Expression profile of oestrogen receptors and oestrogen-related receptors is organ specific and sex dependent: the Japanese medaka *Oryzias latipes* model


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Gene expression of all known subtypes of oestrogen receptor (ER) and oestrogen-related receptor (ERR) in multiple organs and both sexes of the Japanese medaka *Oryzias latipes* was profiled and systematically analysed. As revealed by statistical analyses and low-dimensional projections, the expressions of ERRs proved to be organ and sex dependent, which is in contrast with the ubiquitous nature of ERs. Moreover, expressions of specific ERR isoforms (ERRγ1, ERRγ2) were strongly correlated with that of all ERs (ERα, ERβ1 and ERβ2), suggesting the existence of potential interactions. Findings of this study shed light on the co-regulatory role of particular ERRs in oestrogen-ERs signalling and highlight the potential importance of ERRs in determining organ and sex-specific oestrogen responses. Using *O. latipes* as an alternative vertebrate model, this study provides new directions that call for collective efforts from the scientific community to unravel the mechanistic action of ER-ERR cross-talks, and their intertwining functions, in a cell and sex-specific manner in vivo.

Key words: oestrogen regulation; oestrogenic effects; organs; teleost; vertebrate.

INTRODUCTION

While oestrogen (E2) is generally perceived to be a feminine hormone, its effects are not restricted to females. Besides functioning in the development of ovary and secondary characteristics of females, oestrogen also drives the development of testes as well as male secondary characteristics and behaviour (Eddy *et al*., 1996; Hess *et al*., 1997; Muramatsu & Inoue, 2000). Oestrogen is not only active in the reproductive axis, but is also involved in regulating the central nervous (McEwen & Alves, 1999)
and cardiovascular systems (Stampfer et al., 1991; Mendelsohn & Karas, 1999), cell growth and differentiation (Brannvall, 2002), immune function (Grossman, 1985) and skeletal development (Bilezikian et al., 2001). Oestrogen is thus active in maintaining homeostasis, as well as development and function of the entire spectrum of organs in vertebrates.

Oestrogen exerts its effects mainly through interactions with oestrogen receptors (ER), a class of ligand-dependent receptors in the superfamily of nuclear receptors. Once ERs are activated by binding of oestrogen agonists to the ligand binding domain, the ligand–receptor complex is capable of initiating transcription of over a thousand target genes (Tang et al., 2007) by binding to the promoter regions either directly on oestrogen response elements as homo- or hetero-dimer, or indirectly as a complex with other transcription factors (Björnström & Sjöberg, 2005).

The oestrogen-related receptors (ERR), a class of orphan nuclear receptors bearing high structural homology (particularly in the DNA-binding domain) to ERs, has been postulated to influence E2 signalling by either synergizing or competing with ERs (Horard & Vanacker, 2003) in regulating multiple shared transcriptional targets (Vanacker et al., 1999). Consequently, complete understanding of the mechanisms and functioning of oestrogen signalling cascade will not be feasible without thorough consideration of the direct and indirect involvement of ERRs. The importance of ERRs in E2 responses is already recognized, particularly in clinical applications (Giguère, 2002).

Teleosts have been extensively utilized as model organisms to elucidate effects of oestrogenic compounds that can modulate sex hormone responses, such as oestrogenic endocrine disruptors, in vertebrates (Ankley et al., 1997). While ER sequences of over 90 teleost species are identified and deposited in the NCBI Genbank, the sequences of ERRs have been described for only three bony fishes. Such a contrast reflects a general scarcity of information on teleost ERRs, impeding furtherance of the understanding on the teleostean oestrogen signalling system and its dynamics in response to synthetic oestrogenic chemicals.

The Japanese medaka *Oryzias latipes* (Temminck & Schlegel 1846) is one of the most frequently utilized teleost models for toxicity testing of xenoestrogens and for generalization of the biological effects of oestrogenic compounds in vertebrates (Park et al., 2008; Zhang et al., 2008a; Tompsett et al., 2009). This report summarizes the first systematic illustration of organ and sex dependent expressions of all known ER and ERR subtypes in six major organs (brain, gill, gonad, heart, liver and spleen) of both male and female *O. latipes*. Given the inconsistency in nomenclature of ERs and ERRs among teleost species, a phylogenetic tree was constructed to enable stringent comparisons between the expression profiles of *O. latipes* and other teleost species as well as mammalian models. Statistical analyses were employed to identify potential ER–ERR ‘cross-talks’ for further investigation of their biological significance in vertebrates.

