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Characterization of estrogen receptor β2 and expression of the estrogen receptor subtypes α, β1, and β2 in the protandrous black porgy (*Acanthopagrus schlegeli*) during the sex change process

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ABSTRACT

Estrogens play an important role in many physiological processes in both female and male vertebrates, mediated by specific nuclear receptor, estrogen receptors (ERs). We have isolated a third ER (ERβ2), which was found to contain 2004 nucleotides including an open reading frame that encodes 667 amino acids. We have also cloned ERα and ERβ1 from the published information (GenBank accession nos. AY074780 and AY074779) and investigated the expression pattern of these ER subtypes in the gonads during gonad sex change of black porgy by quantitative polymerase chain reaction. Maturity stages can be divided into five stages during the sex change process from immature male to female (immature male, mature male, male of mostly testis, male of mostly ovary and mature female). The expression of ERα mRNA was highest in the ovary of mature female, followed by the testis of mature male and testicular portion of mostly testis. ERβ1 expression was higher in the mature testis and ovary than in the gonads of other maturity stages. In contrast to that, ERβ2 was highest in the ovary of mature female, and significantly lower levels of ERβ2 expression were observed in the gonads of the other maturity stages. The present study describes the molecular characterization of ERβ2, and documents the expression changes of three ER subtypes during sex change process of the protandrous black porgy.

1. Introduction

In vertebrates, including fish, the steroid hormone estrogen is essential in reproduction, playing important roles in several aspects of sexual maturation and differentiation that include oogenesis, vitellogenesis, and testicular development. In addition, estrogen has been shown to function in non-reproductive tissues such as the bone, brain, and heart (Ishibashi and Kawashima, 2001). The mechanism of estrogen action is mediated by the nuclear estrogen receptors (ERα and ERβ), which function as ligand-dependent transcription factors that regulate transcription of target genes containing the consensus estrogen response element (ERE) in their promoter regions. Also, estrogens exert its actions through a membrane ER (mER), and the various signaling pathways (Ca^{2+}, cAMP, protein kinase cascades) are rapidly activated and ultimately influence downstream transcription factors (Zhang and Trudeau, 2006).

ERs are part of a large superfamily of ligand-activated nuclear receptors that includes receptors for other steroid hormones and thyroid hormone, in addition to a group of so-called orphan receptors (Mangelsdorf et al., 1995). The members of this superfamily share several common features that can be divided into six distinct domains (Krust et al., 1986; Kumar et al., 1987), including the highly conserved C (DNA-binding domain; DBD) and E (ligand-binding domain; LBD) domains, as well as many variable regions at the N and C termini between the DBD and LBD (the A/B, F and D domains) (Choi and Habibi, 2003).

Sequence alignments support the existence of ERα and ERβ subtypes in fish (Chang et al., 1999; Tchoudakova et al., 1999; Xia et al., 1999; Hawkins et al., 2000; Ma et al., 2000; Pakdel et al., 2000; Patiño et al., 2000; Rogers et al., 2000; Socorro et al., 2000; Choi and Habibi, 2003; Halm et al., 2004), including black porgy (Huang and Chang, 2002). In teleosts, a third ER subtype, called ERβ2 (Tchoudakova et al., 1999; Ma et al., 2000; Menuet et al., 2002) or ERγ (Hawkins et al., 2000; Halm et al., 2004), has been cloned that is genetically distinct from the other two. However, no information is available regarding the sequence or expression pattern of ERβ2 mRNA during the reproductive cycle in black porgy.

The discovery of the various ER subtypes and their presence in the female and male reproductive systems in equivalent amounts has generated great interest regarding their potential functions during sexual differentiation and development. In teleosts, ERs are expressed very early during embryonic development and gonadal differentiation,suggesting an important role of estrogen in sexual

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differentiation in fish (Guiguen et al., 1999; Lassiter et al., 2002). However, a comprehensive study, investigating the expression of all three ER subtypes during sex change associated with histological differences is currently lacking for protandrous hermaphrodite fish.

The black porgy (*Acanthopagrus schlegeli*, Perciformes, Sparidae), a widely distributed marine protandrous hermaphrodite, is of particular interest in commercial aquaculture in various parts of Asia, including Korea. Black porgies are well-known to be functional males for the first 2 years of life, and then begin to change sex during the third year. However, only about 40–50% of black porgies change into females during the third spawning season (Chang et al., 1994).

