Molecular characterization of peroxisome proliferator–activated receptors (PPARs) and their gene expression in the differentiating adipocytes of red sea bream *Pagrus major*

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Abstract

To investigate the molecular mechanism of fish adipocyte differentiation, the three subtypes of PPAR genes (α, β and γ) were characterized in a marine teleost red sea bream (*Pagrus major*). The primary structures of red sea bream PPARs exhibited high degrees of similarities to their mammalian counterparts, and their gene expression was detected in various tissues including adipose tissue, heart and hepatopancreas. During the differentiation of primary cultured red sea bream adipocytes, three PPARs showed distinct expression patterns: The α subtype showed a transient increase and the β gene expression tended to increase during adipocyte differentiation whereas the gene expression level of PPARγ did not change. These results suggest that they play distinct roles in adipocyte differentiation in red sea bream. In the differentiating red sea bream adipocytes, mammalian PPAR agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, ciglitazone and fenofibrate did not show clear effects on the adipogenic gene expression. However, 2-bromopalmitate increased the PPARγ and related adipogenic gene expression levels, suggesting the γ subtype plays a central role in red sea bream adipocyte differentiation and in addition, fatty acid metabolites can be used as modulators of adipocyte function. Thus our study highlighted the roles of PPARs in fish adipocyte differentiation and provided information on the molecular mechanisms of fish adipocyte development.

Key Words; adipocyte, cloning, differentiation, fish, gene, in vitro, PPAR, red sea bream

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors which regulate the various genes involved in lipid metabolism (Qi et al. 2000, Kliewer et al. 2001, Lee et al. 2003). PPARs belong to the nuclear receptor superfamily and structure analyses have revealed the presence of DNA and ligand binding regions in their protein sequences (Escriva et al. 1997, Chawla et al. 2001). Three subtypes of PPAR, termed α, β (δ) and γ, have been identified in mammals. The PPARα is predominantly expressed in the liver and mediates lipid catabolism such as the β-oxidation pathway (Kliewer et al. 2001, Lee et al. 2003). PPARβ is ubiquitously expressed in various tissues and is involved in brain and skin functions as well as lipid metabolism (Kliewer et al. 2001, Lee et al. 2003). PPARγ is highly expressed in adipose tissue and activates the adipogenic genes at a transcriptional level by binding to the promoter region directly (Auwerx et al. 1996, Schoonjans et al. 1996). The previous studies have reported that PPARs can be activated by subtype-specific ligands. For example, fibrates are known as specific ligands for α subtype (Kersten and Wahli 2000, Guo et al. 2001). Furthermore prostaglandins and glitazones activate PPARγ in a subtype specific manner (Kersten and Wahli 2000, Houseknecht et al. 2002).

PPARs are involved in adipocyte differentiation. As reported in many studies, PPARγ plays a central role in adipocyte differentiation. During adipocyte differentiation, PPARγ makes a complex with retinoid X receptor and regulates various adipogenic genes involved in lipid uptake, lipid synthesis and glucose utilization in a differentiation-dependent manner (Gregoire et al. 1998, Morrison and Farmer 1999). The retroviral expression of PPARγ in 3T3 fibroblasts stimulates adipocyte differentiation, indicating PPARγ is a critical factor for adipocyte differentiation

(Tontonoz et al. 1994). Moreover, fibrates initiate adipocyte differentiation, indicating that PPARα is involved in the early phase of adipocyte differentiation (Brandes et al. 1986, Pasquali et al. 2004). In preadipocytes, PPARβ is activated by fatty acid ligands such as 2-bromopalmitate (Amri et al. 1994, Bastie et al. 1999), and the activation of PPARβ by fatty acid ligand induces PPARγ gene expression, followed by the terminal differentiation of adipocytes (Bastie et al. 1999).

In fish, the PPARs have been identified and characterized in various species. The overall features including the primary structure, tissue distribution and ligand specificities of PPARs in fish are similar to those of their mammalian counterparts but differ in several respects (Andersen et al. 2000, Ibabe et al. 2002, Boukouvala et al. 2004, Batista-Pinto et al. 2005, Leaver et al. 2005). As reported in various fish species such as zebrafish, sea bass and brown trout, the tissue expression profiles of fish PPARs appeared to be very similar to those in mammals whereas in Atlantic salmon, plaice and gilthead sea bream, PPARγ genes are expressed more widely in fish tissues than in mammals (Andersen et al. 2000, Leaver et al. 2005). Furthermore, the cloning analyses have revealed that the ligand binding regions of fish PPARα and γ contain additional amino acid residues, e.g. extra 23 and 35 amino acids in gilthead sea bream and plaice PPARγ, respectively, in comparison with human PPARγ (Andersen et al. 2000, Leaver et al. 2005, Kondo et al. 2007), suggesting that ligand binding properties in fish PPARs may differ from that in mammals. Furthermore, several studies have reported the ligand specificities of fish PPARs. In primary cultured zebrafish hepatocytes, mammalian PPAR agonists such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (0.3 μM) induce the PPARα and γ protein expression (Ibabe et al. 2005). In Atlantic salmon, fibrates (0.5 mM) stimulate the PPARγ and the related enzyme expression in hepatocytes (Ruyter et al.

1997). Torafugu pufferfish PPARα are activated by mammalian PPARα specific ligand Wy14643 (10 μM), ETYA (5,8,11,14-eicosatetraynoic acid, 1μM) and polyunsaturated fatty acids such as DHA and EPA (50 μM) while they do not affect β and γ subtype activities (Kondo et al. 2007). Cellular transfection assays have revealed that Atlantic salmon PPARβ is activated by monounsaturated fatty acid (100 μM), 2-bromopalmitate (50 μM) and mammalian PPARβ specific ligand GW501516 (10 μM) (Leaver et al. 2007). Furthermore, in plaice and gilthead sea bream, the PPARγ is not activated by mammalian PPARγ specific ligand while PPARα and β exhibit an activation profile similar to that of mammalian PPARs (Leaver et al. 2005).

