Molecular cloning and expression analysis of estrogen receptor betas (ERβ1 and ERβ2) during gonad development in the Korean rockfish, *Sebastes schlegeli*

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**A B S T R A C T**

Estrogen receptors (ER) play a crucial role in mediation of estrogen activities. Here we report the isolation and expression analysis of ERβ1 and ERβ2 from ovary Korean rockfish (*Sebastes schlegeli*), were isolated using reverse transcription-polymerase chain reaction (PCR) and rapid amplification of cDNA ends procedures. The cDNA of this study, ERβ1 (588 amino acids) and ERβ2 (659 amino acids) were identified using reverse-transcriptase PCR (RT-PCR) and rapid amplification of cDNA ends procedures. Structural analysis showed both ERβs contain six typical nuclear receptor-characteristic domains. Phylogenetic analysis indicated that Korean rockfish ERβs were highly conserved among teleost. RT-PCR confirmed that the ERβs were widely distributed in both gonads and extra gonadal tissues. Further, we analyzed the expression patterns of male and female *S. schlegeli* during the reproductive cycle using quantitative real-time PCR (qRT-PCR). The results showed that the highest expression levels were observed in testis at immature sperm stage for both of KrERβ1 and KrERβ2. For female, the expressions of KrERβ1 and KrERβ2 were significantly higher in the ovary at the early-oocyte stage. Cloning these two ERβ subtypes in the Korean rockfish, together with the information on expression levels in adult fish has given us the foundation to investigate their possible role in brain-pituitary-gonad neuroendocrine axis in future studies.

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1. Introduction

It is well known that estrogen is one of the most important hormones in vertebrates, and plays an important role in growth, differentiation, reproductive behavior and pituitary gonadotropin secretion (Cavaco et al., 1998; Kristfalus and Nagler, 2000; Lange et al., 2002). Increasing evidences demonstrate that most estrogen actions are mediated by the member of the steroid receptor super-family which is called estrogen receptors (ERs) (Fairbrother, 2000; Filby and Tyler, 2005; Lange et al., 2002). The protein members of this family have been well recognized that they contain six distinct domains labeled from A to F (Krust et al., 1986; Kumar et al., 1987). Among these, C domain (DNA-binding domain; DBD) and E domain (ligand-binding domain; LBD) are responsible for ligand binding, nuclear localization, and transcriptional activation (AF-2), and they are highly conserved among species (Choi and Habibi, 2003; Ma et al., 2000; Sumida and Saito, 2008). In contrast, A/B domain in the N-terminal and F domain in the C-terminal are less conserved (Filby and Tyler, 2005). In addition, the D domain (hinge region) is necessary for the maintenance of ER three-dimensional structure (Hu and lazar, 1999; Zilliacus et al., 1995). Two subtypes of ER have been described in vertebrates so far, called ERα and ERβ (Hawksins et al. 2000), which are found in the cell or tissue specific context (Choi and Habibi, 2003; Enmark and Gustafsson, 1999). They exhibit distinct differences in ligand binding affinities, transcriptional activities and knockout phenotypes (Nilsson et al., 2001). A current hypothesis suggests that ERαs and ERβs resulted from a whole genome duplication event in ray-finned fishes (Amores et al., 1998). In addition, many studies in zebrafish (Bardet et al., 2002; Menuet et al., 2002), Atlantic croaker (Hawksins et al., 2000), goldfish (Ma et al., 2000; Tchoudakov et al., 1999) suggested that two forms of ERβs existed in teleost. Bardet et al. and Robinson-Rechavi et al. revealed that ERβ1 and ERβ2 were generated by duplication of an ancestral ERβ subtype (Bardet et al., 2002; Robinson-Rechavi et al., 2001).