**MATERIALS AND METHODS**

**FISH CULTURE AND CARE**

Orange-red strain *O. latipes* were maintained in aquaria at the City University of Hong Kong under static conditions (26° C, range ±1° C; pH 7.3, range ±0.1; 7.2 mg O$_2$ l$^{-1}$, 1.5 g l$^{-1}$, 7.8 g l$^{-1}$ salinity, 0.8 g l$^{-1}$ ammonia, 0.8 g l$^{-1}$ nitrite, 0.8 g l$^{-1}$ nitrate).
range ±0.2 mg O$_2$ l$^{-1}$; 14L:10D). Half of the water in each aquarium was replaced daily with dechlorinated, charcoal-filtered tap water. Fish were fed twice everyday with Otohime β1 (Marubeni Nisshin Feed Co.; www.mn-feed.com) and supplemented with brine shrimp Artemia sp. nauplii (Lucky Brand, O.S.I. Marine Lab; www.oceanstarinternational.com) for 3 days every week. Under these husbandry conditions, the fish reached sexual maturity at c. 3 month-post-hatch and laid eggs daily. European Union directive 86/609/EEC was strictly adhered throughout the study. Animal handling procedures as mentioned in this study were accepted by the Animal Ethics Committee, City University of Hong Kong.

**SAMPLING**

Sexually mature *O. latipes* (8 month-post-hatch; 14 males and 14 females) were collected at 6 h after mating and ovulation. Fishes were completely anaesthetized within 15 s by immersion in ice-cold aquarium water. Sedated fish was placed on ice bed and decapitated by making a transverse cut from the gill slit to the spinal cord (immediately caudal to the vagal lobe). The six target organs isolated from the decapitated fish were liver, spleen, gill (both arches), heart (atrium and ventricle), brain (rostral-caudal: from telencephalon to the decapitation position; ventral-most: hypothalamus and inferior lobe) and gonad (i.e. ovary or testis; whole organ was encapsulated by the ovarian or testicular epithelium). Organs from two individuals of the same sex were pooled as one replicate, i.e. a total of seven replicates were analysed for each organ for each sex. Samples were snap-frozen in liquid nitrogen and stored at −80° C.

**TOTAL RNA EXTRACTION AND FIRST-STRAND cDNA SYNTHESIS**

Total RNA was extracted by the use of TRIzol Reagent (Invitrogen; www.invitrogen.com) according to the methods in the manufacturer’s manual. Two microlitres of each of the total RNA samples was electrophoresed on 1.2% agarose gel in 1× tris-acetate-EDTA buffer with GelRed (Biotium; www.biotium.com). RNA integrity was assured by the presence of sharp 28S and 18S rRNA bands in a ratio of c. 2:1. Another 1 µg of the total RNA was treated with RQ1 RNase-Free DNase (Promega; www.promega.com) according to the manufacturer’s protocol. First-strand cDNA was generated from the digestion product following the methods of Yu et al. (2006).

**SELECTION OF REFERENCE GENE**

To maximize the use of the expression profiles as basal reference for future mechanistic studies of oestrogenic compounds, *rpl7*, a house-keeping gene deemed very stable upon E2 exposure (Zhang & Hu, 2007), was specifically chosen as the reference gene. To ascertain *rpl7* is an appropriate endogenous control gene for the present absolute quantitative real-time PCR (qPCR) study, expressions of three other commonly used reference genes (including *mt-rnr2*, *actb* and *gapdh*) were also determined in all organs and both sexes of *O. latipes* (using qPCR as illustrated below). Expression stability across organs and between sexes was gauged using NormFinder (Andersen et al., 2004). *rpl7* was proven the most stable reference candidate.

**ABSOLUTE QUANTIFICATION OF GENE EXPRESSION BY REAL-TIME PCR (qRT-PCR)**

Gene-specific primers were designed using National Center for Biotechnology Information primer basic local alignment search tool (BLAST). Default parameters were used, except that specificity check was conducted for *Oryzias latipes* (taxid:8090) against the nr database. Synthesized primers (1st BASE; www.base-asia.com) were stringently verified for proper efficiency and specificity under routine reaction setup stated below. Sequences of the validated gene-specific primer are listed in Table I.

