Visualization of Estrogen Receptor Transcriptional Activation in Zebrafish

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Estrogens regulate a diverse range of physiological processes and affect multiple tissues. Estrogen receptors (ERs) regulate transcription by binding to DNA at conserved estrogen response elements, and such elements have been used to report ER activity in cultured cells and in transgenic mice. We generated stable, transgenic zebrafish containing five consecutive elements upstream of a c-*fos* minimal promoter and green fluorescent protein (GFP) to visualize and quantify transcriptional activation in live larvae. Transgenic larvae show robust, dose-dependent estrogen-dependent fluorescent labeling in the liver, consistent with *er* gene expression, whereas ER antagonists inhibit GFP expression. The nonestrogenic steroids dexamethasone and progesterone fail to activate GFP, confirming ER selectivity. Natural and synthetic estrogens activated the transgene with varying potency, and two chemicals, genistein and bisphenol A, preferentially induce GFP expression in the heart. In adult fish, fluorescence was observed in estrogenic tissues such as the liver, ovary, pituitary gland, and brain. Individual estrogen-responsive neurons and their projections were visualized in the adult brain, and GFP-positive neurons increased in number after 17β-estradiol exposure. The transgenic estrogen-responsive zebrafish allow ER signaling to be monitored visually and serve as *in vivo* sentinels for detection of estrogenic compounds. **(*Endocrinology* 152: 2690–2703, 2011)**

Sex hormone receptors are ligand-dependent transcription factors that modulate diverse functions. For example, estrogen receptors (ERs) are involved in lipid and glucose metabolism (1–3), bone growth (4, 5), and development of the reproductive tract and function of the female reproductive cycle (6–8). They also directly affect sexually dimorphic behaviors such as mating (9, 10) and aggression (11, 12). ERs are activated by naturally occurring physiological steroids, such as 17β-estradiol, estrone, and estriol (13), or synthetic chemicals, such as bisphenol A (BPA), that may have deleterious effects (14).

To study ER function, transgenic animals and cell culture models have been developed that can report ER activity. Activated ERs bind specific DNA sequences, termed estrogen response elements (EREs) (15). Multiple EREs placed in tandem have been used to drive reporter gene expression in mice (16–20). By placing the luciferase gene under ERE control, Ciana and colleagues (20) demonstrated that the timing of peak activation of ERs differs between tissues during the estrous cycle. Luciferase bioluminescence, however, does not provide the spatial resolution to identify specific cell types or, in the case of the brain, to distinguish the precise regions in which receptors are activated. Mice with EREs regulating expression of the green fluorescent protein (GFP) gene have been produced, but *in vivo* studies have thus far been limited (18).

The zebrafish (*Danio rerio*) is becoming a valuable vertebrate genetic model for endocrinology studies (21). Zebrafish embryos and larvae are transparent and develop outside of the mother, enabling live imaging of fluorescent reporters at single-cell resolution during early developmental stages. The high fecundity of females allows systematic testing of thousands of progeny in chemical or genetic screens. Estrogens, progestogens, androgens, and

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Abbreviations: BPA, Bisphenol A; 4,4′-DDT, 4,4′-dichlorodiphenyltrichloroethane; DES, diethylstilbestrol; DMSO, dimethylsulfoxide; DPN, diarylpropionitrile; EE, 17α-ethynylestradiol; ER, estrogen receptor; ERE, estrogen response element; ERR, estrogen-related receptor; GFP, green fluorescent protein; 4-OHT, 4-hydroxytamoxifen; POA, preoptic area; PPT, propyl pyrazole triol.

their receptors are conserved (22–29), and the organization of the hypothalamo-pituitary-gonadal axis is similar to that of mammals (30–32). Small-molecule endocrine disruptors are effective and easily administered in water (33), and several fish species, including zebrafish, have been used to study their effects (34).

There are three genes in zebrafish that are homologous to mammalian ERs: *erα* (also referred to in zebrafish as *nr3a1* or *esr1*), *erβ1* (*nr3a2a* or *esr2a*), and *erβ2* (*nr3a2b* or *esr2b*). All three zebrafish ER bind to the consensus ERE DNA sequence and activate transcription in cultured cells in an estrogen-dependent manner (25–27), although one study reported that *in vitro* translated ERβ2 had 1.8-fold more affinity for estradiol than ERα or ERβ1 (27). Expression of the three zebrafish genes was found to partially overlap in estrogenic tissues such as the adult liver, gonad, brain, and pituitary (25, 27), similar to what has been described for mammals (35).

Transgenic zebrafish were developed to monitor ER activation (36–39), but their utility has been hampered by their limited response and spatial resolution. For example, the ERE-containing vitellogenin (*vtg1*) and aromatase (*cyp19a1b*) gene promoters are up-regulated by ER directly and were used to drive GFP in zebrafish larvae and adults (36, 38, 39). However, *vtg1* is expressed only in the liver and *cyp19a1b* is brain specific. Tandem EREs alone have also been used to drive the luciferase reporter gene in zebrafish (37), but in contrast to GFP, luciferase-mediated bioluminescence requires the addition of an exogenous substrate to the water, which might interfere with some applications in live fish.

We generated transgenic 5xERE:GFP zebrafish that report ER activation in larval and adult tissues including the brain, liver, and heart. Single estrogen-sensitive neurons and their projections were visible in live larvae and in fixed tissue from adults. GFP expression was sensitive to ER antagonists and was selectively activated in tissues by different estrogenic compounds in a dose-dependent manner. Transgenic zebrafish that report ER activation will serve as a valuable model to explore further the physiological roles of estrogen signaling *in vivo*.

Materials and Methods

Zebrafish

Zebrafish were raised at 28.5 C on a 14-h light, 10-h dark cycle and staged according to hours or days after fertilization (40). The wild-type AB strain (41) was used for all studies unless mentioned otherwise. To inhibit pigment formation, embryos and larvae were incubated in 0.003% 1-phenyl-2-thiourea. For screening and live imaging, embryos and larvae were anesthetized with 0.04% tricaine (40). All procedures were approved by the institutional animal care and use committee.

Generation of the 5xERE:GFP transgene

To place consensus ERE (GGTCACAGTGACC) in tandem, we annealed phosphorylated oligonucleotides (5′-TCGAC-CAGGTCACAGTGACCTAC-3′ and 5′-TCGAGTAGGTC-ACTGTGACCTGG-3′) by combining and denaturing for 5 min in a 95 C heat block and then cooling at room temperature for 2 h. Annealed products were ligated to one another using T4 DNA ligase (New England Biolabs, Ipswich, MA), purified on a G50 column (GE Healthcare, Piscataway, NJ), digested with *Xho*I and *Sal*I (New England Biolabs), gel purified (QIAquick; QIAGEN, Valencia, CA), and ligated into the *Xho*I site of the pT2cfosEGFP vector (42). Ligated 5xERE:GFP products were confirmed by sequencing.