Based on the research in yellow perch, gilthead sea bream, common eelpout, and largemouth bass, ERβs have been detected in a wide range of tissues including kidney, muscle, heart, liver, ovary, testis, gill, pituitary, brain, etc. (An et al., 2008; Andreassen et al., 2003; Lynn et al., 2008; Pinto et al., 2006; Sabo-Attwood et al., 2004). The study of variation of genes expression and serum steroid hormone during gonad development in the ERβ knockout mouse indicated...
that ERβ might regulate expressions of androgen receptor and other ovulation-related factor genes to modulate GnRH release thus further affecting ovulation (Cheng et al., 2002). In teleost, the study of zebrafish ER showed it can be transcriptionally activated by estradiol (E2) (Bardet et al., 2002). ERs expressed early during embryonic development and gonadal differentiation in teleost, suggesting the important role for estrogens in sexual differentiation (Guiguen et al., 1999; Lassiter et al., 2002). Cavaco et al. also suggested that ERs was paramount important for main events during sexual development, such as puberty (Cavaco et al., 1998).

The Korean rockfish (Sebastes schlegeli) is a widely distributed fish, occurs in the coastal areas of the northwestern Pacific Ocean, especially in the East China Sea, Yellow Sea, and the coastal areas of the South Sea of Korea and Japan (Kang and Hwang, 2003). As a typical ovoviviparous fish with the high economic value, it is therefore of strong interest to know more about its molecular mechanisms of endocrine regulatory during the reproductive cycle. In this study, we focused on the two forms of ERs in Korean rockfish since the ERα has been described in our previous work (Shi et al., 2011). We aimed to characterize two subtypes of ERs, for the first time, investigated its tissue distribution and temporal expression in Korean rockfish as a step to further understanding the molecular mechanisms of ER action.

2. Materials and methods

2.1. Experimental fish

Around twenty individuals of adult mature male and female Korean rockfish samples were obtained from Shandong coastal area every 2 months. They were maintained for 3–4 days in indoor culture tanks with natural seawater under controlled conditions (20 ± 0.5 °C; C4 mg/l O2; 14:10 h light; dark cycle). Sexual maturity was determined after excising the gonads defined by the presence of mature ova and sperm, according to Mu et al. (Mu et al., 2013). All fish were anesthetized in 100 mg/l tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). Tissues of stomach, intestine, gill, heart, spleen, kidneys, head kidneys, brain, muscle, pituitary and liver were collected from each fish, and sectioned in two parts, one fixed in Bouin’s solution for hematoxylin and eosin (HE) staining in order to identify the development stages of gonads, the other one was snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.2. Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from Korean rockfish tissue samples using RNAiso reagent (Takara, Japan) following the manufacturer’s instructions. RNA concentration and purity of each sample was quantified in UV spectrophotometer (Ultraspec-2100Pro, Amersham; A260 nm/A280 nm ratios > 1.8). The electrophoresis on ethidium bromide-stained 1.5% agarose gels was applied to check RNA integrity. The first-strand cDNA was synthesized, respectively, with 1 μg total RNA from each sample and d(T)18 primers in 10 μL reactions at 70 °C for 5 min and Reverse Transcriptase M-MLV (Takara, Japan) following the manufacturer’s protocol.

2.3. Isolation and PCR amplification of ERs cDNA fragments

To obtain core partial-length fragments KrERβ1 and KrERβ2 cDNAs, two pairs of degenerated primers were designed by a web-based primer design program-codehop (Chen et al., 2009) from highly conserved amino acid sequences among fish species (Table 1). PCR reaction was carried out in a final volume of 50 μl containing 2 μl of cDNA from ovarian tissue following the manufacturer’s instructions (Takara, Japan). PCR cycling conditions were as follows: 5 min denaturing step at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at a range of annealing temperature from 70 °C to 60 °C (both ERβ1 and ERβ2), decreasing 1 °C each cycle and 30 s at 72 °C, then followed by additional 30 cycles of 30 s at 94 °C, 30 s at 60 °C (both ERβ1 and ERβ2) and 30 s at 72 °C, finally ended with 10 min at 72 °C for extension. The PCR reactions were separated by a 1.5% agarose gel and purified using TIAN gel midi Purification Kit (TIAGEN, China), then cloned into pGM-T vector (Tiangen, China), followed by propagation in Escherichia coli DH5α, clones were subsequently sequenced on the ABI3730XL sequencer (ABI, USA).