Gene-specific PCR products of the target (ERs and ERRs) and reference (*rpl7*) genes were produced by use of validated gene-specific primers (Table I). Copy numbers of these PCR
<table>
<thead>
<tr>
<th>Gene</th>
<th>Template accession number</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>errα</td>
<td>D28954.1</td>
<td>GAGGAGGAGGAGGAGGAGGAG</td>
<td>GTGTACGGTCGGCTCAACTTC</td>
<td>130</td>
</tr>
<tr>
<td>erβ1</td>
<td>NM_001104702.1</td>
<td>GCTGGAGGTGCTGATGATGG</td>
<td>CGAAGCCCTGGACACAACCTG</td>
<td>116</td>
</tr>
<tr>
<td>erβ2</td>
<td>NM_001128512.1</td>
<td>CGCAGTCAAACACACTCAT</td>
<td>TCTTCAGGTCCTCCATCAGG</td>
<td>105</td>
</tr>
<tr>
<td>erra</td>
<td>NM_001104917.1</td>
<td>GCCGGAACCTAGTGATCCAGA</td>
<td>GCACCGTCTCCTCCTGTT</td>
<td>108</td>
</tr>
<tr>
<td>errβ1</td>
<td>NM_001104918.1</td>
<td>CAGTCCGTCTTCGGTCACT</td>
<td>CGTTGGAGTGGCTGTTCAT</td>
<td>101</td>
</tr>
<tr>
<td>errβ2</td>
<td>NM_001163091.1</td>
<td>TCCTACGTGAAGACCGAACC</td>
<td>CGACGTCGATGACGCC</td>
<td>111</td>
</tr>
<tr>
<td>errγ1</td>
<td>NM_001104919.2</td>
<td>AAGACAGAGCGTGCAAGTCC</td>
<td>GAGTCTAAGCCGTGGGATG</td>
<td>125</td>
</tr>
<tr>
<td>errγ2</td>
<td>NM_001163092.1</td>
<td>CTCGGACACCAACTCTAGC</td>
<td>TTGCTGGCCAAGGCTGT</td>
<td>102</td>
</tr>
<tr>
<td>rpl7</td>
<td>NM_001104870.1</td>
<td>GTCGCTCCCTCCACAAGA</td>
<td>AACTTCAAGCCTGCCAACAAC</td>
<td>99</td>
</tr>
</tbody>
</table>

Table I. Gene-specific primer sequences
products (standards) were quantitated by measuring against the MassRuler Low Range DNA Ladder (Fermentas; www.thermoscientificbio.com) and, in parallel, serially diluted (six steps from $10^{-4}$ to $10^{-10}$) and quantitated by qPCR to construct gene-specific standard curves that allowed mapping between template copy numbers and the corresponding threshold cycles ($C_q$). Expression of each gene in each sample was measured by use of qPCR and represented as $C_q$, which was interpolated to absolute copy number according to the aforesaid gene-specific standard curves and normalized to the number of copies of the reference gene, $rpl7$. Triplicates were run for each sample. Reaction condition and cycling setup followed those of Yu et al. (2006), except that Applied Biosystems 7500 Fast Real-Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystems; www.appliedbiosystems.com) were used.

**STATISTICAL ANALYSES**

Principal component analysis (PCA) via singular value decomposition (SVD) was employed to project the ER and ERR expression patterns to lower dimensions after log$_{10}$-transformation, mean-centring and scaling to unit variance. Scores on the first two-components were analysed by use of two-way MANOVA to explore interaction effect between the class variables of organ and sex on patterns of expression. Organ and sex dependent expression patterns were further investigated by the use of partial least-squares discriminant analysis (PLS-DA). Optimal numbers of latent variables retained were determined by minimizing the classification error rates ($p_{error}$) in leave-one-out (LOO) cross-validations. Comparisons between sexes for each individual ER and ERR in every organ were conducted using a $t$-test. Welch's $t$-test or Mann–Whitney rank-sum test was used in place of a $t$-test whenever homoscedasticity or normality assumptions was violated as indicated by Levene’s test on median centres and Shapiro-Wilk’s test, respectively. The relationship between absolute expressions of individual ERs and ERRs was investigated by use of Spearman correlation analysis and bootstrapped hierarchical clustering. $P$-values from multiple comparisons were adjusted to control for false discovery rate (FDR) by use of the Benjamini and Hochberg procedure (Benjamini & Hochberg, 1995). Significance level of 0.05 was used for inferential statistics. All statistical analyses were performed in the R environment 2.14.1 (www.r-project.org).