In this study, we isolated and characterized ERβ2 cDNA and analyzed the mRNA expression of ERα, ERβ1, and ERβ2 in the black porgy to clarify the mechanisms involved in the sexual differentiation and development of this species. We also examined the maturity of the gonads by histological analysis during the sex change process.

2. Materials and methods

2.1. Experimental fish

The study was carried out on immature fish (51.0 ± 2.3 g, 1 year), mature male (220 ± 14.2, 2 year), sex changing fish (489.2 ± 11.5 g, 3 years) and female black porgy (948.5 ± 51.6 g, 4 years). Sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of mature ova and sperm. All fish were netted and anesthetized in tricaine methanesulfonate (MS-222, Sigma-Aldrich, USA), and decapitated prior to tissue collection. Gonad samples from black porgy at the each gonad maturity stage (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary and mature ovary) were removed, immediately frozen in liquid nitrogen and stored at − 80 °C until the total RNA was extracted for analysis.

2.2. Total RNA extraction and reverse transcription (RT)

Total RNA from black porgy at each gonad maturity stages during sex change process were extracted using the TRI REGENT, according to the manufacturer's instructions (Gibco/BRL, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. 3 μg of total RNA was reverse transcribed in a total volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at − 20 °C for use in polymerase chain reaction (PCR) and quantitative PCR.

2.3. Identification of ERβ2 cDNA

Primers for ERβ2 were designed using highly conserved regions of gilthead seabream (GenBank accession no. AJ580048) and killifish (AY570923): bpERβ2 forward primer (5′-GGA AGA GAT CAG ACG AGA GG-3′) and bpERβ2 reverse primer (5′-AGC AGC ATG GTG AGG TGT C-3′). PCR amplification was performed using a 2× Taq Premix I (Solgent, Korea) according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 54 °C for 40 s, and extension at 72 °C for 1 min; followed by 7 min at 72 °C for the final extension. Amplified PCR products were visualized by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Korea) and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoR I (Fermentas, USA). Based on the plasmid DNA, the ERβ2 cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

2.4. Rapid amplification of ERβ2 cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from the gonads using the TRI REGENT (Gibco/BRL, USA). Using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo-d$(T)_{18}$ anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA T$(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Korea).

ERβ2-specific primers were selected from the PCR product obtained by RT-PCR. For the 3′ RACE, the 50 μL of PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mmol/L 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 mmol/L 3′ RACE gene-specific primer (5′-GAA GCT TCA GAG GGA GGA GTA CGT CTG-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by 1 cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μL of PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mmol/L 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mmol/L 5′ RACE gene-specific primer (5′-CGC ACT TCA TCA TGC CCA CTT CGT AGC-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one final extension cycle of 5 min at 72 °C. Amplified PCR products were processed by electrophoresis in a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The transformation was conducted as mentioned above.

2.5. Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length ERβ2 cDNA from various fishes. Amino acid sequences were aligned using the BioEdit Software (Hall, 1999). Sequences used for comparison and their GenBank accession numbers are as follows: black porgy ERβ2 (bpERb2, EU346949), gilthead seabream ERβ2 (gsERb2, CAE30469), largemouth bass ERβ (lbERb, AAO39210), killifish ERβb (kfERbb, AAU44353), Nile tilapia ERβ2 (ntERb2, ABE73151), Mozambique tilapia ERβ2 (mtERb2, ABV55459), rainbow trout ERβ2 (rtERb2, ABB73309), zebrafish ERβb (zfERbb, AAK16741), goldfish ERβ2 (gfERb2, AAF35170) and human ERβ (hmERb, Q62986). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and analyzed using Mega 3.1 software package (Kumar et al., 2001). The degree of support for internal branches was inferred using bootstrapping (1000 replicates) analysis.

