Bacterial Biosensors for Evaluating Potential Impacts of Estrogenic Endocrine Disrupting Compounds in Multiple Species

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ABSTRACT: To study the effects and possible mechanisms of suspected endocrine disrupting compounds (EDCs), a wide variety of assays have been developed. In this work, we generated engineered *Escherichia coli* biosensor strains that incorporate the ligand-binding domains (LBDs) of the β-subtype estrogen receptors (ERβ) from *Solea solea* (sole), and *Sus scrofa* (pig). These strains indicate the presence of ligands for these receptors by changes in growth phenotype, and can differentiate agonist from antagonist and give a rough indication of binding affinity via dose-response curves. The resulting strains were compared with our previously reported *Homo sapiens* ERβ biosensor strain. In initial tests, all three of the strains correctly identified estrogenic test compounds with a high degree of certainly (Z' typically greater than 0.5), including the weakly binding test compound bisphenol A (BPA) ($Z' \approx 0.1$–0.3). The modular design of the sensing element in this strain allows quick development of new species-based biosensors by simple LBD swapping, suggesting its use in initial comparative analysis of EDC impacts across multiple species. Interestingly, the growth phenotypes of the biosensor strains indicate similar binding for highly estrogenic control compounds, but suggest differences in ligand binding for more weakly binding EDCs.
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Keywords: endocrine disruptors; estrogen receptor; bacterial biosensor; *Solea solea*; *Sus scrofa*; *Homo sapiens*

INTRODUCTION

Endocrine disrupting compounds (EDCs) can interfere with normal body function in a variety of ways, including the transport, synthesis and activity of hormones, or by competitive binding to intracellular hormone receptors (Fisher, 2004; Goksoyr, 2006; Soto et al., 2006; Phillips et al., 2008). Regardless of the process, these chemicals produce a number of known and suspected deleterious effects (Oberdorster and Cheek, 2001; Maffini et al., 2006; Caserta et al., 2008). These include developmental and reproductive abnormalities, such as demasculinization and feminization of male species in fish, aquatic fowl, and other wildlife, as well as other disorders (Colborn et al., 1993). Environmentally relevant EDC examples include dichlorodiphenyl-trichloroethane, dieldrin, chlordane, polychlorinated biphenyls, and bisphenol-A (BPA). In general, these EDCs are current or former industrial chemicals and insecticides, and in many cases, thousands of tons have been manufactured and released into the environment. Additional EDC examples include naturally occurring secondary metabolites, such as Daidzein, which is an isoflavone found

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in edible plants with known estrogenic properties. Many additional EDC examples are present in smaller amounts, and include potent drugs and drug-like compounds, such as diarylpropionitrile (DPN) (Meyers et al., 2001) and diethylstilbestrol (DES) (Mori, 2001; Newbold et al., 2006).

Many EDCs have been shown to impact animals, often by acting through the estrogen receptors (ERα and ERβ) of the affected species. Some of these compounds may also have subtle but important effects on human populations, arising from chronic low-level exposures through various routes. To assess the hazards associated with these exposures in both human and animals, several animal-based methods have been developed (deFur, 2004; Vandenbergh, 2004). Unfortunately, many of these methods are expensive, time-consuming, and plagued with confounding variables (Melnick et al., 2002; O'Connor et al., 2002; Stokes, 2004). Further, only a small number of animal species are available that can be used in these assays.

To alleviate some of these limitations, previous investigators have developed a variety of simple cell-based and *in vitro* assays. These assays can include isolated cells from a given animal, or in some cases only the ER gene of the target species, without the requirement for the entire animal (Zacharewski, 1997; Charles, 2004; Martinez et al., 2005; Olsen et al., 2005; Soto et al., 2006; Gillies et al., 2008). Results from these assays suggest that variations in the amino acid sequences of ER ligand-binding domains (LBDs) lead to differences in ligand affinities between species (Matthews et al., 2000). These variations can make evaluations of EDCs in animal models difficult to interpret, especially for predicting impacts on humans. An example is that several studies indicate that ERs of certain piscine species often exhibit different binding affinities for some environmental EDCs as compared with other animal species, suggesting that the risk associated with these compounds may not be constant across interspecies lines.

In previous work, we developed a novel recombinant biosensor protein that includes a human NHR LBD, which is stabilized by an engineered mini-intein splicing domain and joined to a thymidylate synthase (TS) reporter enzyme (Skretas and Wood, 2005a). These components are genetically assembled into a multi-domain biosensor protein and expressed in an auxotrophic *E. coli* strain. This protein to undergoes conformational changes on agonist or antagonist binding to the human ERβ LBD, inducing a change in the TS reporter enzyme activity (Skretas et al., 2007). The change in TS activity is reflected by a change in growth phenotype of the *E. coli* cells, which can be easily quantified by simple growth measurements in a high-throughput format. Importantly, this sensor can differentiate between agonist and antagonist compounds, thus providing more information than would be available via a simple binding assay. We therefore refer to this assay as a pseudotransactivation assay, due to its ability to report the repositioning of helix 12 without the requirement for supplied coactivator or corepressor. Further, the modular design of the biosensor protein allows simple swapping of various NHR LBDs, allowing the creation of orthogonal sensors based on additional NHRs (Skretas and Wood, 2005a, b). The availability of orthogonal sensors in a genetically defined bacterial background can be used to eliminate the possibility of nonspecific growth effects, thus eliminating an important confounder in many whole animal and mammalian cell-based assays.