Thus, recent publications have provided various kinds of information on fish PPARs. However, only limited information is available about the roles of PPARs in fish adipocytes differentiation. In this study, to investigate the roles of PPARs in fish adipocyte differentiation, three subtypes of PPAR genes (α, β, γ) were characterized in a marine teleost red sea bream (*Pagrus major*). Firstly, from red sea bream adipose tissue, cDNA of PPARα, β and γ genes were cloned and their primary structures and tissue distribution were determined. Subsequently, the changes in their expression level during adipocyte differentiation and the effects of mammalian PPAR agonists on differentiating adipocytes were examined *in vitro* by using primary culture system of adipose stromal-vascular cells developed in this species (Oku et al. 2006a). The results showed the distinct characteristics of PPAR subtypes in fish differentiating adipocytes.

2. Materials and Methods

2.1 Experimental fish

For the cloning and RT-PCR experiments, fish (*P. major*) were purchased from a local

121 hatcheries station (Nissin Marine Tech, Aichi, Japan) and reared in our institute (Natl.
122 Res. Inst. Aquaculture, Minami-ise, Mie, Japan) by feeding commercial diet
123 (Higashimaru Co. Ltd, Kagoshima, Japan). The body weight of fish used for the cloning
124 and RT-PCR experiments was approx. 100g.
125 For cell culture experiments, fish weighing 800-1200g were purchased from a local
126 dealer (Nansei Suisan, Minami-Ise, Mie, Japan) and maintained in our institute (Natl.
127 Res. Inst. Aquaculture, Minami-ise, Mie, Japan).
128
129 2.2 Partial cloning of PPAR genes
130 The partial cloning of PPAR genes were carried out as described previously (Oku et al.
131 2002, Oku et al. 2006b). In brief, the total RNA was extracted from visceral adipose
132 tissue of red sea bream by the method of Chomczynski and Sacchi (1987) and the 1 μg
133 of total RNA was used for cDNA synthesis. The oligo-dT primed single strand cDNA
134 was synthesized in 15μL reaction volume with First-Strand cDNA synthesis kit (GE
135 Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.
136 The 0.5μL of synthesized cDNA was subjected to the polymerase chain reaction (PCR).
137 The partial fragments of PPAR genes were amplified with three sets of degenerated
138 primers. The oligonucleotide sequences used for the degenerated PCR are shown in
139 Table 1. The PCR was carried out in 25μL of reaction volume containing 0.5U Taq
140 polymerase (Takara, Tokyo, Japan), 200μM of dNTP, 10mM Tris-HCl pH8.3, 50mM
141 KCl, 1.5mM $MgCl_2$ and 20pmol of oligonucleotide primers. Forty cycles of
142 amplification were carried out for each PCR. Each cycles consisted of a denaturation
143 step at 94°C for 0.5min, an annealing step at 50°C for 1min and an extension step at
144 72°C for 1min. The final extension step was followed by a 3min extension reaction at

145 72°C. The amplified fragments were cloned into plasmid vector (pCR2.1, Invitrogen,
146 Carlsbad, CA, US) and sequenced. The cloning and sequence analyses were carried out
147 as described previously (Oku et al. 2002).
148
149 2.3 3' and 5' RACE
150 The complete nucleotide sequences were determined by the 3' and 5' RACE method.
151 The total RNA was extracted as described above. The cDNA template for 3' and 5'
152 RACE was synthesized with SMART RACE cDNA Amplification kit (Clonetech, Palo
153 Alto, CA, US) according to the manufacturer's instructions. The 3' and 5' RACE
154 fragments were amplified by the first and nested PCR. The adaptors of 3' and 5' cDNA
155 ends and the adaptor primers for RACE were included in the kit. The first PCR was
156 carried out as described in *2.2 Partial cloning of PPAR genes*. After the first PCR, the
157 reaction mixture was diluted to 1:100 and 0.5μL of the diluted products were subjected
158 to the nested PCR in 25μL reaction volume. The nested PCR was carried out under the
159 same conditions as in the first PCR except the annealing temperature was 55°C. The
160 cloning and sequence analyses were carried out as described previously (Oku et al.
161 2002). The oligonucleotide sequences used for gene specific amplification are given in
162 Table 1.

Table 1.

163
164 2.4 Phylogenetic analysis
165 Phylogenetic analysis of amino acid sequences was carried out by the neighbor-joining
166 method. Analysis with 1000 bootstrap was conducted using the software DNASIS Pro
167 version 2.06 (Hitachi, Tokyo, Japan).
168

169 2.5 RT-PCR

170 The RT-PCR was carried out as described previously (Oku et al. 2006b). In brief,
171 visceral adipose tissue, gill, heart, hepatopancreas, immature gonad and dorsal white
172 muscle of young fish (body mass 166g) and mature ovary and testis (body mass 1.8kg)
173 were subjected to RT-PCR. Total RNA was prepared by the method of Chomczynski
174 and Sacchi (1987) and 1μg of the total RNA was used for the random-primed cDNA
175 synthesis in 15μL reaction volume with First-Strand cDNA synthesis kit (GE
176 Healthcare). A 0.5μL of the reaction mixture was subjected to PCR in 25μL reaction
177 volume. Thirty cycles of amplifications were carried out for each PCR. Each cycle
178 consisted of a denaturation step at 94°C for 0.5min, an annealing step at 55°C for
179 0.5min and an extension step at 72°C for 0.5min. The final extension step was followed
180 by a 3min extension at 72°C. Aliquots (5μL) of the products were electrophoresed
181 through 3% agarose gel containing ethidium bromide. The nucleotide sequences of
182 primers for the specific amplification of each PPAR gene are shown in Table 2.