2.6. Quantitative polymerase chain reaction (QPCR)

QPCR was conducted to determine the relative expression of ER subtypes (ERα, ERβ1 and ERβ2) mRNA using total RNA extracted from the gonads of black porgy ($n=3$). Primers for QPCR were designed with reference to the known sequences of black porgy as follows: ERα forward primer (5′-CTA CTA CTC TGC TCC TCT GG-3′), ERα (5′-ATG AAG GGG CTG AGA CGG-3′), ERβ1 forward primer (5′-GCC ATA CCT TTC TAC AGT CC-3′), ERβ1 reverse primer (5′-CCG TGC TGA GGT CGA GCC-3′), ERβ2 forward primer (5′-GTC TGG ATA GGG TTC GTG G-3′), ERβ2 reverse primer (5′-AAG GCA AAT GGC TTC TTG GG-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′) and β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). QPCR

amplification was conducted similar to previous work (Nelson et al., 2007), using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and with the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr green and *Taq* polymerase in buffer (10 mM Tris–HCL [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein) to a total volume of 25 μL. QPCR was carried out as follows: 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin Ct levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from three replicate samples were analyzed with analysis

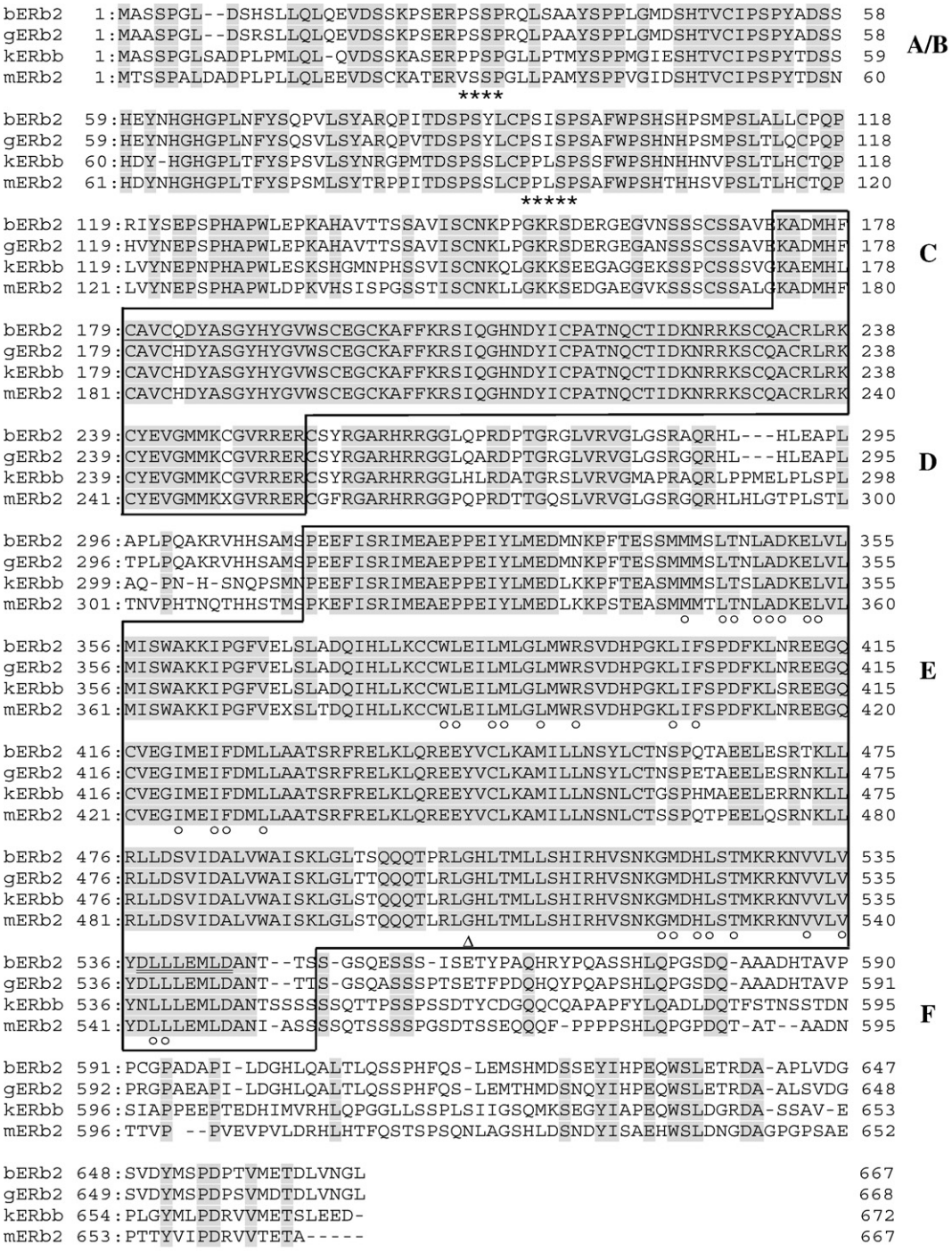

Fig. 1. Comparison of the amino acid sequence of black porgy (*Acanthopagrus schlegeli*) ERβ2, gilthead seabream (*Sparus aurata*) ERβ2, killifish (*Fundulus heteroclitus*) ERβb and Mozambique tilapia (*Oreochromis mossambicus*). ERβ2 was optimally aligned to match identical residues, indicated by the shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The ERβ2 sequences used for alignment are black porgy ERβ2 (bERb2, this paper, EU346949), gilthead seabream ERβ2 (gERb2, AJ580048), killifish ERβb (kERbb, AY570923) and Mozambique tilapia ERβ2 (mERb2, EU140820). ER domains are indicated on the right (C and E boxed in solid line). Motifs shown: putative phosphorylation sites for the MAPK pathway in the A/B domain (*); two conserved zinc-finger motifs in the C domain (underline); amino acids recognized to be involved in dimerization (Δ), ligand interaction (O) and transactivation (AF-2 domain, double underline) in the E domain.

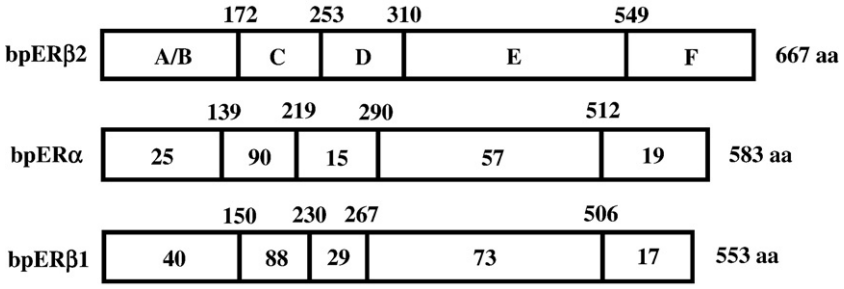