In this work, we generated two new biosensors using the ERβ LBDs from *Solea solea* (sole), and *Sus scrofa* (pig), and tested them with a small group of known and suspected estrogenic compounds. Our new ERβ sensors, compared with the *Homo sapiens* (human) sensor, provide some insight into the similarities and differences these species might exhibit in their responses to various estrogenic compounds. We further present that this method can be adapted for high-throughput screening in a robotically manipulated 96-well format, and can give a rough determination of the potency of the tested compounds through the generation of dose-response curves and calculated EC_{50} values. The results of several experiments confirm that these simple biosensors are able to identify estrogen agonists for these species with high statistical certainty, and the effects predicted by our sensors are consistent with more complex cellular and animal assays.

Abbreviations

BPA	bisphenol A
DES	diethylstilbestrol
DMSO	dimethyl sulfoxide
DPN	diarylpropionitrile
EDC	endocrine disrupting compound
ER	estrogen receptor
LB	Luria-Bertani
LBD	ligand-binding domain
RPTA	relative pseudotransactivation
TS	thymidylate synthase

MATERIALS AND METHODS

Chemicals and Reagents

The estrogen analogs 17β-estradiol (E_2), DES and Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) were purchased from Sigma-Aldrich (St. Louis, MO). Bisphenol A (4,4′-isopropylidenediphenol; BPA) was obtained from ICN Biomedicals (Solon, OH), while DPN was obtained from Tocris Cookson (Ellisville, MO). All compounds were dissolved in ethanol or dimethyl sulfoxide (DMSO) to form 10 mM stock solutions, and stored at -20°C. Before each test, compounds were serially diluted in DMSO or ethanol before addition to growth media to assure a constant solvent concentration throughout each set of experiments.

The -Thy medium used in growth phenotype tests is composed of 10 mL of 20% glucose, 10 mL of Thy Pool (2 mg/mL each of L-Arg, L-His, L-Leu, L-Met, L-Pro,

L-Thr), 10 mL of 10% casamino acids solution, 200 μL of 1% thiamine HCl, 1 mL of 0.1 M $CaCl_2$, 200 mL of 5× Minimal Davis Broth (35 mg/mL dipotassium phosphate, 10 mg/mL monopotassium phosphate, 2.5 mg/mL sodium citrate, 0.5 mg/mL magnesium sulfate, 5 mg/mL ammonium sulfate), 4 mL of 25 mg/mL ampicillin, and q.s. to 1 L using deionized water. The glucose, Thy Pool, casamino acids, and ampicillin components were sterilized by microfiltration through a 0.2 μm membrane. All other stock solutions were sterilized by autoclaving at 121°C for 25 min, and the final medium was mixed after cooling all of the components to room temperature. All media and media stock solutions were prepared using deionized water.

Plasmid Construction

The DNA sequence corresponding to residues Arg251-Lys500 of the porcine ERβ was amplified from the vector pcDNA3.1+ (*Sus scrofa* ERβ GenBank accession number: AF267736, provided by Dr. Holly LaVoie, School of Medicine, University of South Carolina, Columbia, S.C.), whereas the DNA sequence corresponding to the residues Leu43-Ser294 of the sole ERβ was amplified from the pGEM-T Easy vector (*Solea solea* ERβ GenBank accession number: AM286399, provided by Dr. Elisa Caviola, CuTech, Padova, Italy).

The design of the sole and pig biosensor plasmids, referred to here as pMIT::ERβ*(s) and pMIT::ERβ*(p), respectively, followed that of our previously reported human ERβ biosensor plasmid pMIT::ERβ*(h) (Skretas and Wood, 2005a, b). The sequence boundaries of each inserted NHR LBD are shown schematically in Figure 1. To simplify the insertion of the pig and sole sequences, the original ΔI intein (Derbyshire et al., 1997) used in pMIT::ERβ*(h) was modified to include silent *Mlu*I and *Nhe*I restriction sites flanking its unique *Bss*HII site. The pig and sole DNA sequences were polymerase chain reaction amplified with primers encoding silent *Mlu*I and *Nhe*I restriction sites, allowing them to seamlessly replace the human ERβ sequence in pMIT::ERβ*(h). The LBD insertions took place in a pGEM shuttle vector containing the mini-intein sequence. The LBD sequences were then verified by sequencing and transferred into the pMIT::ERβ*(h) plasmid by utilizing the unique *Age*I and *Xho*I restriction sites within the intein sequence.

Determination of Bacterial Growth Phenotypes

As in previous work (Skretas et al., 2007; Skretas and Wood, 2005a), the plasmids pMIT::ERβ*(h), pMIT::ERβ*(s) and pMIT::ERβ*(p) were transformed into the *E. coli* strain D1210Δ*thy*A::Kan^R [F$^-$Δ(*gpt-proA*)62 *leuB*6 *supE*44 *ara*-14 *galK*2 *lacY*1 Δ(*mcrC-mrr*) *rpsL*20 (*Str*^{r}) *xyl*-5 *mtl*-1 *recA*13 *lacI*^{q}] and selected on Luria-Bertani (LB) medium agar supplemented with 100 μg/mL ampicillin and 50 μg/mL thymine. Fresh colonies containing the appropriate biosensor plasmid were used to inoculate 5 mL LB liquid cultures supplemented with 100 μg/mL ampicillin and 50 μg/mL thymine, and incubated with shaking at 37°C until they reached an optical density of 1.3–1.5 at 600 nm (OD_{600}). This typically occurred after 8–10 h of incubation depending on the plasmid. For growth assays carried out in culture tubes, the 5 mL LB cultures were diluted 1:200 into 5 mL of -Thy media supplemented with 5 μL of diluted hormone test compounds (0.1% final solvent concentration) in triplicate. These tubes were then incubated with shaking at 34°C until clear variations in growth relative to hormone or test compound concentration could be observed. This typically occurred after 16 to 20 h of incubation, and growth levels were quantified by measuring the optical density of each sample at 600 nm (OD_{600}) on a GENESYS 2 spectrophotometer with a 1 cm path-length cuvette.