**PHYLOGENETIC ANALYSIS**

To facilitate like-to-like comparisons of the ER and ERR expression profiles between *O. latipes* and other model species, especially mummichog *Fundulus heteroclitus* (L. 1766) and zebrafish *Danio rerio* (Hamilton 1822), which are the only other teleosts having a complete, well studied array of ERs and ERRs, a phylogenetic tree was constructed. Peptide sequences of each ER and ERR subtype from human *Homo sapiens*, mouse *Mus musculus, O. latipes, F. heteroclitus* and *D. rerio* (Table II) were aligned by use of MAFFT 6.864 (Katoh & Toh, 2008) with the E-INS-i strategy and default parameters. Poorly aligned or divergent regions were removed by use of Gblocks 0.91b (Talavera & Castresana, 2007) with default settings, except for the following changes: $-b2 = 17$, $-b4 = 5$ and $-b5 = h$. The phylogenetic tree was then built from the curated alignment using PhyML 3.0 (Guindon et al., 2010) with settings as follows: LG substitution matrix, modelled equilibrium frequency, estimated proportion of invariable sites, estimated gamma shape parameter and number of substitution rate categories $= 4$. A starting tree was first constructed using BioNJ, which was then improved upon by the SPR (subtree pruning and regrafting) algorithm. The constructed tree was mid-point rooted and visualized in iTOL (Interactive Tree of Life) v2.1.1 (Letunic & Bork, 2011).

**RESULTS**

**PHYLOGENETIC ANALYSIS**

The phylogenetic relationship among ERs and ERRs of *O. latipes, D. rerio* and *F. heteroclitus* as well as those of *H. sapiens* and *M. musculus* was illustrated by a
Table II. RefSeq (if not available, then Genbank) accession numbers of the peptide sequences recruited for the construction of phylogenetic tree. The identity and naming of the *Danio rerio* sequences referenced the Zebrafish Information Network (ZFIN; zfin.org; 25-Dec-2011) instead of the original gene cloning publications. Note that genes *erβ*, *errβ* and *errγ* are duplicated in the listed teleost. Nomenclature of these duplicated genes (in parentheses) is inconsistent among species.

<table>
<thead>
<tr>
<th>Homo sapiens</th>
<th>Mus musculus</th>
<th>Oryzias latipes</th>
<th>Fundulus heteroclitus</th>
<th>Danio rerio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>era</em> NP_000116.2</td>
<td>NP_001428.1</td>
<td>BAA86925.1</td>
<td>AAT72914.1</td>
<td>NP_694491.1</td>
</tr>
<tr>
<td>(erβ1)</td>
<td>(erβ1)</td>
<td>(erβa)</td>
<td>(erβa)</td>
<td></td>
</tr>
<tr>
<td>(erβ2)</td>
<td></td>
<td>(erβb)</td>
<td>(erβb)</td>
<td></td>
</tr>
<tr>
<td><em>errα</em> NP_004442.3</td>
<td>NP_001428.1</td>
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<td>AAT72914.1</td>
<td>NP_694491.1</td>
</tr>
<tr>
<td>(errα)</td>
<td>(errα)</td>
<td>(errα)</td>
<td>(errα)</td>
<td></td>
</tr>
<tr>
<td><em>errβ</em> NP_004443.3</td>
<td>NP_001428.1</td>
<td>BAA86925.1</td>
<td>AAT72914.1</td>
<td>NP_694491.1</td>
</tr>
<tr>
<td>(errβ1)</td>
<td>(errβ1)</td>
<td>(errβa)</td>
<td>(errβa)</td>
<td></td>
</tr>
<tr>
<td>(errβ2)</td>
<td></td>
<td>(errβb)</td>
<td>(errβb)</td>
<td></td>
</tr>
<tr>
<td><em>errγ</em> NP_004443.3</td>
<td>NP_001428.1</td>
<td>BAA86925.1</td>
<td>AAT72914.1</td>
<td>NP_694491.1</td>
</tr>
<tr>
<td>(errγ1)</td>
<td>(errγ1)</td>
<td>(errγa)</td>
<td>(errγa)</td>
<td></td>
</tr>
<tr>
<td>(errγ2)</td>
<td></td>
<td>(errγb)</td>
<td>(errγb)</td>
<td></td>
</tr>
<tr>
<td><em>errδ</em> –</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Phylogenetic tree (Fig. 1). Despite the fact that nomenclature of various evolutionary duplicated ERs and ERRs is inconsistent among teleosts (Table II), postfixes of *O. latipes* clearly match with those of *D. rerio* and *F. heteroclitus* in a scheme relating ‘a’ to ‘1’ and ‘b’ to ‘2’. Namely, the *erβ1* of *O. latipes* is orthologous to the *erβa* in *D. rerio*, and *errγ2* in *O. latipes* is orthologous to *errγb* in *D. rerio*. This matching scheme is consistent for most of the concerned receptor genes with the exception of *errβs*, where *errβ1* and *errβ2* of *O. latipes* are orthologous to *errβb* and *errβa*, respectively, in *F. heteroclitus*.