Fig. 2. Domain structure of the bpERβ2 and similarity with bpERα and bpERβ1. The percentage of amino acid identity of each domain relative to the bpERβ2 is indicated within the box representing the corresponding domain.

software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin=97.3%, ERα=97.2%, ERβ1=96.0% and ERβ2=98.2%. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature.

2.7. Gonad histology

The gonad tissues from each developmental group (mature testis, testicular and ovarian portions of the sex changing gonad and mature ovary) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were selected and stained with haematoxylin-eosin for observation under a light microscope (Olympus BS50, Japan) and images captured with a digital camera (Olympus DP-50, Japan).

2.8. Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc., USA). One-way analysis of variance followed by a *post hoc* multiple comparison test (Newman Keul's multiple range test) was used to compare differences in the data at a significance level of $P<0.05$.

3. Results

3.1. Identification of ERβ2 cDNA

RT-PCR was used to clone a fragment of ERβ2 cDNA using total RNA. A single PCR product of the expected size (1075 base pairs [bp]) was obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone full-length cDNA encoding ERβ2. The full-length ERβ2 cDNA contained 2004 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 667 amino acids (GenBank accession no. EU346949) (Fig. 1). Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that ERβ2 amino acids display high identity with those of other species. The amino acid sequence of black porgy ERβ2 was compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid similarities were as follows: 94% with gilthead seabream ERβ2 (gERb2, AJ580048), 75% with Mozambique tilapia ERβ2 (mERb2, EU140820) and 73% with killifish ERβb (kERbb, AY570923). As expected, the amino acid sequence identify of black porgy ERβ2 with other ERs was highest in the DNA-binding domain (DBD or C domain, residues 173–253, about 95%) and in the ligand-binding domain (LBD or E domain, residues 311–549, about 89%) (Fig. 1).

The bpERβ2 amino acid sequence was found to have 38.8 and 49.0%, similarity compared to bpERα and bpERβ1. The bpERβ2 shows a high percentage of conservation in the DBD (88–90%) and LBD (57–73%) when compared to bpERα and bpERβ1 (Fig. 2).