Adaptation to High-Throughput Screening

For each set of assays, 10 mM stock solutions of compounds dissolved in either ethanol or DMSO were serially diluted in 96-well plates (Fig. 2). Each set of serial dilutions for a given compound was made in the same solvent as the stock solution (i.e., ethanol or DMSO), and stored at −20°C. From these plates, 2 μL of each compound at each dilution was transferred to a second 96-well plate, where each well contained 198 μL/well of diluted biosensor cell cultures in -Thy media [constant 1% (v/v) solvent concentration]. Both of these steps were carried out by an automated liquid-handling system (BioMek, 2000, Beckman Coulter). The dilution factor of the LB culture for each strain into the -Thy test medium was adjusted to correct for each strain's basal growth rate in the absence of ligand. The adjusted dilution factors, in terms of LB inoculum to -Thy medium (v/v) were 1:200 for pMIT::ERβ*(h), 1:400 for pMIT::ERβ*(p), and 1:800 for pMIT::ERβ*(s). The plates were then incubated with shaking at 34°C and controlled humidity for the appropriate time to maximize the growth response relative to the test compounds. The OD_{600} of each well was then measured in a UV–vis plate reader (BioTek Synergy2) after ∼16–20 h.

Statistical Analysis of the Results

Growth phenotype changes across ligand concentrations were normalized by subtracting the vehicle control OD_{600} values from the test compound OD_{600} values at each concentration of the test compound (Eq. 1a). The resulting values were then scaled to a percentage response of the full response at the maximum test compound concentration. Dose-response curves were then determined by fitting the scaled values to Eq. 1b, where X is defined as the log of the concentration of ligand in the solution, Y is the

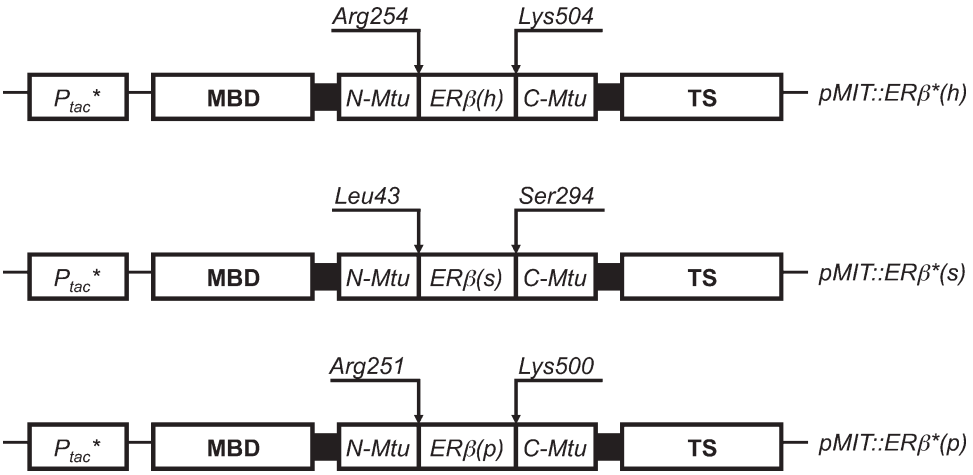

Fig. 1. Schematic representation of human, sole, and porcine biosensor genes. Notations are as follows, P_{tac}* = mutant *tac* promoter associated with hormone-dependent phenotypes; MBD = Maltose Binding Domain; N-Mtu = first 110 residues of the *Mycobacterium tuberculosis* RecA intein (Mtu RecA intein); ERβ = Estrogen Receptor β subtype ligand-binding domain; C-Mtu = last 58 residues of the Mtu RecA intein; TS = bacteriophage T4 thymidylate synthase enzyme. Each ERβ LBD includes the ~250 amino acid residues indicated, as described in Material and Methods. Abbreviations: (h)-human, (s)-sole, (p)-porcine.

corresponding scaled OD_{600} value, and top and bottom refer to the OD_{600} of the highest and lowest values obtained for each compound in a single dose-response experiment. The EC_{50} and HillSlope values were both calculated using nonlinear regression with variable Hill slope. The graphs were obtained by GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA) and optimized by outlier elimination (Q = 1%).

$$Y = \mathrm{OD}_{\mathrm{Sample}} - \mathrm{OD}_{\mathrm{DMSO}} \tag{1a}$$

$$Y = \mathrm{Bottom} + \frac{\mathrm{Top} - \mathrm{Bottom}}{1 + 10^{(\log EC_{50} - X)^{*}\mathrm{HillSlope}}} \tag{1b}$$

The overall significance and quality of the high-throughput assay format results were analyzed using the Z' factor as described by Zhang et al. (1999). For these determinations, two separate tests were each performed in triplicate, including the positive (*e.g.*, E_2 in DMSO) and negative (DMSO only) controls. The Z' factor was calculated as shown in Eq. 2, where SD_{max} is the standard deviation of the OD_{600} values for the test compound (*e.g.*, DMSO + E_2), SD_{min} is the standard deviation of the negative control (DMSO only in all cases), $Mean_{max}$ is the mean of the positive control, and $Mean_{min}$ is the mean of the negative control.

$$Z' = 1 - \frac{3 \cdot (\mathrm{SD}_{\max} + \mathrm{SD}_{\min})}{|\mathrm{Mean}_{\max} - \mathrm{Mean}_{\min}|} \tag{2}$$

The significance of the results was additionally analyzed using a common comparison of signal to noise (S/N) as shown in Eq. 3, as well as signal to background (S/B) ratios as shown in Eq. 4 (Zhang et al., 1999):

$$S/N = \frac{\mathrm{Mean}_{\max} - \mathrm{Mean}_{\min}}{\mathrm{SD}_{\min}} \tag{3}$$

$$S/B = \frac{\mathrm{Mean}_{\max}}{\mathrm{Mean}_{\min}} \tag{4}$$

The relative pseudotransactivation (RPTA) values for each of the ligands were calculated using the response generated by E_2, where RPTA is defined as: $\left(\frac{EC_{50}^{E_2}}{EC_{50}^{\mathrm{ligand}}}\right)$, and %RPTA is defined as $\left(\frac{EC_{50}^{E_2}}{EC_{50}^{\mathrm{ligand}}}\right) \times 100\%$.

