Cloning and expression analysis of follicle-stimulating hormone and luteinizing hormone receptor during the reproductive cycle in Korean rockfish (*Sebastes schlegeli*)

Wei J. Mu · Hai S. Wen · Feng He · Ji F. Li · Miao Liu · Yuan Q. Zhang · Jian Hu · Bao X. Qi

Abstract Full-length cDNA sequences encoding the receptors for follicle-stimulating hormone (FSHR) and luteinizing hormone (LHR) were isolated from ovary of Korean rockfish (*Sebastes schlegeli*) using reverse transcription-polymerase chain reaction (PCR) and rapid amplification of cDNA ends procedures. The cDNA of the KrFSHR encodes a predicted protein of 703 amino acids that showed the greatest homology with European seabass (*Dicentrarchus labrax*) (78 %) and gilthead seabream (*Sparus aurata*) (73 %). The cDNA of the KrLHR encodes a predicted protein of 703 amino acids and exhibited the highest homology with European seabass (*Dicentrarchus labrax*) (79 %) and gilthead seabream (*Sparus aurata*) (76 %). Besides the gonads, expressions of GTHRs mRNA were also obtained in extra gonadal tissues. Seasonal changes in the gonads expression profiles of KrGTHRs mRNA were examined by quantitative real-time PCR, and the present results suggest that levels for KrFSHR mRNA increase during gonadal growth, whereas KrLHR shows high levels during the late gamete generation period. Our study provides molecular characterization of the GTHRs and expressions profile during reproductive cycles, reinforcing previous knowledge of GTHRs important role in the reproductive endocrine regulation of Korean rockfish.

Introduction

It is established that the reproductive cycle in vertebrates is controlled by the brain–pituitary–gonadal axis (Oba et al. 1999a). In addition, it is also well-known that two gonadotropin hormones, follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) secreted by the pituitary gland, play essential roles in the gametogenesis and production of steroid hormone in gonads (Levavi-Sivan et al. 2010).

Gonadotropins (GtHs) are glycoproteins (GPs) composed of a common α subunit. The α subunit links non-covalently to a hormone-specific *β*-subunit that confers the biological activity. These proteins coordinately control gonadal steroidogenesis by binding to their respective receptors (FSHR and LHR) in the gonads. (Gharib et al. 1990) The FSH and LH receptors are G protein-coupled receptors (GPCRs) from the family of rhodopsin-like receptor that includes in addition the TSH (thyroid-stimulating hormone)

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receptor. All of them that mentioned above constitute the subfamily of glycoprotein hormone receptors (GPHRs) (Fredriksson et al. 2003; Gether 2000). The members of this family are characterized by a large extracellular (EC) domain with nine leucine-rich receptor motifs and associated cysteine-rich sequences. This is followed by seven transmembrane (7TM) helices and a carboxy-terminal intracellular tail (Hsu et al. 2000). The cDNA and sequences of the GTHs receptor have been determined for a number of fish species, such as *Oncorhynchus masou rhodurus* (Oba et al. 1999b) and later from many other teleost species such as *Clarias gariepinus* (Bogerd et al. 2001; Kumar et al. 2001a, b; Vischer and Bogerd 2003), *Sparus aurata* (Maugars and Schmitz 2006; Oba et al. 2000), *Danio rerio* (Kwok et al. 2005; So et al. 2005), *Dicentrarchus labrax* (Rocha et al. 2007a), *Anguilla japonica* (Jeng et al. 2007) and *Oncorhynchus mykiss* (Sambroni et al. 2007). Many studies demonstrated that in salmonids, FSH secreted earlier and plays a major regulatory role during early stages of gonadal development that was considered to stimulate early development of ovarian follicle and spermatogenesis in the testes; LH, rising later, triggers the final oocyte maturation and subsequent ovulation in females and spermiation in males (Huang et al. 2009). Gonadotropin-releasing hormones (GnRH), acting as main signaling molecule used by brain, also play an important role in regulating the synthesis and secretion of pituitary gonadotropins (Senthilkumaran et al. 1999). Nowadays, the GTHRs expression studies of man-made hormone-treated fish are much more than the studies under the natural conditions (Mittelholzer et al. 2009), and our result could give more information about researches under the natural conditions.

The Korean rockfish (*Sebastes schlegeli*) is a widely distributed marine ovoviviparous fish, whose development of embryonic stage relies on yolk accumulated in oocytes during vitellogenesis as an energy source (Boehlert and Yamada 1991); during the process, it does not obtain nutrition from mother, but develops by its own yolk. Nowadays, the information about reproductive physiology in ovoviviparous species is scarce. Korean rockfish, with the high economic value, are asked for a high amount in sea fishing these years. Owing to the great value of Korean rockfish both in economic and academic, it is meaningful to clarify the endocrine regulatory mechanisms of reproduction.

The study aimed to clone and characterize the follicle-stimulating hormone receptor and luteinizing hormone receptor in Korean rockfish. In order to understand the possible role of GTHs receptor in the reproductive cycles and in the brain–pituitary–gonad axis of Korean rockfish, we cloned these two genes and generated spatial and temporal profiles in several tissues and gonads during the reproductive cycle.

Materials and methods

Experimental fish

The work was conducted with sexually mature male and female Korean rockfish that were obtained from Shandong coastal area every 2 months. More than twenty individuals were randomly sampled each time. Sexual maturity was determined after excising the gonads defined by the presence of mature ova and sperm (Shi et al. 2011). All fish were anesthetized in tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). Tissues including brain, heart, caeca, liver, gills, head kidney, intestine, stomach, spleen, gonad and pituitary samples at various gonadal-development stages from Korean rockfish were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until extraction of total RNA. Organs were sampled for expression studies of FSHR and LHR.

Total RNA extraction and reverse transcription (RT)

Tissues collected from Korean rockfish were frozen in liquid nitrogen, and the total RNA was extracted from fragment using Trizol reagent (Invitrogen, USA) following the manufacturer's instruction. The conservation and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Total RNA (1 μg) was reverse transcribed in a total volume of 10 μL using random primers and M-MLV reverse transcriptase (Takara, Japan).

Molecular cloning and sequence characterization of Korean rockfish GTHRs cDNA

The strategy for isolating FSHR and LHR cDNA entailed first generated a partial cDNA by reverse transcription-polymerase chain reaction (RT-PCR)

using degenerate primers, followed by amplification of 5′ and 3′-DNA ends by rapid amplification of cDNA ends (RACE) and finally generation of a cDNA encoding the complete coding region in a single set of RT-PCR. The primers of FSHRF/FSHRR and LHRF/ LHRR (Table 1) used for FSHR and LHR fragment amplification were designed using CODEHOP (Chen et al. 2009), which is a web-based primer design program. PCR was carried out in a final volume of 50 μl containing 2 μl of cDNA from ovarian tissue following the manufacture's instructions (Takara, Japan).

