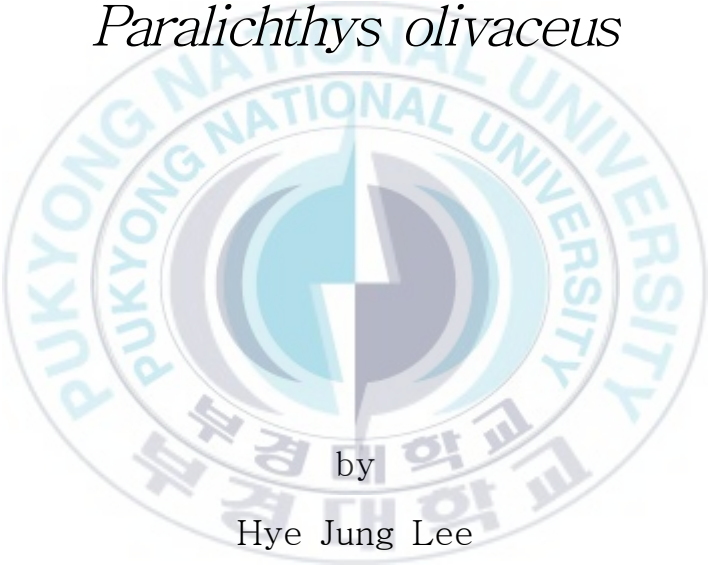
Thesis for the Degree of Master of Fisheries Science

Molecular Cloning of Melanocortin 4 Receptor Gene from Olive Flounder *Paralichthys olivaceus*



by

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February 2008

Molecular Cloning of Melanocortin 4 Receptor Gene from Olive Flounder *Paralichthys olivaceus*

넙치 *Paralichthys olivaceus* 로 부터 Melanocortin 4 Receptor 유전자의 클로닝

Advisor: Prof. Jong Myoung Kim

By Hye Jung Lee

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Fisheries Science

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A dissertation
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Abstract

Melanocortin 4 receptor (MC4R), one of the five MC receptors, is known to be the most monogenic cause of obesity, plays a role in reducing the feeding behavior by binding of MSH peptides to the MC4R. It is belong to the *rhodopsin* family, one of the G protein-coupled receptors (GPCR) that constitute the largest superfamily of cell membrane receptors performing diverse functions including response to neurotransmitters, hormones and other external signals. To study the molecular mechanism of MC4R gene of olive flounder (*Paralichthys olivaceus),* Full-length MC4R gene of *P. olivaceus* was obtained by PCR amplification of genomic DNA as well as cDNA synthesis. Sequence comparison of PCR products of both method indicates that MC4R gene of olive flounder lacks introns. Sequence analysis of the MC4R gene indicates 978 bp DNA fragment encoded 325 amino acids. The sequence alignment of other fish MC4Rs shows the highest degree of 96% identity between *P. olivaceous* and *Verasper moseri,* followed by *Takifugu rubripes* and *Tetraodon nigroviridis* (89%) corresponding to the phylogenetic analysis. Tissue distribution analysis shown that MC4R expressed in liver, eye and brain.

Figure legends

Table legends

Molecular Cloning of Melanocortin 4 Receptor Gene from Olive Flounder *Paralichthys olivaceus*

Ⅰ. Introduction

G-protein coupled receptor (GPCR) is well known to participate in a signalling from outside to inside cells. Upon recognition of a diverse array of endogenous ligands including neurotransmitters, hormones, and exogenous stimuli such as light, odorant, tastes (Gether, 2000), GPCRs begin conformational changes initiating signal transduction cascade through heterotrimeric G-protein activation. This in turn activates or inhibits the secondary messenger leading to the physiological changes. GPCR is one of the largest superfamily of proteins as more than 2000 receptors and 100 subfamilies of GPCR were identified so far. GPCRs has been regarded as one of the most attractive drug targets. While GPCRs recognize diverse arrays of ligands hence involved in various physiological reactions, all GPCRs share a common topology, seven-transmembrane helices segment (7TM), together with conserved regions. This suggests that activation mechanism in GPCRs are similar to each other.

Melanocortin receptors (MCRs) belongs to the *rhodopsin* family of GPCRs. MCRs were known to be activated by ligands including α-, β-, γ-melanocyte stimulating hormone (MSH) and adrenocorticotropic

hormone (ACTH) derived from pro-opiomelanocortin (POMC) precursor mediating various signalling pathways. Five subtypes of MCRs (MCR1-MCR5) were identified so far in mammals. MC1R in melanocyte is known to be involved in skin and hair color determination and anti-inflammatory action in various immunocytes such as leukocytes. MC2R is known to be activated by ACTH mediating physiological reactions in adrenal gland. MC3R and MC4R are involved in food intake and energy balance in animals. This was shown by MC3R and MC4R knock-out mice exhibiting hyperphagia, hyperinsulinemia and maturity-onset obesity that corresponded with agoui obesity syndrome (Huszar *et. al.*, 1997; Chen *et. al.*, 2000). Administration of MCR agonists such as MSHs, natural compound as well as synthetic compound including MTⅡ into mouse showed loss of appetite effect and lean phenotype. Injection of antagonistic ligand of MC3R and MC4R was also shown to block the effect and lead to the gain of the weight (Fan *et. al.*, 1997). In addition, MC4R is the most common monogenic determinant of the obesity. Therefore, MC4R is one of high interest in the pharmaceutical industry for the treatment of anorexia and obesity. MC5R is distributed throughout the peripheral tissue although its physiological functions hasn't been clear yet (Schiöth, 2001).

Olive flounder (*Paralichthys olivaceus*), Japanese flounder, belongs to the Paralichthyidae, Pleuronectiformes. It is one of the major cultured marine fish in Korea produced around 40,000 tons, earned 3,547 billion won (48.8% of the sum total) according to the 2005, The Status of Aquaculture of Marine Fishes (MOMAF, 2006). To study the correlation

between weight gain and MC4R in fish, we isolated and characterized melanocortin 4 receptor gene of the olive flounder.

Ⅱ. Materials and Methods

1. Materials

T4 DNA ligase and *AccuPrep*® Genomic DNA extraction kit were purchased from Bioneer Corporation (Daejeon, Korea). TRI REAGENT™ was obtained from Sigma (Saint Louis, MS). PolyATract® mRNA Isolation System, ImProm-Ⅱ™ Reverse Transcription System and Wizard® *Plus* Maxipreps DNA Purification System were purchased from Promega Corporation (Madison, WI). Various restriction endonucleases were purchased from Bioneer (Daejeon, Korea) and New England Biolabs (Beverly, MA), calf intestine endonucleases were also obtained from New England Biolabs (Beverly, MA). Plasmid Purification mini Kit, Gel Extraction Kit and PCR Purification Kit were purchased from NucleoGen (Seoul, Korea). DNA Walking *SpeedUp*™ Premix Kit Ⅱ were obtained from Seegene (Seoul, Korea). pMD18-T vector of TaKaRa Perfect-T cloning Kit and pGEM®-T Easy Vector system were obtained from Takara (Shiga, Japan) and Promega Corporation (Madison, WI), respectively. Oligonucleotides were synthesized and 5X HiQ-PCRmix were obtained from Genotech (DaeJeon, Korea). PCR primers are listed in the Table 1. MyCycler™ Thermal Cycler used for PCR reaction was obtained from Bio-Rad Laboratories (Hercules, CA).

primer name	sequence (5'→3')	comment
MC4RF3	ATC AAG AGC ATG GAC AAC GT	partial MC4R
MC4RR4	ATC ATG AGG ATG AGG TGG AG	
pMCDWF1	CGC TTG CAC ATG AAG CGC AT	DNA walking PCR
pMCDWF2	AG(G/C) GCG CCA ACA TGA AGG	
pMCDWF3	GGT GTT CGT GGT GTG CTG G	
pMCDWR1	AAG ATG GTG ATG TAG CGG TCG	
pMCDWR2	AGC AGG CTG CAG ATG GAC	
pMCDWR3	GGA GCT GCA GAT CAT AGA GTC A	
RTMC4RF	CTG GAG AAC ATC CTG GTG GTC G	RT-PCR
RTMC4RR	GAA GAT GGT GAT GTA GCG GTC G	
PgfMC4RF	GCAAGAATTCATGAACGCTACAGAACATCCTGGACTG	Full-length of MC4R
PgfMC4RR	GGAGACGGTGGTCGACACACACAGCAGAGCGT(G/A)TGA	
AJactinF	GCA GGT CAT CAC CAT CGG	β-actin
AJactinR	GAG TAT TTG CGC TCA GGT G	

Table 1. List of sequence of oligonucleotides used for the experiment.