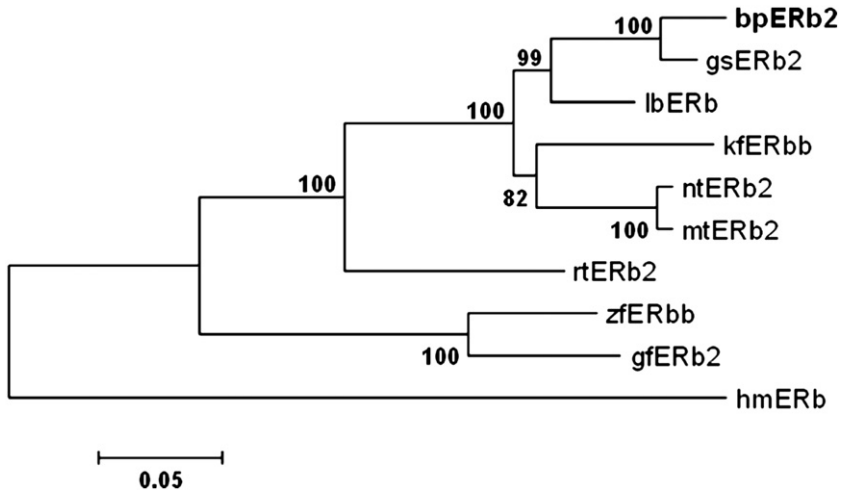

Fig. 3. Phylogenetic tree based on an amino acid alignment for ERβ2 in teleost fish. Bootstrap values (%) are indicated for 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: black porgy ERβ2 (bpERb2, this paper, EU346949), gilthead seabream ERβ2 (gsERb2, CAE30469), largemouth bass ERβ (lbERb, AAO39210), killifish ERβb (kfERbb, AAU44353), Nile tilapia ERβ2 (ntERb2, ABE73151), Mozambique tilapia ERβ2 (mtERb2, ABV55459), rainbow trout ERβ2 (rtERb2, ABB73309), zebrafish ERβb (zfERbb, AAK16741), goldfish ERβ2 (gfERb2, AAF35170) and human ERβ (hmERb, Q62986).

3.2. Phylogenetic analysis

The phylogenetic tree obtained by clustal analysis of the sequences described below was shown in Fig. 3. The phylogenetic analysis indicated that there are four groups of ERβ2 among the teleosts examined: group 1 (black porgy, gilthead seabream and largemouth bass), group 2 (killifish, Nile tilapia and Mozambique tilapia), group 3 (rainbow trout) and group 4 (zebrafish and goldfish). The ERβ2 is the most closely related to gilthead seabream ERβ2 (Fig. 3).

3.3. Quantification of the ERα, β1, and β2 mRNA expression

The expression changes of ERα, β1 and β2 mRNA by QPCR were shown in Fig. 4. The expression of ERα mRNA was highest in mature ovary, followed by mature testis, testicular portion of mostly testis (Fig. 4A). ERβ1 expression was higher in the mature testis and ovary than in the gonads at other maturity stages (Fig. 4B). In contrast to that, ERβ2 was highest in mature ovary, and significantly lower levels of ERβ2 expression were observed in the gonads of other maturity stages (Fig. 4C).

3.4. Histological analysis

The histological examination of the developmental stages of gonad was shown in Fig. 5. Testicular tissue was dominant in the mature testis with few primary oocytes (Fig. 5A). Also, the testicular portion of the gonad was degenerated (Fig. 5B) and the ovarian portion of the gonad was increased (Fig. 5C) during sex changing to female, and we observed oocytes including vitellogenic granule in the mature ovary (Fig. 5D).

4. Discussion

In this study, we cloned the full-length ERβ2 cDNA from the ovaries of mature female black porgy and investigated the changes in expression of ERα, ERβ1, and ERβ2 over time using QPCR. The ERβ2 cDNA from the black porgy contains a total of 2004 nucleotides that encode 667 amino acids in a single ORF. Using the BLAST algorithm (Blastp) from the National Center for Biotechnology Information, we found that the primary sequence of ERβ2 from the black porgy displayed significant identity with that from other species (94% with gilthead seabream ERβ2, 75% with Mozambique tilapia ERβ2, and 73% with killifish ERβb; Fig. 1).

