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Sharing the Roles: An Assessment of Japanese Medaka Estrogen Receptors in Vitellogenin Induction

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Abstract

Teleost fish express at least three estrogen receptor (ER) subtypes. To date, however, the individual role of these ER subtypes in regulating expression of estrogen responsive genes remains ambiguous. Here, we investigate putative roles of three ER subtypes in Japanese medaka (*Oryzias latipes*), using vitellogenin (VTG) I and II as model genes. We identify specific ligand/receptor/promoter dynamics, using transient transactivation assays that incorporate luciferase reporters comprising 3kb promoter/enhancer regions of medaka VTGI and VTGII genes. Four steroidal estrogens (17 β -estradiol, estrone, estriol, and 17 α -estradiol) were tested in these assays. Results indicate that all three medaka ERs (mERs) are capable of initiating transactivation of both VTG I and II, with mER β 2 exhibiting the greatest efficacy. Promoter deletion analysis suggests that ligand-specific receptor transactivation and utilization of regional-specific estrogen response elements may be associated with differential activities of each medaka ER. Further, cluster analysis of *in vivo* gene expression and transactivation suggests that all three ER subtypes putatively play a role in up-regulation of VTG. Results illustrate that preferential ligand/receptor/promoter interactions may have direct implications for VTG gene expression and other ER-mediated regulatory functions that are relevant to the risk assessment of estrogenic compounds.

Keywords

subfunctionalization; estrogen receptors; vitellogenin; molecular initiating events; transactivation assay

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Supporting Information Available: Construct sizes (Table SI-1); Primer list for VTG1 promoter isolation and promoter analysis regions (Table SI-2); tentative estrogen response elements from medaka VTGI and VTGII deletion constructs (Table SI-3); promoter deletion regions (Figure SI-1); medaka exposure and gene expression analysis; qPCR primers (Table SI-4); background fold transactivation of medaka subtypes (Figure SI-2); steroidal estrogen concentration-response curves that have been normalized to E2 β (Figure SI-3); gene expression from *in vivo* medaka exposure (Table SI-5); and transactivation of pVTGs compared to synthetic ERE with the three mERs (Figure SI-4). This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Endocrine active compounds (EACs) are exogenous compounds that alter function of the endocrine system, with the potential to cause adverse effects on individuals, their progeny, or their subsequent progeny.¹ Many EACs have been detected in surface waters throughout the United States, and have become of increasing concern over the past few decades due to impacts on fish populations.^{2–6} Estrogenic EACs, a subclass of EACs, comprise a multitude of chemical classes including: natural estrogens (e.g., 17 β -estradiol [E2 β] and isoflavone), synthetic estrogens (e.g., 17 α -ethynylestradiol and diethylstilbestrol) and estrogen mimics (e.g., nonylphenol and bisphenol A).⁷ Surface waters contaminated with estrogenic EACs have been linked to a number of adverse effects in fish, including the aberrant expression of vitellogenin (VTG), a female specific egg yolk protein, in male fish.^{8–11} Continuous exposure to estrogenic contaminants may result in feminization and/or demasculinization within teleost populations, which may be linked to decreased reproductive output, compromised immunity, altered sex ratios, and ultimately population collapse.^{12–17} Given the potential risk to population, community and ecosystem sustainability, it is critical to gain a better understanding of the molecular initiating events (MIE) leading to adverse effects following exposure to estrogenic EACs.

Estrogenic EACs predominately mediate molecular, biochemical and ultimately physiological activities through the modulation of estrogen receptor (ER) signaling. ERs belong to a superfamily of nuclear receptors that regulate multiple cellular and physiological functions, ranging from bone growth to reproductive maturation.¹⁸ Nuclear receptors are ligand-dependent transcription factors, which facilitate cellular responses to endogenous and exogenous ligands by coordinating complex transcriptional responses.¹⁹ Classically, ligand activated ERs homodimerize, translocate into the nucleus, bind to estrogen response elements (EREs) and facilitate recruitment of co-regulators that govern gene transcription. Non-classically, ERs may heterodimerize with other transcription factors, interact with other DNA response elements, undergo ligand independent transactivation or mediate non-genomic signaling via membrane bound ERs.^{18,20–22} The focus of this study is the classical pathway of gene regulation.

In humans, there are two functional ER subtypes (α and β) that have distinct tissue distribution and physiological roles.^{18,23} In spite of ER β arising from a genome duplication of ER α , ligand selectivity and specificity have diverged between the two receptors.^{21,24–27} Studies also illustrate that target specificity of mammalian ER α and ER β can be further enhanced through preferential interactions with specific EREs.^{21,26} In comparison to humans, teleost fish express at least three ERs (α , β 1, and β 2), with the second ER β paralog arising from a subsequent fish-specific genome duplication event.^{28–32} Similar to mammalian ERs, ligand binding assays indicate that teleost ER subtypes exhibit differential ligand selectivity and specificities, suggesting that receptor subfunctionalization has occurred.^{33–36} Additionally, teleost ER subtypes exhibit distinct tissue (e.g. liver, gonads, brain, muscles, kidney) distribution patterns^{28,30,37–41} and dissimilar tissue specific induction/repression patterns following estrogen exposure.^{37,38,42–44} Although a breadth of knowledge has accumulated since the discovery of a third ER in teleost fish, the respective

function of all ER subtypes in transactivation of estrogen responsive genes remains equivocal.