2. Genomic DNA extraction and cloning of melanocortin 4 receptor

2-1. Isolation of Genomic DNA

Olive flounder genomic DNA was extracted from whole blood using the *AccuPrep*® Genomic DNA extraction Kit according to the experimental protocol. Total 80 μL of blood with 120 μL of PBS [10 mM Tris-Cl, 25 mM EDTA, 150 mM NaCl] was applied to the microcentrifuge tube containing 20 μL of Proteinase K for the lysis. The mixture, added with 200 μL of Binding buffer (GC), was mixed immediately by vortex and incubated at 60℃ for 10 min. After the addition of 100 μL of isopropanol followed by a brief centrifugation, the lysate was transferred carefully to the upper reservoir of the binding column tube supplied by the supplier. The tube was then centrifuged at 13,000 rpm for more than 1 min until the lysate completely passed the column. The Binding column was transferred to a new tube for filtration, washed using washing buffer 1 (W1) and 2 (W2), and centrifuged at 8,000 rpm for 1 min, respectively. To completely remove ethanol, the tube was centrifuged once more at 12,000 rpm for 1 min. Finally, the column was transferred to a new microcentrifuge tube, added 200 μL of distilled water and waited for 2 min until water is completely absorbed into the glass fiber of binding column tube. After that, the tube was centrifuged at 8,000 rpm for 1 min to elute DNA.

The purity of the genomic DNA was confirmed by 1% agarose gel

electrophoresis followed by staining with ethidium bromide (0.5μg/mL). Isolated genomic DNA was stored at -20℃ until its further use.

2-2. PCR for the MC4R partial gene from olive flounder

Olive flounder genomic DNA was used as template in the PCR using 5x HiQ-PCRMix containing buffers [10 mM Tris (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl], 1 mM dNTPs [250 μM each] and improved Taq DNA polymerase. PCR primers were designed from conserved regions of all known fish MC4R genes (rainbow trout, spiny dogfish, spotted green pufferfish, goldfish, zebrafish and fugu (Metz *et al*., 2006)). The forward primer (MC4RF3) was 5'- ATC AAG AGC ATG GAC AAC GT -3' and reverse primer (MC4RR4) was 5'- ATC ATG AGG ATG AGG TGG AG -3'.

For PCR reaction, the mixture (20 μL) contains 2 μL of genomic DNA (0.1 μg/μL), 0.8 μL of MC4RF3 and MC4RR4 primers (10 μM), 4 μL of 5X HiQ-PCRMix and 12.4 μL of distilled water. PCR was carried out with an initial denaturation at 95℃ for 5 min followed by 40 cycles of 45 sec at 94℃, 45 sec at 50/53/55℃, 2 min at 72℃ and final extension of 5 min at 72℃. PCR product of ~450 bp in size was purified from agarose gel and ligated into a pMD18-T vector using TaKaRa T-cloning Kit. Ligation reaction mixture containing 9.5 μL of purified PCR product, 0.5 μL of pMD18-T vector (50 ng/μL) and 10 μL of Ligation Solution was incubated at 14℃ for 2 hrs. Ligated DNA was

transformed into E. *coli* DH5α.

2-3. Transformation of E. *coli* by using recombinant plasmid

2-3-1. Preparation of Competent Cells

Competent cell of E. *coli* was prepared by using Inoue method (Inoue, *et. al.*, 1990). For this, 1/250 volume of E. *coli* DH5α culture previously grown in LB medium was inoculated into 100 mL of LB followed by overnight incubation at 22℃ with moderate shaking (180 rpm). When the OD_{600} of cultured E. *coli* was reached 0.6, the culture was chilled on ice for 10 min and transferred into the centrifuge tube. Upon centrifugation at 2,500 x g for 10 min at 4℃, cell pellet was resuspended in 32 mL of ice-cold transformation buffer (TB) [10 mM Hepes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7] and then incubated on ice for 10 min. Centrifugation was carried out at 2,500 x g for 10 min and the cell pellet was resuspended in 8 mL of ice-cold TB. After the addition of 7% DMSO (560 μL), cells were divided about 500 μL of volume into the microcentrifuge tube and froze in liquid nitrogen. Competent cells were stored in -80℃ until its use.

2-3-2. Transformation of recombinant plasmid into the E. *coli*

Ligation mixture of 10 μL was mixed with 100 μL competent cells and then incubated on ice for 30 min. After heat-shock at 42℃ for 40

seconds, the mixture was rapidly chilled on ice for 3 min. Cells were then mixed with 1 mL of LB broth followed by incubation at 37℃ for 1 hr. Transformed E. *coli* plated onto the LB agar medium containing the Ampicillin (100 μg/mL) and 2% X-gal were incubated overnight at 37℃.

2-4. Identification of recombinant plasmid DNA

Bacterial colonies selected from transformants were inoculated into LB broth medium containing ampicillin (100 μg/mL) followed by overnight incubation at 37℃. Plasmid was isolated from the cells using Plasmid Purification Kit according to the protocol provided by manufacturer. Cells harvested from 1.5 mL of culture by centrifugation at 13,000 rpm for 2 min in microcentrifuge tube were resuspended with 250 μL of Resuspension Solution by using vortex mixer. After the addition of 250 μL of Lysis Solution followed by several times of inversion and incubation for 5 min, the lysate was mixed with 350 μL of Neutralization Solution. Centrifugation was carried out at 14,000 rpm for 10 min. Supernatant containing plasmids was transferred into the spin column supplied from the supplier. After the centrifugation of the column for 1 min, the flow-through was discarded from tube. The column was washed by adding 750 μL of Wash A Solution followed by centrifugation at 13,000 rpm for 1 min. After the removal of the flow-through, the column was centrifuged again for 1 min. The column transferred onto a new 1.5 mL tube was added with 50 μL of distilled

water and waited for 2 min. Plasmids eluted by centrifugation at 13,000 rpm for 1 min were stored at -20℃ until its use.

To identify the insert in the recombinant plasmid, purified plasmids were digested with EcoR Ⅰ or Pvu Ⅱ restriction endonucleases. For digestion reaction, the mixture containing 3 μL of plasmid, 10X NEB reaction buffer provided by the supplier, 0.5 μL of enzymes (10 units/μL), 5.5 μL of distilled water was incubated at 37℃ for 2 hrs. Digested DNA was identified in 1% agarose gel electrophoresis. Selected recombinant plasmids were subjected to sequencing using the M13F sequencing primer.

2-5. Cloning of the 5'- and 3'-end of the MC4R gene

To obtain the 5'- and 3'- ends of olive flounder MC4R genes, DNA amplification was carried out by using the DNA Walking *SpeedUp*™ Premix Kit Ⅱ and three primers of sense and antisense strands (Table 1) complimentary to the MC4R partial genes acquired. Three forward primers used were; pMCDWF1 : 5'- CGC TTG CAC ATG AAG CGC AT -3', pMCDWF2 : 5'- AG(G/C) GCG CCA ACA TGA AGG -3', pMCDWF3 : 5'- GGT GTT CGT GGT GTG CTG G -3' . The reverse primers include pMCDWR1 : 5'- AAG ATG GTG ATG TAG CGG TCG -3', pMCDWR2 : 5'- AGC AGG CTG CAG ATG GAC -3', pMCDWR3 : 5'- GGA GCT GCA GAT CAT AGA GTC A -3'. DNA walking PCR was carried out with one of the primers and universal primers provided from the manufacturer according to the manual.

