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Identification of Differentially Expressed Genes in Improved Rainbow Trout Growth by Treatment with a Fish Myostatin Prodomain Using the Annealing Control Primer System

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ABSTRACT **The present study was conducted to investigate different gene expression profile between treated poMSTNpro and non-treated in rainbow trout and to identify those genes that are specifically or predominantly expressed in treated poMSTNpro by employing annealing control primer (ACP)-based GeneFishing polymerase chain reaction (PCR). We isolated total RNAs in muscle tissues from the treated poMSTNpro fish by immersion bath technique with fish myostatin prodomain (*Paralichthys olivaceus*, poMSTNpro) for one month and the other was non-treated poMSTNpro, and synthesized cDNA using annealing control primers (ACP, Seegene, Korea). Using 20 different ACPs for PCR, were cloned sequenced, and analyzed identities of 2 differentially expressed genes (DEGs). According to BLAST analysis, sequences of 2 clones significantly matched database entries and confirmed by semi-quantitative RT-PCR. The functional roles of one up-regulated gene, cytochrome P450 mono-oxygenases 2K1v2 (CYP2K1v2), and one down-regulated gene was Profilin-1 were identified. We identified distinctive gene expression profiles in improved rainbow trout growth by treatment with a fish myostatin prodomain using ACP-based GeneFishing.**

Key words : Myostatin, myostatin prodomain, Annealing Control Primer (ACP), cytochrome P450 mono-oxygenase, profilin, *Oncorhynchus mykiss*

INTRODUCTION

Myostatin (MSTN) belongs to the TGF-β superfamily and acts as a negative regulator of skeletal muscle development and growth. It is primarily expressed in skeletal muscles in mice (McPherron *et al.*, 1997). MSTN-mutations of cattle and mice result in an increase of skeletal muscle mass with both hyperplasia and hypertrophy (Kambadur *et al.*, 1997; McPherron *et al.*, 1997). In reviewed by Bradley *et al.*, 2008, several studies have reported the regulation methods of the myostatin activity to provide possible therapeutic candidates for human's muscle diseases such as muscular dystrophy and motor neuron disease, and to promote muscle growth for mammalian and marine organisms; Anti-myostatin monoclonal antibodies such as RK35 and JA16, myostatin prodomain, Follistatin, histone deacetylase (HDAC) inhibitors, myostatin peptide and soluble activin IIB receptor. Among them, myostatin prodomain has bound myostatin preventing the release of mature myostatin, and then increased body weight and muscle mass caused by hypertrophy and/or hyperplasia (Bogdanovich *et al.*, 2005). There have been several studies about the functions of myostatin prodomain in mammalian and fish models. In mammalian, overexpression of prodomain in transgenic mice (Lee and McPherron, 2001; Yang *et al.*, 2001; Pirottin *et al.*, 2005) and delivery of myostatin prodomain by an adeno-associated virus vector system in dystrophic mice (Bartoli *et al.*, 2007; Qiao *et al.*, 2008) and dogs (Qiao *et al.*, 2009) were increased in skeletal muscle mass up to two-fold. In fish, transgenic zebrafish overexpressing MSTN prodomain was observed significantly increase in fiber number (Xu *et al.*, 2003) and MSTN prodomain of the marine fish *Sparus aurata* suppressed myostatin activity *in vitro* (Rebhan and Funkenstein, 2008). Recently Lee *et al.* (2010) first reported to the production and expression of soluble fish (*Paralichthys olivaceus*) myo-

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statin prodomain (poMSTNpro) in *E. coli* system to improve muscle growth through blocking mature myostatin. In an *in vitro* gene reporter assay system, the anti-myostatin activity of the soluble myostatin prodomain is similar to one of commercial mouse myostatin prodomain. *In vivo* assay, rainbow trout (*Oncorhynchus mykiss*) has improved the growth in a dose-dependent manner with the maximum growth increase up to 42% by immersion bath method. Therefore, the prodomain of myostatin can be increased a muscle mass of organisms through blocking myostatin to bind it's receptor.

In general, the function, signal pathway and downstream target genes by myostatin like TGF-β superfamily members known as well-established compared to those of myostatin's prodomain. Yang *et al.* (2005) reported the responded genes were identified from myostatin-stimulated myoblasts to understand the pathway of myostatin. However, myostatin prodomain only has been known as a function of binding the mature myostatin to blocking the signal transduction. Elucidation of the functional mechanisms of myostatin prodomain is very important to its application in medicine and animal breeding.