VTG is perhaps the most widely used biomarker of estrogen exposure in oviparous species.^{45–51} Studies in numerous fish species have shown that VTG induction is accompanied by a sharp increase in hepatic ER α expression, with little change in hepatic ER β expression,^{40,43} implying that ER α may be the principle receptor mediating VTG gene induction. Studies in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) suggest that ER β subtypes may play a supporting role in VTG induction by inducing the up-regulation of ER α .^{52,53} Other studies have postulated that ER α is essential for initiating induction of VTG and other estrogen responsive genes (e.g. the egg envelope protein choriogenin [CHG]), while ER β subtypes are necessary for sustaining and enhancing gene expression.^{54,55} These emerging models suggest that ER β subtypes may be critical to vitellogenesis in the normal reproductive cycle of females, as well as to VTG/CHG induction in male fish that are exposed to estrogenic EACs. Yet, to date, few studies have demonstrated a direct role of ER β subtypes in regulating VTG transcriptional activation.

This study seeks to elucidate the roles of the three ER subtypes in driving the transactivation of estrogen responsive genes, using Japanese medaka (*Oryzias latipes*) as a model organism, and VTG as a model gene. Medaka and other teleost fish have two VTG transcripts (VTGI and VTGII), resulting from a genome duplication event.⁵⁶ The roles of medaka (m) ER α , mER β 1 and mER β 2 in driving VTG expression were investigated in a series of transient transactivation assays using putative promoter/enhancer regions of the VTGI or VTGII gene. A VTG promoter/enhancer deletion analysis was also conducted to assess regulatory roles of putative EREs found within VTGI and VTGII promoters. Finally, in order to better examine the putative *in vivo* roles of ER subtypes following estrogen exposure, cluster analysis was used to assess correlations between VTG expression and co-activity/co-expression of ER subtypes. *In vivo* data used in this analysis was derived from a previous publication from our group.⁴⁴ For all assays, test compounds included four steroidal estrogens that are commonly detected in wastewater effluents: 17 β -estradiol (E2 β), estrone (E1), estriol (E3), and 17 α -estradiol (E2 α).

EXPERIMENTAL METHODS

Chemicals

Standards of steroidal estrogens (E2 β , E1, E2 α , and E3) were purchased from Steraloids Inc. (Newport, Rhode Island) and made into 10 mM stock solutions in 100% ethanol (EtOH). Stocks were serially diluted to 1 mM, 0.1 mM, 0.01 mM and 0.001 mM and used across all experiments. All solutions were stored in 2 mL glass amber vials with PTFE-lined solid lids (Thermo Scientific, Waltham, MA), at –20 °C in order to preserve chemical integrity.

Constructs

Medaka ER subtypes were originally received as a generous gift from Dr. Taisen Iguchi (National Institute for Basic Biology, Japan) in pCDNA3.1 vector.³⁸ Each receptor was

further subcloned as full-length open reading frame from the ATG start to the TAG stop sequence in the pSG5 vector (details are provided in Table SI-1). Three kb of the VTGII promoter (pVTGII) from –3005 to +1 of the VTGII translational start site was received as a generous gift from the Dr. Zhiyuan Gong (National University of Singapore, Singapore) in pEGFP⁵⁷. Note this promoter/enhancer was originally described as VTGI regulator, but a refined analysis by our laboratory indicates its position upstream of VTGII within the current medaka genome. Assessment of an established transgenic line with the 3Kb VTGII-eGFP construct in medaka demonstrates concurrent expression of GFP and VTG *in vivo*, illustrating the regional regulation of VTG expression.⁵⁷ The VTGII promoter fragment was further subcloned into pGL4.10 vector using XhoI and HindIII restriction enzymes. To isolate the medaka VTGI promoter (pVTGI), Advantage® 2 polymerase chain reaction (PCR) long distance protocol (Clontech Laboratories Inc, Mountain View, CA) was used to isolate a 3.2 kb fragment –3243 to +1 of the VTGI translational start site from a medaka bacterial artificial chromosome clone (ola-068M06, NIBB/NBRP Medaka, Japan). Primers for pVTGI cloning were designed using Primer3^{58,59} with overhanging restriction enzyme sites (Supporting Information, Table SI-2). Amplicons from PCR reactions were cloned into pCR™2.1-TOPO vector using TOPO® Cloning Reaction Protocol (Life Technologies, Grand Island, NY). Promoter fragments were subsequently subcloned using restriction enzymes (KpnI, XhoI) into the pGL4.10 *Photinus pyralis* (firefly) luciferase reporter vector (Promega, Madison, WI).