The first PCR reaction mixture contains 2 μL of genomic DNA, 10 μL of 2X SeeAmp™ ACP™ Master mix Ⅱ, 5.5 μL of distilled water, 2 μL of 5 μM DW2-ACP primer (supplied, DW2-ACP 1, 2, 3 and 4) and 0.5 μL of 10 μM pMCDWF 1 primer or 0.5 μL of 10 μM pMCDWR 1 primer for the amplification of the 3'-end and 5'-end region, respectively, of the gene. PCR reaction was performed with a cycle of initial denaturation at 94℃ for 5 min, an annealing at 42℃ for 1 min and extension at 72℃ for 2 min, followed by 30 cycles at 94℃ for 30 sec, 58℃ for 30 sec and 72℃ for 100 sec and final extension at 72℃ for 7 min. PCR product was purified using the PCR Purification Kit.

The second PCR mixture contained 2 μL of purified first PCR product as a template, 2 μL of 5 μM DW2-ACPN, 10 μL of 2X SeeAmp™ ACP™ Master mix Ⅱ, 5.5 μL of distilled water and 0.5 μL of 10 μM pMCDWF 2 or pMCDWR 2 primer used for the amplification of the 3'-end and 5'-end region, respectively, of the gene. PCR was carried out with an initial denaturation at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, extension at 72℃ for 1 min and final extension at 72℃ for 7 min.

The third PCR mixture contained 2 μL of second PCR product as template, 1 μL of 5 μM UniP2, 10 μL of 2X SeeAmp™ ACP™ Master mix Ⅱ, 6.5 μL of distilled water and 0.5 μL of 10 μM pMCDWF 3 or pMCDWR 3 primer for the amplification of the 3'- and 5'-end region, respectively, of the gene. PCR was carried out an initial denaturation at 94℃ for 3 min followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing at 65℃ for 30 sec, extension at 72℃ for 80 sec and final extension at 72℃

for 7 min.

PCR products resolved by agarose gel electrophoresis were purified by using Gel Extraction Kit and cloned into the pGEM®-T Easy Vector. Recombinant DNA transformed into E. *coli* were identified by restriction digestion of the plasmids followed by DNA sequencing.

3. Recombination of MC4R gene with expression vector

In order to express the MC4R gene in animal cells, MC4R ORF gene was cloned into pMT4 used for the expression of bovine opsin gene. To confirm the expression of MC4R gene in the animal cells (Cos-1 cell/ HEK293T cell) by immunoblotting, predicted MC4R gene was fused with the region corresponding to the 3'-end of the bovine opsin gene (TETSQVAPA*) known to be recognized by 1D4 antibody. This was carried out by cloning of MC4R gene with PCR primers containing the part of 1D4 sequence together with Sal Ⅰ site and EcoR Ⅰ, respectively. Primer used include PgfMC4RF : 5'- gcaa gaa ttc MNATEHPGL -3' and PgfMC4RR : 5'- S(H/Y)ALLCVSTTVS -3' (Table 1). The PCR reaction mixture contained 4 μL of olive flounder genomic DNA, 1.6 μL of each of 10 μM primer, 8 μL of 5x HIQ-PCRMix and 24.8 μL of distilled water. PCR was carried out with a cycle of reaction with 5 min denaturation at 95℃ followed by 35 cycles of reactions consisted of 40 sec denaturation at 94℃, 40 sec at 55℃, 90 sec at 72℃, and final extension 5 min at 72℃ for 1 cycle. PCR products purified by PCR Purification Kit were subjected to restriction digestions using BssH Ⅱ

restriction enzyme due to the presence of Sal Ⅰ site in the predicted olive flounder MC4R sequences. Upon agarose gel electrophoresis of digested DNAs, DNA fragments of about 650 bp and 350 bp in sizes, corresponding to the 5'- and 3'-end region, respectively, of the gene were purified by Gel Extraction Kit. About 650 bp DNA fragments treated calf intestine endonucleases (CIP) were purified using PCR purification kit followed by digestion with EcoR Ⅰ and Sal Ⅰ restriction enzyme, corresponding to the about 650 bp and 350 bp DNA fragments, respectively. DNA fragments were purified again by Gel Extraction Kit after performing gel electrophoresis and then cloned into EcoRI Ⅰ/Sal Ⅰ digested pMT4 vector (Oprian *et. al*., 1987). Constructs transformed into E. *coli* were identified by BspH Ⅰ restriction digestion of the plasmids isolated from transformants. DNA sequence in the constructs were confirmed by DNA sequencing (Genotech, Korea). Acquired sequences were compared with NCBI database using BlastN & BlastX.

3-1. Large-scale purification of recombinant plasmid

Plasmids containing the predicted olive flounder MC4R gene was subjected to the large scale preparation of plasmid DNA for expression analysis. For this, 2 mL of culture grown on small scale was inoculated into 400 mL of LB broth medium containing ampicillin (100 μg/mL) and incubated for 10 hrs at 37℃ with 200 rpm. Culture transferred to 250 mL centrifuge bottle were centrifuged at 4,000 x g for 20 min at room temperature. Collected cells were resuspended 15 mL of

Resuspension Solution [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 μg/mL RNase A] and mixed well by vortex mixer. Tubes added 15 mL of Lysis Solution [0.2 M NaOH, 1% SDS] were gently mixed by inverting several times. After the incubation for 20 min until solution becomes clear and viscous, 15 mL of Neutralization Solution [1.32 M potassium acetate (pH 4.8)] was immediately mixed by gently inverting several times. Upon centrifugation at 4,000 x g for 30 min, supernatant was filtered through gauze into the 45 mL tube. The filtrate mixed 0.5 volume of isopropanol was centrifuged at 14,000 x g for 10 min in a room temperature. After the supernatant was discard, tube was dried for 2 hrs. DNA pellet resuspended in 2 mL of distilled water was mixed with 10 mL of Wizard® Maxipreps DNA purification Resin for further purification. The mixture transferred the Maxicolumn was passed through with an aid of vacuum. The column was washed with 25 mL of column Wash Solution [80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 μM EDTA, approximately 55% ethanol] and then with 80% ethanol. The column shifted to the 50 ml tube was centrifuged in a swinging bucket rotor at 1,300 x g for 5 min. Maxicolumn was dried completely and placed the Maxicolumn into a new 50 mL tube. For elution of the DNA, 1.5 mL of preheated water was added to the Maxicolumn followed by incubation for 2min. DNA eluted by centrifugation at 1,300 x g for 5 min was filtered using 0.2 μM Syringe filter and then stored in -20℃.

4. Sequence alignment and phylogenetic analysis

The full-length amino acid sequence of olive flounder MC4R was aligned with the other known MCR sequences using ClustalW (Thompson *et. al.*, 1994). The accession numbers of melanocortin receptor sequences searched from NCBI GenBank were : *Homo sapiens* MC4R (NM_005912), *Mus musculus* MC4R (NM_016977), *Gallus gallus* MC4R (AB012211), *Squalus acanthias* MC4R (AY169401), *Danio rerio* MC4R (AY078989), *Oncorhynchus mykiss* MC4R (AY534915), *Takifugu rubripes* MC4R (AY227794), *Tetraodon nigroviridis* MC4R (AY332240), *Carassius auratus* MC4R (AJ534337) and *Verasper moseri* MC4R (AB287975).

Phylogenetic tree was constructed by neighbor-joining method using MEGA v 4.0. Various species of MCRs exhibiting higher homology with predicted olive flounder MC4R gene were selected using the Blastp in NCBI. Assessing tree reliability was tested using a bootstrap with 1000 replicates. Outgroup was human melanocortin 1 receptor.

5. Reverse transcription PCR analysis

In order to confirm the tissue-specific expression of MC4R in fish, RNA was extracted from various internal organs of olive flounder. Isolation of total RNA from kidney, gut, gill, liver, intestine, spleen, heart, eye and brain were carried out as described below. RNA was stored at -80℃ until use.