PCR was performed using the following touchdown PCR cycling conditions: 5 min denaturation step at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at a range of annealing temperature from 70 to 60 °C, decreasing 1 °C each cycle and 35 s at 72 °C, then followed by additional 30 cycles of 30 s at 94 °C, 30 s at 62 °C and 35 s at 72 °C, and finally ended with 10 min at 72 °C for extension. The 5′ and 3′ RACE reactions were obtained by the SMART™ RACE cDNA Amplification Kit (Clontech, USA). Products of the degenerate PCR and RACE reactions electrophoresed on 1.5 % agarose gel. The band of expected size was purified with QIAEX Gel Extraction Kit (QIAGEN, China). The purified fragments were then cloned into PGM-T

vector (QIAGEN, China), propagated in *E. coli* DH5α and were sequenced using an ABI3730XL sequencer.

Phylogenetic analysis and sequence analysis

The presence and location of the putative signal peptide cleavage sites and potential N-glycosylation sites in the amino acid sequences were predicted using the prediction servers of the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). Amino acid sequences were got from Genebank (Altschul et al. 1990), and the sequences of KrGTHRs were aligned with other homologous fish gonadotropin receptors. Multiple protein sequence 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 2.0 (Tamura et al. 2007). A rooted phylogenetic tree was constructed by means of the neighbor-joining algorithm (Saitou and Nei 1987); the data were re-sampled via 1,000 bootstrapping replicates.

Tissue expression of the GTHRs gene

Total RNA was extracted from ovary, liver, kidney, head kidney, brain, heart, spleen, caeca, stomach, fat,

Table 1 Primer sequences used for cloning and mRNA expression analysis

Primers	Sequence (5′–3′)	Position	Usage
FSHRF	CCTGACCTACCCCTCCcaytgytgygc	1212–1239	Degenerate primer
FSHRR	GGCGAAGAAGGAGATGgggngccatrca	1932–1959	Degenerate primer
fshr-C-5-1	GGTCAGCAAAGGCCAAGTGGCACAT	1381–1406	5′-RACE primer
fshr-C-5-2	CTGAAGGCATTGGCGGGGACTATCT	740–765	Nested 5′-RACE primer
fshr-C-3-1	GCGCATGGCCATCCTCATCTTCACCGACT	1872–1901	3′-RACE primer
fshr-C-3-2	CGCACCTTCAGGCGGGATTTCT	2047–2069	Nested 3′-RACE primer
fshr-e-f1	TGCCAGTATCTTTGGCTAGCAC	2486–2508	RT-PCR and qPCR primer
fshr-e-r1	GCCTCTCACACTCAACCGTTTA	2587–2609	RT-PCR and qPCR primer
LHRF	GCCCGACGCCTTCaayccntgyga	1244–1268	Degenerate primer
LHRR	GGCGAAGAAGGAGATGgggngccatrca	1947–1974	Degenerate primer
lhr-c-5-1	GTGCTTACCAATGGACATAGAGACCCC	1716–1733	5′-RACE primer
lhr-c-5-2	GGGAGGTTGTTAAATGTCCTTCTGCCAAT	479–508	Nested 5′-RACE primer
lhr-c-3-1	GGCACACCATCACCAACGCTATGCAGGT	1571–1600	3′-RACE primer
lhr-c-3-2	CAGACACCAAGATTGCAAAGCGCATGGC	1868–1897	Nested 3′-RACE primer
lhr-e-f1	GCCCACCTCACCTACAACAG	922–942	RT-PCR and qPCR primer
lhr-e-r1	GACTTCGTCCTCCACAAGCA	1120–1140	RT-PCR and qPCR primer
18SF	CCTGAGAAACGGCTACCACATC	–	Reference primer
18SR	CCAATTACAGGGCCTCGAAAG	–	Reference primer

gills, intestinal, pituitary of a female fish in late-vitellogenic stage and testis of one male in spermiated stage using RNAiso plus (RNA Extraction Kit, Takara, Japan). Transcription expression of GTHRs genes in various tissues was determined by RT-PCR (reverse transcription PCR). First-strand cDNA was synthesized using oligo (dT) primer. RT-PCR was carried out using the Takara Taq™ (Takara, Japan) according to the manufacturer's guidelines. Semi-quantitative RT-PCR was performed using a Biometra TPersonal Thermal Cycler (Biometra, Germany), and PCR program was 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 10 min (primers were listed in the Table 1). Six microliter of each reaction product was resolved on a 1.5 % agarose gel containing ethidium bromide (EB) and visualized on a Gel system (Tanon, China).

Quantitative real-time PCR (qPCR)

QPCR was conducted to determine the relative expression of GTHR mRNA using the total RNA extracted from gonads of Korean rockfish. PCR analyses were performed using a 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 FSHR (fshr-e-f1 and fshr-e-r1) and LHR (lhr-e-f1 and lhr-e-r1) are listed in Table 1. The total RNA was treated with DNase I (Takara, Japan) and Ribonuclease Inhibitor (Takara, Japan) to prevent the genomic DNA amplification. The FSHR or LHR qPCR conditions were 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, and annealing at 57 °C (for FSHR) or 59 °C (for LHR) of 20 s, respectively, and extension at 72 °C for 20 s. As an internal control, experiments were duplicated with 18SrRNA, and all data were normalized to the 18SrRNA calculated threshold-cycle (Ct) level. The primers were designed to amplify a PCR product of 101 bp (krFSHR) and 198 bp (krLHR). The samples from one stage ($N \geq 3$) with serial dilutions of total cDNA were used as calibrators in this experiment. A dissociation curve with a single peak was used to monitoring the amplified product. The result was analyzed by a comparative Ct method, the mean value of the FSHR at stage V in male was set to 1, and in this