**Organ-dependent patterns of expression of ERs and ERRs**

In *O. latipes*, all ERs, as well as *errα*, were constitutively expressed in all sampled organs from both sexes, whilst the expression of all other ER subtype varied enormously across organs (Fig. 2). Clustering by organ was evident from PCA (Fig. 3). The four major clusters of organs: brain, heart, gill + gonad and liver + spleen were distributed along PC1 (Fig. 3), which was the linear combination comprised mostly of ERRs (loadings: *errα*, 0.32; *errβ1*, 0.51; *errβ2*, 0.37; *errγ1*, 0.49; *errγ2*, 0.44; all ERs ≤ 0.20). Explicit modelling of this seemingly organ-dependent expression pattern by use of PLS-DA generated another low-dimensional projection [Fig. 4(a)], which was analogous to that of PCA. It displayed the same clustering by organs on the first latent vector that was also constructed predominantly from ERRs (loadings: *errα*, 0.25; *errβ1*, 0.52; *errβ2*, 0.38; *errγ1*, 0.50; *errγ2*, 0.45; all ERs ≤ 0.19). Both PCA and PLS-DA projections clearly demonstrate that expressions of ERRs varied among organs, whereas that of ERs was relatively constant. Certain ERRs
Fig. 1. Phylogenetic tree of all known oestrogen receptor (ER) and oestrogen-related receptor (ERR) subtypes from Homo sapiens (human), Mus musculus (mouse), Oryzias latipes (medaka), Fundulus heteroclitus (mummichog) and Danio rerio (zebrafish). Numerical figure shown next to each node is the branch support assessed by approximated likelihood ratio test based on a Shimodaira–Hasegawa-like procedure implanted in PhyML.

were silent in specific organs, for instance, errβ2 and errγ1 were not detected in heart; errβ1, errβ2 and errγ1 were unexpressed in liver; transcripts of errβ1, errγ1 and errγ2 were not detected in spleen (Fig. 2).