5-1. Extraction of Total RNA from olive flounder

Organs dissected from olive flounder were frozen in liquid nitrogen and stored at -80℃. Total RNA was extracted from the organs using the TRI REAGENT™ according to the manufacture's procedure with slight modification. Frozen tissue (500 mg - 1 g) was homogenized using the Glas-Col tube and pestle in the presence of 10 mL of TRI REAGENT™. Homogenized tissue divided into microcentrifuge tubes were centrifuged at 14,000 rpm for 8 min. Supernatant was transferred to a new tube and mixed vigorously with 250 μL of chloroform per 1 mL of TRI REAGENT™ using vortex mixer followed by centrifugation at 14,000 rpm for 10 min. The supernatant was transferred carefully to new RNase-free 1.5 mL tube and mixed with 1 mL of isopropanol by using vortex mixer. Upon centrifugation at 14,000 rpm for 10 min, supernatant was discarded carefully. The pellet was washed two times with 1 mL of 75% ethanol followed by centrifugation at 7,500 rpm for 5 min. Dried RNA pellet was dissolved in 75 μL of 0.05% diethyl pyrocarbonate (DEPC)-treated water. Quality and quantity of extracted total RNA were estimated by 1% agarose gel and spectrophotometry.

5-2. Isolation of mRNA

Isolation of mRNA was carried out by using the PolyATract®

mRNA Isolation System according to the protocol provided by the manufacturer based on the property of eukaryotic mRNA composed of 3' poly(A) tail. Biotinylated-oligo(dT) probe was added to the total RNA and selectively hybridized with mRNA. Streptavidin coupled to paramagnetic particles were used to collect biotinylated-oligo(dT) probe hybridized with mRNA.

SSC solutions (0.5X and 0.1X SSC) were prepared by the dilution of 20X SSC with RNase-Free water. For the experiment, total RNA diluted with RNase-free water to 500 μL was incubated at 65℃ for 10 min and then mixed with 3 μL of Biotinylated-Oligo(dT) probe and 13 μL of 20X SSC followed by incubation at room temperature until completely cooled. Streptavidin-Paramagnetic Particles (SA-PMPs) were prepared by gently flicking the bottom of the tube and captured at the side of the tube by placing next to the magnetic stand. Supernatant was carefully removed and the SA-PMPs were washed three times with 300 μL of 0.5X SSC and then resuspended in 100 μL of 0.5X SSC. Mixture containing total RNA was added to the SA-PMPs and incubated at room temperature for 10 min. Tube was gently inverted every 1-2 min. SA-PMPs was captured using magnetic stand and supernatant was discarded. Particle was washed four times with 300 μL of 0.1X SSC and the mRNA was eluted with 200 μL of RNase-free water.

5-3. Synthesis of first strand cDNA

First strand cDNA was synthesized by using mRNA as a template

and oligo(dT)$_{13}$ as a primer using the ImProm-II™ Reverse Transcription System. For this, 7 μL of mRNA mixed with 7 μL of dT_{13} primer were incubated at 70℃ for 5 min and then chilled on the ice for 5 min. The reaction mixture for cDNA synthesis containing 6 μL of ImProm-II™ 5X Reaction Buffer, 7.2 μL of 25 mM $MgCl_2$ (final concentration 6 mM), 1.25 μL of 10 mM dNTPs (final concentration 0.5 mM), 0.75 μL of Recombinant RNasin® Ribonuclease inhibitor (30 units), 1 μL of ImProm-II™ Reverse Transcriptase, and 14 μL of mRNA and dT_{13} primer was annealed at 25℃ for 5 min and then extended the strand by incubation at 42℃ for 1 hr. The reaction was stopped by inactivation at 70℃ for 15 min and then stored in -20℃ until further use.

5-4. PCR amplification of MC4R genes by using cDNA

Amplification of MC4R gene was also carried out by using first strand cDNA as a template and specific primers derived from full-lengh olive flounder MC4R sequence, PgfMC4RF and PgfMC4RR (Table 1). The reaction mixture (20 μL) contains 2 μL of genomic DNA or cDNA, 0.8 μL of primers, 4 μL of 5X HiQ-PCRmix and 12.4 μL of distilled water. Gradient PCR was carried out as follows ; initial denaturation for 5 min at 95℃ followed by 40 cycles consisting of incubations for 40sec at 94℃, 40sec at 45℃/51℃/55℃, 90sec at 72℃, and final extension for 5 min at 72℃. PCR products were confirmed by 1% agarose gel electrophoresis.

5-5. Reverse transcription PCR

In order to confirm tissue-specific expression of MC4R gene, RT-PCR was carried out by using first strand cDNA as template and specific primers derived from full-length olive flounder MC4R sequence. These include forward primer (RTMC4RF) 5'- CTG GAG AAC ATC CTG GTG GTC G -3' and reverse primer (RTMC4RR) 5'- GAA GAT GGT GAT GTA GCG GTC G -3'. The size of the PCR product was predicted to be approximately 280 bp. Reaction conditions used for PCR include denaturation 3 min at 95℃ for 1 cycle, 30 cycles of 40 sec at 94℃, 40 sec at 60℃, 40 sec at 72℃ and then final extension 5 min at 72℃ for 1 cycle using the 5X HiQ-PCRMix. PCR was also performed with genomic DNA used as positive control and with water as negative control. PCR result was confirmed from 1.2% agarose gel. As an internal positive control, PCR amplification of β-actin gene was carried out 3 min at 95℃ for 1 cycle, 25 cycles of 40 sec at 94℃, 40 sec at 60℃, 40 sec at 72℃ and final extension 5 min at 72℃. PCR primers used for the amplification of β-actin gene were derived from Japanese eel (A. *japonica*). AJactinF : 5'- GCA GGT CAT CAC CAT CGG -3', AJactinR : 5'- GAG TAT TTG CGC TCA GGT G -3'.

6. Site-directed mutagenesis of MC4R variants

To study the structure and function of MC4R, cloning of MC4R mutants containing point mutations (D125Q, D145Q, R146Q, T174A, L249Y) were constructed by using the overlap extension PCR (Urban *et. al.*, 1997; Fig. 1). For this, 4.2 ng of constructed wild-type olive flounder MC4R was used as a template together with sense and antisense primer (PgDMC4RF, PgDMC4RR, F & R primers, respectively) that could make complete MC4R are common to all mutants. Specific primers with complementary oligonucleotides (33-mer) including desired mutation region (sense primer : f, antisense primer : r) were also included for PCR (Table 2). For this the first PCR reaction was performed using F primer and r primer and the other PCR was used f primer and R primer. PCR reaction mixture contains 1.5 μL of plasmid containing MC4R gene, each of 1 μL of 10 μM primers, 4 μL of 5X HiQPCR-MIX and 12.5 μL of distilled water. PCR was carried out with 1 cycle of 95℃ for 2 min, 25 cycles of 94℃ for 40 sec, 55℃ for 45 sec, 72℃ for 1 min and 1 cycle of 72℃ for 5min.

Second PCR was carried out with two PCR reaction products as templates. The reaction mixture contained 0.5 μL of each PCR product, 1 μL of primers (PgDMC4RF and PgDMC4RR), 5 μL of 5X HiQPCR-MIX and 17 μL of distilled water and the PCR condition was identical as the first PCR reaction. PCR products were purified using Gel Extraction Kit after the digestion with EcoR Ⅰ/Sal Ⅰ restriction endonucleases. DNA fragments were cloned into EcoR Ⅰ/Sal Ⅰ digested pMT4 (Oprian *et. al.*, 1987) for the expression and identified by plasmid isolation and DNA sequence analysis.