2.4. 3’ and 5’ RACE-PCR

The 3’ and 5’ ends RACE PCR were applied using the SMART™ RACE cDNA amplification Kit (Clontech, USA). Specific primers and nested primers for amplification of 5’ and 3’ ERβ cDNA ends were listed in Table 1. The first-strand cDNA synthesis and RT-PCR were used 1 μg of total RNA and 1 μm L−1 each primers. PCR was performed using the cycling conditions: 5 min denaturing step at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 68 °C (ERβ1-C-3-2), or at 69 °C (ERβ2-C-5-1; ERβ2-C-5-2), or at 70 °C (ERβ1-C-5-1; ERβ1-C-3-1; ERβ2-C-3-1), then followed by additional step at 72 °C for 1 min, finally ended with 10 min at 72 °C for extension. The PCR reactions were separated by a 1.5% agarose gel and purified using TIAN gel midi Purification Kit (TIAGEN, China), then cloned into pGM-T vector (Tiangen, China), followed by propagation in Escherichia coli DH5α, selected clones were sequenced on the ABI3730XL sequencer (ABI, USA).

2.5. Phylogenetic analysis and sequence analysis

Multiple protein sequence of ERβ’s cDNA were obtained from Genbank, and alignments were aligned by the ClustalX version 1.81 (Thompson et al., 1997). Phylogenetic analyses, of full length amino acid sequences, were conducted using MEGA version 4.0 (Tamura et al., 2007). A rooted phylogenetic tree was constructed by means of the Neighbor-Joining algorithm (Saitou and Nei, 1987), and the data were re-sampled via 1000 bootstrapping replicates (Felsenstein, 1985). Protein sequence analysis was performed with the ExPaSy Molecular Biology Server (http://www.expasy.ch/) scanning all known PROSITE motifs based on PROSITE database (Bairoch et al., 1997). Percent identities of proteins motifs between Korean rockfish and other species were calculated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.6. Tissue distribution analysis by reverse transcription (RT)-PCR

The expression profiles of ERβ1 and ERβ2 mRNA in different tissues were examined using RT-PCR (reverse transcriptase-polymerase chain reaction) assays. Total RNA was extracted from tissues of ovary, liver, kidney, head kidney, brain, heart, spleen, caeca, stomach, fat, gut, intestinal and pituitary of one female fish in late-vitellogenic stage and testis from one male fish at spermiated stage. To avoid genomic contamination, extracted RNA was treated with RNA DNase I before reverse transcription. Total RNA of those organs were extracted and reverse-transcribed as described above (see 2.2). Specific primers designed based on the sequences generated were listed in Table 1 (ERβ1-e-f and ERβ1-e-r; ERβ2-e-f and ERβ2-e-r). Tissue expression was normalized using 18S rRNA (Table 1) as an internal control. Semi-quantitative RT-PCR was performed using a Biometra TPersonal Thermal Cycler (Biometra, Germany), the PCR cycling conditions were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 5 s, 58 °C for 30 s (both for ERβ1 and ERβ2), 72 °C for 30 s, finally 72 °C for 10 min. Reaction product was checked by 1.5% agarose gel (containing ethidium bromide) electrophoresis and visualized on a Gel system (Tanon, China).
there was lack of typical MAPK kinase phosphorylation sites in this domain. In the C domain, motifs of D-box (EGCKAFF), P-box (PATNQ), PKA (protein kinase A) and eight cysteine residues of ERβ were completely conserved compared with O. mykiss ERs and Sparus aurata ERs. A conserved motif of CK-2 as well as a protein kinase C phosphorylation site was found in ERβ, another CK-2 motif was in ERα. In the E domain, two PKC sites and a ligand-dependent transactivation function motif (AF-2) were conserved in both of ERα and ERβ, and a motif of CK-2 was found in ERβ.

Amino acid sequences of KrERβ/1 and KrERβ/2 showed an overall identity of 58%. The two ERs share 38% identity in the A/B domain, 89% in the C domain (the DNA-binding domain), 17% in the D domain, 73% in the E domain (the ligand-binding domain), and 17% in the F domain (Tables 2 and 3). In comparison of Korean rockfish ERβ/1 with other species, KrERβ/1 shared 79–21, 96–87, 71–7, 93–66, and 75–8% identities in the A/B, C, D, E and F domains, respectively (Tables 2 and 3), showing the high conserved features of C domain.