Table 2

183

184 2.6 Culture media

185 The plating medium for the preparation of red sea bream stromal-vascular cells (SV
186 cells) consisted of DMEM/Ham's F12 supplemented with 65mM NaCl, 10% fetal
187 bovine serum, 100μg/mL streptomycin and 100U/mL penicillin (Invitrogen, Carlsbad,
188 CA, USA). The induction medium for adipocyte differentiation consisted of
189 DMEM/Ham's F12 supplemented with 65mM NaCl, 100μg /mL streptomycin and
190 100U/mL penicillin (Invitrogen), ITS mixture (1×ITS: 5μg/mL bovine insulin, 50μg
191 /mL transferrin and 5ng/mL sodium selenite, Sigma-Aldrich, St. Louis, MO, USA) and
192 50ng/mL hydrocortisone (Sigma). The 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ2)

193 (Cayman, Ann Arbor, MI, US), ciglitazone (Cayman) were prepared in DMSO and
194 fenofibrate (Sigma-Aldrich) were prepared in ethanol, and added to the induction
195 medium. 2-bromopalmitate (Sigma-Aldrich) were prepared in ethanol and added to the
196 induction medium with fatty acid free bovine serum albumin (final conc. 1%).
197
198 2.7 Preparation of SV cells of red sea bream
199 The cell culture was carried out as described previously (Oku et al. 2006a). In brief,
200 the red sea bream SV cells were prepared by enzymatic digestion of visceral adipose
201 tissue. The visceral adipose tissue was minced in PBS containing 5% bovine serum
202 albumin, followed by the digestion with 1mg/ml type I collagenase (Invitrogen) for 1hr
203 at room temperature. The digested tissue suspension was filtered through 200μm nylon
204 mesh and centrifuged at 800×g for 3min to separate the mature adipocytes and SV
205 fraction. The SV fraction was suspended in plating medium and seeded in 35mm culture
206 dishes. The cell density was approximately $4.3\times10^4/cm^2$. The seeded cells were cultured
207 in the plating medium at 25°C in 5% CO_2 in air. After 2days, the attached SV cells were
208 washed three times with the plating medium without fetal bovine serum, followed by
209 additional 2days cultivation in the plating medium. Adipocyte differentiation was
210 initiated by switching the medium to the induction medium. The SV cells were
211 maintained at 25°C in 5% CO_2 in air. The culture medium was changed every 2 or
212 3days. The culture periods and the addition of PPAR agonists are indicated in the figure
213 legends.
214
215 2.8 Cytological observations
216 The cultured SV cells were fixed in 10% formaldehyde and subjected to Sudan black B

and nuclear fast red staining.

2.9 RNA extraction, cDNA synthesis and quantification of the adipogenic gene transcripts

The expression levels of PPARs and other adipogenic genes identified in red sea bream were measured by real time PCR method. The oligonucleotide sequences and GenBank accession numbers of genes analyzed in this study are indicated in Table 2.

The RNA extraction, cDNA synthesis and quantification of the transcripts by real time PCR were carried out as described previously (Oku et al. 2006a). In brief, the total RNA extraction and random primed cDNA synthesis were performed with SV total RNA isolation system (Promega, Madison, WI, USA) and Omniscript RT PCR kit (Qiagen, Hilden, Germany). Quantity of isolated RNA was determined by the absorbance at 260nm. The total RNA extraction and cDNA synthesis were carried out according to the manufacturer's instructions. Measurements of the gene transcripts of interest were made by a real time PCR with iCycler iQ real time PCR detection system (Biorad, Hercules, CA, US). Aliquots of 20μL of the reaction mixture were prepared with iQ SYBR Green Supermix (Biorad) and the specific primers at a final concentration of 1μM. The oligonucleotide sequences used in this study are given in Table 2. The real time PCR protocol consisted of 3min at 95°C, 0.5min at 50°C and 0.5min at 72°C. Reactions were carried out in duplicate and each transcript level was calculated as copies/μg input RNA. Standard curve was generated by amplification of serial dilutions of known quantities of cDNA fragment of each gene prepared from red sea bream.

2.10 Statistical analyses

241 Statistical analyses of differences among treatment means were carried out by ANOVA
242 and Tukey multiple comparison test by using Kyplot 4.0 (Kyens Lab, Tokyo, Japan).
243 Differences were considered significant if $P<0.05$.
244
245 **3. Results**
246 3.1 Molecular cloning of red sea bream PPARs
247 Three kinds of PPAR cDNA fragments were cloned by degenerated PCR, followed by
248 3' and 5' RACE. By phylogenetic analyses, the identified three PPAR genes were
249 assigned, respectively, as PPARα (Genbank accession number **AB298547**), PPARβ
250 (**AB298548**) and PPARγ (**AB298549**) (Fig. 1). The obtained 1948 bp sequence of red
251 sea bream PPARα contained 1407 bp of an open reading frame (ORF) that is translated
252 into 469 amino acids (Fig. 2). The 1990 bp of red sea bream PPARγ contained 1566 bp
253 of an ORF that is translated into 522 amino acids (Fig. 2). By the RACE analyses, while
254 we could not determine the complete sequence of red sea bream PPARβ, the 1201 bp of
255 nucleotide sequence which corresponds to 399 amino acids in ORF was obtained (Fig.
256 2). Fig.1
257 The three subtypes of red sea bream PPAR share high degrees of sequence similarities
258 to each other (55 - 63 %) and to their mammalian counterparts (55-61 % to human
259 PPARγ, Fig. 2). In comparison with human PPARγ, the 24 additional amino acid
260 residues (res.298-321) were found in the ligand binding region of red sea bream PPARγ
261 (Fig. 2). Fig.2
262
263 3.2 Tissue-specificities
264 The tissue-specificities of red sea bream PPARs were determined by RT-PCR. The

gene expression of red sea bream PPARs were detected in various tissues (Fig. 3). In red sea bream, all three subtypes of PPAR genes were expressed in the adipose tissue, gill, heart, gonad, hepatopancreas of young fish and ovary of adult fish while the expression of α subtype in testis and γ subtype in muscle were not detected (Fig. 3).

3.3 The changes in the gene expression levels during red sea bream adipocyte differentiation

By using primary cultured SV cells prepared from visceral adipose tissue of red sea bream, the changes in PPAR gene expression levels during adipocyte differentiation were investigated. In this experiment, taken together with PPAR genes, the gene expression levels of adipogenic genes were analyzed. Additionally, β-actin gene, which have been reported to show the decreased expression during murine adipocyte differentiation (Spiegelman et al. 1983, Cornelius et al. 1988), were also examined.