Transient Transfection

HeLa cells (human cervical adenocarcinoma cells) were used due to the absence of endogenous ERs.⁶⁰ Cells were maintained in phenol red-free Dulbecco's Modified Eagle Medium (DMEM; Corning Inc, Corning, NY) fortified with 10% vol/vol fetal bovine serum (FBS; Corning Inc), 2.0mM L-glutamine (Corning Inc), and 1% antibiotic/antimycotic (Sigma- Aldrich, St. Louis, MO). During the assay, cells were maintained in hormone-free media containing 10% dextran/charcoal striped FBS (DCC-FBS; Corning Inc). Cells were seeded at a density of 10⁵ cells per well in 96-well plates (Corning Inc) and allowed to attach overnight in 37°C incubator with 5% CO₂ and humidity. Cells were then transfected with 50 ng of pGL4-luc reporter construct (pVTGs-firefly) and 100 ng of pSG5-ER (mER α , mER β 1 or mER β 2) using Lipofectamine 2000 (Life Technologies, Grand Island, NY). To control for variations in transfection efficiency, 20 ng of pRL-tk-luc (Promega), a *Renilla reniformis* (renilla) luciferase gene with constitutively active herpes simplex virus thymidine kinase promoter was co-transfected. Twenty-four hours post-transfection, cells were dosed with compounds of interest or solvent control (EtOH), the amount EtOH per well did not exceed 0.1% EtOH. Following a 24-hour dosing period, luciferase activity was determined using a Dual-Glo Luciferase Assay System (Promega) and FLOUstar Omega Filter-based multi-mode microplate reader (BMG Labtech, Ortenberg, Germany). Luciferase readings were initially normalized to *Renilla* luciferase to obtain firefly:renilla ratio. This ratio was further normalized to EtOH response (dividing by ETOH firefly:renilla ratio) to obtain transactivation.

Transactivation of mERs

The capacity of steroidal estrogens to transactivate mERs was assessed by transient transfection assay described above. Assays were conducted with E2 β , E1, E3 or E2 α concentrations ranging between 0.0001 and 10,000 nM. Sigmoid concentration-response curves were generated for each compound with all receptor/reporter pairs. Transactivation was plotted against log transformed concentration and fitted to the following symmetric logistic function,

$$y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\text{HillSlope}(\text{LogEC}_{50} - \text{Log}x)}} \quad (\text{Eq. 1})$$

with y as transactivation, x as concentration and bottom values constrained to 1, using Prism 5.0 software (GraphPad, La Jolla, CA; note: top values were not constrained because of difference in ligand/receptor efficacies). Steroidal estrogen responses were expressed as concentration that evoked half the maximum response (EC₅₀). The maximum efficacy (E_{MAX}; i.e., top) of each compound is additionally reported for each compound/receptor/reporter combination. Each compound-dose was run as three technical replicates/plate, and each experiment was repeated 3–4 times.

Relative Potency of Steroidal Estrogens and Receptors

To compare potency between compounds with different E_{MAX} values, data was further normalized to top and bottom of the E2 β concentration-response curve for each receptor by calculating percent induction as described for yeast estrogen screen in a previous study,⁴⁴

$$\text{Percent Induction} = \frac{y - \text{Bottom}_{\text{E2}\beta}}{\text{Top}_{\text{E2}\beta} - \text{Bottom}_{\text{E2}\beta}} \times 100\%, \quad \text{Eq. 2}$$

with y as transactivation. Percent induction was plotted against log transformed concentration, which was fitted to Eq. 1, with bottom constrained to 0. Percent induction of E1 and E2 α did not exceed 50%. To account for the range in E_{MAX} among the compounds, the concentration that produced 20% response (EC₂₀) was used to calculate the potency relative to E2 β (relative estrogenic potency, or REP),

$$\text{REP} = \frac{\text{EC}_{20} \text{ of E2}\beta}{\text{EC}_{20} \text{ of } x} \times 100. \quad \text{Eq. 3}$$

To compare potency of the four steroidal estrogens across the three ER subtypes, EC₂₀ was used to calculate the potency at each receptor relative to mER α (mER α to mERx ratio),

$$\text{mER}\alpha:\text{mER}x = \frac{\text{EC}_{20} \text{ of mER}\alpha}{\text{EC}_{20} \text{ of mER}x}. \quad \text{Eq. 4}$$

VTG Promoter Deletion Analysis

Estrogen response elements (EREs) within both medaka VTGI and VTGII promoter/enhancer regions were identified using NUBIscore V2.0 (University of Basel, Basel, Switzerland). The location of eight inverted repeat 3 (IR3) response elements were identified within the cloned 3.2 kb fragment of the pVTGI promoter and eleven IR3 response elements were identified within the cloned 3.0 kb fragment of the pVTGII promoter (Table SI-3). Putative EREs were used to establish deletion constructs comprising 100%, 50% and 25% of the ~3kb proximal regulatory sequence of each pVTG via PCR, using Advantage® 2 PCR kit protocol and primers designed in Primer3 (Table SI-2). Amplified regions of each promoter were cloned into pCR™2.1-TOPO vector (as described above) and subsequently subcloned into pGL4.10. The VTGI 100%, 50% and 25% promoters contained eight, six and four putative EREs, respectively. The VTGII 100%, 50% and 25% promoter contained eleven, seven and three putative EREs, respectively (Figure SI-1). Transactivational capacities of mERs with promoter fragments were assessed by transient transfection assay described above. Transactivational analysis was conducted with 1,000 nM of each steroidal estrogen to ensure maximal induction, and data was analyzed as fold transactivation. Each compound was run in triplicate/plate, and mean fold transactivation was calculated based on 2–3 assay replicates.