### 2.7. Quantitative real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR (qRT-PCR) was conducted to determine the relative expression of ERs in mRNA using the RNA extracted from gonads of Korean rockfish. PCR analyses were performed using Eppendorf iCycler iQ multicolor real-time PCR detection system (Eppendorf, Hamburg, USA) and the iQ SYBR Green Supermix (Takara, Japan) according to the manufacturer's protocol. The primer sequences for ERβ/1 (ERβ/1-e-f and ERβ/1-e-R) and ERβ/2 (ERβ/2-e-f and ERβ/2-e-R) are listed in Table 1. As an internal control, 18S rRNA was amplified under the same conditions using Korean rockfish-specific primers (Table 1) and no significant changes were observed in the 18S rRNA level during gonadal development. The mRNA was treated using DNase I (Takara, Japan) and Ribonuclease Inhibitor (Takara, Japan) to remove trace genomic DNA and prevent potential genomic DNA amplification. The ERs' qPCR conditions were as follows: 1 cycle of denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 20 s, and extension at 72 °C for 20 s. Three PCR reactions were performed for each sample and then averaged. Relative expression levels (ERβ: reference gene) were determined using a development of the arithmetic comparative method, 2−ΔΔCT method (Livak and Schmittgen, 2001). The samples from one stage with serial dilutions of total cDNA were used as calibrators to calculate the expression. ΔΔCT = (Avg. CtTarget − Avg. CtReference gene) − (Avg. Ctcalibrator gene − Avg. CtReference gene).

### 3. Results

#### 3.1. Isolation and characterization of ERβ/1 cDNA

The full-length KrERβ/1 cDNA (2451 bp, FJ466610.3) was found to have an open reading frame (ORF) of 1683 bp which began with the first ATG codon at position 588 bp and ended with a TGA stop codon at position 2273 bp. It encoded 561 amino acids (Fig. 1). The cDNA of KrERβ/2 (2338 bp, HQ452829.1), was found to have an open reading frame (ORF) of 1977 bp which began with the first ATG codon at position 182 bp and ended with a TGA stop codon at position 2161 bp. It encoded 659 amino acids (Fig. 2). Both of them were lacking a typical polyadenylation signal in the 3′-UTR but containing a poly (A) tail.

The KrERβ/1 and KrERβ/2 sequences can be classified into six domains (A/B, C, D, E and F) based on its sequence identity to other species' ERβ/1s (Krist et al., 1986) (Fig. 3). In A/B domain of ERβ/2, there was a PCK (protein kinase C) phosphorylation site which was considered to make up for ligand-independent transactivation function motif (AF-1), and there was lack of typical MAPK kinase phosphorylation sites in this domain. In the C domain, motifs of D-box (EGCKAFF), P-box (PATNQ), PKA (protein kinase A) and eight cysteine residues of ERβ were completely conserved compared with O. mykiss ERs and Sparus aurata ERs. A conserved motif of CK-2 as well as a protein kinase C phosphorylation site was found in ERβ/2, another CK-2 motif was in ERα. In the E domain, two PKC sites and a ligand-dependent transactivation function motif (AF-2) were conserved in both of ERβ/1 and ERβ/2, and a motif of CK-2 was found in ERβ/2.

Amino acid sequences of KrERβ/1 and KrERβ/2 showed an overall identity of 58%. The two ERs share 38% identity in the A/B domain, 89% in the C domain (the DNA-binding domain), 17% in the D domain, 73% in the E domain (the ligand-binding domain), and 17% in the F domain (Tables 2 and 3). In comparison of Korean rockfish ERβ/1 with other species, KrERβ/1 shared 79–21, 96–87, 71–7, 93–66, and 75–8% identities in the A/B, C, D, E and F domains, respectively (Tables 2 and 3), showing the high conserved features of C domain.

#### 3.2. Phylogenetic analysis

The phylogenetic analysis was conducted using the amino acid sequences for detection of the evolutionary relationship among ERβ/1s (Fig. 4). All ERβ/1s proteins appeared to be clustered in two distinct clades (ERαs and ERs). Three subclasses including fish ERβ/1, fish ERβ/2 and tetrapod ERβ were classified into ERβ/1 clades. In the teleost ERβ/1 clade, KrERβ/1 had the highest similarity with Perca flavescens ERβ/1a (86% similarity). Inside the teleost ERβ/2 clade, KrERβ/2 had the highest similarities with Micropterus salmoides ERβ/2 (88% similarity).