Generation and identification of transgenic zebrafish

One-cell-stage embryos were injected with 5xERE:GFP DNA together with Tol2 transposase RNA (43) and raised to adulthood (F0 generation). Approximately 33% of adult F0 fish produced estrogen-responsive progeny, enabling us to recover nine independent transgenic lines. We focused on characterizing four lines that displayed the brightest fluorescence, lines c258, c259, c262, and c263. Each line was independently derived from a different F0 founder and maintained by successive matings to wild-type for six to eight generations. Transgenic carriers were identified by incubating larvae in 100 μg/liter estradiol at 3 or 4 d and selecting GFP-positive individuals at 5 d. GFP-positive larvae were washed and raised to adulthood in untreated water. This brief exposure to estradiol at 3–5 d is unlikely to bias sex ratios because it occurred well before gonad development and differentiation (44). We saw no obvious difference between the sex ratio of fish treated with estradiol at 3–5 d compared with untreated fish.

Mapping transgenic insertions

Genomic positions of two 5xERE:GFP transgenic insertions used extensively in this study were mapped by the ligation-mediated PCR, as described (45). Total genomic DNA was purified from tail fin clips of c262 and c263 adult fish and digested with *Nla*III. Sequences flanking the Tol2 arms were used to position and orient the insert within the zebrafish genome (Ensembl zebrafish genomic sequence database release Zv8). Each insertion was mapped from both the left and right arms of the transgene to confirm the integration site.

Chemical treatments

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) except for 4-hydroxytamoxifen (4-OHT) (EMD Chemicals, Gibbstown, NJ) and ICI 182,780 (Tocris Bioscience, Ellisville, MO). Diethylstilbestrol and 4-OHT were dissolved in methanol. ICI 182,780, 4,4′-dichlorodiphenyltrichloroethane (4,4′-DDT), atrazine, enterolactone, propyl pyrazole triol (PPT), and diarylpropionitrile (DPN) were dissolved in dimethylsulfoxide (DMSO). All other chemicals were dissolved in ethanol. For all treatments of embryos and larvae, chemical stocks were diluted 1000-fold into dechlorinated fish water to reach final concentration. Embryos and larvae were incubated in chemical or vehicle control (ethanol, methanol, or DMSO diluted 1:1000), and GFP fluorescence was visualized 1–3 d later.

For diethylstilbestrol (DES) treatment of adults, no more than six fish were placed together in a 3-liter tank (Aquatic Habitats, Apopka, FL). Diethylstilbestrol (100 μl of 10 mg/ml) was added to each tank twice daily for 3 d. Fresh water flowed into tanks at approximately 8 ml/min except at night, when tanks were maintained with no water flow. After treatment, water containing DES was mixed with Dri-Zorb (Lab Safety Supply, Janesville, WI) loose granular absorbent and discarded as solid hazardous waste.

In situ hybridization and immunofluorescence

For synthesis of RNA probes, full-length *erα, erβ1*, and *erβ2* cDNA (25) was subcloned into pCRII vector (Invitrogen, Carlsbad, CA). *Vasotocin* and *otpa* RNA probes were synthesized using previously described cDNA plasmids (46). Full-length *isotocin* was amplified by PCR from cDNA (prepared from total RNA from 5-d larvae using RETROscript first-strand synthesis kit and random decamer primers; Applied Biosystems/Ambion, Austin, TX) using primers 5′-GGTGTCAGCCTTGGTGAATA and 5′-TACAAAAGTGGGTGGCGAGT and subcloned into pCRII. Aromatase (*cyp19a1b*) was amplified from cDNA using primers 5′-ACGGCAGGTCTGAGCTGTATGTTC-3′ and 5′-CAAGGAATTTACTCTGTGCGCC-3′ and subcloned into pCRII. Full-length *gfp* was derived from EGFP-1 plasmid (Clontech, Mountain View, CA). All clones were verified by sequencing. Digoxigenin-labeled antisense RNA was transcribed using Sp6 or T7 polymerase (Roche, Indianapolis, IN). Whole-mount *in situ* hybridization was performed on larvae (47) and whole brains from adult fish (48), with the following modifications for double labeling experiments with *otpa* and GFP. The *otpa* transcript was visualized using tyramide signal amplification (49) and cyanine 5 (Perkin-Elmer, Boston, MA). Larvae were then incubated in antibody blocking solution (50) followed by rabbit anti-GFP antibody (Torrey Pines Biolabs, East Orange, NJ) diluted 1:500. GFP antibody labeling was detected using goat antirabbit-Alexa fluor 488 (Invitrogen) diluted 1:1000. Larvae were embedded in 4% low-melting-temperature agarose (Cambrex, East Rutherford, NJ), sectioned to 50 μm using a vibrating microtome (VT1000S; Leica Microsystems, Wetzlar, Germany), and mounted in ProLong Gold antifade reagent (Invitrogen) on Superfrost Plus slides (VWR, Radnor, PA). To identify GFP-expressing neurons, *Tg(5xERE:GFP)* adults were anesthetized in tricaine and killed by rapid immersion in ice water. Dissected brains were fixed overnight in 4% formaldehyde at 4 C and sectioned to 50 μm as above. Sections were deposited on slides, incubated at room temperature for 15 min in NeuroTrace fluorescent Nissl stain 435/455 (Invitrogen) diluted 1:100 in PBS, and washed three times for 10 min each in PBS and mounted as above.

Imaging

Live larvae were visualized using Leica MZ16F or MZFLIII stereomicroscopes equipped with Leica DC500 digital cameras. For multiphoton confocal imaging, live larvae were mounted in 1.2% low-melting-temperature agarose on a glass-bottom petri dish (Electron Microscopy Sciences, Hatfield, PA) and imaged using a ×40 water immersion objective (NA 0.85) and a Spectra-Physics MaiTai HP Ti:Sapphire laser (output from 690-1020 nm) with a nondescan detector (Leica Microsystems). Colorimetric *in situ* hybridization images of whole larvae and adult brain sections were collected using a Zeiss Axioskop microscope equipped with an AxioCam HRc digital camera (Carl Zeiss Microimaging, Thornwood, NJ). Cells expressing *gfp* in adult brain sections were counted using the PointPicker plug-in on ImageJ software (National Institutes of Health, Bethesda, MD). Fluorescent images of fixed tissue were collected using a Leica SP5 confocal microscope. Images were pseudocolored and superimposed using the Metamorph program (Molecular Devices, Sunnyvale, CA). Adjustments and cropping were performed using Photoshop CS5 and InDesign CS5 (Adobe Systems Inc., San Jose, CA).

Fluorescence quantification

For quantification of fluorescence intensity, live larvae were visualized using an Olympus MVX10 stereomicroscope (Center Valley, PA) equipped with a Leica DFC500 digital camera. The green channel was extracted, and then background was subtracted based on a region of identical size and shape from each image using a statistical correction (Metamorph). Thresholds for fluorescence intensity were set for each image to include fluorescence in the liver and exclude autofluorescence from the yolk. Average fluorescence intensity (total fluorescence intensity divided by area of fluorescence, arbitrary units) was measured for the liver of each larva using Metamorph.

Statistical analyses

For quantitative analysis of fluorescence in response to various estradiol levels between multiple groups, results were evaluated for significance using the Kruskal-Wallis test followed by the Steel-Dwass posttest due to unequal variance. For comparing the number of *gfp*-positive cells in the preoptic area (POA) between estrogen-treated and control animals, a Student's *t* test was performed. Statistical analyses were performed using JMP version 9 software (SAS, Cary, NC).