Fig. 1 Amino acid alignment of Korean rockfish FSHR with ▶ other teleosts (**a**). Comparison of the deduced peptide sequence of the Korean rockfish FSHR (kr) (Genebank Accession NO: JN5365) to European seabass (es) (Genebank Accession NO: AAV48628), rainbow trout (rt) (Genebank Accession NO: AAQ04551), channel catfish (cc) (Genebank Accession NO: AAK16067) and gilthead seabream (gs) (Genebank Accession NO: AAT01413), which are extracted from the Genebank. The identical residues are indicated by *dots* and the gaps introduced are shown by *dashes*. The *arrowhead lines* denote the site of predicted signal peptide and C-terminal cysteine region. The ten motifs (X–L–X–L–X) of the leucine-rich repeats (LRRs) identified by *gray boxes*. Five potential N-linked glycosylation sites, conserved in the krFSHR, are indicated by *open boxes*. The position of seven transmembrane helices is shown as *black boxes*. Conserved cysteine residues are indicated by *black arrowheads*. Amino acid alignment of Korean rockfish LHR with other teleosts (**b**). Comparison of the deduced peptide sequence of the Korean rockfish LHR (kr) (Genebank Accession NO: HQ712166) to European seabass (es) (Genebank Accession NO: AAV48629), rainbow trout (rt) (Genebank Accession NO: AAQ04550), channel catfish (cc) (Genebank Accession NO: AAK16066), and gilthead seabream (gs) (Genebank Accession NO: AAT01412), which are extracted from the Genebank. *Dots* indicate the identical residues, and *dashes* show the gaps introduced. The *arrowhead lines* denote the site of predicted signal peptide and C-terminal cysteine region. The nine motifs (X–L–X–L–X) of the leucine-rich repeats (LRRs) identified by gray boxes. Three potential N-linked glycosylation sites, conserved in the krLHR, are indicated by *open boxes*. The position of seven transmembrane helices is shown as *black boxes*. Conserved cysteine residues are indicated by *black arrowheads*

method, the target gene expression was normalized against 18SrRNA expression, generating the ΔCt value (ΔCt = target Ct − reference Ct). Then, the relative expression was calculated according to $2^{-\Delta\Delta Ct}$ method (Fig. 5).

Result

Characterization of FSHR cDNAs

The cloned FSHR cDNA (Accession NO. JN165365) consists of 2,851 nucleotides, including ORF that is predicted to encode a protein of 703 amino acids. The receptor displays the typical structure of a G members of the protein-coupled receptor family with an extra-cellular domain (ECD), followed by a 7 transmembrane domain (TMD) and an intracellular C-terminal domain.

The FSHR ECD contains 378 amino acids, which the first 20 amino acids is predicted to constitute the putative signal peptide (Fig. 1a). There are ten leucine-