**ORGAN-SPECIFIC DIFFERENCES IN EXPRESSION OF ER AND ERR BETWEEN SEXES**

Applying PLS-DA to model differences in expression patterns of ERs and ERRs with sex as a class variable resulted in a coalesced projection [Fig. 4(b)], which did not feature any distinct grouping of sexes or organs. This classification was unrelated to dimension reduction as the full model training $p_{\text{error}}$ was as low as 0.61. That is, at best, the classification of sexes was erroneous 61% of time. The fact that there was no gross difference in expression of ERs and ERRs between males and females was further supported by symmetry of the expression profiles (Fig. 2). Two-way MANOVA of the scores on the first two principal components, however, demonstrated a statistically significant interaction between the two class variables, sex and organ (Wilks’ lambda = 0.629, approximate $F_{5,72} = 3.49$, $P < 0.001$). This was consistent with the conclusion that differences in ER and ERR expressions between males and females were not general, but rather organ-specific. As observed in the projections of PCA (Fig. 3) and the PLS-DA that modelled organ-dependent expressions [Fig. 4(a)], individuals of different sexes could be segregated into distinct
clusters in liver as well as in gonad (which was unsurprising since testis and ovary are two entirely different organs). Only \( \text{era, er} \beta \)1, \( \text{err} \beta \)2 and \( \text{err} \gamma \)2 exhibited sex difference for some organs (Table III). Whenever a statistically significant difference in expression of a particular ER or ERR gene was detected between males and females, expression was always higher in females (Fig. 2). Conversely, exceptional sex difference was evident for hepatic \( \text{err} \gamma \)2, which was not expressed in females and detectable only in males (Fig. 2).

**Correlations between individual ERs and ERRs**

Correlation of every pair of ERs and ERRs showed two distinct expression clusters [Fig. 5(a)], which were characterized by significant, positive correlations between receptors of the same class (i.e., among ERs or among ERRs) and the lack of strong correlations between those from different receptor classes (i.e., between ERs and ERRs). For across-class correlation, only \( \text{err} \gamma \)1 and \( \text{er} \beta \)2 were correlated, and \( \text{err} \gamma \)2
was the only ERR that exhibited statistically significant correlation with all subtypes of ER [Fig. 5(a)].

Sub-clusters within the two major clusters (ERs and ERRs) were also evident, yet, unexpectedly, the duplicated ER or ERR subtypes, with the exception of errβ, were not clustered together [Fig. 5(b)]. Namely, in terms of profiles of expression, errβ2 was more similar to era than errβ1 (bootstrap: 96%); similarly, errγ2 was very different from the rest of ERRs (bootstrap: 99%).

**DISCUSSION**

This is the first report that has profiled and correlated gene expression of the complete array of ERs and ERRs in multiple organs of both sexes of a vertebrate. Earlier studies have endeavoured profiling ER or ERR gene expression in other teleosts such as Atlantic croaker *Micropogonias undulatus* (L. 1766) (Hawkins et al., 2000), fathead minnow *Pimephales promelas* Rafinesque 1820 (Filby & Tyler, 2005), gilthead seabream *Sparus aurata* L. 1758 (Socorro et al., 2000), goldfish *Carassius auratus auratus* (L. 1758) (Tchoudakova et al., 1999; Ma et al., 2000; Choi & Habibi, 2003), largemouth bass *Micropterus salmoides* (Lacépède 1802) (Sabo-Attwood et al., 2004), the inbred QurtE strain of *O. latipes* (Chakraborty et al., 2011), *F. heteroclitus* (Tarrant et al., 2006; Greytak & Callard, 2007), rainbow

![Graph showing two-dimensional projection of expression profiles.](image-url)
Biplot of the first two latent vectors from partial least-squares discriminant analysis (PLS-DA) classifications in an attempt to model (a) organ-dependent and (b) sex-dependent expression patterns of oestrogen receptors (ER) and oestrogen-related receptors (ERR) in *Oryzias latipes*. Scores (samples) were linearly scaled to have absolute maximum of 1 and overlaid with the loadings (receptor genes). Male and female samples are denoted as ♂ and ♀. (a) The three-component classification model (the last latent vector is not shown for the sake of simplicity) performed satisfactorily in modelling the organ-dependent expression pattern (training \( p_{\text{error}} = 0.05 \); cross-validated \( p_{\text{error}} = 0.20 \)), while capturing adequate variation in the expression dataset (\( R^2_X = 0.78 \)). Note that this projection is very similar to that of principal component analysis. (b) The modelling of sex-related expression pattern was rather incompetent (two-components: training \( p_{\text{error}} = 0.90 \), cross-validated \( p_{\text{error}} = 0.97 \), \( R^2_X = 0.40 \); Optimal five-components: training \( p_{\text{error}} = 0.61 \), cross-validated \( p_{\text{error}} = 0.85 \), \( R^2_X = 0.83 \)). Organs are annotated in different colours: ⁎, brain; ♂, gill; ♀, gonad; ♂, heart; ♂, liver; ♂, and ♀, spleen.