Results

Recovery of 5xERE:GFP transgenic zebrafish lines

To detect ER activity in zebrafish, we generated a construct containing five tandem consensus ERE sequences (15) upstream of a mouse c-*fos* minimal promoter (51) and the *gfp* gene. Beginning at 1 d after fertilization, injected embryos were incubated in water containing 100 μg/liter (367 nM) 17β-estradiol (estradiol) or with vehicle control (0.1% ethanol). At 4–5 d, larvae were examined for fluorescence. Only estradiol-treated larvae displayed fluorescence in the liver, demonstrating that the 5xERE:GFP construct reports ER activation.

To produce stable transgenic lines, injected embryos were raised to adulthood and mated to wild-type fish. We recovered multiple founders and focused on four that displayed the brightest fluorescence in the presence of estradiol. Each independently derived F0 founder was outcrossed to a wild-type fish to establish the c258, c259, c262, and c263 stable transgenic lines. All lines generate larvae that show robust, estrogen-dependent fluorescence. Levels of GFP labeling have remained consistent for six

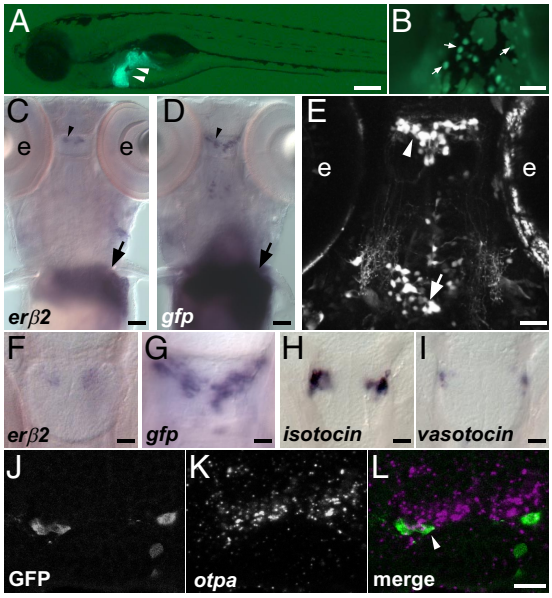

FIG. 1. *Tg*(*5xERE:GFP*) activation in larval zebrafish. A, Lateral view of live 5-d larva, line c258, showing fluorescent liver (*arrowheads*) after incubation in 100 μg/liter estradiol. B, Ventral view of live 4-d larva, line c262, incubated in untreated water, showing dim fluorescent cells in the skin (*arrows*). Six-fold longer exposure times and brightness/contrast enhancements are required to visualize these cells. C, *erβ2* is expressed in the liver (*arrow*) and POA of the brain (*arrowhead*) of 5-d larvae assayed by whole-mount RNA *in situ* hybridization. D, Estradiol-treated larvae from line c262 show *gfp* expression in the same tissues. E, Confocal two-photon projection of fluorescence in the POA (*arrowhead*) and presumptive hypothalamus (*arrow*) after estradiol treatment in 5-d c259 larvae. F–I, High magnification of gene expression in the POA. J–L, Fluorescent *in situ* hybridization with GFP antibody labeling showing some overlap (*arrowhead*) between GFP-positive cells (Alexa 488, *green*) and cells expressing *otpa* (Cy5, *magenta*), a marker of the POA. Coronal sections (50 μm) are from 5 d larvae, line c262. C–I, Dorsal view, anterior to the top. e, eye. *Scale bars*, 200 μm (A), 50 μm (B–D), 20 μm (E–I), and 10 μm (J–L).

generations, and we have seen no evidence of transgene silencing. Using linker-mediated PCR, lines c262 and c263 were determined to contain a single insertion of the transgene on different chromosomes (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), and these lines were used for most experiments.

In the absence of exogenous estrogen treatment, dimly fluorescent puncta were distributed on the surface of larvae (Fig. 1B; see also Fig. 4C) in a pattern reminiscent of epidermal mucosal cells (52). Because these faintly GFP-positive cells were detected in independently derived transgenic lines and their fluorescence intensity was not altered by estradiol or ER antagonist ICI 182,780 treatments (not shown), we suspect that the transgenic construct harbors low-level promoter activity in only these specific cells. This ectopic fluorescence was not apparent in adults (not shown) and, owing to the low level of labeling and superficial position of these cells, did not interfere with the visualization of induced fluorescence in estrogen-responsive larval tissues.

ER-dependent fluorescence in diverse larval tissues

When transgenic larvae were incubated in estradiol and assayed for transgene activity, GFP labeling was found in the brain and liver, the same tissues where *erβ2* is expressed (Fig. 1, C–E). We did not detect *erα* or *erβ1* mRNA in embryos or larvae through 5 d by colorimetric *in situ* hybridization (not shown).

Live, two-photon imaging of fluorescence in the brain revealed two distinct regions containing GFP-positive cells (Fig. 1E), anatomically consistent with the POA and hypothalamus (53). Using *in situ* hybridization, we compared *erβ2* and *gfp* expression with that of isotocin, vasotocin ,and orthopedia homolog a (*otpa*), genes known to be expressed in the larval POA (32, 46, 54). Transcripts for *erβ2* and *gfp* were found in the same brain regions at 5 d (Fig. 1, F–I) and ER-responsive cells showed some overlap with *otpa* expression (Fig. 1, J–L).

Although 5xERE:GFP fish reproducibly exhibited GFP labeling in the liver and brain, in tissues where ER are expressed, we also observed fluorescence in several regions where we did not detect *er* transcripts. GFP-positive cells in the ventral fin adjacent to the cloaca, in the developing olfactory organ, and in the heart (Figs. 2 and 3) were observed in 3- to 5-d larvae incubated in estradiol. The cells in the olfactory region contained prominent cilia (Fig. 2, A and B), suggesting that they are ciliated olfactory sensory neurons (55). We also detected faint fluorescence in the anterior telencephalon that likely corresponds to olfactory neuron axons terminating at an olfactory glomerulus (Fig. 2B). To identify where in the heart GFP is expressed, we examined live larvae at high magnification and found two distinct populations of fluorescent cells that move in concert with blood flow, indicating that GFP is expressed in cells associated with the heart valves (Fig. 2, C and D). Fluorescent cells were present at the junction between the atrium and the ventricle and in the bulbus arteriosus (56), the chamber that connects the ventricle to the ventral aorta, and in proximal blood vessels (Fig. 2E). GFP labeling was not observed in other regions of the heart or in blood cells.