A

signal peptide   LRR1   LRR2   LRR3
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krFSHR  MMMVTILIMLTILMIKMATASAPDTEMDLKPGADECMAKQTLTICYQLKFG-VTDIPSSISSY-TPCLEVKQTEIAVIPQGSIYGLQHLRRRARRYYTTQMGNISALAFASLPRLTEIFISENAALKGIGDSAFSDLPELT
esFSHR  ....M...M....T....V.GP...V...VETSL..R..SF.....-..E......N-T.....V....ALNS.....KLTIWENDKLES.NEF.....SQ..D...G.V...N..AF.....
rtFSHR  -..KMKK..KML.CMLGCVCVS---------Q.EVA.VNSGT.FT.LCMGNTI.HM.TH.PKN-.TD..F..H.R.F.R---------------E..TN.QQ..A.VLT..GM.ES..AF..AN..R..
ccFSHR  -..CF..SW.MMHAGN.CLG.Y---------A.L.N-GT.RSFLCLGSK.HQM.YH.PKN-.TYV.I.L.R.IML.S---------------R.MS..HD.KR.LV...GV.QR.EAY..AN.TK.E
gsFSHR  -.RTAPPLL.FLIVLISCGWKS---------TSGFVCPRICRCFANTIRCNN..QGSALTMEHRDKR.FLYHLPLHT.SS---------------HS.EG.KGVQR.D.AQSVT.ET.ETL..NN.LN.S
```
LRR4   LRR5   LRR6   LRR7   LRR8   LRR9
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krFSHR  KITIIKSQHLRHIRHDAFKNMVKLQYLKIADTGLRRFPNFTKIHSAASNFMFYLQDNRHIKIVPANAFRGLCLQAIKYLRLSKNAIREVASGGFNGAKMHRLFLRGNEQLTHINSSAFVGCRNLFVQDISQTNLSSLPDN
esFSHR  E...T..K..T..NP....DI..K..T..N...L.D.....TG-LLL.D.H..S..ER.....K..T.PEI..TR.G.K...DA...T......K....PN....SSE.V.L...A.....Y.
rtFSHR  E...T..K..VI.H.Q..IGLP...SH.T.CN....VL..SR.....LT.LLD.....V..V.I.S...K..TTNT.DE...T..G.S..E.HA...T.I.K....M..L..S.MHNNS.K.AEGPGFL...R.A.....ES
ccFSHR  E...T..KN.VCMDRHT.WGLP...R..T.SN...TVL.D.S.VQ...FE.L.D.E..M..EVI.S...A..TSGT.TE...T..G.T..DRNA...T.IEK....M..Q..KR.HNY..L.AEGPL.L...R.AI....E.
gsFSHR  E.S.QNTRS.MR.GRGT.N.LP...R..S.SN...ITV..DI.S.Y.LEPE.ILDIY..LYLLEI.P...I..TKEYVTMN-..YN.G...IHDHA...T.IDK.V.KN.RN.RG.HRD...K.ATGPE.L.V.A.A.KK..AE
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LRR10
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krFSHR  ILGGLKTLFAESTLLKELPPPQLFTKLSQANLTYPSHCCPFQLMSHNES-RWHPLCSHPDAPT------------------------------DRNFQQ--DYCFNFSSVACSPTPDDFNPCEDIMS
esFSHR  ....QK.I..APN.....LE.....H......S...A.HNIH..R.-K.NS.....QG------------------------------NLH.YR-.--..S.ST.II.T..Q.......
rtFSHR  V..EVEH.S.V.VFS.RA...LS......R..A.HKHQ..RTF.MTSA.FK.G.QN------------------------------NLH.FM--..F.L.WT.....A..A.......G
ccFSHR  M.RR...L.I.T.VYS.RW..NLEI..E.T......A.KNFKK.K.-EKNR..NDSTIRNQ------------------------------EPY.FE--EH.KDVIE.R.Y.E..G
gsFSHR  G.ESVLV...Q.AYA..S...L.GLWS.RE.H...N....ALLSW.THRDFTFN.AWNNGSTSCDESDSTARVQHVIGGSAEGTLPKDLNIFSDADLFVDDESFG.V..HYPEL.F.QTRPTLL.T.EA.A.....AG
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krFSHR  AVPLRVLIWIISILALLGNAAVLLVLLGSRSKLTVPRFLVCHLAFSDLCMGIYLVVIATVDMLTRGGLYYNYAIDWQTGLGCSAAGFFTVFASELSVFTLTAIITLERWHTINHALRLDRKLRLRHACIVMTAGWIFSSIAA
esFSHR  ................TV.........T............Q.Q..H......V..................T............AV...
rtFSHR  SA.........T.V....TI.......A.M......S.A..V..........H.S..A..DI.........C..T............A..AT..A..CL..
ccFSHR  FTF.......F..V..V..FT......S..T..K......A.F..L..LI.GS..LQ..SH....G.E...V..GT..L........Y............TY.M..E.Q..H..S..AF..L..VL..
gsFSHR  FSF...A..F.N...IT..LT....SFT...N....F..N...A...I...LM...LR.H.H.SQH..E...P.....LS..GG....Y..ST..........TN..QVE.H.L.TQ.ASI.A...LI.LGMG
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krFSHR  LLPTVGVSSYSKVSICLPMDVESLGAQVYVVSLLLLNILAFFCVCGCYLSIYLTVHNPSSAPAHADT-RVAQRMAILIFTDFLCMAPISFFAISAALKLPLITVSDAKLLLVLFFPINSCSNPFLYAFFTRTFRRDFFLL
esFSHR  ...........G......F..S...........R..........V.....V............-...V...V..............Y...
rtFSHR  ..........PS..F.MF.....VV..L...V....S.R.S..P..S.E.S.M...........L.........S....LY...A....GLC...
ccFSHR  .T.VI....M.T.......TASS.A.IML..I..V...VT..A..VR....RH.A.V.DS..A-..K...V.V.............Q...H..V....A...KS.KQ...V.
gsFSHR  M..L.......M.....I.TPL...TFIIII..F.VG...IV..V...VL...A.K..EVPRRS...-KM.K..V...............F.V...TNS.I...........I...KA..K.AYQ.
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c-terminal cyceine region
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krFSHR  TARFGLFKTRAQIYRTESSSYQQPAWT--SPKSS-HVMLYSLANTLSLDGKQEC---
esFSHR  A..............C.....-.......A..E..P.F--
rtFSHR  A..Y...T.K..V....FPV..A..IQM....V.-..GT.C------------
ccFSHR  .S...C.ES..RV....T..LHNGPRMR-...N.-DGT....GHVAHPH------
gsFSHR  MSAL..CC.SK.SVH.MNAHCGEKAINFGS..Y.G.GTAVRLAVMEGQ.HHP.E.GELT
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B

signal peptide   LRR1   LRR2   LRR3   LRR4
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krLHR   MWASLPAFLFLSVLFLYGCKC--ASGFACPRICRCFSNTIRCNNVTFMM-SAQTTDHRDKRLFLHHLTLHTISSHSFEGLKGVQGIEITQSVTLETIEALAFNNLRNLSEISIQNTRSLMHIGRRTFNNLPKLHYLSIS
esLHR   --..T....L....GF.....--..P..G.....QG-..LMM.....FY..S.N.....D.....R..A.........L.....S.......L...R.....
rtLHR   -..SI..LFLFYPY..LFF.FG.GYT.S.V..G....SA.....I..K-..VP.SE-..GP..V.K....MS..A..T.D..RR..H...G...A...T......L..N..F.K.I...V..A.......R.....
ccLHR   MVSRC.VA.L.LAV.MR.SRGG-----A.T..P..S.TAD.LS.TAQ..RRTRVPSSTTSTN.R.A..P.KEVQ.GT.R..IN.SR...S..DSIRK.KGE..LSIH.VI...L.IIH.SAMEKGA.TD..R.R....C
gsLHR   -..RTAP.LL...I.ISC.W.S--T...V...G....-.C...L.ME.....G-..V.K....R.D.A.......T......L..........R.....G.....R.
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LRR5   LRR6   LRR7   LRR8   LRR9
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krLHR   NTGITTFPDIISINSLES---EFILDICDNLYLLEIPPNAFTGMTKEYVTMNLYNNGIREIHDHAFNGTKIDKLVLKNNRNLRVIHRDAFKGATGPGVLDVSATALAKLPPQGLESVLVLLAQSAYALKSLPPLQGLWSL
esLHR   .....L....TY.....---.................I.L.........V...Y...E.............T.........
rtLHR   .....V...MT..H..PWNQN.V..........S..V..V...T..TA...F.......Q.Y......N..........E...V..RI....S..IET..SH.N..VE.V.RT..G..R...FRD.GN.
ccLHR   ..RH...FSR.S..GL----..F..MG..IQ.NN...A..Q.IS.ADMY...VR..FTQ.ES....RLE..IM.D.WH.IK..D..E..E.TL....S..KS..TR..RG.K..I.R.TPS..T....DN.AE.
gsLHR   .....V..T..Y..P----.......Y.........I.L.....G..................E....K..AE......F.
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krLHR   REAHLTYNSHCCALLSWN-THRDFPINSAWNNGS--TYCDESDPSARVQRVIGGSADNTLVMDMPVFSDVDLLVEDEVFGNVNFHYPELDA-CQTRPALVCTPEADAFNPCEDNAGIQFLRVAIWLINILAITGNLTVLL
esLHR   .........-..S..P.Y..D.....-RGQL.............T.......F....SED-.G..D.....F-....T..........I.FS.....F.......
rtLHR   QK.........T.D-...S...A.QH...RP....D.-..EKFPAGMVD..S.TS.LVEI---HGTNED...SY.G.D.Q.....GLN....T.Q........I..FS.....F......I...
ccLHR   Q..D...P....FHT.KRNN.ETAVFDRFK.LT--------MLCNMDDQ.N-----------NMDPSGDDLNDI....D.EL-.ASSSSFK....P.....LL.HT...AIT.IVTVF.LV...A...
gsLHR   .........-...TF.P...-..S..ST....H.....EG..PK.LNI...A..F.D.S..D.....F-....T.L.........I..FS.....F......
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krLHR   VFFTSRNKLTVPRFLMCHLALADLCIGVYLLMIATVDLRTHGQYSQHAIEWQTGPGGSAAGFLSVFGGELSVYTLSSITLERWHTITNAMQVERHLVLTQAASIMAAGWLICLGMGMLPLVGVSSYTKVSMCLPMDIETP
esLHR   .S...........L.....G......C.........S.............T.......L.......I......F....A.......
rtLHR   I.....C.......F..F.........A..H.R.H..E..D...A...........T.......H.L.L.KR.G.A...G..G.....A.........SR.....VK..
ccLHR   .LLL.HQ..S.S.....N..F....M..F...L..VA.Y.SRQE.YN..TD....M..GV....T..AS..I...TM........H...MS.R.R.RHMVTM..I..GFSFVIAL..V......S..I.......
gsLHR   .S.......F..N..F.....I.......H.................T......L...........S.........S..........
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krLHR   LAQAFIIIILLFNVAAFVVVCVSYVLIYLAVKNPEHPSGRSADTKIAKRMAVLIFTDFLCMAPISFFAISCQGLSMPPHTGSHSDTLLIFMHQPYSLCNPFLYTALTRGFRTDACLLLRRCGCCTPNADFYRSQTLASHLN
esLHR   ......L....G......C........F.R--........AAFKV.LI.VTN.KI..VLFFPIN.CA....AIF.KA...K..YQ.MSAM...KSK.SV..MKAYC.ENA
rtLHR   .LLL......G..L..C......R..QF.-...A............L........AAFKV.LI.VTN.KI..VLFFPIN.CA....AIF.KA..K.VY...SNM...ENK.NM..MKAYC.ENL
ccLHR   VS.GYVVAV.VI....LM..S..AG...S.R..NV.T-.HGH.RM........AA.H..LISV.Q.KI....LFYPIN......IF..A..R.M...S.....HAQ.E.......G.LGFTL
gsLHR   ...T........G..I...C......V.R-....M.........AAFKV.LI.VTN.KI..VLFFPIN.CA....AIF.KA...K..YQ.MSAL...KSK.SVH.MNAHCGEKA
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c-terminal cyceine region
```
krLHR   CTQKTSNPLPHSLSFYAYHIKVKDCFLNKGAT------
esLHR   IKSNLGSNNKE.GTGGRRAAVAGQSHHIL.EERELT--
rtLHR   VKSSSG.KGTLICTTLRMDPLPLQSQQT.DDGDLGTI
ccLHR   APR.KRVRK...KN.R...V.LQG.IF..A.-------
gsLHR   IN--FGSSYKG.GTAVRLAVMEGQSHHP.EEGELT--
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rich repeats (LRRs) on the amino- and carboxy-sites by cysteine-rich sequences. The krFSHR protein possesses 6 potential N-linked glycosylation sites ([100]NISA, [177]NFTK, [252]NSSA, [271]NLSS, [310]NLTY, [326]NESR) and 13 conserved cysteine residues, five of them in an N-terminal cluster ([317]C, [318]C, [350]C, [358]C and [368]C) eight in a TMD ([414]C, [422]C, [454]C, [558]C, [560]C, [596]C and [632]C). The intracellular loop shows a potential protein kinase C phosphorylation site (407T). Phosphorylation site predictions identified ten potential phosphorylation sites, [161]Ser, [170]Ser, [265]Ser, [269]Ser, [300]Ser, [4]Thr, [36]Thr, [37]Thr, [282]Thr and [302]Thr, in the extracellular domain, and 9 potential phosphorylation sites, [462]Ser, [463]Ser, [465]Ser, [611]Ser, [612]Ser, [613]Ser, [621]Ser, [625]Ser and [585]Thr, in the TMD.