Fig.4

By culturing red sea bream SV cells in the induction medium, the accumulated lipid droplets in the cytoplasm were observed within 10days (Fig. 4). In association with adipocyte differentiation, the changes in the expression levels of adipogenic genes were observed (Fig. 5). The gene expression for lipid uptake (LPL1 and LPL2), fatty acid synthesis (FAS) and fatty acid desaturation (d6DES, SCDa and SCDb) were increased during adipocyte differentiation (Fig. 5). On the other hand, the Glut1 and β-actin gene expression were significantly decreased whereas the Glut3 gene expression level did not change during adipocyte differentiation in red sea bream (Fig. 5).

Fig.5

During adipocyte differentiation in red sea bream SV cells, the PPAR gene expression was regulated in a subtype specific manner (Fig. 6). The red sea bream PPARα showed a transient increase during the induction period. The PPARα gene expression level

289 reached maximum after 7days of induction and then decreased to the initial level after
290 10days (Fig. 6). The gene expression level of PPARβ showed a tendency to increase
291 during adipocyte differentiation whereas that of PPARγ was not affected during the 10
292 day cultivation (Fig. 6).

Fig.6

293

294 3.4 The effects of mammalian PPAR agonists on the adipogenic gene expression in the
295 differentiating adipocytes.

296 To obtain insights into the roles of PPARs in the expression of adipocyte function, the
297 effects of mammalian PPAR agonists (15d-PGJ_2, ciglitazone, fenofibrate and
298 2-bromopalmitate) on the PPARs and related adipogenic genes in differentiating
299 adipocytes of red sea bream were examined. The gene expression levels were analyzed
300 by culturing red sea bream SV cells in the presence of each agonist for 3days.

Fig.7

301 During the treatment, cytotoxic effects such as cell death and decrease in cell density
302 were not observed in our experimental conditions (Fig. 7). The 15d-PGJ_2 and
303 ciglitazone treatments, respectively, resulted in the decrease and increase in the PPARβ
304 and PPARγ gene expression levels while the adipogenic gene expression was not
305 affected significantly (Fig. 8). Fenofibrate did not show any significant effects on the
306 PPARs and adipogenic gene expression (Fig. 8). In the presence of 2-bromopalmitate,
307 the PPARγ expression was significantly increased whereas PPARα and PPARβ were
308 decreased in their gene expression levels (Fig. 8). In association with the changes in the
309 gene expression levels of PPARs, the activation of various adipogenic genes including
310 LPL1, FAS and d6DES, SCDa and SCDb were observed in the differentiating red sea
311 bream adipocytes (Fig. 8).

Fig.8

312

313 **4. Discussion**

314 In this study, we characterized the three subtypes of PPAR genes (α, β and γ) in red
315 sea bream. The amino acid sequences of red sea bream PPARs showed high degrees of
316 similarity to those of mammals (Fig. 2) and the RT-PCR analyses revealed that all three
317 subtypes of PPAR genes were widely expressed in various tissues in red sea bream (Fig.
318 3). During adipocyte differentiation, the red sea bream PPARα, β and γ genes were
319 distinctly regulated *in vitro*. In the differentiating red sea bream adipocytes, mammalian
320 PPAR agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, ciglitazone and fenofibrate did not
321 show clear effects on the adipogenic gene expression but 2-bromopalmitate increased
322 the PPARγ and related adipogenic gene expression levels (Fig. 8). Thus our study
323 highlighted the roles of PPARs in fish adipocyte differentiation and provided
324 information on the molecular mechanisms of fish adipocyte development.

325 In our results, the red sea bream PPARα, β and γ gene expression were detected in
326 various tissues including adipose tissue, gill, hepatopancreas and heart (Fig. 3). Also in
327 other fish species, it has been reported that the PPAR genes are expressed in various
328 tissues while their expression levels are different in each tissue (Andersen et al. 2000,
329 Boukouvala et al. 2004, Batista-Pinto et al. 2005, Leaver et al. 2005, Raingeard et al.
330 2006). These results indicate that fish PPARs are responsible for the lipid metabolism in
331 various tissues. Recent molecular genetic studies have revealed that the genomic
332 organizations of PPAR subtypes in some fish species are different from that of
333 mammals (Robinson-Rechavi et al. 2001, Maglich et al. 2003, Leaver et al. 2007). For
334 example, Atlantic salmon PPARβ contains two isotypes (β1 and β2) and these two
335 PPARβ genes show distinct tissue specificities: salmon PPARβ1 is predominantly
336 expressed in liver whereas the PPARβ2 gene predominates in gill (Leaver et al. 2007),

337 suggesting there are functional differences between the two isotypes. For a better
338 understanding of red sea bream PPAR functions, the elucidation of entire genomic
339 organization of PPAR genes and characterization of each gene are required in this
340 species.

341 By using primary cultured SV cells of red sea bream, we analyzed the PPAR gene
342 expression during adipocyte differentiation. The red sea bream SV cells derived from
343 visceral adipose tissue could undergo the adipocyte differentiation *in vitro* with a lipid
344 accumulation in the cytoplasm (Fig. 4) and the differentiation-dependent gene
345 expression (Fig. 5). During adipocyte differentiation, the three subtypes of PPAR genes
346 were regulated differently in red sea bream SV cells (Fig. 6), suggesting each subtype
347 plays a distinct role in adipocyte differentiation. The red sea bream PPARα showed a
348 transient increase in the expression level within 7days (Fig. 6), suggesting this subtype
349 is involved in an early stage of adipocyte differentiation as reported in mammals
350 (Brandes et al. 1986, Pasquali et al. 2004). In mammalian adipocytes, PPARγ is a
351 critical factor for adipocyte differentiation. During adipocyte differentiation, PPARγ is
352 activated and induces the downstream adipogenic gene expression (Gregoire et al. 1998,
353 Morrison and Farmer 1999). Like in mammalian adipocytes, it has been reported that
354 PPARγ protein expression in cultured salmon adipocytes was induced in association
355 with adipocyte differentiation (Vegusdal et al. 2003). Indeed, the activation of PPARγ
356 gene with 2-bromopalmitate was linked to the expression of adipogenic genes in red sea
357 bream (Fig. 8), suggesting the activation of PPARγ is required for adipocyte
358 differentiation. However, in contrast to this result, the time course study revealed that
359 red sea bream PPARγ gene expression level was not linked to adipocyte differentiation
360 whereas PPARβ gene expression showed a trend to increase (Fig.6). This fact allows us