To confirm that the observed fluorescence was due to ER activation, we tested whether the ER antagonist ICI 182,780 (fulvestrant) reduced fluorescence in these unexpected sites of reporter activity. Progeny from heterozygous c262 matings were incubated in 10 μg/liter estradiol together with DMSO (vehicle control) or 6 mg/liter ICI 182,780. After estradiol treatment, 79% of larvae displayed fluorescence in the head, heart, and liver and ad-

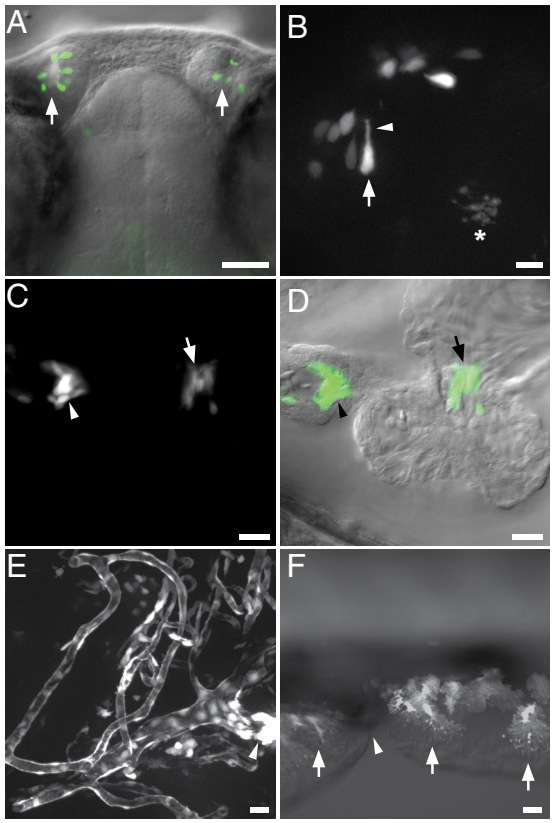

FIG. 2. Fluorescence in tissues where *er* transcripts were not detected. A–F, Fluorescence in live larvae after 100 μg/liter estradiol treatment beginning at 3 d. A, Dorsal view of head at 4 d, anterior to the top; *arrows* indicate olfactory organs. B, High-magnification image of a single olfactory organ; *Arrow* indicates cell body, *arrowhead* indicates cilium, and *asterisk* indicates putative glomerulus in olfactory bulb. C, Heart at 5 d. Valves between atrium and ventricle (*arrows*) and ventricle and aorta (*arrowhead*) are fluorescent. D, Fluorescence image overlayed onto differential interference contrast. E, Aortic valve (*arrowhead*) and branches are fluorescent at 5 d. F, Fluorescent cells (*arrows*) near the cloaca (*arrowhead*). All fluorescence was sensitive to ICI 182,780. Images are from transgenic line c262 except for A, line c263. *Scale bars*, 50 μm (A), 10 μm (B), and 20 μm (C–F).

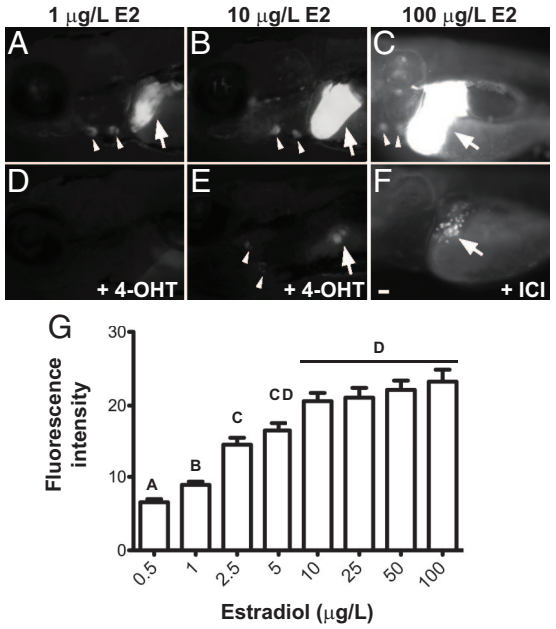

FIG. 3. Estradiol-induced fluorescence is dose dependent and sensitive to ER antagonists. Panels A–F, Larvae were treated with the indicated dose of 17β-estradiol (E2) alone or together with 775 μg/liter (2 μM) 4-OHT or 6 mg/liter (10 μM) ICI 182,780 (ICI) at 4 d, and fluorescence was visualized 1 d later. *Arrows* indicate cells in the liver; *arrowheads* indicate the heart valves. Lateral views are anterior to the left. Panel G, Embryos 6–8 h old were incubated in indicated concentration of E2, and average fluorescence intensity was measured in livers at 3 d, n = 10–17 per treatment group, mean ± SEM. Levels not connected by the *same letter* are significantly different ($P < 0.05$, Kruskal-Wallis test with Steel-Dwass posttest). Larvae are from line c262 (A, B, D, E, and G) or c258 (C and F). *Scale bar*, 50 μm.

jacent to the cloaca (n = 30). In contrast, all larvae doubly treated with estradiol and ICI 182,780 lacked GFP-positive cells in the olfactory area and in the brain (n = 37, not shown), and fluorescence in the heart was reduced or abolished (Fig. 3, D–F). This finding indicates that in tissues where *er* expression was not detected by RNA *in situ* hybridization, reporter activity is nonetheless dependent on ER activation.

ICI 182,780, a pure antiestrogen that does not activate ERs (57), also reduced the robust estradiol-induced fluorescence observed in the larval liver. When 1-d embryos were maintained in 1 μg/liter estradiol, 71% had GFP-positive cells in the liver 4 d later. However, when incubated in 1 μg/liter estradiol together with 6 mg/liter (10 μM) ICI 182,780, only 5% of 4-d larvae showed any GFP-positive cells in the liver (Supplemental Table 2 and Fig. 3, C and F).

Another selective ER modulator, 4-OHT, decreased estradiol-dependent fluorescence in transgenic larvae. In mammals, 4-OHT can act as an ER agonist or antagonist, depending on the cell type (58). Fluorescence in the livers of c262 larvae was markedly reduced in the presence of 4-OHT (Fig. 3, A, B, D, and E, and Supplemental Table 2), indicating that the response of *Tg(5xERE:GFP)* fish can be blocked by different ER modulators.

ER reporter activity is dose dependent

In addition to their sensitivity to ER antagonists, if *Tg(5xERE:GFP)* fish are valid reporters of ER activation, then the level of fluorescence should vary in proportion to the amount of ER agonist. To test whether reporter activity is dose dependent, 6- to 8-h-old embryos were maintained in varying concentrations of estradiol (0–100 μg/liter), and GFP labeling was visualized 3 d later. The average fluorescence intensity in the liver increased with higher doses of estradiol (Fig. 3, A, B, and G), increasing 2.3-fold between 1 and 10 μg/liter ($P = 0.0003$). Fluorescence intensity peaked at 10 μg/liter. Between 10 and 100 μg/liter, the mean fluorescence intensity was not significantly different (fluorescent signal was not saturated). The lowest concentration of estradiol at which fluores-