The aim of this study was to find differentially expressed genes in treated poMSTNpro and non-treated rainbow trouts for one month by Annealing Control Primer (ACP)-based RT-PCR.

MATERIALS AND METHODS

1. Rainbow trout sample

We examined two groups of the rainbow trouts; one group was the poMSTNpro treated fish by immersion bath technique with fish myostatin prodomain (*Paralichthys olivaceus*, poMSTNpro) for one month and the other was non-treated poMSTNpro. The muscle samples of 10 fishes at each group were immediately frozen in liquid nitrogen and then stored at −70°C before use. Rainbow trout was selected as our model fish because it was easily available at the time of our experiment.

2. Genefishing reverse transcription polymerase chain reaction

To identify poMSTNpro-related genes in rainbow trout, the mRNAs from the muscle of both samples, poMSTNpro and non-treated ones, were extracted and applied to ACP RT-PCR analysis using 20 arbitrary primers (from ACP1 to ACP20). Total RNA was isolated from the muscle of non- and poMSTNpro treated-rainbow trout with TRIzol Reagent (Invitorgen, USA) following the manufacturer's. The RNA quality and quantity were verified using spectrophotometry (Ultrosepc 3100pro, Amersham Biosciences). The RNAs were used for the first-strand cDNA synthesis by reverse transcription. Reverse transcription was carried using GeneFishing™ DEG kits (Seegene, Korea). Reverse transcription was performed for 90 min at 42°C in a final reaction volume of 20 μL containing 3 μg of total RNA, 4 μL of 5 x RT buffer (Invitorgen, USA), 5 μL of 2 mM dNTP, 2 μL of 10 μM dT-ACP1 (5′-CGT GAATGCTGCGACTACGATIIIIIT (18)-3′), 0.5 μL of RNase inhibitor (40 U/μL, Promega, USA) and 1 μL of MMLV reverse transcriptase (200 U/μL, Invitorgen). First-strand Cdnas were diluted with 80 μL of RNase-free water for the GeneFishing™ PCR and stored at −20°C until use.

The differentially expressed genes were screened by ACP (Annealing Control Primer)-based PCR method using the GeneFishing™ DEG kits (Seegene, Korea). The second-strand cDNA was synthesized at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 μL containing 3 μL (about 50 ng) of first-strand cDNA, 1 μL of 10 μM dT-ACP2 (5′-CGTGAATGCTG CGACTACGATIIIIIT (15)-3′), 2 μL of 5 μM arbitrary ACP and 10 μL 2 x master mix (Seegene). The PCR protocol for the second-strand synthesis was one cycle at 94°C for 5 min, followed by 50°C for 3 min and 72°C for 1 min. After the second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 sec, followed by 65°C for 40 sec, 72°C for 40 sec and final extension at 72°C for 5 min.

3. Cloning and sequencing

The PCR product bands showing differential expression on 1.5% agarose gel between poMSTNpro treated and non-treated rainbow trout samples were excised and purified by agarose gel extraction kit (Bioneer, Korea) and cloned into a pGEM-T easy vector (Promega) for sequencing. The DNA sequences were identified by BLAST search program at the National Center for Biotechnology Information (NCBI) GenBank.

4. Semi-quantitative reverse transcription polymerase chain reaction analysis

The differential expression of DEGs was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) using each gene specific primer pair. The primer sets and annealing temperatures of two genes are shown in Table 1. The cDNA was amplified using primers derived from the sequence of the DEGs and β-actin gene as a control reference. The PCR reaction was conducted in a final volume of 25 μL containing 3 μL (about 50 ng) of diluted first-strand cDNA, 2.5 μL of 10X reaction buffer, 1.5 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTP (10 μM), 0.5 μL of PCR primers (10 μM) and 0.2 μL of Taq DNA polymerase (5 unit). The PCR amplification protocol was an initial 3 min denaturation at 94°C, followed by 20∼25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for

30 s, and a 7 min final extension at 72°C. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel. The bands were photographed using Polaroid film under ultraviolet light after ethidium bromide staining. The optical density of each band was analysied by densitometry. The relative amount of mRNA was determined by calculating the ratio of the amount of each mRNA relative to the amount of β-actin.