361 to speculate that the β subtype is involved in the differentiation-linked adipogenic gene
362 expression in red sea bream adipocytes. Thus there is a contradiction between the
363 implications of two experiments (Fig.6 and Fig.8) and the conclusion on the roles of
364 PPARs in fish adipocyte differentiation has not yet been fully elucidated. In our
365 previous (Oku et al 2006a) and present studies, the differentiation of red sea bream
366 adipocytes was induced by the hormonal treatment in a serum free medium. Under this
367 experimental condition, the functions of PPARγ and PPARβ maybe complementary and
368 interchangeable in the time course study. To explain the roles of PPARβ and γ in the red
369 sea bream adipocyte differentiation, further analyses, e. g. the analyses under the same
370 culture and differentiation conditions as used for salmon adipocytes, are required.
371 It has been reported that fish PPARα and γ contain additional amino acid residues in
372 the ligand binding region in comparison with human PPARγ (Andersen et al. 2000,
373 Leaver et al. 2005, Kondo et al. 2007). In red sea bream, like in those of other fish
374 species, the additional 24 amino acids (res.298-321) were found in the ligand binding
375 region of PPARγ (Fig. 2), suggesting that ligand binding properties of fish PPARs may
376 differ from those in mammals. Our research group is interested in the control of
377 adipocyte development and adipocyte function in fish. In this study, we examined the
378 effects of mammalian PPAR agonists but 15d-PGJ_2 (0-0.3 μM), ciglitazone (0-0.3μM)
379 and fenofibrate (0-5 μM) did not show clear effects on the adipogenic gene expression
380 (Fig.8). Among the treatments with the PPAR agonists, 2-bromopalmitate (0-30 μM)
381 could activate various adipogenic gene expression including LPLs, FAS and SCDs (Fig.
382 8), suggesting fatty acid metabolites can be used as modulators of adipocyte function in
383 this species. In the presence of 2-bromopalmitate, the increase in the expression level of
384 PPARγ gene was observed (Fig. 8), but it remains to be determined if 2-bromopalmitate

385 acts on PPARγ directly in red sea bream. In Atlantic salmon, 2-bromopalmitate activates
386 PPARβ (Leaver et al. 2007). Furthermore, it has been reported that the activated PPARβ
387 by 2-bromopalmitate induces the PPARγ expression and the subsequent adipocyte
388 differentiation (Amri et al. 1994, Bastie et al. 1999). The activation of the PPARγ gene
389 by 2-bromopalmitate in the differentiating red sea bream adipocytes maybe promoted
390 through the action of PPARβ.
391 In this study, we characterized three subtypes of PPAR genes in red sea bream and
392 investigated the gene expression during adipocyte differentiation *in vitro*. The
393 elucidation of the mechanisms of adipocyte differentiation in fish is important for the
394 eventual control of adiposity in cultured species. Our results in the present study will
395 facilitate further investigation on the molecular mechanisms of the fish adipocyte
396 differentiation.
397

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Captions to Figures and Tables

Figure 1.

Phylogenetic analysis of PPARs.

The phylogenetic tree was constructed using DNASIS Pro version 2.06 software (Hitachi, Tokyo, Japan). The sequences of red sea bream are boxed. The Genbank accession numbers used in this analysis are as follows: red sea bream PPARα (**AB298547**), red sea bream PPARβ (**AB298548**), red sea bream PPARγ (**298549**), human PPARα (**NML001001930**), bovine PPARα (**AF229356**), mouse PPARα (**NML011144**), chicken PPARα (**PPL539467**), torafugu PPARα1 (**AB275885**), torafugu PPARα2 (**AB275886**), human PPARβ (**NML177435**), bovine PPARβ (**AF229357**), mouse PPARβ (**NML011145**), chicken PPARβ (**AF163810**), salmon PPARβ (**AF342945**), zebrafish PPARβ (**AF342938**), medaka PPARβ (**AY055372**), torafugu PPARβ (**AB275887**), human PPARγ (**L40904**), mouse PPARγ (**NML011146**), bovine PPARγ (**AY179866**), chicken PPARγ (**AF163811**), salmon PPARγ (**AJ416951**), flounder PPARγ (**AJ249075**), plaice PPARγ (**AJ539469**), and torafugu PPARγ (**AB275888**).

Figure 2.

Comparison of the deduced amino acid sequences among human PPARγ and red sea bream (RSB) PPARα, β and γ.

Dots (·) indicate identical residues. Gaps are introduced to maximize sequence similarities. The DNA binding region (underline) and ligand binding region (double underline) are indicated.

577

578 **Figure 3.**

579 **Tissue-specificities of red sea bream PPARα, β and γ.**

580 The expression of each gene was detected by RT-PCR. The RNA samples were
581 extracted from 1) adipose tissue, 2) gill, 3) heart, 4) hepatopancreas, 5) gonad, 6)
582 muscle, 7) ovary, and 8) testis. The cDNA samples were prepared with reverse
583 transcription (RT+) and negative controls for contamination of genomic DNA were run
584 without reverse transcription (RT-).

585

586 **Figure 4.**

587 **The lipid accumulation in the cytoplasm during the differentiation of red sea**
588 **bream SV cells.**

589 The cells were fixed in 10% formaldehyde and stained with Sudan black B and nuclear
590 fast red after 0, 3, 7 and 10 days of the induction of adipocyte differentiation. The bar
591 indicates 50μm.

592

593 **Figure 5.**

594 **The changes in the adipogenic gene expression levels in the red sea bream SV cells**
595 **during adipocyte differentiation.**

596 The cells were sampled after 0, 3, 7 and 10days of the induction of adipocyte
597 differentiation. The expression levels are indicated as copies/μg input RNA. The bars
598 represent standard error. The experiment was repeated six times with isolated cell
599 cultures derived from six different fish. Means not sharing a common superscripts are
600 significantly different ($P<0.05$). The abbreviations of each gene are shown in Table 2.