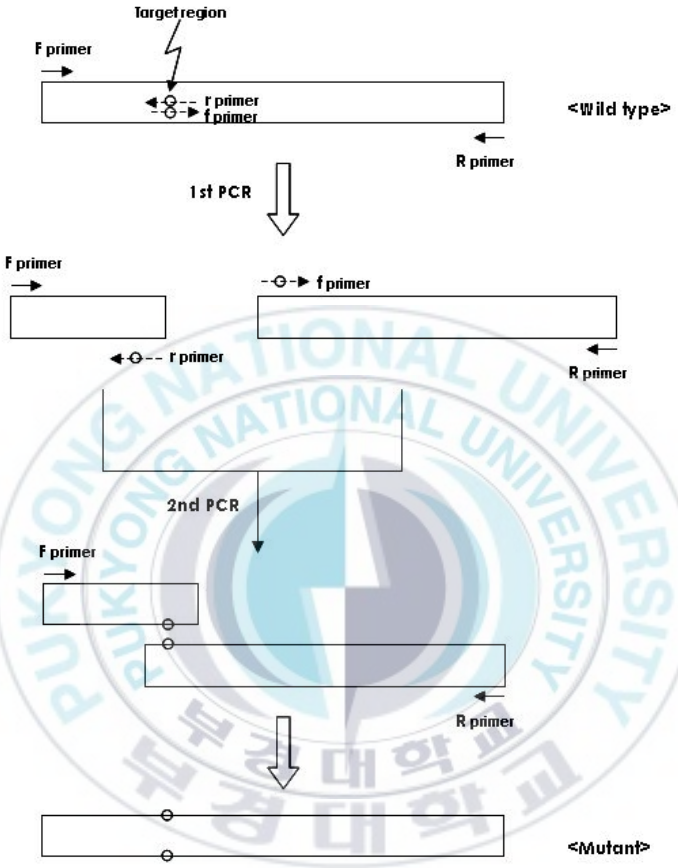

Figure 1. Scheme of Overlap Extension-PCR.

primer name	sequence (5'→3')
po_D125QF	ATG GAC AAC GTG TTT **CAA** TC**G** ATG ATC TGC AGC
po_D125QR	GCT GCA GAT CAT CGA TTG AAA CAC GTT GTC CAT
po_D145QF	CTC GCC ATC GCA GTC **CAA** CGC TAC ATC ACC ATC
po_D145QR	GAT GGT GAT GTA GCG TTG GAC TGC GAT GGC GAG
po_R146QF	GCC ATC GCA GT**T** GA**T** **CAG** TAC ATC ACC ATC TTC
po_R146QR	GAA GAT GGT GAT GTA CTG ATC AAC TGC GAT GGC
po_T174AF	ATC AGC AGC ATC TGG **GCA** TGC TGC ATC GTG TCG
po_T174AR	CGA CAC GAT GCA GCA TGC CCA GAT GCT GCT GAT
po_L249YF	ATC ACC CTC ACC ATC **TAC** CT**A** GGG GTG TTC GTG
po_L249YR	CAC GAA CAC CCC TAG GTA GAT GGT GAG GGT GAT
PgfMC4RF	GCA AGA ATT CAT GAA CGC TAC AGA ACA TCC TGG ACT G
PgfMC4RR	GGA GAC GGT GGT CGA CAC ACA CAG CAG AGC GT(G/A)TGA

Table 2. List of oligonucleotide sequences used for site-direct mutagenesis. Each mutant has sense and antisense primer located in the region. Underline indicated the restriction endonuclease site as well as target region. Bold letter indicates the mutation site in each of sense oligonucleotide.

Ⅲ. Results and Discussion

Amplification of the genomic DNA was first carried out with genomic DNA as a template. Genomic DNA was isolated from the whole blood of olive flounder. Agarose gel electrophoresis was carried out to examine the integrity of the isolated DNA. High molecular weight of the genomic DNA (Figure 2) indicated the integrity of the genomic DNA.

For the amplification of MC4R gene, oligonucleotides corresponding to the conserved sequences of known MC4R gene were designed (Figure 3).

Cloning of the PCR products followed by its sequence analysis indicated that partial MC4R products (Figure 4), PgMC3 and PgMC4, were obtained by PCR amplification by using genomic DNA as template and degenerate primers (MC4RF3 and MC4RR4) derived from the conserved regions of MC4R of other fish. Both fragments PgMC3 and PgMC4 were identical in terms of 454 bp in size exhibiting high similarity with other MC4R gene as shown by search using BlastN and BlastX. In order to obtaing sequence information for the full length of the gene, DNA walking PCR was performed with target specific sense or antisense primers (Table 1) based on the sequence information of PgMC3 and PgMC4. Upon rounds of amplification of using the system, several PCR products were obtained. DNA sequence analysis of the products indicated that 715 bp and 634 bp fragments corresponding to

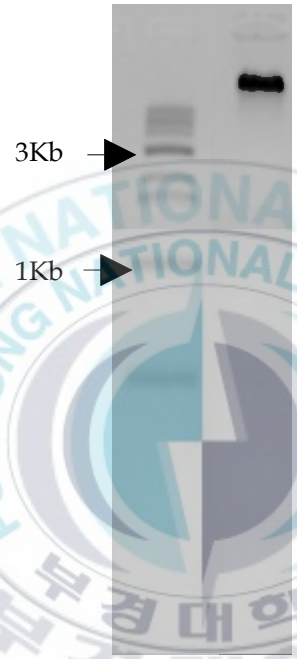

Figure 2. Genomic DNA isolated from whole blood of olive flounder *(P. olivaceus)* was subjected to 1% agarose gel electrophoresis. The right lane includes genomic DNA snd the left lane includes KB ladder (Bioneer, Korea).

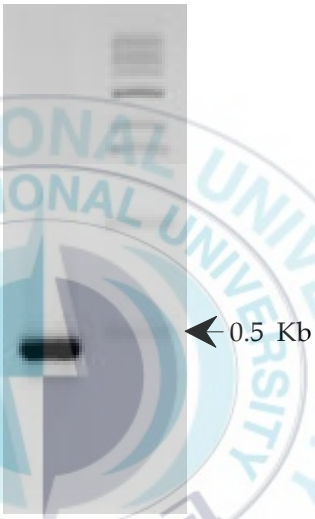

Figure 3. PCR product based on genomic DNA and primers corresponding to the conserved sequences of known fish MC4R genes. The left lane includes PCR product and the right lane includes KB ladder (Bioneer, Korea).

Figure 4. Predictied MC4R partial sequence from olive flounder *(P. olivaceus)*. Square boxes indicated primers used for PCR amplification of MC4R gene. Six double lines indicated the position and sequence of primers used for DNA walking PCR.

the 5'-end and 3'-end, respectively, regions of the gene were obtained (Figure 5). DNA sequence of the full length MC4R gene together with the flanking region sequences were confirmed from the analysis.

DNA sequence information based on the analysis of the partial gene products amplified by PCR and DNA walking experiments were compiled to obtain full length MC4R gene. It was obtained 978 bp, 325 amino acid residues MC4R full-length sequences from olive flounder (Figure 6). It also showed that highest similarity of MC4R amino acid sequence alignment is 96% with barfin flounder *(V. moseri)*, followed fugu (89%), zebrafish (80%), human (67%) using Clustal W program (Thompson *et. al.,* 1994). Amino acid sequences was compared to those of other species MC4R genes (Figure 7). It also showed the presence of the predicted seven transmembrane regions, one of the characteristics of GPCRs. The percentage of identity between other MC4R genes was shown in Table 3. Comparison of PCR products obtained from the amplification of the genomic DNA and cDNA from brain as template were shown in Figure 8. MC4R gene of olive flounder also showed other characteristics of GPCRs including putative coding region lacks introns and ERY/DRY motif at the end of the TM3 in all MC4R genes. Putative N-glycosylation site were also existed at Asn^{2}, Asn^{15}, Asn^{95} and Asn^{109}. Alignment of MC4R genes also indicates the presence of characterized PMY motif in ICL 1 (Ringholm *et. al.*, 2002; Cerdá-Reverter *et. al.*, 2005) and short extracellular and intracellular loops. In general the regions corresponding to the seven-transmenbrane

