2	E3
ERα1	EC50 (nM)	0.49	0.0059	0.2
	Fold-Activation (± SEM) *	1.62 (± 0.16)	1.66 (± 0.04)	1.57 (± 0.09)
	Ratio**	0.98	1.00	0.95
ERα2	EC50 (nM)	0.29	0.016	0.31
	Fold-Activation (± SEM) *	1.51 (± 0.12)	1.79 (± 0.12)	1.82 (± 0.13)
	Ratio**	0.84	1.00	1.02
ERα3	EC50 (nM)	0.25	0.0093	0.29
	Fold-Activation (± SEM) *	1.55 (± 0.11)	1.75 (± 0.08)	1.87 (± 0.15)
	Ratio**	0.89	1.00	1.07
ERβ	EC50 (nM)	0.14	0.001	0.02
	Fold-Activation (± SEM) *	2.87 (± 0.16)	3.23 (± 0.26)	3.49 (± 0.26)
	Ratio**	0.89	1.00	1.08

*Fold activation values were calculated based on the 10 nM values.
**Ratio values were calculated by dividing the fold-activation value of estrogens by the value of E2.

4. Discussion

Significant advances have been made in elucidating the evolution of estrogen signaling in animals [1, 2, 9, 11, 19, 23, 24]. Amphioxus, a chordate that is a close ancestor of vertebrates, contains an ER and a steroid receptor (SR) that is an ortholog of receptors for 3-keto-steroids, including testosterone, progesterone, cortisol, and aldosterone [11, 25]. Contrary to expectations, the amphioxus ER does not bind E2 or other steroids [11, 24, 25], while E2 and E1 are transcriptional activators of the SR [11, 25]. Atlantic sea lamprey, a jawless fish, contains an ER [19] that is activated by E2 and E1 [11, 24].

The estrogen receptors in Chondrichthyes, cartilaginous fishes with jaws, are basal to lobe-finned fishes and are ancestors of terrestrial vertebrates [14]. These ERs are still not fully characterized [15, 26]. The identification of ERα1, ERα2, ERα3, ERα4, and ERβ from the sequencing of the elephant shark [14] provided an opportunity to investigate estrogen signaling in a basal jawless vertebrate that evolved about 525 million years ago. We investigated the response to E1, E2, and E3 of ERα1, ERα2, ERα3, and ERβ from elephant shark expressed in HEK293 cells (Figure 5). These studies revealed that elephant shark ERα1, ERα2, ERα3, and ERβ are activated by E1, E2, and E3. We note that we found that ERα4 did not respond to an estrogen, which we propose is due to a sequence deletion in the DBD (data not shown). Thus, we did not continue studies of estrogen activation of ERα4.

Our data with estrogen activation of ERα1, ERα2, ERα3, and ERβ align with the established ligand binding characteristics of ERs in other vertebrates, thereby substantiating the conserved nature of estrogen signaling pathways [25, 27-29]. Estrogen-dependent transcriptional activity of the ERs from various species, including other sharks, revealed that the ERs from all species have a stronger response to E2 than to E3 [16, 30-34].

Instead of only one ERα gene, elephant shark contains three ERα genes with very similar sequences, which is unexpected. The strong conservation of ERα4, which is not lig-and-activated, also is unexpected. Further research is needed to gain a more comprehensive understanding of the physiological function of estrogen and the multiple estrogen receptors in elephant sharks. This data should provide clues for understanding the physiological function(s) of each isoform.

In summary, this is the first report with evidence for the conservation of elephant shark ERα and ERβ compared to human ERα and ERβ, which provides a valuable perspective on the early evolutionary history of these critical nuclear receptors in vertebrates. The findings from

this study not only confirm the conserved nature of ER-mediated estrogen signaling across vertebrates but also open new avenues for research into the functional diversification of these receptors across vertebrates. These findings furnish researchers with critical molecular data to examine the role of ERs in future studies, such as those examining gonadal development, reproductive biology, and developmental and evolutionary endocrinology.

Author Contribution

Y.A.: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, and Data curation. **H.N.:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **W. T.:** Resources**.** **S.H.:** Resources**.** **M.E.B.:** Writing – review & editing, Project administration, Validation, Supervision, Conceptualization. **Y.K.:** Writing – review & editing, Project administration, Visualization, Validation, Funding acquisition, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data is freely available on request to Dr. Katsu or to Dr. Baker.

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