Assessment of Co-expression and Co-activation of mER subtypes with VTG

In a previous study,⁴⁴ our group conducted an analysis of *in vivo* hepatic gene expression in male medaka, in which the fish were exposed to four different steroidal estrogen treatments: 0.64 nM E2 β , 1.42 nM E1, 89.20 nM E3, and 21.59 nM E2 α , as well as a 0.01% EtOH control. The expression of several estrogen-responsive genes was evaluated using quantitative polymerase chain reaction (qPCR), including VTGI and VTGII. See Supporting Information for more details. To better assess *in vitro* co-activation of ER subtypes and *in vivo* co-expression of VTG, transactivation assays were repeated using the same ligand concentrations that were used in the *in vivo* exposures. This was done under the assumption that estrogen concentrations at the cellular level in fish were directly related to the exposure concentrations in water. Although this assumption does not take potential ADME (absorption, distribution, metabolism, and excretion) effects into account, we can only assume that the effects observed in fish were related to the exposure concentration.

Transactivation assays were conducted with each mER (mER α , mER β 1, mER β 2) and ~3kb VTGI or VTGII reporter constructs. Each compound was run in triplicate/plate and mean transactivation was calculated based on 2–3 assay replicates. Mean fold transactivations were then used in a bioinformatics summary described below.

Statistical Analysis

Graphs were developed and statistical analysis was conducted in Prism 5.0. Shapiro-Wilk test and Bartlett's test were used to test data for normality and equal variances, respectively. Transactivation from promoter deletion analysis and *in vitro/in vivo* comparison failed the normality test, so Wilcoxon pairwise analysis was conducted to determine effects of treatment and promoter region on transactivation. Two-way hierarchical clustering (Ward's method) of *in vitro* transactivation and *in vivo* gene expression from medaka exposure was

conducted using JMP Pro 12 statistical software (SAS, Cary, NC). Cluster analysis included five treatments (0.01% EtOH, 0.64 nM E2 β , 1.42 nM E1, 89.20 nM E3 and 21.59 nM E2 α), *in vitro* transactivation of three ERs (mER α , mER β 1, and mER β 2) and *in vivo* expression of five genes (mER α , mER β 1, mER β 2, mVTGI and mVTGII).

RESULTS

Transactivation of mERs

Analysis of mER subtype activity with EtOH revealed that background transactivation was not significantly different (F-test, $p > 0.05$) among three mER subtypes for both VTGI and VTGII (Figure SI-2). Assessment of ER transactivation revealed that each steroidal estrogen (E2 α , E2 β , E1 and E3) transactivated all three receptor subtypes (mER α , mER β 1, mER β 2) with both pVTGI and pVTGII reporter constructs (Figure 1). Consistently, E2 β exhibited the highest potency of the four steroidal estrogens regardless of receptor subtype (Table 1). E1 was generally the least potent compound. There was a noted exception: E1 and E2 α exhibited similar potency in mER β 2 transactivation with both VTG reporters. Similarly, E3 and E2 α exhibited similar potency in transactivation of mER α and mER β 1 with both VTG reporters.

Analysis of E_{MAX} (Figure 1 and Table 1) revealed that regardless of ligand or pVTG reporter, the transactivational activity of mER β 2 was consistently greater than mER β 1 and mER α receptor subtypes. Similarly, E_{MAX} of mER β 1 was larger than mER α , independent of ligand or reporter. Within each receptor, E_{MAX} values suggests that steroidal estrogens functioned as either full or partial agonist. In comparison to the efficacy of E2 β (considered a full agonist), E1, E3 and E2 α were generally partial agonists. There were a few exceptions: E1 exhibited full agonist activity with mER β 1-pVTGI; E3 exhibited full agonist activity with mER α -pVTGI, mER α -pVTGII, and mER β 1-pVTGII; and E2 α exhibited full agonist activity with mER α -pVTGI.

Relative Potency of Steroidal Estrogens and Receptors

To compare the potency among the four steroidal estrogens and three receptors with different E_{MAX} values, data was normalized to E_{MAX} of E2 β within each receptor subtype (Figure SI-3) and EC_{20} values (Table 2) were used as described in experimental methods. Assessment of REPs revealed that E1, E3, and E2 α were less potent than E2 β across all three receptors and with both pVTGI and pVTGII reporters (Table 2). Rank order of potency were: E2 β > E2 α $\approx$ E3 > E1 for mER α ; E2 β > E3 $\approx$ E2 α > E1 for mER β 1; and E2 β > E3 > E2 α $\approx$ E1 for mER β 2. Analysis of compound potency with each receptor relative to mER α (mER α :mER x) revealed that the potency of E2 β and E3 was 3.0–17.3 times greater with the mER β s relative to mER α . In contrast, the potency of E1 and E2 α was similar or lower with the mER β s relative to mER α , with one exception: with pVTG1, the potency of E2 α was 6.7 times greater with mER β 1 relative to mER α .