<A>

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GAG TTT AGG TCC AGC GTC CGT GGG GGG GGA CGT TCT GCT TCG CTC TGC 48
TCC GTT CGT CAG GAC TCT TCA GAG AAC GTT CTC ACT TCG CAG TCT GTG 96
AGA GCG GAG AGG AGA GCG GGG GGG GGG CGA GGA CGG ATC ATC CTT GGC 144
TGA GAG AAA CAC CAG CGG CAG GTG TTT GGA GGA AAT AGT TTC TCT GCA 192
TGG GAA GAA GAA AAA GAA AAG ATT CTT TCT TTC TCT CTG AGG ACA TGT 240
TGA CGT CCT CAC AAT GAG GAC GAT TGA CAG CCG TAC TCA CTG AGA CCA 288
GAT GGA AAA CTA AGG ACA GAC AAC AGG AGA CAT TAT GAA CGC TAC AGA 336
ACA TCC TGG ACT GAT CCA AGG CTT CCA CAA CCG GAG CCA GAC CAC GCC 384
GTC ACC GAA CGA GGA CTT TTC CGC CCA GGA CAA GGA CTC GTC AGC AGG 432
ATG CTA CGA GCA GCT GCT GAT CTC CAC CGA GGT CTT CCT CAC CCT GGG 480
CAT CGT CAG CCT GCT GGA GAA CAT CCT GGT GGT CGC TGC CAT AAT CAA 528
GAA CAA GAA CCT TCA CTC GCC GAT GTA CTT CTT CAT CTG CAG CCT TGC 576
CGT TGC TGA CAT GCT CGT CAG CGT CTC CAA CGC CTC TGA GAC TAT CGT 624
CAT CGC GCT CAT CAA CGG AGG AAA CCT GAC CAT CCC CGT CAC GTT GAT 672
TAA AAG CAT GGA CAA CGT GTT TGA CTC TAT GAT CTG CAG CTC C       715
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<B>

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GGT GTT CGT GGT GTG CTG GGC GCC GTT TTT CCT CCA CCT CAT CCT CAT 48
GAT CAC GTG CCC GAG GAA CCC GTA CTG CAC CTG CTT CAT GTC CCA CTT 96
CAA CAT GTA CCT CAT CCT CAT CAT GTG CAA CTC CGT CAT CGA CCC CAT 144
CAT CTA CGC CTT CCG CAG CCA GGA AAT GAG GAA AAC CTT CAA GGA GAT 192
TTT CTG CTG CTC AGA CGC TCT GCT GTG TGT GTG AGA CGT CAG CAG AGC 240
GAC GGC CTT GAC GGC AAC GAT CTG CAG TTT AAA TGC CGG TCG GCC AAC 288
GAG GAC TTT GAT GTT TCG AGC TGC TCT CAC AGT CGC TGT AAA TGA TTG 336
ATG ATT GCA GCG TTT GCC TGT GGC GCC GTG AGT CAG GGT TCC GAG GGG 384
GGA AGG TTT ACA AAC AGC TTC TGA CCT GGA GCG TCG CTC TGC AGG CGA 432
ACG CTG CGA CTG CTC TGC TGC TCT CAA GTG CAA TTT GAA GTA AAT GTG 480
ACC AAC ATG TGT TCA AAC CAT CAA ACA AAC TGT TGT TTC ATG ACG TCA 528
TCA TTC ATC CGA CTG ATT CTT TCT ACT TTC ACG TGT TCA AAT CTA TCG 576
CGG TCA CCG TCC CCC CAC GGA CGC TGG ACC TAA ACT CAC GGA CGC TGG 624
ACC TAA ACT C                                                   634
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Figure 5. Sequences of MC4R gene corresponding to the 5'-end and 3'-end region by DNA walking PCR. <A> shows 715 bp fragment corresponding to the 5'-end region and a gray box indicates the start codon. <B> shows 634 bp fragment corresponding to the 3'-end region. The gray box indicates the stop codon.

5‘-CGAGGACGGATCATCCTTGGCTGAGAGAAACACCAGCGGCAGGTGTTTGGAGGAAATAGTTT
CTCTGCATGGGAAGAAGAAAAAGAAAAGATTCTTTCTTTCTCTCTGAGGACATGTTGACGTCCT
CACAATGAGGACGATTGACAGCCGTACTCACTGAGACCAGATGGAAAACTAAGGACAGACAACA
GGAGACATT

ATGAACGCTACAGAACATCCTGGACTGATCCAAGGCTTCCACAACCGGAGCCAGACCACG 60
M N A T E H P G L I Q G F H N R S Q T T 20
CCGTCACCGAACGAGGACTTTTCCGCCCAGGACAAGGACTCGTCAGCAGGATGCTACGAG 120
P S P N E D F S A Q D K D S S A G C Y E 40
CAGCTGCTGATCTCCACCGAGGTCTTCCTCACCCTGGGCATCGTCAGCCTGCTGGAGAAC 180
Q L L I S T E V F L T L G I V S L L E N 60
ATCCTGGTGGTCGCTGCCATAATCAAGAACAAGAACCTTCACTCGCCGATGTACTTCTTC 240
I L V V A A I I K N K N L H S P M Y F F 80
ATCTGCAGCCTTGCCGTTGCTGACATGCTCGTCAGCGTCTCCAACGCCTCTGAGACTATC 300
I C S L A V A D M L V S V S N A S E T I 100
GTCATCGCGCTCATCAACGGAGGAAACCTGACCATCCCCGTCACGTTGATTAAAAGCATG 360
V I A L I N G G N L T I P V T L I K S M 120
GACAACGTGTTTGACTCTATGATCTGCAGCTCCCTGCTGGCGTCCATCTGCAGCCTGCTC 420
D N V F D S M I C S S L L A S I C S L L 140
GCCATCGCAGTCGACCGCTACATCACCATCTTCTACGCGCTGCGATACCACAACATCGTC 480
A I A V D R Y I T I F Y A L R Y H N I V 160
ACGCTGCGGCGGGCGATGCTGGTCATCAGCAGCATCTGGACGTGCTGCATCGTGTCGGGC 540
T L R R A M L V I S S I W T C C I V S G 180
ATCCTCTTCATCATCTACTCGGAGAGCACCACGGTGCTCATCTGCCTCATCACCATGTTC 600
I L F I I Y S E S T T V L I C L I T M F 200
TTCACCATGCTGGTGCTCATGGTGTCGCTGTACGTGCACATGTTCCTGCTGGCGCGCTTG 660
F T M L V L M V S L Y V H M F L L A R L 220
CACATGAAGCGCATCGCGGCGCTGCCGGGCAACGCGCCCATCCAACAGCGAGCCAACATG 720
H M K R I A A L P G N A P I Q Q R A N M 240
AAGGGCGCCATCACCCTCACCATCCTCCTCGGGGTGTTCGTGGTGTGCTGGGCGCCGTTT 780
K G A I T L T I L L G V F V V C W A P F 260
TTCCTCCACCTCATCCTCATGATCACGTGCCCGAGGAACCCGTACTGCACCTGCTTCATG 840
F L H L I L M I T C P R N P Y C T C F M 280
TCCCACTTCAACATGTACCTCATCCTCATCATGTGCAACTCCGTCATCGACCCCATCATC 900
S H F N M Y L I L I M C N S V I D P I I 300
TACGCCTTCCGCAGCCAGGAAATGAGGAAAACCTTCAAGGAGATTTTCTGCTGCTCAAAC 960
Y A F R S Q E M R K T F K E I F C C S N 320
GCTCTGCTGTGTGTGTGA 978
A L L C V * 325

(continue to next page)

GACGTCAGCAGAGCGACGGCCTTGACGGCAACGATCTGCAGTTTAAATGCCGGTCGGCCAACGA
GGACTTTGATGTTTCGAGCTGCTCTCACAGTCGCTGTAAATGATTGATGATTGCAGCGTTTGCC
TGTGGCGCCGTGAGTCAGGGTTCCGAGGGGGGAAGGTTTACAAACAGCTTCTGACCTGGAGCGT
CGCTCTGCAGGCGAACGCTGCGACTGCTCTGCTGCTCTCAAGTGCAATTTGAAGTAAATGTGAC
CAACATGTGTTCAAACCATCAAACAAACTGTTGTTTCATGACGTCATCATTCATCCGACTGATT
CTTTCTACTTTCACGTGTTCAAAT-3‘

Figure 6. The complete open reading frame sequence of MC4R gene of olive flounder (*Paralichtyhys olivaceus*). The bold letters indicates amino acid sequences and the numbers of sequences are shown to the right.