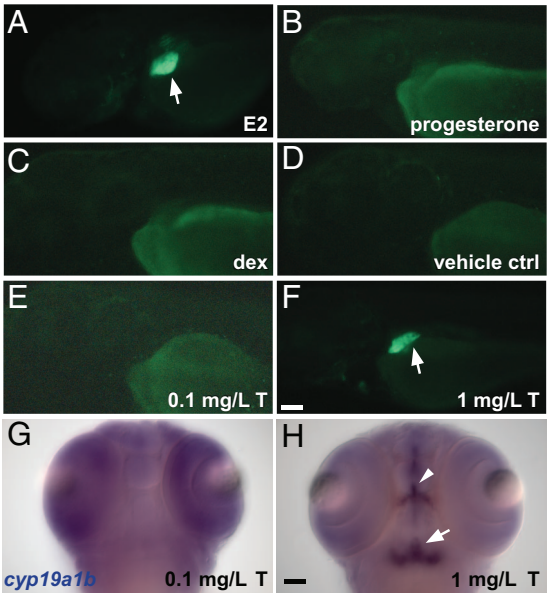

FIG. 4. Nonestrogenic steroids fail to activate GFP. A–F, Fluorescence in 3-d live larvae, line c262, after treatment with 100 μg/liter (367 nM) estradiol (E2), 3.15 mg/liter (10 μM) progesterone, 9.81 mg/liter (25 μM) dexamethasone (dex), vehicle control, or testosterone (T) as indicated. *Arrows* indicate cells in the liver. Lateral views are anterior to the left. G and H, Aromatase (*cyp19a1b*) gene expression in the POA (*arrowhead*) and hypothalamus (*arrow*) is increased after high-dose testosterone treatment. Larvae were assayed by whole-mount RNA *in situ* hybridization at 3 d. Dorsal views are anterior to the top. *Scale bars*, 100 μm (A–F) and 50 μm (G and H).

cence was observed was 0.5 μg/liter. Larvae from all four independently derived transgenic lines displayed similar responses to ER agonists and antagonists (Supplemental Table 2).

Tg(5xERE:GFP) fish respond to estrogenic compounds

Selectivity of the 5xERE:GFP reporter was evaluated by exposing embryos and larvae to progesterone (315 μg/liter, 1 μM), dexamethasone (9.81 mg/liter, 25 μM), and testosterone (100 μg/liter, 350 nM), steroid hormones that are structurally similar to estradiol but do not directly activate ERs. At these doses, GFP labeling was not detected (progesterone, n = 49 larvae; dexamethasone, n = 20 larvae; testosterone, n = 50 larvae; Fig. 4, A–E), strongly suggesting that the *5xERE:GFP* transgene is a selective reporter of ER activation.

A higher dose of testosterone (1 mg/liter), however, induced fluorescence in the larval liver (Fig. 4F) and brain (not shown). Previous studies demonstrated that similar high doses of testosterone increased expression of the *cyp19a1b* gene (59, 60), which encodes an aromatase that converts testosterone to estradiol in the larval brain. Consistent with this finding, 5xERE:GFP larvae showed dramatic up-regulation of *cyp19a1b* expression in the brain after exposure to high levels of testosterone (Fig. 4, G and H). This suggests that in the presence of high testosterone, aromatase-mediated production of estradiol is increased, leading to activation of the 5xERE:GFP transgene.

Tissue-specific differences in reporter activation by ER modulators

We compared the response of 5xERE:GFP embryos to 11 known and potential estrogenic compounds: DES, BPA, 17α-ethynylestradiol (EE), PPT, DPN, genistein, enterolactone, 4-nonylphenol, methoxychlor, 4,4′-DDT, and atrazine (Table 1).

The synthetic estrogen DES is a carcinogen (61, 62) and potent ER agonist (63). More than 80% of larvae incubated in 26.8 μg/liter DES displayed GFP-labeled livers and hearts (n = 30, Fig. 5, A and B). BPA, used in the production of polycarbonate plastics, is also an ER agonist (14, 64, 65). Robust fluorescence was observed in the liver and heart of more than 90% of larvae treated with 2.3 mg/liter BPA (n = 44, Fig. 5, C and D). Strikingly, larvae incubated in a lower dose of BPA (0.23 mg/liter) displayed fluorescence only in the heart and not the liver (Fig. 5E). Larvae incubated in BPA together with 775 μg/liter 4-OHT did not display any fluorescent cells (n = 43, Fig. 5F), indicating that BPA-induced fluorescence is ER dependent. We observed a similar result with genistein, an isoflavone from plants (66). GFP was observed in the liver and heart with 500 μg/liter and higher doses, whereas 50 μg/liter activated the reporter only in the heart (Fig. 5, G and H).

EE, a synthetic estrogen widely used in female oral contraceptives, activated the transgene in the larval liver and heart, similar to DES and estradiol (Fig. 5, I and J). At 10 ng/liter EE, all fluorescent larvae expressed GFP in the liver and in the heart (n = 15). At 0.1 ng/liter EE, however, 59% of larvae expressed GFP only in the liver and not in the heart (n = 17). This suggests that EE preferentially activates ER in the liver, in contrast to BPA and genistein.

Larvae incubated in 39 mg/liter PPT, an ERα-specific agonist in mammals (67), displayed fluorescence only in the liver (Fig. 5K) and brain (not shown; n = 88), whereas 240 μg/liter DPN, an ERβ-specific agonist in mammals (68), induced fluorescence in the liver, heart, proximal blood vessels, and brain (Fig. 5L, n = 14), similar to larvae treated with estradiol. Other presumed estrogenic agonists enterolactone, methoxychlor, 4,4′-DDT, 4-nonylphenol, and atrazine failed to activate the transgenic reporter.

These results suggest that 5xERE:GFP transgenic zebrafish are useful for screening compounds for their potency as ER agonists and for tissue-specific differences in their activity.

TABLE 1. Tissue-specific differences in reporter activation by ER modulators

Compound	Class	Dose[a]	Reporter activation
17β-Estradiol	Endogenous steroidal estrogen	1–100 μg/liter (3.67–367 nM)	Liver, heart, aorta, brain, olfactory area, ventral fin
		0.5 μg/liter	None
Estrone	Endogenous steroidal estrogen	10–1000 μg/liter (37 nM to 3.7 μM)	Liver, heart, aorta, brain, olfactory area, ventral fin
		1 μg/liter	None
EE	Synthetic steroidal estrogen	10 ng/liter (33.7 pM)	Liver, heart
		0.1 ng/liter	Liver
DES	Synthetic nonsteroidal estrogen	100 ng/liter-200 μg/liter (372.5 pM −745 nM)	Liver, heart, aorta, brain, olfactory area, ventral fin
		5–10 ng/liter	Liver, heart
		1 ng/liter	None
BPA	Synthetic nonsteroidal estrogen	2.3 mg/liter (10 μM)	Liver, heart
		0.23 mg/liter	Heart
PPT	Synthetic nonsteroidal ERα-selective agonist	39 mg/liter (100 μM)	Liver, brain
		3.9 mg/liter	None
DPN	Synthetic nonsteroidal ERβ-selective agonist	0.24–24 mg/liter (1–100 μM)	Liver, heart, aorta, brain, olfactory area, ventral fin
Genistein	Phytoestrogen isoflavone	500–5000 μg/liter (1.85–18.5 μM)	Liver, heart
		50 μg/liter	Heart
Enterolactone	Phytoestrogen lignan	6 mg/liter (20 μM)	None
Methoxychlor	Organochlorine pesticide	100 μg/liter (289 nM)	None
4,4′-DDT	Organochlorine pesticide	3.55 mg/liter (10 μM)	None
4-Nonylphenol	Alkylphenol surfactant/pesticide	1 mg/liter (4.5 μM)	None
Atrazine	Triazine herbicide	10.8 mg/liter (50 μM)	None

Embryos 6–8 h old were incubated as indicated for 3 d, and fluorescence was visualized.