VTG Promoter Deletion Analysis

Transfection studies with VTG promoter deletion constructs (Figure SI-1) for pVTGI indicates that all three promoter constructs (100%, 50% and 25%) were active with each ER

subtype and each ligand tested (Figure 2A–C) with one exception: E2 α did not transactivate mER α -50%pVTGI. Two general patterns of transactivation emerged among the promoter/enhancer regions of pVTGI. The most common pattern was 25%pVTGI < 50%pVTGI < 100%pVTGI, in which transactivation with 25%pVTGI was significantly lower than 100%pVTGI, but 25%pVTGI and 100%pVTGI were not significantly different from 50%pVTGI. The other pattern was 25%pVTGI $\approx$ 50%pVTGI, with both being significantly lower than 100%pVTGI. There were two exceptions to these patterns. For E2 α -mER α , transactivation with 50%pVTGI was significantly lower than 100%pVTGI, and transactivation of both 50%pVTGI and 100%pVTGI were not significantly different from 25%pVTGI (Figure 2A). For E1-mER β 1, transactivation with 25%pVTGI was significantly lower than both 50%pVTGI and 100%pVTGI (Figure 2B).

Transactivation data for pVTGII demonstrates that only 100% and 50% promoter constructs were active, with the following exceptions: E2 β -mER α , E3-mER β 2 and E2 α -mER β 2 were active with the 25%pVTGII (Figure 2D–F). For most receptor/ligand combinations, a general pattern was observed in which transactivation with 50%pVTGII was lower than 100%pVTGII (although generally not significantly lower), and both were significantly greater than 25%pVTGII.

Assessment of Co-expression and Co-activation of mER subtypes with VTG

Our group has previously published an analysis of the *in vivo* response of male medaka to estrogenic ligands, including E2 β , E1, E3, and E2 α .⁴⁴ Gene expression results from this study are summarized in Table SI-5. To provide an assessment of co-expression and co-activation, a second set of transactivation assays were conducted using the same steroidal estrogen concentrations that were utilized in the *in vivo* medaka exposures. In these assays, fold transactivation was generally larger with E2 β and E3 compared to E2 α and E1, independent of receptor and pVTG reporter (Table 3). In addition, among the three receptors, fold transactivation was consistently larger with ER β 2, followed by ER β 1.

Two-way hierarchical cluster analysis was conducted to establish putative relationships between gene expression following steroidal estrogen treatments (Table SI-5) and transactivation of mER subtypes (Table 3). For pVTG1, cluster analysis of steroidal estrogen treatment groups resulted in two empirical clusters of C1 [E2 β and E3 treatments] and C2 [E1, E2 α treatments and EtOH]. Within C2, E1 and E2 α formed a sub-cluster independent of EtOH (Figure 3A). Hierarchical clustering of data generated using the VTGII reporter (Figure 3B) resulted in three empirical clusters of C1 [E2 α , E1 and E2 β], C2 [EtOH] and C3 [E3] and within C1, E2 α and E1 formed a sub-cluster independent of E2 β .

The organization of individual “assay activities” (fold induction of *in vivo* gene targets and *in vitro* transactivation of the mER subtypes) within VTGI and VTGII clusters were identical. Results demonstrate two empirical clusters of C1 [*in vivo* expression of mER α , mVTG, and transactivation (T) of T-mER α , T-mER β 1, T-mER β 2] and C2 [*in vivo* expression of mER β 1, mER β 2]. Within C1, three sub-clusters were present SC1 [*in vivo* expression of mVTG and T-mER β 2], SC2 [T-mER α and T-mER β 1] and *in vivo* expression of mER α clustered independent of all other activities in C1.

DISCUSSION

To elucidate the differential roles of teleost ER subtypes in estrogen-induced gene induction, we investigated the transactivation capacity of three mER subtypes, using VTGI and VTGII as prototypic target genes. Through the use of novel luciferase reporter constructs incorporating 3 kb upstream regions of mVTGI and mVTGII genes, we illustrate distinct ligand potency and receptor efficacy patterns among three mER subtypes. To our knowledge, this is the first study to demonstrate interaction and functionality of all three ER subtypes with tangible promoter/enhancer regions of the VTGI and VTGII genes, which contain multiple EREs.

For VTGI and VTGII, our results show that mER β 2 exhibits greater efficacy than mER β 1 and mER α . This pattern was consistent across all four steroidal estrogens analyzed (E2 β , E1, E3 and E2 α), suggesting that mER β 2 may play a larger role in up-regulation of VTGs genes. This observation is similar to previous studies examining transactivational activity of medaka, tilapia and zebrafish ERs, which found that efficacy was greater with ER β 2 relative to ER β 1 and ER α .^{38,42,61} However, other species of fish, such as largemouth bass and carp, exhibit an opposite pattern of receptor activity, with ER α having a greater efficacy compared to ER β s.^{29,30} This discrepancy implies that interspecies differences may exist with regards to ER subfunctionalization.

We also observed that ligand potencies remained relatively consistent across each of the three mERs and across both of the pVTG reporters, with E2 β consistently found to be the most potent ligand. This was followed by E3 and E2 α , which were frequently equipotent. The least potent estrogen was often E1. This is consistent with steroidal estrogen activities in carp,³⁰ where E1 was generally the least potent or equal in potency to E3. Conversely, E1 has been shown to be equipotent to E2 β in roach,⁶² further implying that ER subfunctionalization may not have co-evolved across teleost fish.