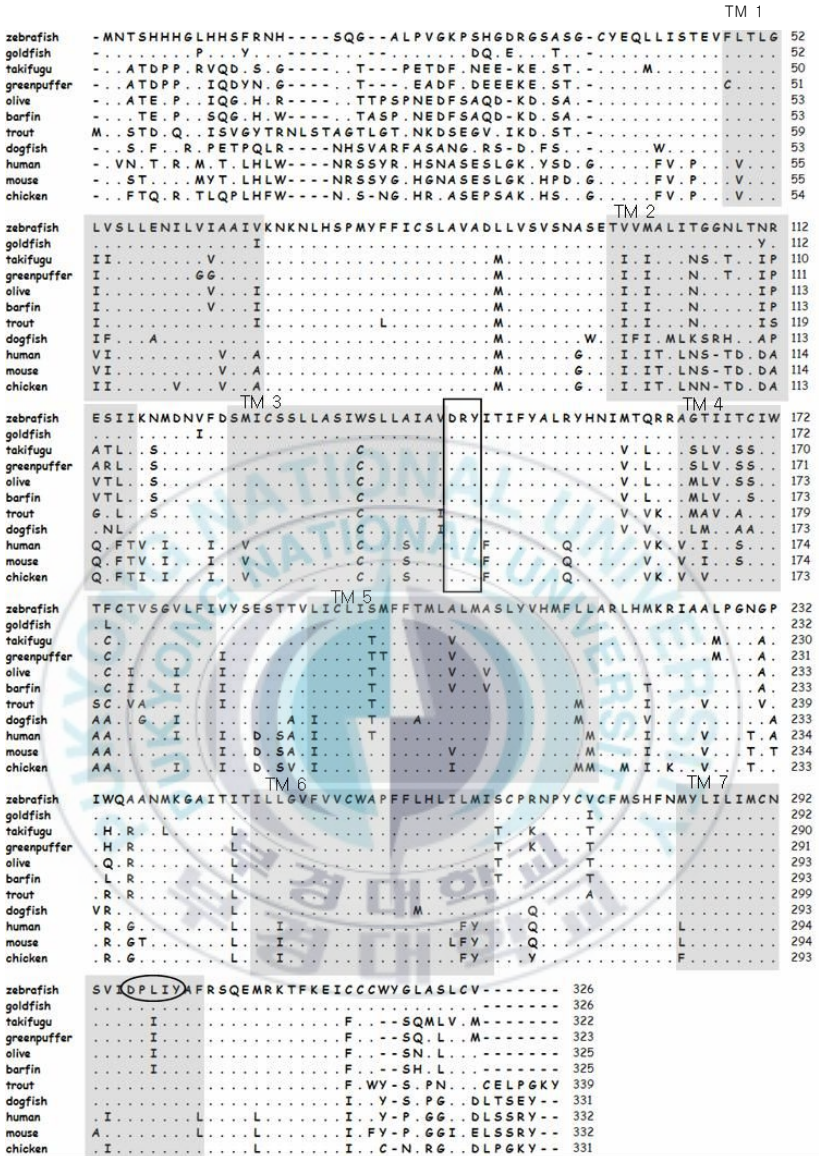

Figure 7. Alignment of MC4Rs from olive flounder together with zebrafish, goldfish, fugufish, greenpufferfish, barfin flounder, rainbow trout, spiny dogfish, human, mouse, chicken. Conserved sequences indicate as dots. Regions corresponding to the seven transmembranes are indicated by shadow boxes. DRY motif is indicated as square box and DPXXY motif is indicated as oval shape.

	hMC	mMC	chMC	dMC	zMC	tMC	fMC	pMC	gMC	oMC	bMC
hMC	100	93	87	70	69	68	66	65	70	67	68
mMC		100	85	67	70	69	65	66	69	68	67
chMC			100	67	68	71	65	65	67	68	68
dMC				100	77	74	73	72	73	74	73
zMC					100	78	76	78	96	80	80
tMC						100	78	79	78	82	80
fMC							100	94	76	89	88
pMC								100	77	89	88
gMC									100	78	79
oMC										100	96
bMC											100

Table 3. Percentage of identity of the MC4R amino acid sequences between different species.

Figure 8. Comparison the MC4R PCR product between genomic DNA and synthesized cDNA. Middle lane contains 1 kb molecular weight marker (Bioneer, Korea) together with PCR product obtained from the genomic DNA (left) and cDNA (right) as templates.

regions are more conserved as compared to that of the N- and C-terminals showing more variability. For DPXXY motif present at the end of 7TM region as common feature of MC4R, most of MC4R genes have DPLIY motif. However, olive flounder, barfin flounder, fugu and green pufferfish showing a higher homology than others, have DPIIY motif. This was also confirmed in phylogenetic analysis (Figure 9) performed to examine the evolutionary relationships of MCRs using the neighbor-joining method. Moreover, olive flounder MC4R was revealed fifteen cystein residues in all ORF region corresponding to the number and residue locations of barfin flounder and fugu MC4R (Kobayashi *et. al.*, 2007). Human MC4R also has similar pattern that is composed of 15 cysteine residues on the similar locations except the Cys^{172} (172 residue of barfin flounder, fugu and olive flounder is serine and they were located Cys^{175} instead of 172 residue). It is possible that these cystein residues may exert important role in MC4Rs function such as crucial role for the pharmacology of MC5R by the presence of a disulphide bridge between the EC loops (Schiöth, 2001).

Tissue distribution of olive flounder MC4R gene was examined by reverse transcription PCR as shown in Figure 10. For the internal positive control of RT-PCR, primers corresponding to the β-actin gene were included (Fig. 10B). MC4R gene was detected in liver, eye and brain. Interestingly, expression of MC4R gene in the liver was just only reported from barfin flounder in which showed a higher expression in fasted fish than in fed fish (Kobayashi *et. al.*, 2007). Both olive flounder and barfin flounder belonging to the order of Pleuronectiformes showed