Characterization of LHR cDNAs

The cloned LHR cDNA (Accession NO. HQ712166) consists of 2918 nucleotides. The KrLHR ECD is predicted to contain 377 amino acids, which the first 21 amino acids are predicted to constitute the putative signal peptide (Fig. 1b). There are nine leucine-rich repeats (LRRs) on the amino- and carboxy-sites by cysteine-rich sequences. The LHR displays the typical structure of a G members of the protein-coupled receptor family with an extracellular domain (ECD), followed by a 7 transmembrane domain (TMD) and an intracellular C-terminal domain. The krLHR protein possesses 3 potential N-linked glycosylation sites ([30]NVTE, [91]NLSE, [187]NGTK) and 14 conserved cysteine residues, six of them in an N-terminal cluster ([33]C, [40]C, [283]C, [284]C, [369]C and [379]C), five in a TMD ([425]C, [433]C, [465]C, [540]C, [569]C, [608]C and [670]C) and one in a intracellular terminal domain ([671]C). The intracellular loop shows a potential protein kinase C phosphorylation site (407T). Phosphorylation site predictions identify thirteen potential phosphorylation sites [72]Ser, [133]Ser, [150]Ser, [153]Ser, [271]Ser, [311]Ser, [314]Ser, [324]Ser, [337]Ser, [4]Thr, [36]Thr, [50]Thr, [51]Thr, [291]Thr, [306]Thr and [328]Thr in the extracellular domain, six potential phosphorylation sites, [453]Ser, [586]Ser, [622]Ser, [643]Ser, [629]Ser and [592]Thr in the TMD and one potential phosphorylation site [682]Thr in the intracellular C-terminal domain.

Phylogenetic analysis

The phylogenetic analysis, based on deduced amino acids, shows that the GTHRs are divided into two groups composed of an FSHR cluster and an LHR cluster in the phylogenetic tree (Fig. 2).The GTHRs of Korean rockfish were clustered within the teleost GTHRs group with the other mammals GTHRs. Among teleost FSHR, Korean rockfish (*Sebastes schlegeli*) FSHR shows a high sequence identity with the European seabass (*Dicentrarchus labrax*) (78 %) and gilthead seabream (*Sparus aurata*) (73 %), which cluster in the same branch with a high bootstrap value. The krLHR exhibits highest homology with European seabass (*Dicentrarchus labrax*) (79 %) and gilthead seabream (*Sparus aurata*) (76 %) that form a cluster at the same branch.

Tissue distribution

The tissue expression pattern of the krFSHR and krLHR was analyzed by RT-PCR (Figs. 3, 4) The tissues included ovary, liver, kidney, head kidney, brain, heart, spleen, caeca, stomach, fat, gills, intestinal, pituitary of a female fish and testis of a male one. It is revealed that the krFSHR gene was expressed in the gonads, spleen, head kidney, caeca, and, at low levels, in the heart, liver, gill, kidney and intestine tissues. Expressions in the stomach, fat, brain and pituitary are hardly detectable. On the other hand, the krLHR shows the highest expression in head kidney and kidney, and lower expression levels are detected in gonads, brain, pituitary, caeca and gill. Expressions in the heart, stomach, fat, liver and intestine are not detectable.