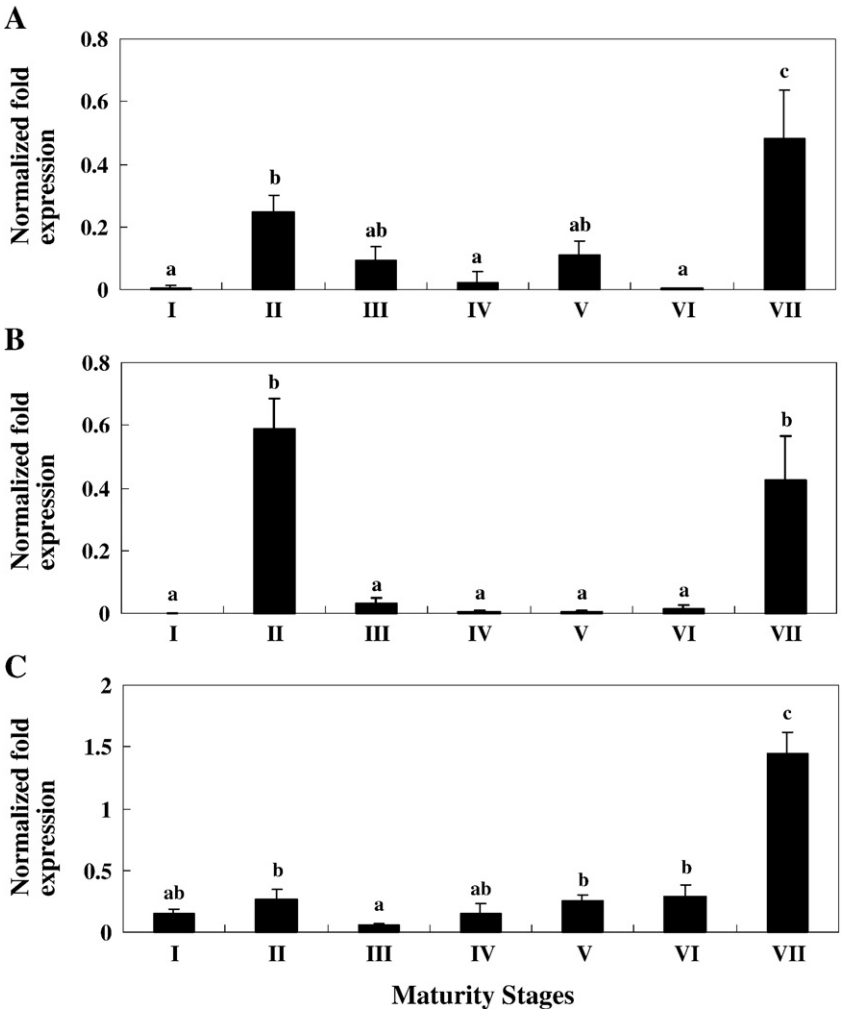

Fig. 4. Expression of ERα (A), ERβ1 (B) and ERβ2 (C) mRNA in the gonad of black porgy by quantitative real-time PCR. 3 μg of total RNA prepared from gonad was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex change process from male to female (I: immature testis, II: mature testis, III: testicular portion of mostly testis, IV: ovarian portion of mostly testis, V: testicular portion of mostly ovary, VI: ovarian portion of mostly ovary, VII: mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