Although we observed general trends in potency among the four steroidal estrogens, our results also suggest that there are distinct activity groupings among the estrogens with respect to the different ER and promoter subtypes. In almost all cases, E2 β and E3 exhibited greater potency with mER β 1 and mER β 2 than with mER α . This coincides with ligand affinity data in medaka and Atlantic croaker (*Micropogonias undulatus*),^{33,44} which found that E2 β and E3 had higher affinity for the ER β s compared to ER α . Furthermore, E1 and E2 α had similar transactivation patterns, having equivalent or lower potencies with mER β 1 and mER β 2 relative to mER α . Ligand binding data also illustrate that both compounds have greater affinity for ER α than the ER β s.^{33,44} Possibly, the estrogens within these two pairs (E2 β /E3 and E1/E2 α) elicit similar structural and transactivational modifications (such as recruitment of co-regulators) to mERs that result in comparable functional activities. Tohyama et al.⁶³ illustrate that binding to specific residues within the binding pocket of mERs confers ligand-specific activity. Similarly, through promoter deletion analysis we revealed that ligand-receptor pairs utilize specific and distinct regions of DNA regulatory regions, and this varied between VTGI and VTGII. In humans, ER subtypes bind to specific and sometimes different chromatin binding regions,²⁶ and binding to specific EREs is proposed to effect co-regulator recruitment and receptor configurations, ultimately leading to

enhanced/repressed transcriptional activity.²¹ A similar mechanism may explain differential and specific transactivational activities among the mER subtypes. Further investigation of co-regulator recruitment and chromatin binding assays may shed more light on the divergences among the three ER subtypes.

Ultimately, we set out to investigate the respective roles of teleost ER subtypes following estrogen exposure. Our bioinformatics analysis indicated a strong association between transactivational activity of mER β 2 and *in vivo* VTG expression levels. In addition, *in vivo* VTG expression was clustered, although not as closely, with expression levels of mER α and transactivation activity of mER α and mER β 1. This corroborates previous studies illustrating that both ER α and ER β s are necessary for vitellogenesis in medaka, zebrafish and goldfish.^{52–54} Yamaguchi and colleagues also illustrate that all three mER subtypes are capable of regulating mVTG and mCHG expression, in a study which used mER α - and mER β -selective agonists (orthoester-2k and 2-(4-hydroxyphenyl)-5-hydroxy-1,3-benzoxazole [HPHB], respectively).^{54,55}

Several recent studies have proposed hypotheses on the respective roles of ER α and ER β in the up-regulation of estrogenic biomarkers in teleost fish. Yamaguchi and colleagues propose that mER α plays a role in initiating the expression of mVTGII and mCHG-Heavy (H), while mER β s enhance and sustain expression of these genes.^{54,55} Using increasing concentrations of orthoester-2k and a constant concentration of HPHB, they illustrate that the mER β significantly enhances the up-regulation of mCHG-H and mVTGII by mER α . They document the inverse results with mCHG-Light (L), suggesting mER α may enhance the up-regulation of mCHG-L by mER β .^{54,55} Other studies have proposed that hepatic ER β s play a role in up-regulating the expression of ER α upon estrogen stimulation, and thus are important for priming hepatocytes for vitellogenin production.^{52,53} Our bioinformatics and transactivational assessments with mVTGI and mVTGII, suggest that indeed all three ERs are likely involved in this process.

In our previous study,⁴⁴ we demonstrated that *in vivo* exposure to steroidal estrogens results in a large significant increase in mER α gene expression, and a smaller but significant decrease in mER β s. This occurred simultaneously with an increase in mVTG and mCHG, similar to findings in several other fish species.^{37,43,64–66} This is reflected by the correlation between expression levels of mER α and mVTGs, observed in the cluster analysis. The fact that mER α exhibits significant yet low transactivational activity with endogenous estrogenic steroids, is up-regulated simultaneously with mVTG, and clusters close to VTG expression, could support the hypothesis that mER α is associated with the initial induction of VTG. Furthermore, the high transactivation capacity of ER β s (especially ER β 2) and clustering of mER β 2 transactivation to mVTG expression, could support the notion that mER β s (possibly just mER β 2) maintain the ability to directly sustain and/or enhance expression of VTGs. Additional studies, perhaps using selective mER α - and mER β -specific agonists, would be necessary in order to fully delineate these roles. Regardless, this combined evidence suggests that VTG up-regulation is likely driven by interplay between these multiple ER subtypes.

The patterns of receptor-based ligand potency and efficacy observed here and in other recent studies also implies that classical methods of screening surface waters for estrogenic activity may not be adequate for the ecological assessment of fish health. Estrogenic activity of environmental media is often assessed using bioassays such as the yeast estrogen screen, T47D-KBluc, and E-Screen. Each of these assays report activation of human ERs, and often only utilize ER α .^{46,67–70} Our observations suggest that mammalian ER based assays may not recapitulate estrogenic responses in teleost. For example, the four estrogens tested in this study generally function as full agonists in mammalian cell-based estrogen screening assays.^{71,72} In contrast, we demonstrate that E1, E3, and E2 α generally functioned as partial agonists in the medaka model. With regards to the potencies of estrogenic ligands, most mammalian ER studies report a rank order of E2 β >E1>E2 α >E3.^{35,71,72} In contrast, we found that E1 was generally the least potent of these steroidal estrogens across all three mERs. This supports a divergence between mammalian and teleost ERs as demonstrated by Davis et al.⁴² These differences in ligand potency and efficacy, in conjunction with the lack of conservation with selective ER agonists and antagonists between mammalian and teleost models, suggest that ER responsiveness may have diverged over the course of teleost-mammalian ER evolution.