Expression profiles analysis

The temporal expression of FSHR and LHR during reproductive cycle of ovary and testis was analyzed by qPCR (Fig. 5). The Korean rockfish testis and ovary developmental phase were seen in Shi et al. (Shi et al. 2011). The expression in stage V in male FSHR was set as the control group. In male, the expression level of FSHR was 18.07 ± 8.07 at stage II (spermatogonia stage), increased to its highest of 28.43 ± 9.38 (approximately 28.4-fold that of the control) at stage III (testes full of immature sperm) and dropped sharply from stage III to 1.04 ± 0.48 at stage V ($P < 0.05$). In female, the expression level of FSHR was increased from 2.4 ± 0.15 at stage II (perinucleolus stage) to the highest level of 11.03 ± 5.17 at stage IV–V (post-
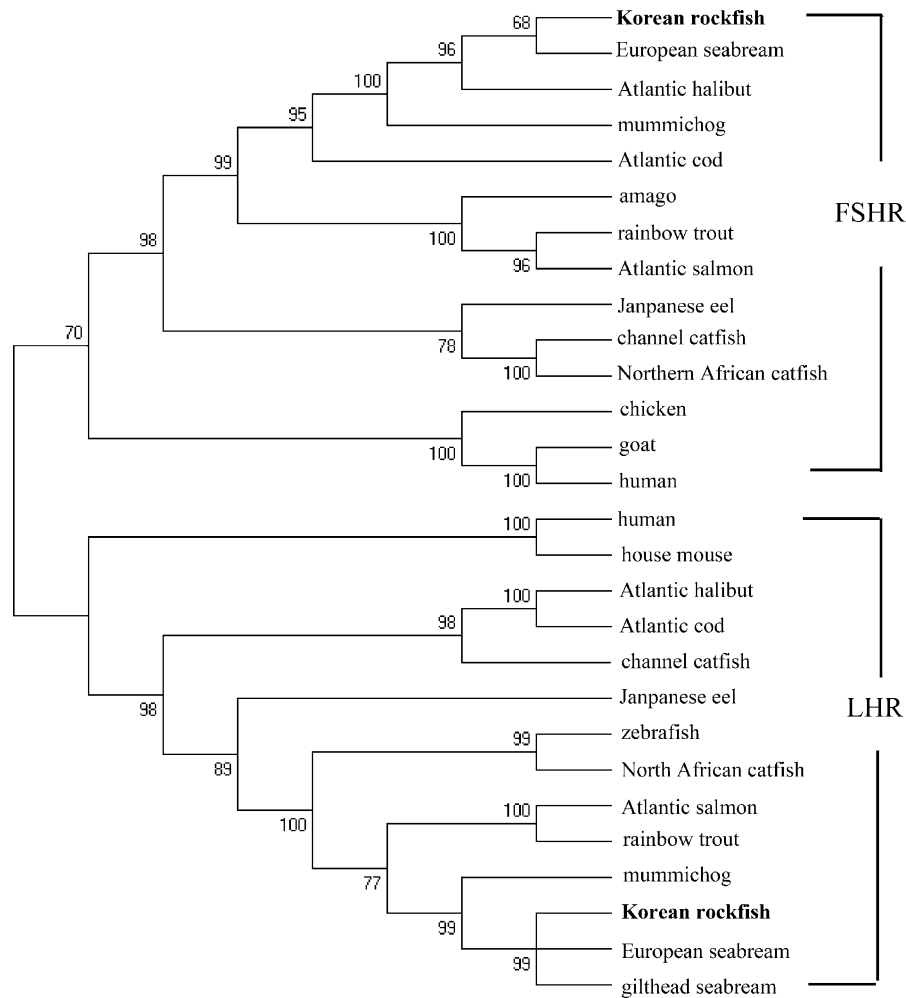
Fig. 2 Phylogenetic analysis of GTHR amino acid sequences inferred from the neighbor-joining method. Accession protein number: Korean rockfish FSHR (AEJ33654), European seabass FSHR (AAV48628), channel catfish FSHR (AAK16067), amago FSHR (BAA86898), Atlantic salmon FSHR (CAD98923), rainbow trout FSHR (AAQ04551), gilthead seabream FSHR (AAT01413), Atlantic halibut FSHR (ACB13177), mummichog FSHR (BAF48336), Japanese eel FSHR (AAU90017), North African catfish FSHR (CAB51907), Atlantic cod FSHR (ABD62885), chicken FSHR (AAC60030), goat FSHR (ACF24872), human FSHR (AAA52478); Korean rockfish LHR (ADV59773), Atlantic salmon LHR (CAE30288), European seabass LHR (AAV48629), rainbow trout LHR (AAQ04550), channel catfish LHR (AAK16066), zebrafish LHR (AAV31154), gilthead seabream LHR (AAT01412), Atlantic halibut LHR (ACB13176), mummichog LHR (BAF48337), Japanese eel LHR (AAU90018), North African catfish LHR (AAN75752), Atlantic cod LHR (ABD62886), house mouse LHR (EDL38652), human LHR (AAB19917). Bootstrap values are indicated (1,000 replicates)

oocyte) (approximately 11-fold that of the control) and decreased sharply from stage IV–V ($P < 0.05$) to 1.30 ± 0.21 at stage VI. However, the expression pattern of male and female was quite different. In male, the expression levels of LHR were maintained at low levels from 2.9 ± 0.61 to 4.58 ± 2.71, from stage II to stage V (post-spermiation), but increased dramatically to the highest level of 13.00 ± 0.23 at stage V ($P < 0.05$) (approximately 13-fold that of the control). In female, the expression levels of LHR maintained at high levels of 5.97 ± 1.49 in stage II and significantly decreased to 1.53 ± 0.64 at stage III (early-oocyte). Thereafter, LHR levels increased sharply to 6.77 ± 2.08 at stage IV–V and dropped markedly to the lowest level of 1.13 ± 0.47 at stage VI (gestational ovary) ($P < 0.05$).
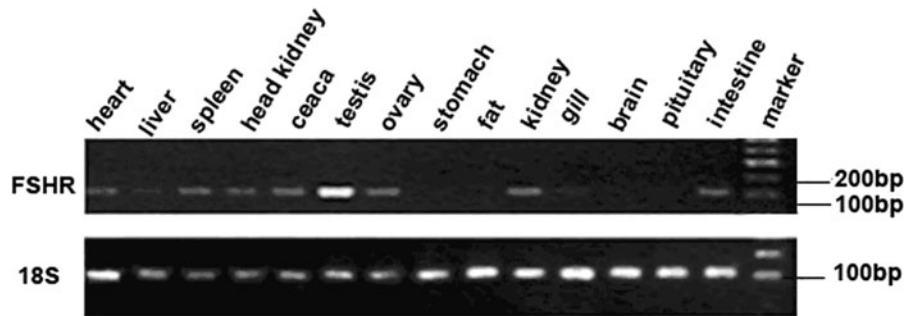
Fig. 3 Expression pattern of FSHR in different adult tissues as detected by RT-PCR. Various tissue-specific expressions of the krFSHR gene were determined by RT-PCR, using the primers fshr-e-f and fshr-e-r. The integrity of the RNA from the each tissue was ensured by uniform amplification of 18SrRNA transcripts (*lower panel*). *H* heart, *L* liver, *S* spleen, *He* head kidney, *C* caeca, *T* testis, *O* ovary, *St* stomach, *K* kidney gill, *G* gill, *B* brain, *P* pituitary, *I* intestine
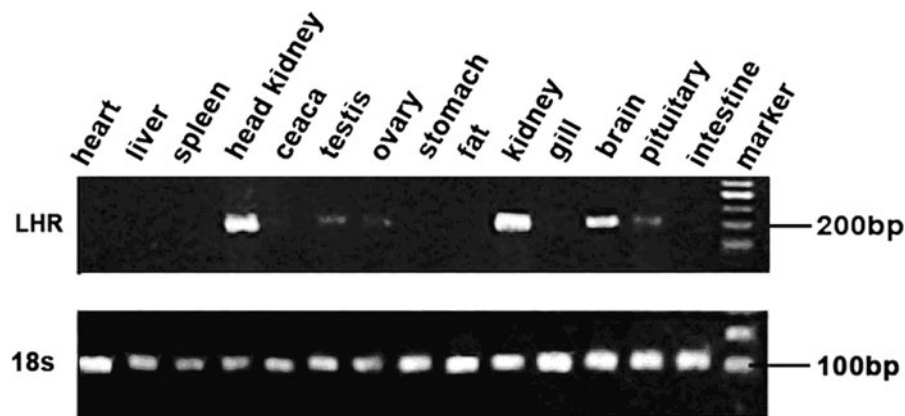
Fig. 4 Expression pattern of LHR in different adult tissues as detected by RT-PCR. Various tissue-specific expressions of the krLHR gene were determined by RT-PCR, using the primers lhr-e-f and lhr-e-r. The integrity of the RNA from the each tissue was ensured by uniform amplification of 18SrRNA transcripts (*lower panel*). *H* heart, *L* liver, *S* spleen, *He* head kidney, *C* caeca, *T* testis, *O* ovary, *St* stomach, *K* kidney gill, *G* gill, *B* brain, *P* pituitary, *I* intestine