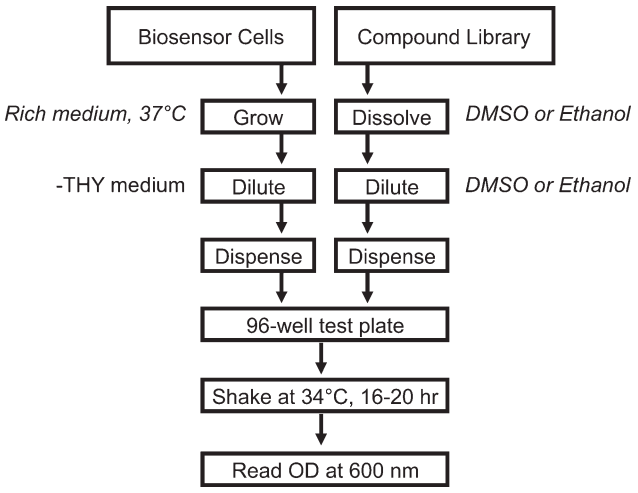

Fig. 2. Schematic representation of high-throughput screening method as described in Materials and Methods.

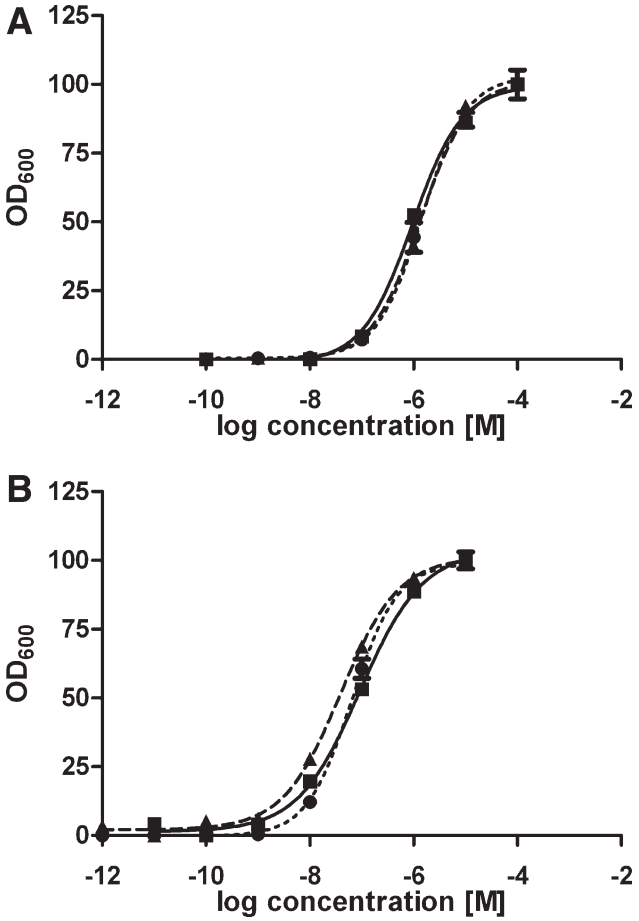

Fig. 3. Growth response curves for biosensor strains exposed to varying concentrations of (a) 17-β-estradiol (E_2) and (b) Diethylstibestrol (DES) in culture tubes. Solid lines = Human (*Homo sapiens*) responses; Dashed lines = Porcine (*Sus scrofa*) responses; Dotted lines = Sole (*Solea solea*) responses. Calculated EC_{50} values are as follows: (a) For E_2 Human, $EC_{50} = 9.4 \times 10^{-7}$ M (95% CI: 6.03×10^{-7} to 1.47×10^{-6} M; $R^2 = 0.99$); Porcine, $EC_{50} = 1.3 \times 10^{-6}$ M (95% CI: 1.15×10^{-6} to 1.53×10^{-6} M; $R^2 = 0.99$); Sole, $EC_{50} = 1.4 \times 10^{-6}$ M (95% CI: 1.14×10^{-7} to 1.82×10^{-6} M; $R^2 = 0.99$). (b) For DES, Human (*Homo sapiens*), $EC_{50} = 9.3 \times 10^{-8}$ M (95% CI: 5.66×10^{-8} to 1.52×10^{-7} M; $R^2 = 0.99$); Porcine (*Sus scrofa*), $EC_{50} = 3.9 \times 10^{-8}$ M (95% CI: 2.91×10^{-8} to 5.37×10^{-8} M; $R^2 = 0.99$); Sole (*Solea solea*), $EC_{50} = 6.5 \times 10^{-8}$ M (95% CI: 5.31×10^{-8} to 8.05×10^{-8} M; $R^2 = 0.99$).

RESULTS

Validation of Animal Biosensors in Culture Tubes

The human, sole and pig biosensor proteins, encoded by pMIT::ERβ*(h), pMIT::ERβ*(s), and pMIT::ERβ*(p), respectively, were tested using the estrogenic control compounds E_2 and DES dissolved in ethanol as a delivery vehicle. The resulting dose-response curves for E_2 and DES were plotted (Fig. 3) and EC_{50} values were calculated. The bacterial biosensor assay confirmed agonistic effects of E_2 and DES in all three species, indicated by an increase in the growth of our sensor strains in -Thy medium. Notably, with the pure ethanol control (vehicle-only), the sole biosensor strain demonstrated significant growth, but this growth was accelerated by the addition of agonist. In contrast, the porcine and human biosensors exhibited very little growth in the absence of agonist. As has been observed previously, DES was found to be more potent than E_2. The RPTA trend for DES *versus* E_2, calculated as the ratio $\left(\frac{EC_{50}^{E_2}}{EC_{50}^{DES}}\right)$, was as follows: 33 for pMIT::ERβ*(p) > 22 for pMIT::ERβ*(s) > 10 for pMIT::ERβ*(h).