[a] Highest dose represents maximum tolerated dose or dose at which compound was soluble in fish water.

Tg(*5xERE*:*GFP*) adult fish report endogenous ER activation

We did not observe fluorescence in untreated *Tg*(*5xERE*:*GFP*) larvae at 5 d. However, at this stage, the ovary, a major source of estrogens, has not yet formed. In adult females, the ovary is mature and produces estradiol (69), and in contrast to larvae, the 5xERE:GFP transgene is constitutively active in known estrogenic tissues.

ERs are expressed in the liver, ovary, and pituitary gland of adult zebrafish, where they are thought to regulate reproduction (27, 70). Consistent with receptor localization, GFP labeling was observed in these tissues dissected from untreated adult fish (n = 10). Constitutive GFP labeling was dim in livers from untreated female fish (Fig. 6, A and B) and was not detected in the livers of untreated males (data not shown). However, incubation of transgenic adult fish (n = 6) in DES, a synthetic ER agonist more potent than estradiol (63), strongly activated GFP expression in the livers of both sexes (Fig. 6, C and D, males not shown). Ovaries from 90% of the untreated fish were highly fluorescent (Fig. 6E), as were the pituitary organs from both untreated male (not shown) and female (Fig. 6, F and G) adults.

ERs are also expressed in the hypothalamus and POA of the adult brain and play a key role in regulating mating behavior and reproduction in vertebrates (9, 71). Although we detected fluorescence in the brain of 5xERE:GFP larvae only in the presence of exogenous estrogens, robust labeling was observed in the brains of untreated adults. Nineteen brains were examined, representing three independently derived transgenic lines. Eighteen brains contained GFP-positive cells in the POA, consistent with the location of estrogen-activated fluorescence in the larval brain and *er* gene expression in the adult (Fig. 7, A–C). In addition to the POA, fluorescence was detected in several other regions of the adult brain, including the caudal and ventral zones of the periventricular hypothalamus (not shown). To confirm that GFP-positive cells in the POA were neurons, we exposed brains from untreated c263 adults to a fluorescent Nissl stain that specifically labels neurons. GFP-positive cells were Nissl positive (n = 40 cells), demonstrating that ER are active in neurons in the POA (Fig. 7, D–F).

To determine whether increased levels of estrogen would alter the number of responsive cells in the POA, we administered DES to adult fish and assayed for GFP expression. The number of GFP-positive cells in the POA was significantly increased in DES-treated fish compared with vehicle controls [237.5 ± 71.22 (SD), n = 4, *vs.* 41.5 ± 18.59, n = 4; $P = 0.0097$, Student's *t* test; Fig. 7, G and H]. Thus, 5xERE:GFP transgenic zebrafish can effectively re-

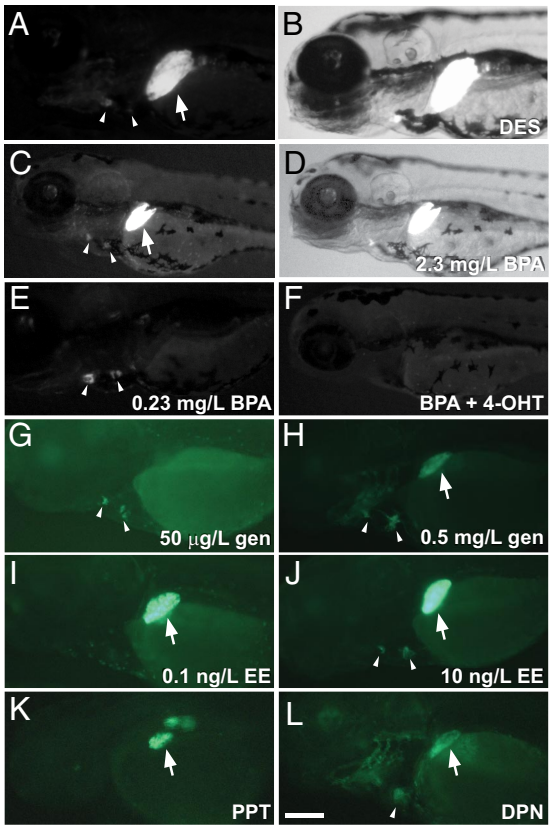

FIG. 5. Potential endocrine-disrupting compounds activate GFP. A, C, and E–L, Fluorescent images; B and D, superimposed with white light images of live larvae treated with 27 ng/liter (100 nM) DES (A and B), BPA (C–E), 2.3 mg/liter BPA plus 775 μg/liter (2 μM) 4-OHT (F), genistein (gen) (G and H), EE (I and J), 6 mg/liter (100 μM) PPT (K), and 0.24 mg/liter (1 μM) DPN (L). *Arrows* indicate cells in the liver; *arrowheads* indicate heart valves. Lateral views are anterior to the left. All larvae are from line c262 except for C, D, and F (c263). A–F, 5 d; G–L, 3 d. *Scale bar*, 200 μm.

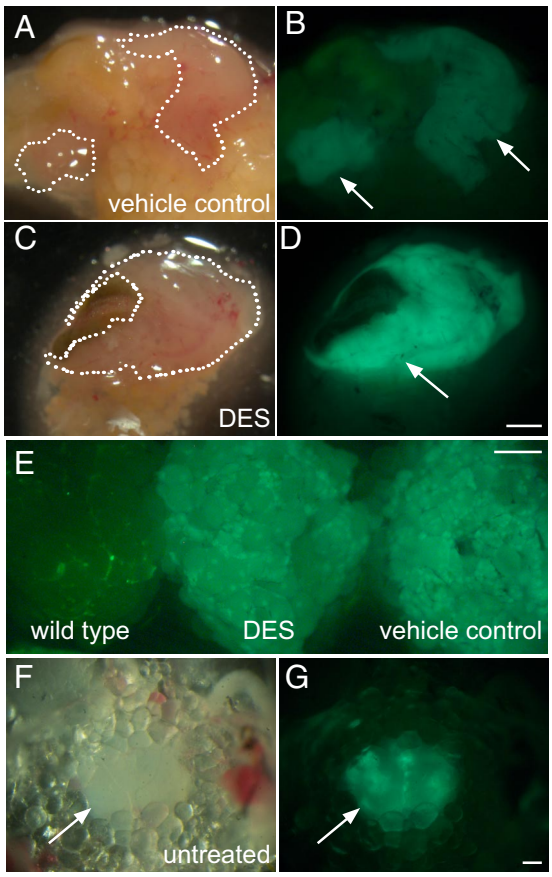

FIG. 6. Endogenous ER activation in adult liver, ovary, and pituitary. A–E, Adult fish were treated with DES or vehicle control, and fluorescence was visualized in dissected liver and ovaries. A and C, Bright-field images of liver (*outlined in white*) and associated internal organs from line c262. B and D, Corresponding fluorescence images; *arrows* indicate liver. E, Fluorescence is visible in ovaries from transgenic (DES- and vehicle-treated) but not wild-type fish. F and G, Fluorescence is visible in the pituitary (*arrows*) of untreated fish, line c263. F, Bright-field image; G, corresponding fluorescence image. *Scale bars*, 1 mm (A–E) and 100 μm (F and G).

port endogenous as well as induced ER activation in the adult brain.