Discussion

In our study, the FSHR and LHR mRNA of Korean rockfish were cloned, their structures were analyzed, and tissues distributions were examined. Analysis of amino acid sequences revealed that FSHR and LHR showed typical structural features of glycoprotein receptors: a relatively long ECD with nine or ten LRRs, which links to the rhodopsin-like G protein-coupled receptor. Each unit of LRRs shows the highly conserved L–X–X–L–X–L motif, which consists of a short β-strand and an α-helix element on the convex side (Kobe and Kajava 2001). These β-strand contain the LRR consensus sequences that usually provide a versatile structure framework for protein–protein interaction (Kajava et al. 1995).

Study on many fish revealed that there were nine LRRs in FSHR, and human FSHR contained 10 imperfect LRRs (Smits et al. 2003). It suggested that there was an additional LRR located in the hFSHR N-terminal cysteine-rich domain (Levavi-Sivan et al. 2010). However, the Korean rockfish shows an additional LRR that is located in the ECD. There are some other fish showing a similar condition, such as European seabass (Rocha et al. 2007a, b), half-smooth tongue sole (Chen et al. 2010a, b) and trout (Sambroni et al. 2007). The additional LRR would change the shape of hormone-binding domain, so the hormone recognition, specificity and activation would be influenced (Mittelholzer et al. 2009). However, it is not clear about the specificity of this motif, and Maugars et al. pointed out that an extra LRR could potentially

A

B

FSHR

C

D

LHR
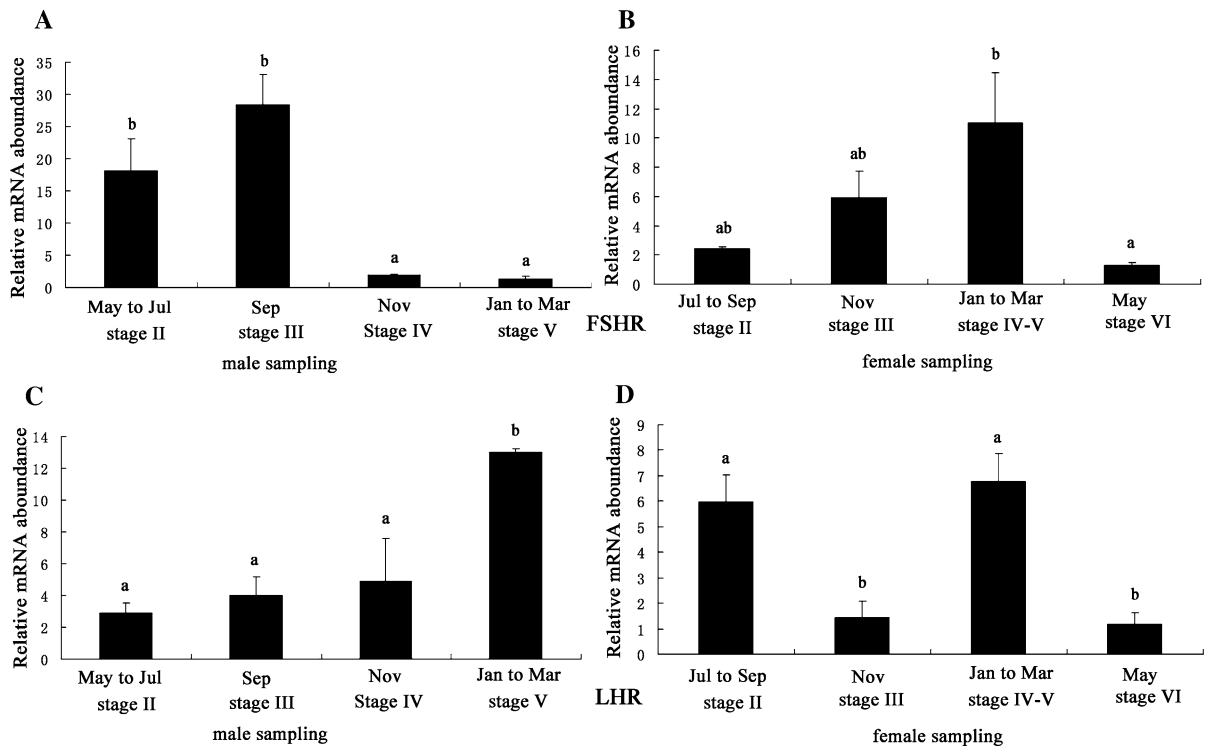
Fig. 5 FSHR and LHR mRNA expressions during the reproductive cycle. Relative mRNA abundance of FSHR (**A**, **B**) and LHR (**C**, **D**) are measured during the reproductive cycle by real-time PCR. Results are expressed as normalized fold expression with respect to 18SrRNA levels for the same sample. *Vertical bars* represent mean ± SEM ($n \geq 3$); groups with *different letters* are significantly different ($P < 0.05$, one-way ANOVA, followed by Duncan's test). 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–V* post-oocyte; *stage VI* gestational ovary

affect the ligand-binding mode (Maugars and Schmitz 2006). In addition, we identified 9 LRRs in the Korean rockfish LHR, which shows the same condition with mammalian or human LHR. What is more, seven transmembranes are generally considered to have the function of signal transduction and G protein-coupled (Vassart et al. 2004; Ulloa-Aguirre et al. 2007), which also exists in GTHRs of Korean rockfish.