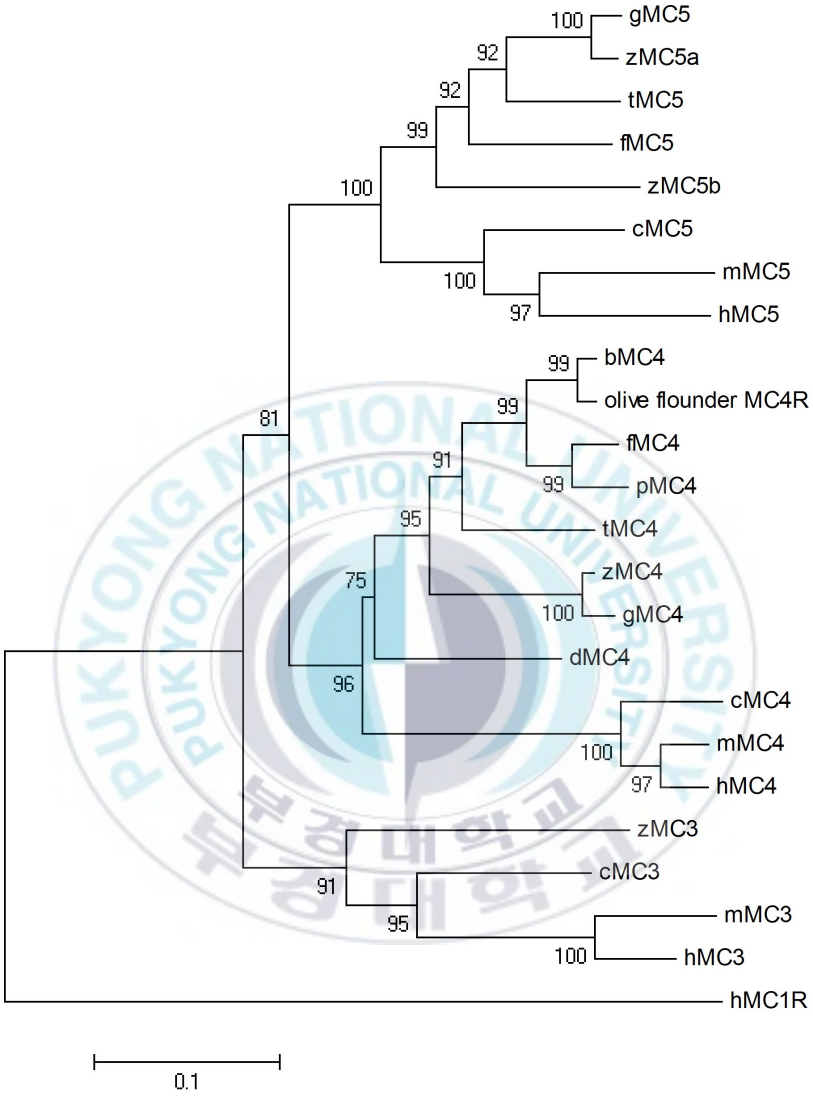

Figure 9. Phylogenetic tree for the MC receptors using comparison of the full-length amino acid sequences. zMC: zebrafish MCR, hMC: human MCR, mMC: mouse MCR, fMC: fugu MCR, pMC: pufferfish MCR, gMC: goldfish MCR, tMC: rainbow trout MCR, dMC: spiny dogfish MCR, cMC: chicken MCR, bMC4: barfin flounder MC4R.

abundant expression in the liver. It is possible that reducing the feeding causes increase the expression of the MC4R in the liver because the MC system is associated with liver functions (Yada *et. al.*, 2000; Kobayashi *et. al.*, 2007). Expression of MC4R gene from the olive flounder is revealed in liver, eye and brain but that not in the kidney, heart, intestine, stomach, gill and spleen. In the mammalian MC4R is only found in the brain (Adan *et. al.*, 2006) although others reported expression of MC4R gene in peripheral tissues in rainbow trout (Haitina *et. al.*, 2004) and in the head kidney in fugu (Klovins *et. al.*, 2004). It was also reported the expression of the gene in the gill, ovary and spleen of goldfish (Cerdá-Reverter *et. al.*, 2003a) as well as peripheral tissue wide expression in zebrafish and chicken (Ringholm *et. al.*, 2002; Takeuchi *et. al.*, 1998; Teshigawara *et. al.*, 2001). This suggests that the expression pattern of the MC4R gene in fish is hint of wider role of receptor during early vertebrate evolution. In other words, it seems that MC4R role in more specific central nervous system has undergone evolutional development.

In order to amplify the coding region sequence of the MC4R gene, oligonucleotides PgfMC4RF and PgfMC4RR containing DNA sequences corresponding to the 5'- and 3'-end of the gene together with the restriction endonuclease recognition sequences were designed to facilitate the cloning into the expression vector. PCR amplification of the genomic DNA using these primers resulted in the amplification of 1 Kb products. This, together with expression in animal cells followed by enzyme-immunoassay of cAMP accumulation will be used for the

screening of anti-obesity compound.

Figure 10. Analysis of MC4R gene expression in various tissues of olive flounder (P. *olivaceus*) by using RT-PCR as described in Methods. <A> Expression of MC4R gene. <B> β-actin PCR (internal positive control) i; intestine, l; liver, s; spleen, g; gill, k; kidney, e; eye, h; heart, st; stomach, b; brain, (+); positive control, olive flounder genomic DNA, (-); negative control, water. Molecular weight marker: DNA 123 bp ladder (Sigma, USA)

Abstract (Korean)

넙치 *Paralichthys olivaceus* 로부터 melanocortin 4 receptor 유전자의 클로닝

이 혜 정

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5개의 subtype을 가진 melanocortin receptor의 하나인 Melanocortin 4 receptor (MC4R)는 비만의 가장 큰 단일유전적 원인 물질로 알려져 있으며 MSH 펩티드가 수용체에 결합함으로써 음식섭취를 감소시키는 역할을 한다. Melanocortin 4 receptor는 G-protein coupled receptor (GPCR)의 family 중의 하나인 *rhodopsin* family에 속한다. GPCR은 neurotransmitters 와 hormones, 다양한 생리학적 신호 등을 인식하는 등 다양한 기능을 수행하는 세포막 단백질 중에서 가장 큰 수용체 집단이다. 양식 대상종인 넙치 (*Paralichthys olivaceus*)의 melanocortin 4 receptor 유전자의 분자적 기작을 연구하기 위하여, 넙치의 genomic DNA와 합성한 cDNA로부터 PCR 증폭을 통하여 전체 MC4R 유전자를 획득하였다. 두 방법으로 획득한 PCR product의 염기서열 비교를 통하여 넙치 MC4R 유전자는 intron이 존재하지 않음을 확인 하였으며, MC4R 유전자의 sequence 분석으로 978 bp, 325 아미노산을 확인하였다. 이미 알려진 다른 어류의 MC4R 염기서열과 비교분석한 결과 노랑가자미 (*Verasper moseri*)와 넙치가 가장 높은 상동성을 나타내었고, 자주복 (*Takifugu rubripes*)과 초록복어 (*Tetraodon nigroviridis*)가 89% 상동성으로 그 뒤를 이었다. 이러한 결과는 Neighbor-joining 방법을 이용한 계통도 분석에서도 일치한다고 나타났다. 넙치 MC4R의 조직특이적 발현분석에서 MC4R은 간과 눈, 그리고 뇌에서 발현되는 것을 확인하였다.

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V. 감사의 글

그저 막연한 관심만 있었을 뿐, 아무것도 모른 채 하나하나 시작했던 일들이 비록 미숙하나마 작은 결실을 맺게 되었습니다. 어디서부터 시작해야 하는지 모른 채 허우적거릴 때에도, 스스로의 한계에 부딪히며 좌절할 때에도 항상 옆에서 지켜봐주시며 끊임없는 관심을 베풀어주시고 학문적 기틀을 잡아주신 김종명 지도 교수님께 가장 큰 감사를 드립니다.

학부생활 4년과 대학원생활 2년 동안 다양하고 많은 가르침을 주신 손철현 교수님, 허성범 교수님, 장영진 교수님, 김동수 교수님, 조재윤 교수님, 배승철 교수님, 김창훈 교수님 그리고 남윤권 교수님께도 깊은 감사를 드립니다.

또한 하루의 절반이상을 함께 지내며 동고동락해 온 성완선배를 비롯한 실험실 식구들에게도 고마운 마음을 전합니다.

무엇보다 소중한 내 삶의 오아시스, 친구들- 서울로 보령으로 멀리 떨어져 버린 세경이, 서영이, 우리 수학천재 나리, 자상하고 다정하고 멋진 민진이, 미영이, 미래를 향해 열심히 공부중인 아름이, 하나, 정은이, 동해처녀가 된 은선이, 힘든 결정을 내리고 이제 새로이 시작하는 아방이, 일본에서 멋진 대학원 생활을 즐기고 있을 미영이, 지금은 미국에서 온몸으로 부딪혀 가며 미래를 만들어 가고 있는 다린이, 1인 3역 이상을 소화해 내는, 항상 마음으로부터 걱정하는 현진이. 대학원생활 내내 옆에서 힘이 되어 준 상엽이. 니네들이 없었으면 어떻게 살았을까 싶다.

임용고사 준비로 얼굴이 반쪽이 되어버린 사랑하는 내 동생 준형이, 3월부터 시작하는 군 생활 별탈없이 무사히 보내길 바란다.

마지막으로, 행여나 힘들까 항상 노심초사하시는, 떨어져 있어도 그 크나큰 마음의 목소리가 들리는 부모님께 사랑과 존경을 드리며 이 작은 결실의 기쁨을 함께 하고 싶습니다.

6년 동안 배운 많은 가르침들을 감사한 마음으로 간직하고, 앞으로 제가 어디서 무엇을 하든 소중한 밑거름으로 삼아 더 크게 성장하도록 노력하겠습니다.