Discussion

We capitalized on the properties of the zebrafish to generate a powerful tool for visualizing ER activity *in vivo* during development and in adulthood. The ability of 5xERE:GFP transgenic lines to respond to ER activation was validated by pharmacological experiments. *Tg(5xERE:GFP)* larvae report ER activity in a dose-dependent manner and are sensitive to two well-characterized ER antagonists, ICI 182,780 and 4-OHT. A variety of estrogenic compounds, but not the nonestrogenic steroids progesterone and dexamethasone, activated GFP, demonstrating the specificity of the response to estrogens.

High-dose testosterone also activated the transgene, most likely in an ER-dependent manner. High-dose testosterone treatment up-regulates aromatase gene expression in the brain (our results) (59, 60). Aromatase converts testosterone to estradiol, which binds ERs and activates the 5xERE:GFP reporter in the brain. Estradiol can potentially exit the central nervous system via the blood, leading to reporter activation in the liver and heart. Alternatively, testosterone could be converted into the estrogenic metabolite 5α-androstane-3β,17β-diol (59, 72). This reaction requires 5α-reductase and 3β-hydroxysteroid dehydrogenase, both of which are expressed in 5-d zebrafish larvae (52, 73). Conversion of testosterone into 5α-androstane-3β,17β-diol does not require aromatase and could account for induction of GFP expression in the liver and heart, tissues where aromatase genes are not expressed.

Assaying the induction of ER target genes is commonly used to report ER activation; however, many ER target genes, such as aromatase (74) and vitellogenin (36), display limited spatial expression patterns. Although c-*fos*

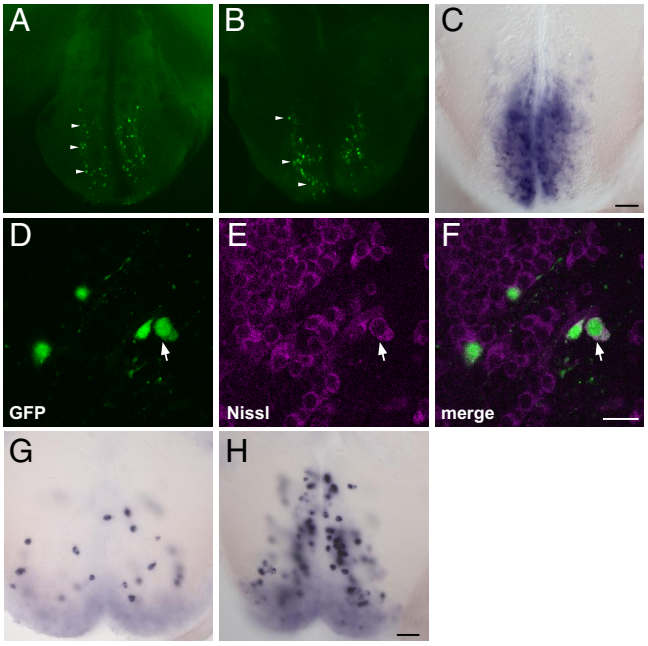

FIG. 7. Endogenous ER activation in adult brain. A–H, Coronal sections from POA of adult female brains. A and B, Endogenous fluorescence is visible in line c259 (A) and in line c262 (B). C, *In situ* hybridization showing abundant *erβ2* transcripts in the same brain region. D–F, Endogenous GFP-positive cells (*green*) in line c263 are Nissl-positive neurons (*magenta*). G, *In situ* hybridization for *gfp* on brain section from untreated c262/+ fish. H, Increase in the number of *gfp*-positive cells after estrogen treatment. *Scale bars*, 100 μm (A–C), 10 μm (D–F), and 40 μm (G and H).

gene expression is also used as a reporter of ER activation (75), such expression is not always specific to ER activation. *Tg(5xERE:GFP)* fish are a direct and more widespread reporter of ER activation because GFP is expressed in an ER-dependent manner throughout the animal.

In *Tg(5xERE:GFP)* larvae exposed to exogenous estrogens, we observed estrogen-dependent GFP labeling in the liver and POA of the brain, the same regions where *er* genes are expressed. However, we also found estrogen-dependent GFP expression in areas where *er* gene expression was not detected, notably in heart valves and olfactory neurons. In light of the observation that androgen receptors are expressed in the olfactory area of larval zebrafish (48), it is possible that androgens and estrogens influence zebrafish development and/or behavior via olfactory neurons as early as 3 d after fertilization. In another fish species, the round goby, olfactory cells can detect estrogens (76), which may act as pheromones in adults (77, 78). High levels of estrogens in the water bias larval and juvenile zebrafish to develop as females (79–82), whereas androgens bias zebrafish to develop as males (70, 83), but the mechanism by which this occurs is not well understood. It is an intriguing speculation that olfactory neurons sense estrogens or androgens in the water and signal to the brain to promote female or male sex differentiation.

In heart valves, the potential for ER activation was unexpected. Although estrogen signaling has been implicated in cardiovascular function (84), it has not been previously described to be active in heart valves. We also detected estrogen-dependent fluorescence in cells adjacent to the cloaca, in the presumptive hypothalamus, and in a small subset of cells in the caudal hindbrain (not shown) that likely correspond to neurons in the vagus nerve. Treatment with an ER antagonist reduced or eliminated fluorescence in all of these regions, confirming that the observed GFP labeling is dependent on ER activity and is not due to ER-independent activation of the transgene.

There is a discrepancy between our analysis of *er* gene expression and the results of previous reports (85, 86). We failed to detect *er* transcripts in the neuromasts of the lateral line or in the larval hypothalamus. This could be due to different properties of the RNA probes used for *in situ* hybridization. In our study, probes corresponding to the full-length open reading frame of each *er* gene were used, whereas other studies employed shorter probes (250–400 bp) that included untranslated regions. Embryonic and larval *er* gene expression should be examined further to resolve this issue. Irrespective of *er* expression, the pharmacological assays we performed demonstrate that ER are present and competent to activate the 5xERE:GFP reporter in the larval POA, hypothalamus, olfactory neurons, and heart.

Treatment with exogenous estrogens showed that cells in the brain and liver are competent to respond to estrogens; however, fluorescence was never observed in these tissues in untreated *Tg(5xERE:GFP)* larvae. One possibility is that, between 3 and 5 d of development, endogenous estrogen levels are too low to be detected by the 5xERE:GFP transgene. The gonad, a major (but not exclusive) source of estrogens in adults, is not yet formed at 5 d, and the levels and distribution of ER ligands in zebrafish larvae are unknown. Aromatase, the enzyme that generates 17β-estradiol, is also expressed at very low levels in the brain and has not been found in other tissues at this stage (74, 87) (our unpublished observations). A second possibility is that ERs are activated at larval stages but function to repress, rather than to induce, ERE-mediated transcription. This is likely occurring in the posterior lateral line primordium, where *erα* down-regulates *cxcr4b* expression (88). As designed, the 5xERE:GFP transgene cannot report ERE-mediated transcriptional repression, and accordingly, GFP labeling of the lateral line was not observed in transgenic larvae.