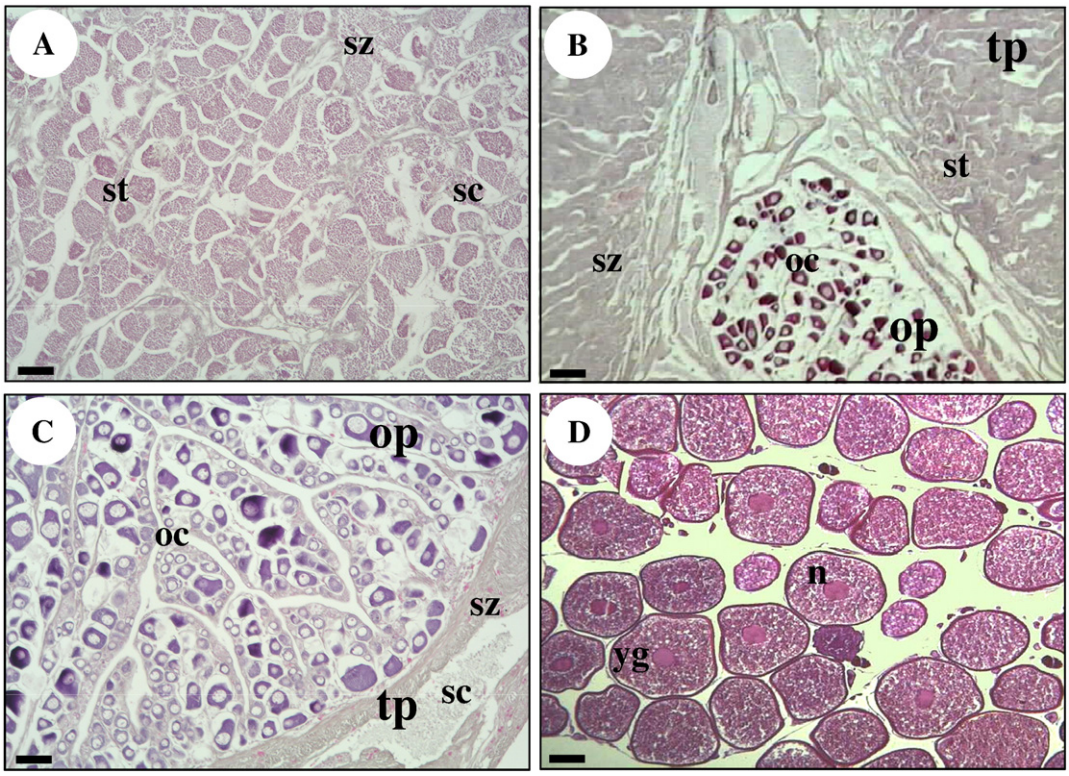

Fig. 5. Photomicrograph of gonad maturity stages during the sex change of black porgy. A: mature testis, B: mostly testicular gonad (III and IV in Fig. 4), C: mostly ovarian gonad (V and VI in Fig. 4), D: mature ovary, tp: testis part, st: spermatids, sz: spermatozoa, sc: spermatocytes, op: ovary part, oc: oocytes, n: nucleus, yg: yolk granules. Scale bar = 100 μm.

Black porgy ERβ2 has a similar domain structure to human, which was divided into six distinct (A–F) domains by Krust et al. (1986). Black porgy ERβ2 shows a high degree of conservation in its C (88–90%) and E (57–73%) domains with those in black porgy ERα and ERβ1 (Fig. 2), while the A/B domain (residues 1–172), which is similar in length with that in other fish, has two potential phosphorylation sites for mitogen-activated protein kinase (MAPK) ($PX_{1or2}SP$; residues 27–30 and 92–96; Fig. 1), suggesting that it may be phosphorylated in response to the MAPK pathway, a mechanism that has been shown to enhance the transcriptional activity of mammalian ERα and ERβ in a ligand-independent manner (Tremblay et al., 1997; Lannigan, 2003). Moreover, two zinc fingers (residues 179–200 and 215–234) are present in the DBD. The percent identity of ERβ2 from black porgy with that from other fish was highest in the DBD (domain C, residues 173–253, about 95%) and LBD (domain E, residues 311–549, about 89%). In addition, the activation function (AF)-2 domain (DLLLEMLD, located in the LBD at residues 537–544) (Kumar et al., 1987; Danielian et al., 1992; Pinto et al., 2006), which is involved in receptor dimerization, ligand-binding, and ligand-dependent transactivation, was well conserved in black porgy ERβ2 (Pinto et al., 2006) (Fig. 1).

The function of the F domain, which is similar in length to that in other species, is not well understood; however, it may interact with cofactors required for ER/Sp1 activity (Kim et al., 2003), which controls the magnitude of gene transcription (Montano et al., 1995) and inhibits dimerization of the receptor through an interaction with the E domain (Peters and Khan, 1999). Therefore, black porgy ERβ2 is similar to other members of the ERβ2 family in terms of its general features, and it contains all of the recognized consensus sequences.

Phylogenetic analysis of ERβ2 identified four subgroups (group 1: black porgy, gilthead seabream, and largemouth bass; group 2: killifish, Nile tilapia, and Mozambique tilapia; group 3: rainbow trout; and group 4: zebrafish and goldfish). The ERβ2 in black porgy was most closely related to that in gilthead seabream (Fig. 3). Notably, a third ER, called ERγ in the Atlantic croaker (Hawkins et al., 2000), was named ERβ2 in sea bass (Halm et al., 2004), goldfish (Ma et al., 2000), and gilthead seabream (Pinto et al., 2006). Based on our proposed nomenclature and phylogenetic analysis, we have designated the protein ERβ2.