Compared with Atlantic cod FSHR (six) and African catfish FSHR (seven), the ECD of Korean rockfish contains five potential N-linked glycosylation sites. There are three potential sites in the mammalian homologous FSHR but only two in the human. Three potential N-linked glycosylation sites are located in the Korean rockfish LHR, whereas this is quite different with mammalian that observed six.

Two cysteine residues ([36]C and [45]C) in the N-terminal cysteine-rich region are located in Korean rockfish FSHR. This structure permits the formation of a single disulfide bridge in KrFSHR, it is different

from that in hFSHR where have been found the location of two bridges (Fan and Hendrickson 2005). It may describe a different folding situation of this receptor.

Also, the two cysteine residues of the extracellular loops 1 and 2, [454]C and [529]C in Korean rockfish FSHR, [464]C and [539]C in Korean rockfish LHR, have been conserved through the glycoprotein receptor lineage and have been demonstrated to be essential for surface expression of the receptor (Kawate and Menon 1994; Zhang et al. 1996).

There are several analogous cysteine residues in the hinge region that positioned at 317, 318, 350, 358, 368 of KrFSHR and 283, 284, 369, 379 of KrLHR, and these residues are considered to be participated in the KH-fold architecture of the hinge region in vertebrate. Based on study in mammals, the presence of these cysteine residues was considered to be required for protein cell surface expression of an integer protein (Moyle et al. 2004). And this regain may play an

important role in the specific binding and the induction of signal response (Maugars and Schmitz 2008).

Compared with other fish from the amino acid sequence alignment, the KrFSHR and KrLHR shared the greatest homology with European seabass (*Dicentrarchus labrax*); it was 78 and 79 %, respectively.

In addition, the conservation in transmembrane region demonstrates that both FSHR and LHR show a well-conserved homology in LRR3, LRR4 and LRR6. The homology of second and fifth intracellular is differed largely in KrGTHRs, and this may interact mostly with activation of G protein by hormone receptor. The C-terminal domain that is rich of potential phosphorylation sites is quite different in many species, and this may be involved in the regulation of activity in GTHRs (Gether 2000).

As many species of fish, the expression profiles of KrGTHRs are found in the ovary and in the testis. GTHRS of Red dot salmon was specifically expressed in gonads (Oba et al. 1999a, b). It is interesting that GTHRs also express in non-gonadal tissues. Based on our study of Korean rockfish, besides gonads, GTHRs expression profiles are found in several extragonadal tissues. Similarly, extragonadal expression has been found for other fish species (Kumar et al. 2001b; Vischer and Bogerd 2003; Kwok et al. 2005) and in tetrapods (You et al. 2000; Ascoli et al. 2002). Kwok et al. (2005), who used RT-PCR, discovered that FSHR and LHR expression were different in zebrafish; meanwhile, their result of study demonstrated that both FSHR and LHR are highly expressed in the ovary and in the testis; further, expression of LHR showed high expression levels in the kidney and the head kidney. However, the study of channel catfish indicated that there was a high expression level only in the head kidney (Kumar et al. 2001a). LHR expression in KrLHR is found higher in kidney and head kidney than gonads that are in line with reports in channel catfish. The LHR gene is transcribed in low quantities in brain, but FSHR is not detected in brain, and this result is similar with Atlantic salmon (Maugars and Schmitz 2006). Many studies on fish tissue expressions demonstrated that GTHRs expressed in other non-gonadal tissues and found new features of these hormones. FSH/FSHR may participate in bone building by the increasing of osteoclast formation and function (Sun et al. 2006), and hCG/LH receptors pathway may be involved in implantation process (Pakarainen et al. 2007). Despite many reports on extragonadal tissues'

expression profiles, the specific biological properties in these tissues are still unclear (Pakarainen et al. 2007). Study on gonadotropin receptor expression in the fish reproductive cycle shows that fish gonadotropin receptors play a role in a specific time of gametogenesis.

GTHRs transcripts levels varied with reproductive stages in both males and females. Based on our research, the seasonal changes of gene expression showed that in the female, KrFSHR transcript abundance increases with oocytes growth, and higher abundance during early stage is significantly visualized than that during the late stage. A similar expression pattern was reported in amago salmon (Oba et al. 1999a, b). Hirai et al. (2000) found that female expression of FSHR increased in vitellogenesis, peaking in mid-vitellogenesis, and started to decline in the maximum oocytes stage. Zebrafish has the same expression pattern, with the period of yolk development into the vitellogenesis. FSHR expression levels were significantly increased and dropped after reaching a certain stage (So et al. 2005). In early vitellogenesis stage, tilapia FSHR expression also had the most abundant level. All of these studies demonstrated the FSHR may play an important role in the ovary growth and maturation. However, in channel catfish, FSHR expression showed low level in the whole reproductive cycle and demonstrated a sudden increase after ovulation, which was different with the majority of fish (Hirai et al. 2000). The early development stage of the male FSHR expression was significantly higher than the late stage, which decreased after a certain level, match with expression pattern in Atlantic salmon (Maugars and Schmitz 2006), showing its role in the testis early developmental stage.

KrLHR transcript levels in females are peaked in the ovulation period, which is in line with reports in zebrafish; during this period, the expression level reached the highest and oocytes is full of yolk granules (Kwok et al. 2005). Interestingly, in contrast to LHR of many species , which has low expression level in the early development stage, KrLHR expression shows high level in stage II (perinucleolus oocyte stage). This may reflect species difference in various stages of reproduction. Moreover, two genomic regions were classified as the CpG island in the transmembrane domain of LHR, where the methylation pattern could influence the gene expression. KrLHR in the male

possessed the similar expression pattern with other male fish species, displaying increased expression with testis growth followed by peak transcript levels at the stage V (spermiation period). In the male Red Seabream, LHβ predominates around ovulation and spermiation (Gen et al. 2000), and our research of the increase in the LHR around the time of ovulation fits well with this model. The seasonal changes in gene expression that is determined by real-time PCR revealed that KrFSHR involves the initiation of the endogenous vitellogenesis and early spermatogenesis, regarding KrLHR; its gene expression is consistently supporting important role in final oocyte maturation and spermiation processes.

In summary, present study describes the cloning and expression analysis of FSHR and LHR during the reproductive cycle of an oviviviparous Korean rockfish. Our results revealed high transcript levels of FSHR and LHR during early gamete and late gamete maturation respectively. These findings can be used to further understanding the endocrinological mechanism in the teleost. Furthermore, our next work is to explore the function of GTHRs in Korean rockfish by in situ hybridization and immunohistochemistry.

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