*Fig. 4.*

**Table III.** \( P \)-values of two-sample statistical tests (\( t \)-test – Mann–Whitney \( U \)-test) examining the null hypothesis of no sex difference in the average normalized expression of each gene in each organ. All \( P \)-values were adjusted to control for false discovery rate using the Benjamini & Hochberg procedure.

<table>
<thead>
<tr>
<th>organ</th>
<th>( er )</th>
<th>( er\beta 1 )</th>
<th>( er\beta 2 )</th>
<th>( err )</th>
<th>( err\beta 1 )</th>
<th>( err\beta 2 )</th>
<th>( err\gamma 1 )</th>
<th>( err\gamma 2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.152</td>
<td>0.022*</td>
<td>0.481</td>
<td>0.935</td>
<td>0.062</td>
<td>0.086</td>
<td>0.064</td>
<td>0.709</td>
</tr>
<tr>
<td>Gill</td>
<td>0.800</td>
<td>0.006**</td>
<td>0.481</td>
<td>0.314</td>
<td>0.934</td>
<td>0.507</td>
<td>0.212</td>
<td>0.074</td>
</tr>
<tr>
<td>Gonad</td>
<td>0.314</td>
<td>0.027*</td>
<td>0.934</td>
<td>0.285</td>
<td>1.000</td>
<td>0.011*</td>
<td>0.152</td>
<td>0.006**</td>
</tr>
<tr>
<td>Heart</td>
<td>0.774</td>
<td>0.624</td>
<td>0.285</td>
<td>0.934</td>
<td>0.950</td>
<td>NA</td>
<td>NA</td>
<td>0.800</td>
</tr>
<tr>
<td>Liver</td>
<td>0.008**</td>
<td>0.861</td>
<td>0.437</td>
<td>0.861</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.688</td>
</tr>
<tr>
<td>Spleen</td>
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<td>0.073</td>
<td>0.314</td>
<td>0.994</td>
<td>NA</td>
<td>0.285</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, undetectable in both sexes. \( *p < 0.05 \), \( **p < 0.01 \).
are consistent with the results of studies concerning mammals and other teleosts. All subtypes of ER were expressed by all organs studied in H. sapiens (Kuiper et al., 1997), M. musculus (Couse et al., 1997) and teleosts. ERRα was also found in virtually all organs of H. sapiens, M. musculus (Giguère et al., 1988; Shi et al., 1997; Sladek et al., 1997) and F. heteroclitus (Tarrant et al., 2006). Previous studies in H. sapiens, rodents and F. heteroclitus have also shown that ERRβ and ERRγ expressions are restricted to specific organs. For instance, in rat Rattus sp., ERRβ was only expressed in brain (cerebellum and hypothalamus), heart and testis, but not liver (Giguère et al., 1988); in F. heteroclitus, both errα and errβb were detected in most organs studied but absent from liver and spleen (Tarrant et al., 2006). Likewise, in H. sapiens, M. musculus and F. heteroclitus, ERRγ was expressed in diverse organs but at a much lesser magnitude or entirely absent in the liver (Heard et al., 2000; Süsens et al., 2000; Tarrant et al., 2006). The highly conserved ER and ERR profiles and oestrogen biology among vertebrates indicate that O. latipes can serve as a viable alternative vertebrate model for the study of generalized oestrogen responses and signalling system in vivo.

It has been proven in O. latipes that modulation of gene expression in response to oestrogenic compounds is cell, tissue and organ-dependent (Inoue et al., 2002; Zhang et al., 2008a, b). Such a phenomenon is commonly attributed to conjectural cell-specific ‘cross-talk’ between ERs and ERRs that controls transcription of specific arrays of oestrogen target genes. Thus far, no experimental evidence in vivo has been provided to substantiate such claim (Giguère, 2002). Conceivably, it is time-consuming and expensive, yet absurd to study speculative interactions between all combinations of ERs and ERRs without knowing whether such subtype ER–ERR interactions occur naturally in specific cells, tissues and organs. This study demonstrates that while all ER subtypes and errα were ubiquitously expressed, expressions of errβ and errγ isoforms (i.e. errβ1, errβ2, errγ1 and errγ2) varied enormously across specific organs. The results lead to reasonable postulation that the observed organ-specific oestrogen responses are defined by the expressions of selected errβ and errγ isoforms, which dispose specific pairing and cross-talk with the ERs in specific organs.