RESULTS AND DISCUSSION

Previously works, we expressed the soluble fish myostatin prodomain (poMSTNpro) in *E. coli* system. The expressed poMSTNpro showed the activity of myostatin inhibition both *in vitro* and *in vivo* assays. Especially, *in vivo* test, the growth of the poMSTNpro-treated rainbow trout was improved in a dose-dependent manner with the maximum growth increased up to 42% for one month compared to non-treated one (Lee *et al.*, 2010). Fig. 1 showed two differentially expressed genes between two groups on the basis of the differential expression levels of the mRNA fragments observed on the agarose gel. The expression levels of one (cytochrome P450 mono-oxygenase2K1v2; CYP2K1v2) were found to be markedly up-regulated gene in poMSTNpro-treated rainbow trout, while the other band (profilin-1) was down-regulated.

We confirmed different expressions of two genes using sequence-specific primers designed to amplify products with lengths ranging from 100 to 200 bp (Table 1). Quantitative expression patterns of two differentially expressed genes that CYP2K1v2 was up-regulated and profilin-1 was down-regulated in poMSTNpro-treated rainbow trouts.

Understanding the molecular mechanisms of poMSTNpro requires investigation of differentially expressed genes. In this study, we used a ACP that specifically targets sequence hybridization to a template via a polydeoxyinosine linker using poMSTNpro-treated rainbow trout samples. The structure of ACP comprises a 3′ end region with a target core nucleotide sequence that substantially complements the template nucleic acid for hybridization, a 5′ end region with a non-target universal nucleotide sequence, and a polydeoxyinosine linker bridging the 3′end and 5′ end sequences. Using dT-ACP2 (reverse primer) and 20 arbitrary ACPs (forward primer) for PCR amplification, we were able to display two of differentially expressed genes. The genes were sequenced and screened by a BLAST search. For comparative analysis, their expression was quantified by semi-quantitative RT-PCR.

We found that Cytochrome P450 mono-oxygenases 2K1v2 (CYP2K1v2) was expressed significantly more highly in treated samples than in non-treated samples (Fig. 2). Cytochrome P450 mono-oxygenases (CYPs) catalyze oxidation of a wide range of drugs and other xenobiotics and are responsible for the metabolism of endogenous compounds, including steroids (breakdown including estrogen and testosterone synthesis and metabolism) (Thum and Borlak, 2000; Borlak and Thum,

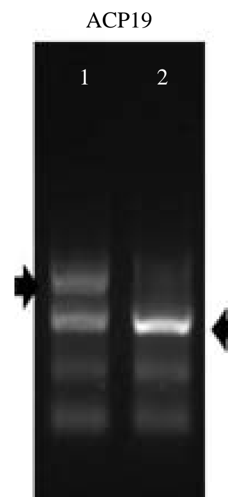

Fig. 1. Annealing control primer (ACP)-based polymerase chain reaction (PCR) for the identification of differentially expressed genes from treated poMSTNpro and non-treated in rainbow trout. mRNA from treated poMSTNpro and normal tissues was used for the synthesis of first strand cDNA using dT-ACP1. Using a combination of dT-ACP2 (reverse primer) and 20 arbitrary ACPs (forward primer), second-strand cDNA sequences were amplified during second-stage PCR, separated for differentially expressed genes on 1.5% standard agarose gels, and stained with ethidium bromide for visualisation. Bands were excised from the gel for further cloning and sequencing. 1, mRNA from non-treated muscle tissue; 2, treated poMSTNpro muscle tissue. Arrows indicate differential cDNA bands and ACP19 indicate the ACP number.

Table 1. Sequence of primers used in the cloning and RT-PCR

Gene	GeneBank number	Primer sequence (5′-3′)	Annealing temperature (°C)	PCR product size (bp)
Profilin-1 putative mRNA	NM001165157	F: GATCTGTGGTTCTGATGCGG R: AGGGGATCAGTAGCAGTGGG	55	234
cytochrome P450 monooxygenase CYP2K1v2	AF045052	F: TACAAGACGGTCAAGCAGGC R: TTGGAGACGGCATAAAGCAC	55	290

Findicates forward primer and R indicates reverse primer.