Figure 6.

The changes in the gene expression levels of red sea bream PPARs during the adipocyte differentiation in red sea bream SV cells.

The cells were sampled after 0, 3, 7 and 10days of the induction of adipocyte differentiation. The expression levels are indicated as copies/μg input RNA. The bars represent standard error. The experiment was repeated six times with isolated cell cultures derived from six different fish. Means not sharing a common superscripts are significantly different ($P<0.05$).

Figure 7.

The phase contrast micrographs of the red sea bream SV cells after treatment with mammalian PPAR agonists.

The cells were cultured in the induction medium for 3days in the absence (control) or presence of 3μM of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), 0.3μM of ciglitazone (Cg), 5μM of fenofibrate (FF) and 30μM of 2-bromopalmitate (Br-palmitate). The bar indicates 100μm.

Figure 8.

The changes in the expression levels of PPARs and adipogenic genes by the treatment with mammalian PPAR agonists in red sea bream SV cells.

The cells were cultured in the induction medium for 3days in the presence of 0, 0.3, 3μM of 15d-PGJ_2 (PG), 0, 0.03, 0.3μM of ciglitazone (Cg), 0, 0.5, 5μM of fenofibrate (FF) and 0, 3, 30μM of 2-bromopalmitate (Br-palmitate). Values (mean ± SEM) are

reported as percentage to the mean value of the control (0 μM) and taken as 100%. Means not sharing a common superscript are significantly different ($P<0.05$).

Table 1.

The oligonucleotide sequences for degenerated PCR and RACE.

* The amino acid sequence of PPARγ is indicated in Fig.2.

Table 2.

The oligonucleotide sequences for RT-PCR and real time PCR.

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650

651 Fig.1

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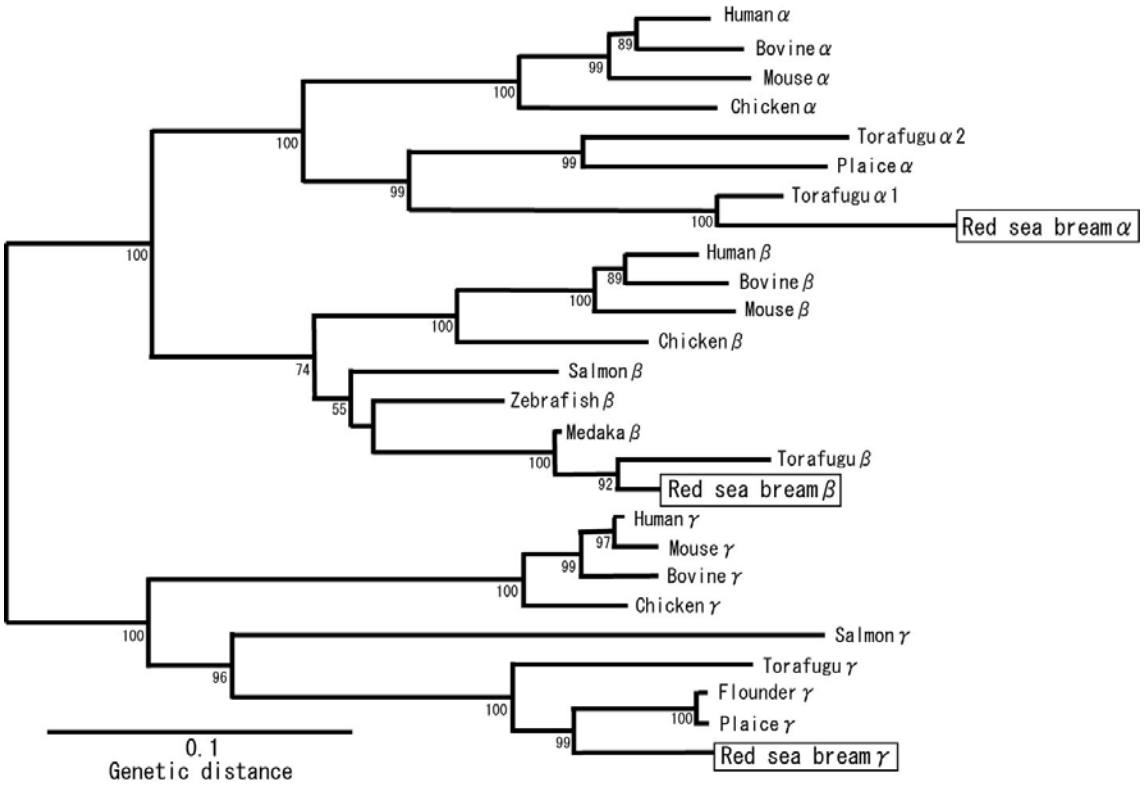
653