Another factor that may impact the predictive capabilities of ER-based screening assays is that fish species may differ from one another with regards to ER activity. As discussed above, studies in other fish species have observed ligand potencies and ER efficacies that contrast with our observation in medaka. This is corroborated by observations by Lange et al.,⁷³ who documented differences in sensitivities to five estrogens (E2 β , E1, E3, 17 α -ethynylestradiol and diethylstilbestrol) among six fish species (medaka, carp, zebrafish, fathead minnow [*Pimephales promelas*], roach and stickleback [*Gasterosteus aculeatus*]), using in vitro and in vivo models. Taken together, these variations suggest that interspecies differences may exist in the activity of teleost ERs, suggesting that assays using ERs from a single species may not be sufficient in assessing potential risk to fish populations and communities.

A third factor that may impact the predictive capabilities of ER-based screening assays is the use of synthetic EREs in these assays. As stated previously, our study employed transactivation assays that were regulated by endogenous VTG gene promoter sequences, while other recent studies have employed transactivation assays that are regulated by synthetic EREs. We observed that the efficacy of steroidal estrogens in our assays was considerably greater than that observed in other recent studies examining ER transactivation.^{30,38,42,61,65} To further assess these differences, we performed a follow-up experiment in which we evaluated the transactivation of all three mERs using a synthetic ERE reporters in the presence of E2 β (data in Supporting Information). Analysis revealed that for all three mER subtypes, efficacy was larger with both pVTG reporters relative to synthetic ERE (Figure SI-4). This provides suggestive evidence that reporter assays driven by synthetic EREs may be underestimating functional activity. This is yet another factor that may impact *in vitro* to *in vivo* extrapolation for estrogenic EACs, and another limitation of the *in vitro* assays that are classically used to evaluate estrogenic activity.

In sum, we employed novel transactivation assays and provide evidence that all three mERs have the capacity to regulate mVTG expression. Efficacy and potency patterns suggest subfunctionalization occurred among the mER subtypes, which may be critical in initiation and enhancement of estrogen responsive genes. Through this novel approach, we were able to enhance the current understanding of MIEs following estrogen exposure in medaka. Simultaneously, we shed light on several potential concerns over using classical screening assays to evaluate estrogenic activity in surface water: discrepancies between mammalian and piscine ER subtypes, interspecies differences within fish populations, and potential inefficiencies with synthetic EREs. These variables should be kept in mind when considering the hazards posed to fish populations by estrogenic EACs. Given possible interspecies discrepancies in ER subtypes (among teleost fish and between mammals) and growing knowledge on the ER β s activity, there is a necessity to re-examine current toxicity testing methods used in ecological assessments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Steroidal estrogen regulated fold transactivation of medaka estrogen receptor alpha (mERα; A and D), mERβ1 (B and E) and mERβ2 (C and F) with vitellogenin promoter I (pVTGI; A–C) and pVTGII (D–F). Transcriptional activity of 17β-estradiol (E2β – solid black line with closed circles), estrone (E1 – solid dark grey line with squares), estriol (E3 – dotted light grey line with triangles) and 17α-estradiol (E2α – dashed black line with open circles) show differential patterns.

Figure 2.

Mean ($\pm$ standard error) fold transactivation of 25% (white), 50% (white with diagonal stripes) and 100% (black) of pVTGI (A–C) and pVTGII (E–F) Promoters with medaka subtypes. Transcriptional activity of medaka ER α (A and D), ER β 1 (B and E) and ER β 2 (C and F) generated with fixed concentrations of 17 β -estradiol (E2 β), estrone (E1), estriol (E3) and 17 α -estradiol (E2 α). Letters denote a significant difference (Wilcoxon post hoc test, $p < 0.05$) among promoter regions within each treatment for each receptor. Asterisks denote significant difference (Kruskal-Wallis, $p < 0.05$) of compound from EtOH treatment for each region.

Figure 3.

Hierarchical clustering analysis of genes analyzed and fold transactivation (T) for VTGI (A) and VTGII (B) from medaka exposure. Relative up-regulation of genes (*in vivo*) and high transactivation are expressed from high (grey) to low (black) relative to each parameter. A two-way cluster was conducted with the five treatments: 17β-estradiol (E2β), estrone (E1), estriol (E3) and 17α-estradiol (E2α) and activity: gene expression and transactivation using Ward's method. C# and SC# denote clusters numbers and sub-cluster numbers.