#### 3.3. Expression of ERβ/1s in different adult tissues

Primers (ERβ/1-eF and ERβ/1-eR; ERβ/2-eF and ERβ/2-eR, listed in Table 1), were applied to determine tissue expression of KrERβ/1s. In order to avoid cross amplification, the primers were constructed from stretches of sequence that exhibit significant differences between ERs. The length of generated PCR products of ERβ/1 and ERβ/2 were 249 bp and 389 bp respectively (Fig. 5). Both of ERβ/1 and ERβ/2 displayed a widely distribution. The results showed that ERβ/1 and ERβ/2 were found in pituitary, brain, kidney, gonads, head-kidney and spleen. However, ERβ/1 was expressed in the kidney, intestine, liver as well as kidney which was common found the expression in all kind of fish, but ERβ/2 was detectable in these tissues.
3.4. Expression profiles during the reproductive cycle

Variations of the temporal expression of ERβs during reproductive cycle in ovary and testis were analyzed by qRT-PCR, showing in Fig. 6. According to Shi et al. (2011), the Korean rockfish testis and ovary developmental phase were divided into four phases. In the male fish, the ERβ1 expression level was $1.00 \pm 0.51$ during May to July (stage II), increased to the highest level to $4.62 \pm 1.00$ in the September (stage III), then dropped sharply from September, finally, the expression level was slightly increased to $1.80 \pm 0.55$ during January to March (Stage V). However, the expression of ERβ2 in male fish varied gently with the development of gonad. The ERβ2 expression level was $0.90 \pm 0.19$.
during May to July (stage II), then peaked to 1.33 ± 0.12 during September, dropped to 1.05 ± 0.21 during January to March (stage V), and finally slightly increased to 1.02 ± 0.14.

In the female, the expression pattern of ERβ1 and ERβ2 was similar. During July to September (stage II), the expression level of ERβ1 and ERβ2 was 0.92 ± 0.35 and 2.49 ± 0.49 respectively. The highest level of ERβ1 was 3.73 ± 1.48 during November (stage III), and ERβ2 was 5.99 ± 1.78. Finally, the level of ERβ1 and ERβ2 dropped to the lowest level of 0.18 ± 0.04 and 1.05 ± 0.21 during May (stage V).

Fig. 2. Nucleotide and deduced amino acid sequence of KrERβ2 isolated from ovary. Two zinc-finger motifs in DNA binding domain were underlined, and eight cysteines in the same domain were also shaded. The initiation codon and termination codon were boxed.

Initiation codon: ATG Termination codon: TGA
Amino acid identities between ERβ1 in Korean rockfish and ERs in fish and mammals (see Section 2.5 for sequence references. The total score of amino acids and the number of residues per domain are marked in brackets).

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<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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<td>71(38/40)</td>
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<td>79(106/152)</td>
<td>95(45/85)</td>
<td>67(41/40)</td>
<td>94(228/238)</td>
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<td>70(149/152)</td>
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<td>65(40/40)</td>
<td>95(238/238)</td>
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<tr>
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<td>32(40/40)</td>
<td>89(238/238)</td>
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4. Discussion

Two full-length cDNA of ERβ1s in the Korean rockfish were cloned in this study. The KrERβ11 cDNA was found to contain an ORF of 1683 nucleotides encoding the protein of 588 amino acids, and the ERβ2 cDNA was 1977 bp, encoded the protein of 659 amino acids. The feature that ERβ1 protein of Korean rockfish contained more amino acids than ERβ1 was found in many teleosts. The two sequences possess the domain structure (A, B, C, D, E domains) which are typical for ERs, and the highly-conserved zinc-finger motif, including the P- and D-boxes, are indispensable for DNA-binding (Schwabe et al., 1993). It is a common feature that ERα has a mitogen-activated protein kinase (MAPK kinase site) in the A/B domain (Halm et al., 2004; Shi et al., 2011). However, the lack of typical MAPK kinase phosphorylation site in both KrERβ1s in the A/B domain was similar with the S. aurata (Pinto et al., 2006), showing the different species may have different signal transduction pathways according to their evolutionary status. Furthermore, it is supposed that the lacking of MAPK (AF-1) in A/B domain in ERβ1s may be a reason that Korean rockfish ERs and ERα have different functions. The two zinc-finger motifs in the C domain as well as the estrogen-dependent activation domain AF-2 (DLLEMLD) in the E domain were completely conserved, the latter one was related to ligand dimerization, ligand binding and ligand-dependent transcription activation function (Pinto et al., 2006). The function of E domain was unknown, however, it was considered to control gene transcription interacting with nuclear cofactors by affected ER/Sp1 action (Kim et al., 2003; Montano et al., 1995).