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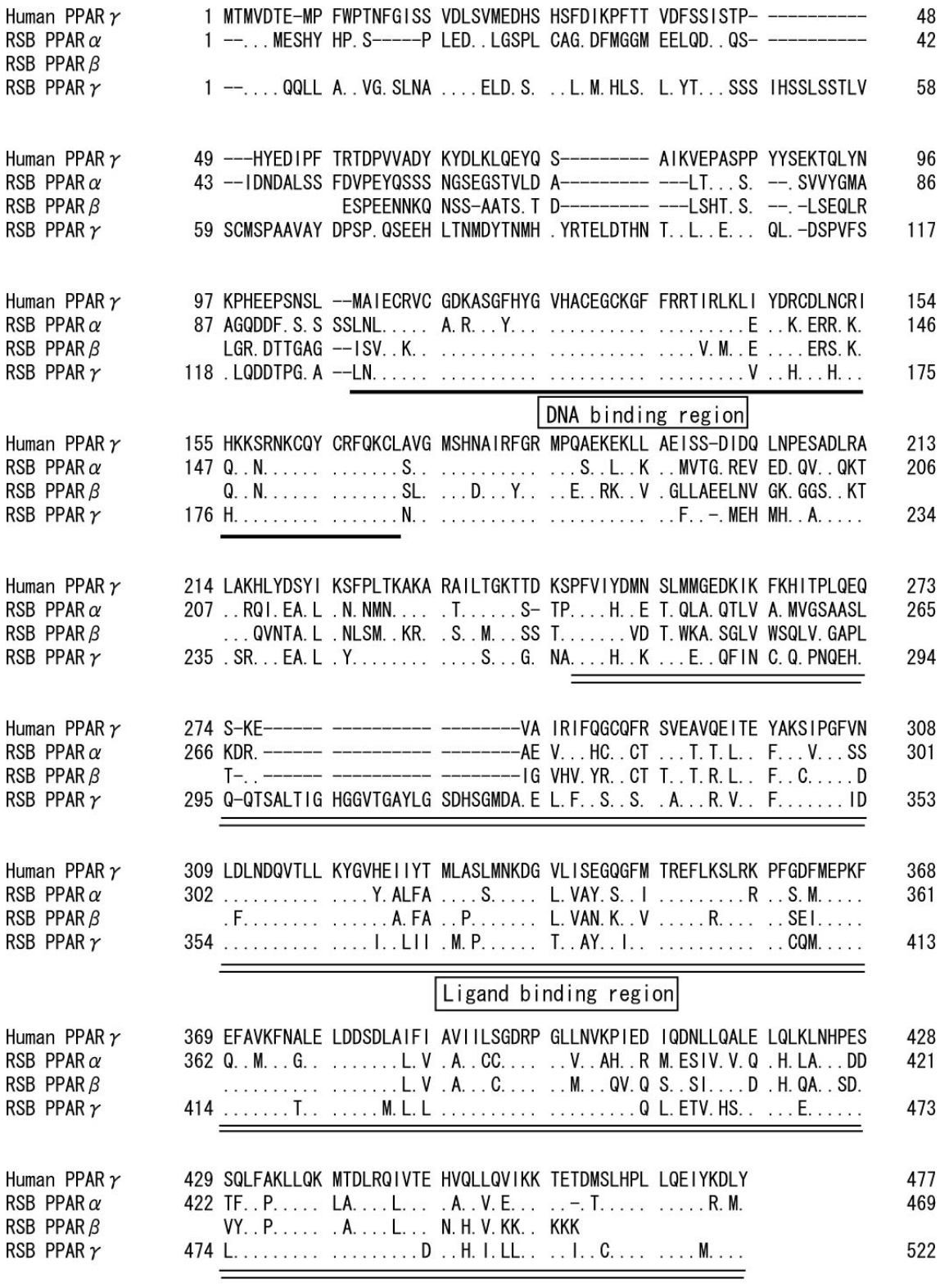
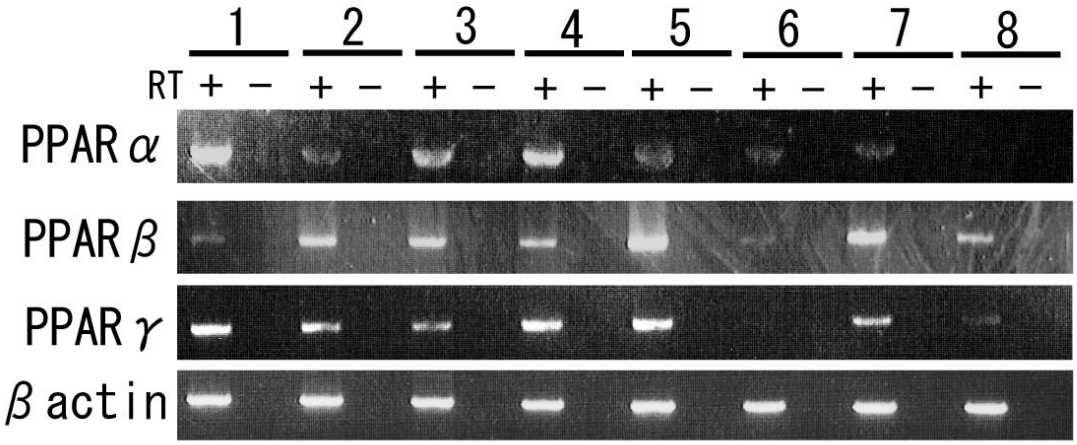
663 Fig.2