Validation of Animal Biosensors in 96-well Microtiter Plate Format

The culture tube volume was scaled down by a factor of 25 for growth phenotype determinations in a 96-well microtiter plate, and growth responses were analyzed for the sole, human and porcine biosensors. For microtiter plate phenotype evaluations, the solvent vehicle was changed from ethanol to DMSO, and the final solvent volume in the growth medium was increased to 1% (v/v). For comparative purposes, growth phenotype evaluations with E_2 as the ligand were performed simultaneously in tubes and plates, using 0.1% (v/v) ethanol or 1% (v/v) DMSO as the solvents, respectively.

It was observed that the use of DMSO with E_2 generally increased the sensitivity of the biosensor strain by approximately one order of magnitude, whereas the use of plates also increased the sensitivity by approximately the same amount (data not shown). Overall, the EC_{50} for E_2 decreased from 940 nM using a tube-based assay with ethanol solvent, to 15 nM using DMSO solvent in a microtiter plate assay. The calculated EC_{50} values for pig and sole were similarly affected, dropping from 1300 nM to 16 nM and 1400 nM to 56 nM respectively (Figs. 3 and 4, Table I).

Species Response Comparisons with Additional Control Compounds

To further examine the ability of the new sensors to accurately report estrogenic potency in a microtiter plate format, they were tested with the additional test compounds DPN, Daidzein and BPA. In these tests, the relative ranking of estrogenic potency of these compounds for human and pig was identical (E_2 > DPN > Daidzein > BPA), whereas the trend for sole was E_2 > DPN > BPA > Daidzein. Overall, the highest potency of E_2 was observed for human ($EC_{50} = \sim 1.5 \times 10^{-8}$ M) and the lowest for BPA across all of tested

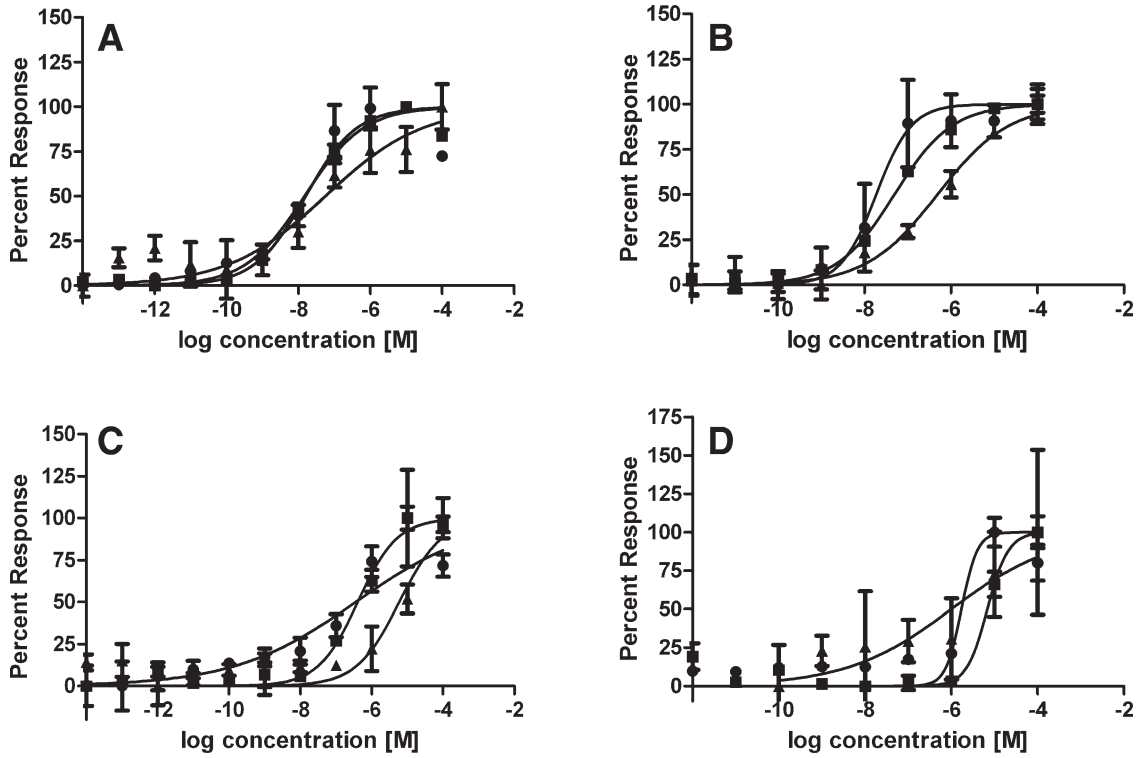

Fig. 4. Ligand effects on the growth phenotype of *Escherichia coli* carrying the vector D1210Δ*thy*A pMIT::ERβ* for (a) 17-β-estradiol; (b) Diarylpropionitrile (DPN); (c) Daidzein and (d) Bisphenol-A (BPA). Experiments took place in 96-well microtiter plates as described in the Methods. Symbols: triangles = Sole (*Solea solea*); squares = Porcine (*Sus scrofa*); circles = Human (*Homo sapiens*).

species ($EC_{50} = \sim 10^{-6}$ M) as well as Daidzein for sole ($EC_{50} = \sim 10^{-6}$ M; Table I).