The premise that ERR isoforms may functionally interact with ERs is reinforced by the systematic statistical analyses exemplified in this study. Peculiar correlation between expressions of errγ2 and all ERs, as well as between errγ1 and errβ2 has been identified. It is attested that functionally related genes having highly correlated expression profiles are often being co-regulated (Allocco et al., 2004) and are likely to interact with each other physically (Teichmann & Babu, 2002). Conceivably, the statistically significant correlation between expressions of ERs and errγ subtypes may indicate possible existence of co-regulatory mechanisms and, potentially, functional interactions in vivo. Further research on functional interactions between ERs and errγs, in particular erβ1-errγ2, erβ2-errγ2 and erβ2-errγ1, is urgently needed to define biological significance of these ER-ERR cross-talks at conferring cell, tissue and organ-dependent responses upon receiving oestrogenic stimuli.

It is recognized that whole organ qPCR analysis is unable to demonstrate definite co-expression of ERs and ERRs within the same cell, which is prerequisite to potential interaction. Recently, two histological devices: the embryo chip (Cheung et al., 2012) and whole adult histoarray (Kong et al., 2008; Park et al., 2008, 2009; Padilla et al., 2009; Tompsett et al., 2009), were developed to enable localization
Fig. 5. Correlogram and hierarchical clustering of the oestrogen receptor (ER) and oestrogen-related receptor (ERR) expressions. (a) Correlogram showing the correlation coefficient (Spearman’s $\rho$) of every pairing of the analysed genes. Significance of the correlation coefficients after controlling for false discovery rate: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The two prominent green blocks at the upper left and lower right sectors are the pairings of ERs and ERRs, respectively. (b) Average linkage clustering of the ERs and ERRs using $1-[\text{Spearman’s } \rho]$ as the measure of dissimilarity. Numerical figures above the nodes were cluster support (%) based on multiscale bootstrap resampling ($B = 10000$). ERs and ERRs were agglomerated into two distinct clusters (annotated as Cluster 1 and Cluster 2) with very high statistical confidence (i.e. bootstrap: >95%). Note that duplicated ER and ERR subtypes were not necessarily clustered together.
and quantification of multiple gene and protein expressions at the cellular level across multiple organs of the same fish simultaneously using in situ hybridization (ISH) and immunohistochemistry (IHC). Alternatively, qPCR analysis of cells removed from histologic sections using laser capture microdissection (LCM) can also be performed to localize gene expression at the tissue and cellular levels with sufficient resolution. Studies are ongoing to screen antibodies that can be applied to co-localize cellular expression of the above target ER and ERR proteins in O. latipes. Concurrently, in vitro assays are being developed to demonstrate possible interaction between O. latipes ER and errγ proteins on transcriptional regulation of oestrogen responsive genes.

The existence of a differential profile of erβs (i.e. erβ1 and erβ2) in teleost species was not uncommon (Filby & Tyler, 2005; Hawkins et al., 2005; Greytak & Callard, 2007; Nagler et al., 2007). This has been attributed to neo- and sub-functionalization processes that ultimately diverged each of the erβ duplicates to provide distinct functions, and hence was expressed by specific cells and tissues (Hawkins et al., 2000, 2005). This is the first study to report divergence in expressions for ERR duplicates (i.e. errβ1 and errβ2; errγ1 and errγ2), suggesting potential neo and sub-functionalization of ERR duplicates in vertebrates. Interestingly, errγ2 expression in liver exhibited unique sex-specific pattern (i.e. detectable in males only), which is distinct from that of all other ERs and ERRs (i.e. generally higher in females than in males). Establishment of knockout mutant of errγ2, together with other ERRs, would be useful to uncover their novel roles in oestrogen signalling, especially in a sex-specific manner.

PERSPECTIVES

The data presented by this study highlight the potential importance of ERRs in defining organ and sex-specific responses to oestrogenic substances in vertebrates. The findings provide useful insights into functional importance of ERRs in oestrogen signalling. Possible research directions were put forward to aid revealing biological significance of ERs and ERRs divergence in O. latipes/vertebrates. In view of technically demanding and laborious nature of functional analyses, communal efforts are certainly deemed necessary.

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