Table 1

Mean (shown in bold) potency (EC₅₀) and efficacy (E_{MAX}) from dose response curves of steroidal estrogens, with 95% confidence interval (shown in italics)

	E2β	E1	E3	E2α
mERα	1.1 (0.6–2.1)	73.8 (38.0–143.2)	27.8 (10.4–74.2)	18.3 (8.8–38.2)
pVTGI mERβ1	0.1 (0.1–0.2)	37.5 (14.6–96.2)	1.4 (0.8–2.6)	3.9 (1.4–11.2)
mERβ2	0.2 (0.1–0.5)	45.9 (21.4–98.4)	5 (3.2–7.9)	37.2 (20.0–69.2)
mERα	3.3 (1.4–7.5)	120 (31.4–458.3)	36.4 (20.4–64.9)	18.1 (6.7–48.9)
pVTGII mERβ1	0.2 (0.1–0.5)	57.7 (30.6–108.8)	1.6 (0.8–2.9)	6.3 (4.4–9.2)
mERβ2	0.1 (0.1–0.3)	66.7 (37.4–119.0)	3.7 (1.8–7.5)	64.9 (45.0–93.5)
mERα	7.2 (6.3–8.1)	4 (3.5–4.5)	6.2 (5.0–7.4)	6.6 (5.6–7.6)
pVTGI mERβ1	19.9 (18.4–21.5)	17.6 (15.0–20.2)	13.6 (12.3–14.9)	13.1 (10.5–15.6)
mERβ2	217.5 (186.7–248.4)	154.7 (133.1–176.2)	155 (137.6–172.3)	115.3 (94.0–136.5)
mERα	14.5 (12.4–16.5)	7.7 (5.8–9.7)	12.2 (10.0–14.5)	9.9 (7.4–12.4)
pVTGII mERβ1	28.6 (24.9–32.3)	17 (14.9–19.1)	23.1 (20.4–25.8)	15.1 (13.5–16.7)
mERβ2	165.6 (143.6–187.7)	98.8 (87.5–110.0)	107.8 (85.5–130.1)	109.9 (90.4–129.5)

mER: medaka ER. E2β: 17β-estradiol. E1: estrone. E3: estriol. E2α: 17α-estradiol. pVTG: vitellogenin promoter. EC₅₀: 50% maximal response concentration. E_{MAX}: maximum efficacy.

Table 2

Relative estrogenic potency (REP) and mER α :mERx ratio for steroidal estrogens at each mER subtype and VTG promoter construct.

	EC ₂₀			REP			mER α :mERx		
	mER α	mER β 1	mER β 2	mER α	mER β 1	mER β 2	mER α	mER β 1	mER β 2
E2 β	0.31 (0.20–0.53)	0.04 (0.02–0.04)	0.03 (0.02–0.08)	100	100	100	1.0	7.1	10.6
E1	17.69 (12.09–38.21)	5.38 (3.23–8.09)	9.20 (5.56–14.06)	1.8	0.8	0.3	1.0	3.3	1.9
E3	6.24 (4.24–13.23)	0.46 (0.26–0.62)	2.10 (1.21–2.85)	5.0	9.5	1.4	1.0	13.5	3.0
E2 α	4.23 (2.38–5.45)	0.63 (0.46–1.71)	11.05 (5.57–11.22)	7.4	7.0	0.3	1.0	6.7	0.4
E2 β	0.81 (0.52–1.44)	0.08 (0.05–0.13)	0.05 (0.03–0.11)	100	100	100	1.0	10.7	17.3
E1	20.10 (15.37–65.40)	30.17 (23.01–97.01)	22.67 (12.49–27.83)	4.0	0.3	0.2	1.0	0.7	0.9
E3	12.98 (6.88–14.62)	0.87 (0.39–0.71)	2.30 (1.80–8.17)	6.3	8.8	2.0	1.0	15.0	5.6
E2 α	7.44 (4.37–10.58)	2.75 (1.14–1.95)	37.12 (15.66–27.09)	10.9	2.8	0.1	1.0	2.7	0.2

EC₂₀: 20% maximal response concentration. REP: relative estrogenic potency.

Mean ($\pm$ standard error, italicized) fold transactivation of medaka estrogen receptors, at the exposure concentrations used for comparison with in vivo data.*

Table 3

Treatment	VTGI			VTGII		
	mER α	mER β 1	mER β 2	mER α	mER β 1	mER β 2
0.64 nM E2 β	4.6 (1.9) ^{a,b}	15.5 (3.3) ^a	106.4 (23.0) ^a	3.2 (1.1) ^{a,c}	42.0 (5.7) ^a	160.0 (28.8) ^a
1.42 nM E1	2.1 (0.7) ^{b,c}	2.4 (0.5) ^b	3.2 (0.8) ^b	1.3 (0.4) ^{b,c}	2.1 (0.8) ^b	3.2 (0.6) ^b
89.20 nM E3	8.6 (2.4) ^a	17.9 (3.2) ^a	130.7 (25.0) ^a	12.2 (2.4) ^b	112.6 (38.9) ^a	169.8 (28.5) ^a
21.59 nM E2 α	3.7 (0.9) ^{a,b}	11.2 (2.3) ^a	15.1 (3.5) ^f	2.2 (0.4) ^a	25.0 (8.8) ^a	29.9 (6.5) ^f
0.01% EtOH	1.0 (0.1) ^f	1.0 (0.2) ^b	1.0 (0.1) ^d	1.0 (0.2) ^f	1.0 (0.1) ^b	1.0 (0.1) ^d

* Letters denote significant differences (Wilcoxon post hoc test, $p < 0.05$) among treatments for each receptor/reporter pair (along the column).