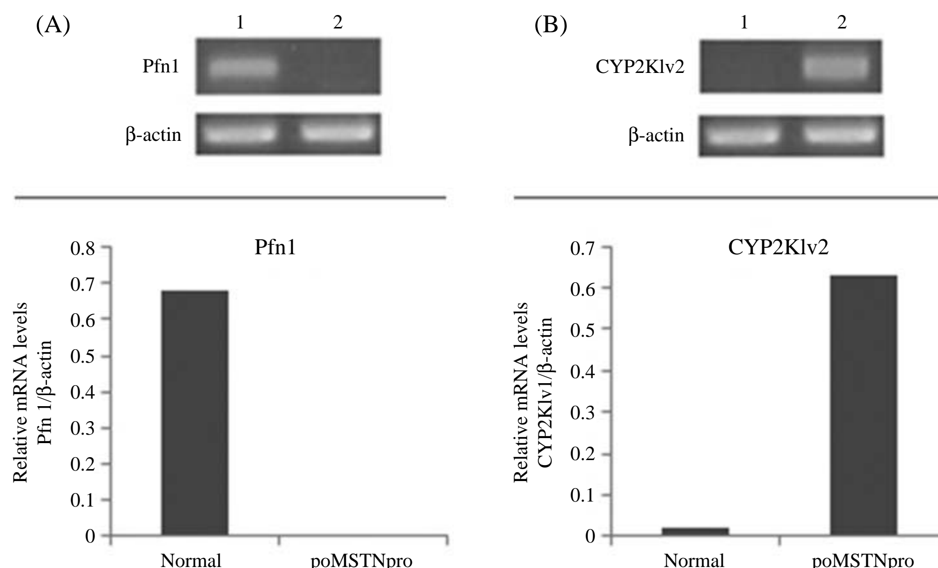

Fig. 2. Confirmation by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of the differential mRNA expression pattern of two genes that were identified by sequence-specific primers as being differentially expressed genes. RT-PCR and densitometric analysis. The comparison of the expression patterns of the two differentially expressed genes (1: normal and 2: treated-poMSTNpro) was determined by semi-quantitative RT-PCR. The amplified DNA products were separated on 1.5% agarose gels and stained with ethidium bromide. Quantitative analyses of expression patterns of bands were carried out. β-actin was used as a control to confirm the integrity of the mRNA samples and each band was scaled to the intensity of beta actin in the respective lane. (A) Profilin-1 (Pfn1) gene expression pattern. (B) Cytochrome P450 mono-oxygenase 2K1v2 (CYP2K1v2) gene expression pattern.

2001), cholesterol, bile, fatty acids (Simpson, 1997; Capdevila *et al.*, 2000), bradykinin (Fulton *et al.*, 1995), and certain vitamins (Omdahl, 2001). In mammals, proteins belonging to the first families (CYP1-CYP4) are highly associated with drug activation and metabolism. However, studies on CYP function and modulation in non-mammalian vertebrate systems are much less defined, and the function is often inferred from mammalian data, assuming similar function across vertebrate taxa. CYP families and subfamilies, with the exception of the CYP2 subfamilies, are nearly identical in vertebrate taxa and the total numbers of CYP genes in vertebrate species is similar (Thomas, 2007). The mammalian CYP2 family is regulated through a number of receptors, including the retinoic acid receptor (RAR), the constitutive androstane receptor (CAR) and hepatocyte nuclear factor 4 (Honkakoski and Negishi, 2000; Lewis *et al.* 2002). Similar induction pathways exist across vertebrate classes and with the exception of CAR, all of these nuclear receptors have been identified in fish (Moore *et al.,* 2002, 2003; Maglich *et al.,* 2003). Thum and Borlak (2002) show metabolism of testosterone to be increased in hypertrophic hearts and induction of CYP mono-oxygenases to be linked to enhanced production of certain testosterone metabolites. Androgens seem to have strong negative impact on myostatin expression, which might be a key factor in the weight regulation of LA muscle (Mendler, 2007). From the higher expression of CYP2K1v2 in treated samples in this study, it was suggested that CYP2K1v2 might play an important role in mediating the muscle growth of treated rainbow trout.