Using QPCR, changes in the mRNA expression of ERα, ERβ1, and ERβ2 were investigated in the gonads of black porgy during the change in sex from male to female. A high level of ERα mRNA expression was observed in the mature testis and mature ovary, respectively; similarly, a high level of expression was observed in those gonads containing a large testicular portion during the sex change process. These results are in agreement with those from studies on the gilthead seabream (Socorro et al., 2000) and black porgy (He et al., 2003). In this study, ERα expression was higher in the mature ovary than in the testis, which contradicts the result of a previous study (He et al., 2003) in which higher expression was observed in the testis. These opposing results are presumed to be due to differences in the age and level of maturity of the black porgy used in each set of experiments. Although little published data are available on ERα expression in the gonads during sex change, ERα can be deduced from the results of this study to play an important role in the sexual development of black porgy because ERα transcription took place not only in the ovary of mature females, but also in the testis and testicular portion of the gonads of mature males during the sex change process. A high level of ERβ1 mRNA expression was observed in the mature testis and mature ovary, similar to the trend seen for ERα. Also, given that high levels of ERβ expression are important during sexual differentiation and development (Byers et al., 1997; Halm et al., 2004), high ERβ1 mRNA expression in mature testis of black porgy indicates that ERβ1 functions in the development and maturation of the gonads. Furthermore, we observed high levels of ERα and ERβ1 mRNA expression not only in mature ovary, but also in testis. This result corresponds to that of a previous study in goldfish (Choi and Habibi,

2003) and gilthead seabream (Pinto et al., 2006) showing high levels of receptor expression in the testis. This is an interesting result that underscores the potential functions of ERs during sexual differentiation and development as well as the novel function of estrogen in the male reproductive system (Couse and Korach, 1999; Halm et al., 2004).

In contrast to the above findings, ERβ2 was highly expressed only in mature ovary, with levels slowly increasing from testicular portion of mostly testis through to mature ovaries. This is in accordance with the results of Choi and Habibi (2003) and Halm et al. (2004), which showed greater ERβ2 mRNA expression in the ovaries than in the testis of mature goldfish and European sea bass respectively. Although ERβ1 is known to play an important role in the sexual differentiation and development of mammalian gonads (Byers et al., 1997), considering the high level of ERβ2 expression in the ovaries of mature female black porgy, ERβ2 appears to play an important role in the mature ovary.

Since both ERα and ERβ1 are expressed highly in mature testis and ovaries, we can speculate that they are involved in the maintenance and normal function of mature gonadal tissue. In contrast, ERβ2 expression is at its lowest at the onset of sex change, and slowly increases through the process, reaching a maximum in mature ovaries. This indicates that 1) ERβ2 may be directly involved in sex change and 2) it is likely to contribute along with ERα and ERβ1 to the maintenance and function of mature ovarian processes.

Histological analysis of each stage of the sex change process during the spawning season revealed the presence of sperm in the gonads, indicating the existence of functionally mature gonads, and oocytes containing numerous vitellogenic granules, which are indicative of impending ovulation, were observed in the ovaries of the mature females. Moreover, as the switch to ovaries occurred in the bisexual gonads, degeneration of the testicular tissues was observed as were oocytes with chromatin in the nucleolus and peri-nucleolus stages. Oocytes with secondary yolk (i.e., globule stage), which is characteristic of mature oocytes just before ovulation (Wu et al., 2005), were also observed in the mature ovaries (Fig. 5). We found mature oocytes in the ovaries of the mature female at the same time as we detected high levels of ER mRNA expression. This is in accordance with the results of a previous study (Filby and Tyler, 2005) showing a higher level of ER mRNA expression in the testis and ovary than in the immature gonad of male and female fathead minnows (*Pimephales promelas*).

In summary, full-length ERβ2 cDNA was separated from the ovary of mature female black porgy, and using QPCR, the mRNA expression of three ER subtypes (ERα, ERβ1, and ERβ2) in the gonad was compared at each stage of the sex change process. Our results indicate a high level of expression in the mature testis and ovary, from which we deduce that ERs play an important role in gonadal maintenance and function while ERα and ERβ1 are down-regulated during sex change, implicating the role of ERβ2 as a potential modulator of sex change since its transcript slowly increases. Additional studies will be necessary to determine the roles of ERs in gonadal development and maturation. Furthermore, the results of this study can be used to elucidate the endocrinological mechanism of sex change in black porgy.

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