A statistical analysis of the data in Table II shows that the closest correlation of EC_{50} values for the tested ligands were for human and pig; P value for two tail 95% confidence was 0.011. However, the least similar effect of the ligands was found for sole compared with human and pig: $P = 0.986$ (no correlation) and $P = 0.840$ (no correlation), respectively.

The assay quality was statistically evaluated over two separate experiments, comprising a total of five replicates, to determine Z' factor, signal to noise and signal to background ratios for the strongly binding control compound E_2 and the weaker binding BPA (Table IV). The signal to background ratio between the mean values of the maximal response obtained for specific ligands and vehicle control was between two and four for all of the ligands using the human and pig biosensors, and greatly exceeded the noise in these measurements. A smaller signal to background ratio was observed for the sole biosensor, particularly in the presence of the weak agonist BPA. Even the weak response of the human biosensor to BPA, however, exhibited a Z' factor of 0.04 and a signal to noise ratio of 3.3, indicating an unambiguous and statistically significant result (Table III).

DISCUSSION

This study investigates a novel approach for examining differences in agonistic ligand effects on the ERβ LBD derived from multiple species. Engineered allosteric biosensor fusion proteins, coupled with the TS reporter system, are a key component of these methods. In previous work, we have demonstrated that these biosensors are capable of recognizing hormone like compounds for various human NHR targets, and can differentiate agonistic and antagonistic compounds (Skretas et al., 2007). In several cases, drug-like compounds initially identified by our bacterial sensors, including subtype-selective agonists and antagonists, have been confirmed in subsequent human cell assays (Skretas et al., 2007; Hartman et al., 2009).

Our system is distinct from conventional transactivation assays, which require the presence of coregulators and detection systems for various reporter genes. Coregulators are not present in our system; however, ligands binding to the ERβ LBD produce a conformational change, which can be quantified via the TS reporter effects on phenotype. Our previous work with human NHR LBDs suggests that these conformational changes are correlated with those that lead to coactivator recruitment. Therefore, we refer to our system as a pseudotransactivation assay. Further, previous work and the tests

TABLE I. Median effect (EC_{50}) observed for *E. coli* D1210ΔthyA cells with (a) pMIT::ERβ*(h), (b) pMIT::ERβ*(s), and (c) pMIT::ERβ*(p) bound to E_2, BPA, DPN, and Daidzein

Compounds	Qualitative Response (Bacterial Sensor/Literature)	EC_{50} (CI) [μM]	R^2	%RPTA [%]
(a) *Homo Sapiens* ERβ Biosensor				
E_2	Agonist/Agonist (Awais et al., 2004; Breinholt and Larsen, 1998; Escande et al., 2006; Jordan and O'Malley, 2007; Zysk, 1995 #521; Meyers et al., 2001)	1.5×10^{-2} (6.7×10^{-3} to 3.3×10^{-2})	0.90	100.00
DPN	Agonist/Agonist (Escande et al., 2006; Meyers et al., 2001; Motylewska et al., 2009)	1.9×10^{-2} (1.1×10^{-2} to 3.2×10^{-2})	0.92	78.95
Daidzein	Agonist/Agonist (Breinholt and Larsen, 1998; Escande et al., 2006; Fokialakis et al., 2004; Kuiper et al., 1998)	3.6×10^{-1} (1.3×10^{-1} to 9.8×10^{-1})	0.87	4.17
BPA	Agonist/Agonist (Kuiper et al., 1998; Nagel et al., 1997; Tsai, 2006)	1.7 (1.8×10^{-1} to 17)	0.82	0.88
(b) *Sus Scrofa* ERβ Biosensor				
E_2	Agonist/Agonist (De Wilde et al., 2004; Traupe et al., 2007)	1.6×10^{-2} (1.3×10^{-2} to 1.92×10^{-2})	0.99	100.00
DPN	Agonist/Agonist (Traupe et al., 2007)	4.9×10^{-2} (4.05×10^{-2} to 6.01×10^{-2})	0.99	32.65
Daidzein	Agonist/Agonist (De Wilde et al., 2004; Whitten and Patisaul, 2001)	4.1×10^{-1} (3.9×10^{-1} to 5.35×10^{-1})	0.98	3.90
BPA	Agonist/Agonist (Okano et al., 2007)	6.9 (4.88–9.71)	0.99	0.23
(c) *Solea Solea* ERβ Biosensor				
E_2	Agonist/Agonist (Olsen et al., 2005; Smeets et al., 1999)	5.6×10^{-2} (2.09×10^{-2} to1.53×10^{-1})	0.86	100.00
DPN	Agonist/Agonist (Leanos-Castaneda and Van Der Kraak, 2007)	4.8×10^{-1} (2.48×10^{-1} to 9.67×10^{-1})	0.91	11.67
Daidzein	Agonist/Agonist (Matsuoka, 2005; Milligan et al., 1998)	6.0 (3.25–11.0)	0.94	0.93
BPA	Agonist/Agonist (Larsen et al., 2006; Olsen et al., 2005; Smeets et al., 1999)	1.0 (0.10–9.85)	0.86	5.60

The EC_{50} values were obtained based on the normalized response curves with the bottom of the curve set up as the lowest value and top of the curve as the highest value. Each point of the concentration was done in triplicate. The cell growth time for each of the assay was 16–24 h. Abbreviations: R^2 = goodness of fit determined for EC_{50} curve; RPTA = Relative Pseudotransactivation value; CI = 95% Confidence Intervals; E_2 = 17-β-estradiol; BPA = bisphenol-A; DPN = diarylpropionitrile. The Literature results shown for sole are based on tests with carp and rainbow trout when available as described in Discussion.