In human ERα, 64 amino acids residues (M342-L354, W383-R394, L402-L410, V418-L428, M517-M528 and L539-H547) in the E domain formed the 17α-estradiol binding cavity (Brzozowski et al., 1997). In KrERβ1 and ERβ2, 48 and 50 amino acids residues of these 64 were conserved, respectively. Furthermore, 15 amino acids residues (L346, A350, L387, L391, R394, S395, E397, L402, L403, F404, A405, P406, L408, L410 and L525) also in this domain were essential for ligand contact within binding pocket of human ER-α. There were 12 of them could be identified in KrERβ1 (except E397, L403 and A405), and 11 of them were found in KrERβ2 (except E397, L403, A405 and L408). In addition, four residues (Leu189, Met221, Tyr252, and Cys530) changes which existed in KrERs were at positions surrounding the human ERα pocket, in accordance with the study by Hawkins and Thomas.
Fig. 4. Phylogenetic tree based on amino acid sequences for ERβs in tetrapods and teleosts. The human estrogen receptor α is used as an outgroup. Bootstrap values are indicated (1000 replicates) (see Materials and methods for sequence references and abbreviations).

Fig. 5. RT-PCR expression analysis of KrERβs in tissues. The integrity of the RNA from each tissue was ensured by uniform amplification of 18S transcripts (lower panel).
(2004). All of these significant conserved residues showed the proposed function for determining species and subtype-specific ligand binding characteristics in ERβs. By comparing the functional domains, we found that ERβs in various species were highly conserved. The motifs of EFCKAF and CPATNQC, highly conserved in all ERβs, were important for species binding to ERE (estrogen response element) on target genes and receptor dimerization respectively (Forman and Samuels, 1990; Ma et al., 2000). These results indicate there was repetition in combination of target DNA with ER, redundancy function of starting target genes transcription. It also suggested these two subtypes of ERβs were capable to combine with different ligands, thus exerting different effects on the biology activity.

The ER genes expressed in various tissues showed an important significance for estrogen action. Although the structures were similar, these ER subtypes had the different expression patterns. RT-PCR was used to measure the tissue distribution of ERβs mRNA in maturing Korean rockfish. As is shown in Fig. 3, RT-PCR analysis exhibited that both of KrERβs mRNA were highly detected in E2 target tissues that were known related to the reproductive function, such as the pituitary, ovary and testis. And this result was similar with goldfish ERβ2 (Choi and Habibi, 2003) and sea bream ERβs (Socorro et al., 2000). Study in mammals showed that ERβ was expressed at relatively high levels in the reproductive system, including prostate, epididymis, testis, ovary and uterus, but level in the pituitary was low (Kuiper et al., 1996). The high levels of ERβs expression in the gonads and pituitary of teleost fish showed ERβs may play an important role in sexual differentiation and/or development (Halm et al., 2004). In addition, the high level of ERβs transcript levels in the adult zebrafish ovary indicated a special role for ERβs in reproductive tissue as well as ERβs in mammals (Byers et al., 1997). The high expression of ERβs is also found in Korean rockfish brain, suggesting some relationship between ER and neuroendocrine functional control. Studies in other teleosts, such as pejerrey, goldfish and zebrafish, also detected the ERβs expressed in brain, suggesting the preoptic area and hypothalamus was the main target site for E2, which may involved in the regulation of neuroendocrine related gene expression (Marlatt et al., 2008; Menuet et al., 2002; Stroble-Mazzulla et al., 2008). The interesting thing is that the high level expression of KrERβ2 but undetectable expression of Kr ERβ1 was found in the liver. Most of teleosts showed the three subtypes of ER mainly expressed in liver, such as sea bream, goldfish and father minnow (Choi and Habibi, 2003; Filby and Tyler, 2005; Pinto et al., 2006). However, there was a high expression level of ERα and ERβ2 but low level of ERβ1 in liver of fathead minnow, which indicated the interspecific differences existing in the genes of ERs expression. Some studies showed that the high mRNA levels of ERα1 and ERβ2 were accordance with the effect of estrogen on liver to induce vitellogenesis (Campbell and Idler, 1980; Sumpter and Jobling, 1995). Leaños-Castañeda and Kraak found the vitellogenin production was mainly mediated through ERβ2 in male fish liver (Leaños-Castañeda and Kraak, 2007). Our research may suggest that KrERβ2 play the predominant role and that ERβ1 may have no function or major effect in this process. In this study, in contrast to ERβ1, expression of ERβ2 was more wide-spread in Korean rockfish tissues. Furthermore, the ERβ1 in Korean rockfish was expressed abundantly in kidney, moreover, the low level of mRNA expression was found in fat, head-kidney and heart. However, the KrERβ2 mRNA was almost highly expressed in intestine, kidney, caeca, head-kidney and spleen. The abundance transcripts of ERβ2 were reported in the sea bream intestine, suggested that it involved in the modulation of calcium transport, even though the mechanism was not yet well explained (Guerreiro et al., 2002). All of these results