In contrast to larvae, GFP-labeled cells were visualized in tissues of the adult in the absence of exogenous estrogen. Additionally, many GFP-negative cells in the brain were competent to respond to estrogens, because a significant

increase in *gfp*-expressing cells was found throughout the POA after treatment with DES. The capacity to respond to estrogens suggests that transcriptional cofactors and other molecules required for ER-mediated transcription are present. However, the availability of endogenous ligand may normally be restricted, accounting for *gfp* expression in only a subset of cells in the POA. Strikingly, in untreated adults, we found areas of the POA where a single cell, but none of its neighbors, expressed *gfp*. It is possible that ligand is synthesized only in those cells in which ER are active (but not adjacent cells) or that ligand is systemic but is prevented from activating ER in some cells and not others. The zebrafish POA could serve as a useful model for understanding how recruitment of estrogen-responsive neurons is regulated.

Although *Tg(5xERE:GFP)* fish can be used to visualize ER activation, it is impossible to identify which ER subtype is active, because all three zebrafish ERs bind the ERE (25–27). Tiefenbach and colleagues (89) used an alternative ligand trap approach to detect nuclear hormone receptor transcriptional activation in zebrafish. The ligand trap method reports ligand binding to a specific receptor of interest. Once the individual ER transgenic lines are generated, a ligand trap method could be used to complement the 5xERE:GFP approach and discriminate between ERα, ERβ1, and ERβ2 transcriptional activation.

Although our experiments were directed at monitoring ER activity, we cannot rule out that some GFP labeling results from activation of an estrogen-related receptor (ERR) protein. ERRs are similar in sequence to ER but have no known endogenous ligand. *In vitro*, they constitutively bind to ERE DNA sequence and other DNA-binding sites specific to ERRs (90), although it is not clear how frequently ERRs activate transcription via EREs *in vivo* (91). Five *err* genes have been characterized in zebrafish, but their expression in the adult brain has not been examined (90). Future studies could compare *er* and *err* expression in the POA, or GFP expression could be assayed in *Tg(5xERE:GFP)* fish treated with an ERR-specific agonist (92) or dominant-negative ERR.

We anticipate many applications for the ER reporter fish. In addition to characterizing estrogen-responsive tissues, *Tg(5xERE:GFP)* zebrafish could potentially serve as sentinels of aquatic pollution. We were able to detect GFP in the liver of 3-d-old larvae exposed to as little as 0.1 ng/liter EE, an environmentally relevant concentration (93). However, weaker estrogenic compounds either did not activate GFP (*e.g.* 4-nonylphenol, enterolactone, methoxychlor, 4,4′-DDT) or activated GFP at higher doses than those found in the environment. For example, 5xERE:GFP larvae did not activate GFP at less than 50 μg/liter genistein, a higher dose than was found in streams in Iowa (8 ng/liter) (94) or pulp mill effluent in Ontario, Canada (10 μg/liter) (95), but within levels found in rivers in Osaka, Japan (24–143 μg/liter) (96). Comparing the lowest chemical concentrations at which fluorescence was observed, we infer that EE is the most potent estrogen we tested. This is consistent with results in other fish and mouse models (36, 66, 97). Genistein, which has relatively weaker estrogenic properties *in vitro* compared with EE (35), also generated a weaker response in 5xERE:GFP embryos and larvae (lowest observed effect at 50 μg/liter, compared with 0.5 μg/liter estradiol). Comparable responses were reported in transgenic zebrafish that report estrogen-dependent vitellogenin expression in the liver (*vtg-GFP*), where potent estrogens such as DES and EE induced GFP, but weak estrogenic compounds, including nonylphenol and genistein, did not (36). Because 5xERE:GFP embryos were incubated in weak estrogenic compounds for only 3 d, a longer incubation period might induce GFP expression. It is also possible that the lipophilic properties of some weak estrogens, such as methoxychlor, causes them to be trapped within the lipid-rich yolk, preventing their diffusion to additional embryonic tissues. Incubation beginning at later stages of development, such as 6 d, when much of the yolk has been absorbed, might enable GFP induction.

Tissue-specific differences in ER activation are also revealed using *Tg(5xERE:GFP)* fish, as was the case for embryos incubated in genistein, BPA, and PPT. At low doses, genistein preferentially activated receptors in the heart, whereas at higher doses, it also activated receptors in the liver. However, in an ERE-Luc mouse (97), the opposite was observed: at lower doses, genistein activated luciferase in the liver and not in the heart. This discrepancy could be due to differences in the developmental stages of the mice (adults) and zebrafish (embryos) assayed or to species-specific differences in response. An advantage of the 5xERE:GFP reporter is that because of the high spatial resolution of GFP and the transparency of larvae, we were able to identify the cell type in the heart activated by genistein and other estrogens.

The preference of BPA and genistein for activating ER in the heart is reminiscent of the mechanism of action of selective ER modulators. For example, the degree to which tamoxifen acts as an ER agonist or antagonist in breast cancer cells is dependent upon levels of the transcriptional corepressor N-CoR (98). A similar mechanism could underlie the tissue-specific effects of BPA and genistein in zebrafish larvae, in which the identity and/or ratio of coactivator and corepressor proteins in the heart may be different from in the liver, or ER subtypes may be differentially activated by the two compounds.

DPN, an ERβ-specific agonist in mammals (68), induced GFP in the heart, whereas PPT, an ERα-specific agonist in mammals (67), did not. This result suggests that the heart expresses only ERβ subtypes. If ERβ1 is more sensitive to genistein and BPA than ERα, this could explain why genistein and BPA preferentially induced GFP in the heart. However, it is unknown whether PPT and DPN display similar selective affinities toward zebrafish ER subtypes as they do toward mammalian ER subtypes. In Mozambique tilapia fish, PPT had higher affinity for ERα than for ERβ subtypes, but in contrast to results observed in mammals, DPN was able to activate both ERα and ERβ subtypes (99). Future studies are necessary to elucidate to what extent PPT and DPN selectivity are conserved among fish species.

The 5xERE:GFP transgenic fish will have many applications in addition to toxicology research. For example, estrogens have been shown to modulate central nervous system inflammation (100), and the *Tg(5xERE:GFP)* reporter will facilitate the identification of the cell types where ERs act in response to injury or disease. The transgenic fish will also be useful for mutagenesis screens to identify genes and small molecules that regulate ER transcriptional activity and estrogen-responsive cells. By replacing the GFP reporter gene with Gal4, it will be possible to capitalize on existing transgenic zebrafish lines that use the Gal4/UAS transcriptional activation system (45) to drive expression of, for example, a toxin to preferentially ablate estrogen-responsive cells, or a recombinase such as Cre to map the fate of estrogenic cells from the larval brain.

Our results also lay the groundwork for tracking estrogen-responsive neurons and their projections during development and in adulthood and potentially for mapping receptor activity in the brain during sexually dimorphic behaviors. Additional GFP-positive neurons in the POA of the brain were recruited by increased estrogen levels, suggesting that the 5xERE:GFP reporter will allow changes in the neuronal response to estrogens to be detected during mating, stress, aggression, and other social behaviors. Ultimately, similar transgenic methods can be expanded to visualize transcriptional activation of androgen and progesterone receptors, providing a powerful approach to investigate sex hormone receptor activity *in vivo*.

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