TABLE II. Significance of the differences of the response of ligands for ERβ (E_2; DPN; Daidzein; BPA) across species was calculated based on data shown in Table I: The *P* value (two-tailed with 95% confidence) shows significant correlation between effects (EC_{50}) of estrogenic compounds on human ERβ and porcine ERβ, and no correlation between results obtained in presence of human ERβ and sole ERβ as well as sole ERβ and porcine ERβ

	Human ERβ Response	Porcine ERβ Response	Sole ERβ Response
Human ERβ Response	–	0.011	0.986
Porcine ERβ Response	0.011	–	0.840
Sole ERβ Response	0.986	0.840	–

presented here indicate reproducible, statistically significant ligand responses for the ERβ sensor across several species, with a dimensionless Z' factor typically between 0.5 and 0.7.

We have attempted to validate our biosensor proteins by comparing our results with those of previous researchers using other assays, and have generally found good correlations (Skretas and Wood, 2005a, b; Skretas et al., 2007; Hartman et al., 2009. For example, the effect of compounds on human ERβ has been increasingly tested since its discovery in 1996. Studies performed using a yeast-based human ERβ transcriptional assay indicate the following EC_{50} trend for our test compounds: DES (3381) $\gg$ E_2 (100) $\gg$ Daidzein (0.4) $>$ BPA (0.2) (Escande et al., 2006; Chu et al., 2009). Our trend is similar: DES (1000) $\gg$ E_2 (100) $>$ Daidzein (4.2) $>$ BPA (0.9), where the relative

TABLE III. Significance of the differences of the response of ligands for ERβ (E_2; DPN; Daidzein; BPA) across species was calculated based on data shown in Table I. Comparison of the data for all of the species was analyzed for S/N-Signal to Noise ratio, S/B-Signal to Background ratio and Z' factor, the statistical value which determines the quality of the assay

	Homo Sapiens ERβ Biosensor			*Sus Scrofa* ERβ Biosensor			*Solea Solea* ERβ Biosensor		
Compounds	Z' Factor	S/N	S/B	Z' Factor	S/N	S/B	Z' Factor	S/N	S/B
E_2	0.68	16.75	2.50	0.67	17.99	3.42	0.13	8.57	1.73
DPN	0.62	16.45	2.47	0.59	19.39	3.61	0.37	9.6	1.82
Daidzein	0.51	8.95	3.77	0.64	13.92	4.14	0.53	14.34	1.67
BPA	0.04	3.28	2.02	0.43	11.67	3.63	0.08	7.88	1.37

DES to E_2 potency was measured using culture tubes and ethanol as the solvent (Table I). Most notably, a close correlation between the EC_{50} values obtained from the yeast and our bacterial assays was observed for BPA and Daidzein in microtiter plates: 2.5×10^{-7} M (yeast assay) *versus* 1.7×10^{-6} M (bacterial assay) and 1.45×10^{-7} M (yeast assay) *versus* 3.6×10^{-7} M (bacterial assay), respectively (Chu et al., 2009). It has also been observed that DPN is 50 times less potent than E_2 in these and similar assays (Escande et al., 2006; Chu et al., 2009), while our human ERβ assay indicated nearly 20% lower potency of DPN *versus* E_2. Finally, our results suggesting that BPA is a weak human ERβ agonist are consistent with extensive previous work from other investigators (Kuiper et al., 1998; Shi et al., 2001; Kurosawa et al., 2002; Nendza and Wenzel, 2006; Dobbins et al., 2008; Chu et al., 2009).

Comparisons of our system with animal models are somewhat more difficult due to lack of available data. In a few cases, however, we can make comparisons. For example, previous work in several laboratories shows a connection between ERβ in the human cardiovascular system and a cardioprotective estrogenic effect seen in human as well as pigs (Mendelsohn and Karas, 1999; Christian et al., 2006; Kim and Levin, 2006; Traupe et al., 2007; Arnal et al., 2009). Interestingly, in one of these studies, it was found that the DPN had a twofold weaker effect than E_2 on coronary artery relaxation in pig after one hour of exposure at 10 μmol/L each (Traupe et al., 2007). Our system shows a threefold lower effect of DPN *versus* E_2 using our pig ERβ biosensor. In addition, our biosensors indicated ninefold lower effect for DPN *versus* E_2 in fish.

TABLE IV. Statistical analysis of larger set of assays to asses the quality of biosensors using (a) strong and (b) weak estrogenic compounds (E_2 and BPA). The calculations are based on the fifteen readings

	S/N	S/B	Z' Factor
(a)			
E_2-Human ERβ	15.2	2.8	0.6
E_2-Porcine ERβ	10.2	4.1	0.5
E_2-Sole ERβ	31.5	1.7	0.4
(b)			
BPA-Human ERβ	9.61	2.30	0.15
BPA-Porcine ERβ	8.39	4.19	0.32
BPA-Sole ERβ	25.91	1.32	0.25

Abbreviations: S/N = Signal to Noise ratio; S/B = Signal to Background ratio; Z' Factor-dimensionless value which determines the quality of the assay; ideal ($SD_{min\&max} = 0$ and $Z' = 1$), very good ($1 > Z' \geq 0.5$), fair ($0.5 > Z' > 0$). Finally, when $Z' = 0$, the assays are able to distinguish between signal and no signal, but when $Z' < 0$, the assay indicates no separation between signal and base line (*e.g.*, DMSO and DMSO with ligand) (Zhang et al., 1999).