Fig. 6. Expression of ERβ1 in testis (A) and ovary (B) and ERβ2 in testis (C) and ovary (D) mRNA of Korean rockfish during the annual reproductive cycle. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference (P < 0.05, one-way ANOVA, followed by Duncan’s test) (n ≥ 3) In male fish: stage II spermatogonia stage; stage III testes full of immature sperm; stage IV mature testes; stage V post-spermiation. In female: stage II perinucleolus stage oocyte; stage III early-oocyte; stage IV post-oocyte; stage VI gestational ovary.
above showed the potential complexity function of ERβ subtypes in these organs. Further studies were needed to elucidate any more bio-
logical effects of these subtypes in various tissues.

Using quantitative real-time PCR, high levels of ERα's mRNA in Korean rockfish were observed in early development stage of testis, being different with that of trend of ERs (Shi et al., 2011), which sug-
gested that those two kinds of ER played different roles during the testis development. In addition, the study in fathead minnow indicat-
ed different mechanisms of regulation for different ERs (Filiby and Tyler, 2005), which may be another result for the different pattern of ERs. In the early stage of spermatogenesis in male Korean rockfish, the ERs mRNA level increased as well as the plasma E2 level (Shi et al., 2011), which showed that the ERs was involved the regulation of estrogen-dependent spermatogenesis. The fact that some researches revealed the high level ERβ mRNA expression during early testis develop-
ment stage suggests that ERβ was important for gonads development and maturation (Byers et al., 1997; Filiby and Tyler, 2005; Halm et al., 2004). The study of European sea bass suggested that the ER had initiated

References


Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the boot-


Hawks, M.B., D., 2004. Cloning, characterisation, and expression of three oestrogen rece-

Hawks, M.B., 2004. The unusual binding properties of the third dis-
tinct teleost estrogen receptor subtype Erβ is accompanied by highly conserved amino acid changes in the ligand binding domain. Endocrinology 145, 2968–2977.

Hawks, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., 2000. Iden-
tification of a third distinct estrogen receptor and reclassification of estrogen rece-


Kim, K.Y., K. N. Saville, R. Safe, S., 2003. Domain of estrogen receptor alpha (ERα) re-

Krisfalusi, M., Nagler, J.J., 2000. Induction of gonadal intersex in genotypic male rainbow-


Leaños-Castañeda, O., Kraak, G.V.D., 2007. Functional characterization of estrogen re-
ceptors subtypes, ERα and ERβ, mediating vitellogenin production in the liver of rain-

time quantitative PCR and the 2-DDCT method. Methods 25, 322–408.

Lynne, S.G., Birgle, M.J., Molecular characterization and sex-specific tissue expression of estrogen receptor α (esr1), estrogen receptor β (esr2A) and ovarian arsenic (cyp19a1a) in yellow perch (Perca flavescens). Comp. Biochem. Physiol. B. 149, 126–147.


pression profiling of 17β-estradiol action in the neuroendocrine axis of male gold-