The level of profilin-1 (Pfn1) was expressed significantly down-regulated in treated samples (Fig. 2). Profilins (Pfns) belong to a class of small G-actin-binding proteins comprising of four members identified to date: Pfn1 (ubiquitously expressed in almost all cell types), Pfn2 (mainly expressed in nervous system in vertebrates), Pfn3 and 4 (expression restricted to kidney and testis). Besides binding to actin, Pfns also interact with a multitude of other ligands including various phosphoinositides and proteins containing proline-rich motifs that are involved in actin cytoskeletal regulation, endocytosis, and gene transcription. Pfn1, the founding member of the protein family, promotes actin polymerization in cells by virtue of its ability to (i) catalyze nucleotide exchange factor (ADP to ATP) on G-actin, (ii) shuttle G-actin to the barbed ends of actin filaments, and (iii) interact with almost all major protein families that are known to be involved in nucleation and/or elongation of actin filaments (Witke, 2004; Jockusch *et al.*, 2007). Cell-cycle progression is tightly regulated by coordinated activities of cyclin/cyclin-dependent kinase (CDK) complexes. The interactions of cyclins with their partner CDKs are negatively regulated by CDK inhibitors (CDKIs). Two families of CDKIs, namely the CIP/KIP ($p21^{Cip1/Waf1}$ (p21), $p27^{Kip1}$ (p27), and $p57^{Kip2}$ (p57)) and INK4 ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) cause cell-cycle arrest at GI phase (Sherr, 1994; Sherr and Roberts,

1995). Myostatin functions by binding to two different type of serine/threonine kinase receptors, which leads to the phosphorylation of Smad2 and Smad3 (Lee and McPherron, 2001; Rebbapragada *et al.*, 2003). Phosphorylated Smad2 and Smad3 form a complex with Smad4 that translocates into the nucleus, where it is involved in regulating the transcription of target genes (Shi and Massague, 2003). In vitro, an addition of MSTN to muscle cell culture inhibits cell proliferation by up-regulation of p21 and Cdk2, which control the cell cycle progression process (Thomas *et al.* 2000; Joulia *et al.* 2003). Profilin-1 overexpression inhibits proliferation of MDA-MB-231 breast cancer cells partly through $p27^{kip1}$ upregulation (Zou *et al.*, 2010). Decrease Profilin-1 might play a role in the muscle growth that was the hallmark of treated samples.

In conclusion, we found differential expression of two genes (CYP2K1v2 and Pfn1) in relation to drug, steroids metabolism and proliferation in treated poMSTNpro rainbow trouts using the GeneFishing™ PCR technique. Although the detailed function of these genes remain to be determined, they could be important and deserve further investigation, and their identification in this study provides preliminary data for further study of the molecular mechanism underlying treated poMSTNpro in rainbow trout.

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Annealing control primer system을 이용한 어류 재조합 myostatin prodomain 단백질에 의해 성장이 증가된 무지개송어의 특이적 발현 유전자 탐색

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요 약 : 이전 연구에서 넙치유래 재조합 마이오스타틴 프로도메인을 무지개송어에 한달간 침지법을 통하여 처리한 결과 대조군에 비하여 무게가 최대 약 42% 증가되었다. 따라서 본 연구는 재조합 마이오스타틴 프로도메인을 침지법에 의해 처리된 무지개송어와 대조군의 근육으로부터 발현되는 cDNA를 제작하여 마이오스타틴 프로도메인에 의해서 유도된 특정유전자를 선발하기 위하여 ACP (annealing control primer)를 이용한 DDRT법을 통하여 분석하였다. 총 20가지의 ACP를 이용한 결과 2개의 특정 유전자를 분석하였으며, NCBI BLAST분석결과 Cytochrome P450 mono oxygenase와 Profilin으로 판명되었다. 이 중 Cytochrome P450 mono oxygenase는 대조군보다 발현량이 증가하였으며, Profilin는 대조군에 비해서 발현량이 감소하였다. 이러한 결과를 재확인하기 위하여 두 유전자의 primer를 각각 제작하여 semi-quantitative RT-PCR를 시행한 결과 DDRT법에 의한 분석과 동일하였다. 본 결과는 어류의 성장에서 마이오스타틴 프로도메인의 기능 및 메카니즘에 대한 연구에 유용한 자료가 될 것으로 사료된다.

찾아보기 낱말 : 마이오스타틴, 마이오스타틴 프로도메인, Annealing control primer, cytochrome P450 mono-oxygenase, profilin, 무지개송어