Comparisons are more difficult for sole, since we were unable to find any studies on the effects of our test compounds on this species. Several studies exist on other piscine species, however, including carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). These assays include direct binding assays on purified recombinant receptors (Matthews et al., 2000, 2001), as well as transcriptional assays in yeast (Petit et al., 1995) and human cells (Matthews et al., 2001), and assays based on estrogen-induced secretion of vitellogenin in piscine hepatocytes (Smeets et al., 1999). Our results compare favorably to these assays, generally reproducing the overall binding trends and relative binding affinities. In the case of carp, for example, the BPA effect on hepatocyte secretion of vitellogenin was very weak, precluding the determination of a precise EC_{50} value (Smeets et al., 1999). In these tests, vitellogenin secretion could be induced at a BPA concentrations of 50–100 μM, which is several times higher than EC_{50} of BPA for sole as determined by our tests (0.10–9.85 μM), but consistent with the overall weakness of BPA as a ligand with the sole ERβ biosensor. For E_2, however, the EC_{50} varied between 50 and 150 nM in the vitellogenin test, which is very close to our sole biosensor finding of an EC_{50} between 21 and 153 nM.

Competitive binding assays using radiolabeled E_2 have been used to evaluate several compounds, including Daidzein and BPA, for potential effects on human and rainbow trout ERβ (Matthews et al., 2000, 2001). These studies report that the trout ERβ exhibited the strongest affinity for E_2 compared with these xenobiotic estrogens, where the affinity of each compound is reported using its relative binding affinity (RBA) to E_2. Daidzein and BPA were found to bind very weakly to the trout ERβ, with RBA values

smaller than 0.1% for both compounds (E_2 is set to 100% in this case). These findings correlate fairly well with the decreasing potency of $E_2 \gg$ BPA $>$ Daidzein reported by our sole ERβ biosensor (%RPTA: 100, 5.6 and 0.9, respectively). Other previous studies also report relatively weak binding for phytoestrogens to trout ERβ receptors compared with E_2 (Smeets et al., 1999; Matthews et al., 2001).

Additional cell-based and transcriptional assays have been used to compare human and rainbow trout responses to various estrogenic compounds. In general, these studies also report trends and affinities that are consistent with those exhibited by our bacterial sensors. For example, a yeast-based transcriptional assay used to compare E_2 activation of trout and human ERα reported K_d values 3–5 fold higher in trout than human. The authors note that these results correspond well with the 3–6 fold lower affinity measured by other yeast and mammalian cell-based methods (Petit et al., 1995; Matthews et al., 2001). These results are also consistent with our findings, where the potency of E_2 decreased about four-fold between the sole and human ER. A cell-based assay using human MCF-7 cells and male rainbow trout hepatocytes against BPA indicated EC_{50} values of 0.3 μM and 3.5 μM, respectively (Dobbins et al., 2008), which are also similar to those reported by our biosensors (0.18–17 μM and 0.10–9.85 μM, respectively). Finally, a displacement assay using a trout ER nuclear extract reported an RBA value of 0.22 for DPN, compared with 100 for E_2 (Leanos-Castaneda and Van Der Kraak, 2007). The same study confirmed vitellogenin elevation caused by DPN and E_2 and showed DPN to be an rtERβ selective agonist. This is qualitatively consistent with our results, which indicate that DPN is an ERβ agonist in sole, with significantly reduced binding affinity relative to E_2.

Interestingly, our biosensor exhibited relatively higher RPTA values for weaker binding compounds such as Daidzein and BPA. This unexpected phenomenon was observed in our previous work with the human ERα and ERβ biosensors, where our assay sensitivity was substantially lower than those of conventional assays for strongly binding compounds, but converged with even the most sensitive assays for very weakly binding compounds (Skretas et al., 2007). We have hypothesized that this effect arises from the direct link between ligand binding and TS activation in a single allosteric sensor protein molecule, which generates an immediate and proportional signal without the need for transcription of a reporter protein (Skretas and Wood, 2005a; Skretas et al., 2007). This is in contrast to conventional transcription assays, where strongly binding compounds can induce reporter transcription and generate strong signals at very low concentrations, while weakly binding compounds may not be able to induce the steps required for reporter transcription, and thus generate no signal at all. Conversely, strongly binding compounds will generate reduced signals in our system, due to the finite number of reporter proteins present, but weakly binding compounds will almost always generate a measureable signal through some level of direct TS activation. Finally, the stringency of the TS genetic selection system is also highly tunable by temperature (Derbyshire et al., 1997; Skretas and Wood, 2005a), allowing us to optimize its sensitivity for a range of binding affinities.

Most importantly, this biosensor system is capable of accepting multiple LBDs from many species, allowing accurate identification of potential endocrine disruptors. We attribute this ability to the presence of the mini-intein splicing domain, which we have hypothesized acts as a stabilizing factor for the inserted LBDs (Skretas and Wood, 2005a; Skretas et al., 2007). In this case, the intein domain constrains the termini of the inserted LBD, while at the same time the configuration of the LBD affects the stability of the intein and the activity of the associated TS domain. In the case of the sole biosensor, we hypothesize that the LBD may be somewhat unfolded in the absence of ligand, which would destabilize the intein and allow higher background activity of the TS enzyme. This would lead to higher basal cell growth rates in the absence of ligand, as was observed in our study. Clarification of this effect is planned for future work, and should aid in the development of additional sensors that can be used at a wider range of growth temperatures.

This study also showed that solvent and growth conditions have a substantial affect on the sensitivity of the assay. In particular, the sensitivity of the assay increased greatly in the presence of 1% DMSO as the carrier solvent for the test compounds. This is presumably due to increased transport of the test compounds through the cell membranes, and suggests that free diffusion, rather than active transport, dominates internalization of the test compounds. In addition, some compounds may dissolve better in DMSO than in ethanol, although no precipitant was observed in any cases in these experiments. The increased sensitivity arising from the use of plates over tubes is likely due to increased oxygen transfer owing to the smaller incubation volumes. Because the assay is based on modulations of TS activity and its effect on DNA synthesis rate, subtle changes in growth conditions are likely to have a significant impact on sensitivity. Regardless, however, the modification of the method to the high-throughput format resulted in highly reproducible results, with the unexpected benefit of a significantly increased sensitivity for test compounds.

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