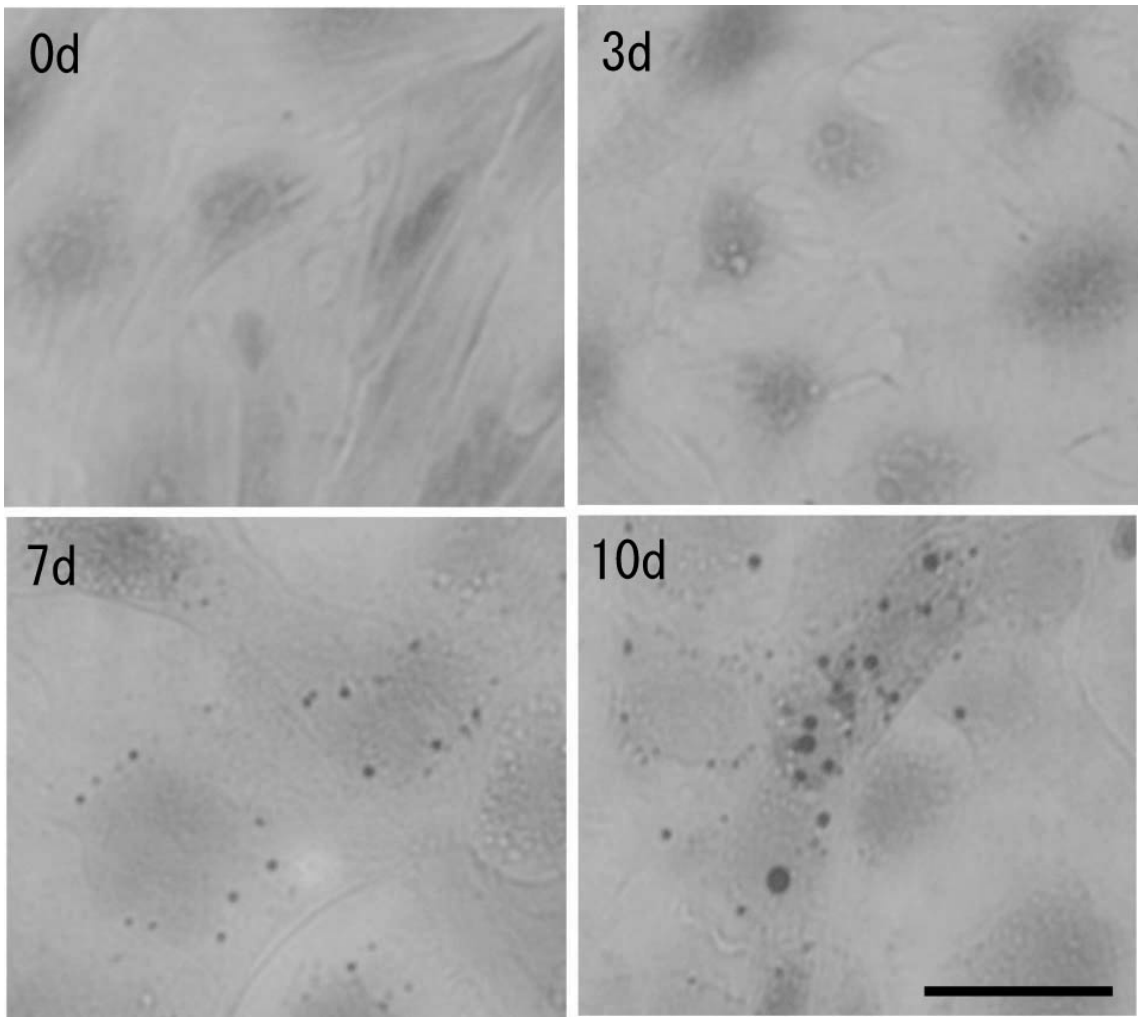

Human PPARγ
RSB PPARα
RSB PPARβ
RSB PPARγ
DNA binding region
Ligand binding region

664

Fig.3



683 Fig.4



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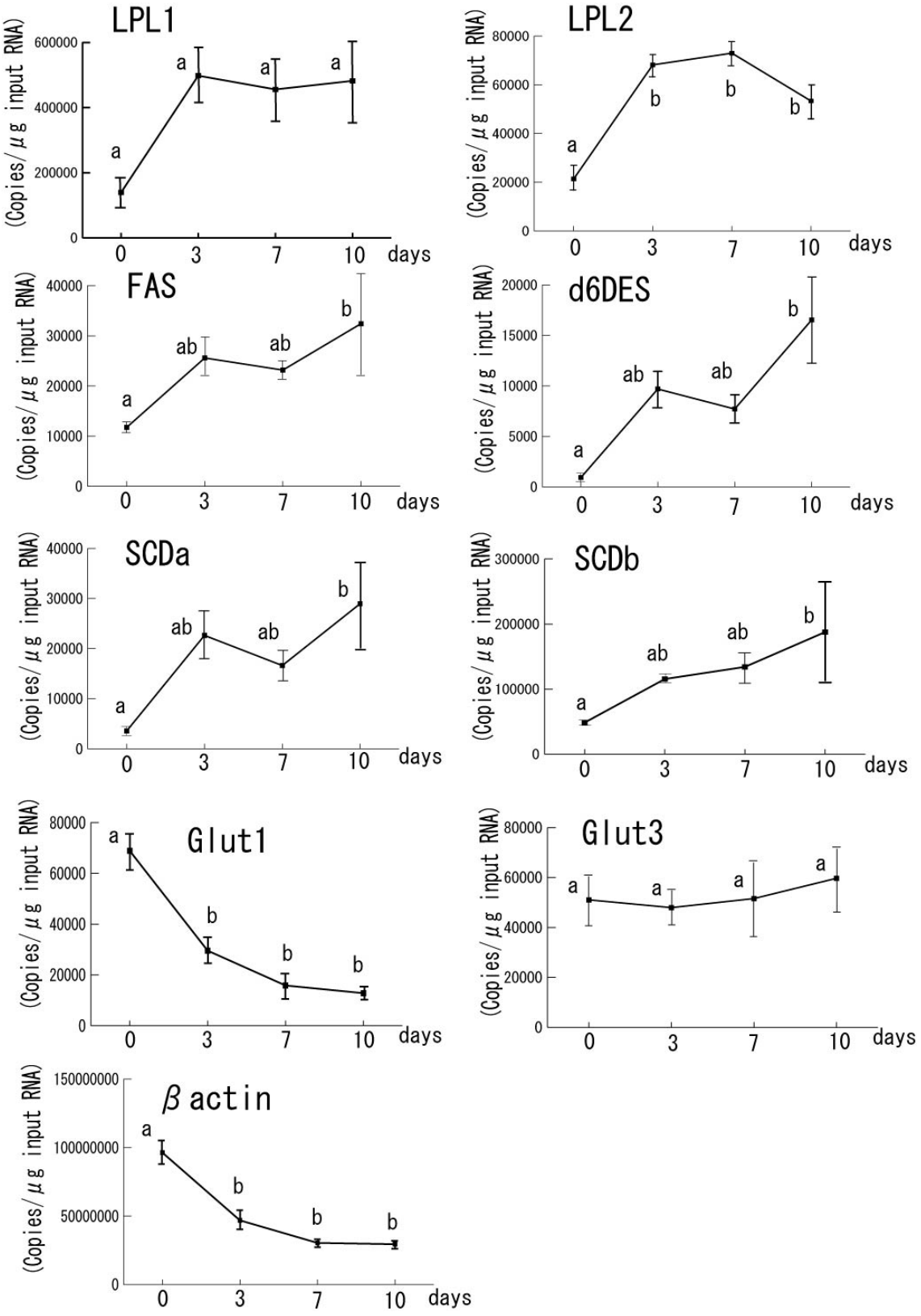
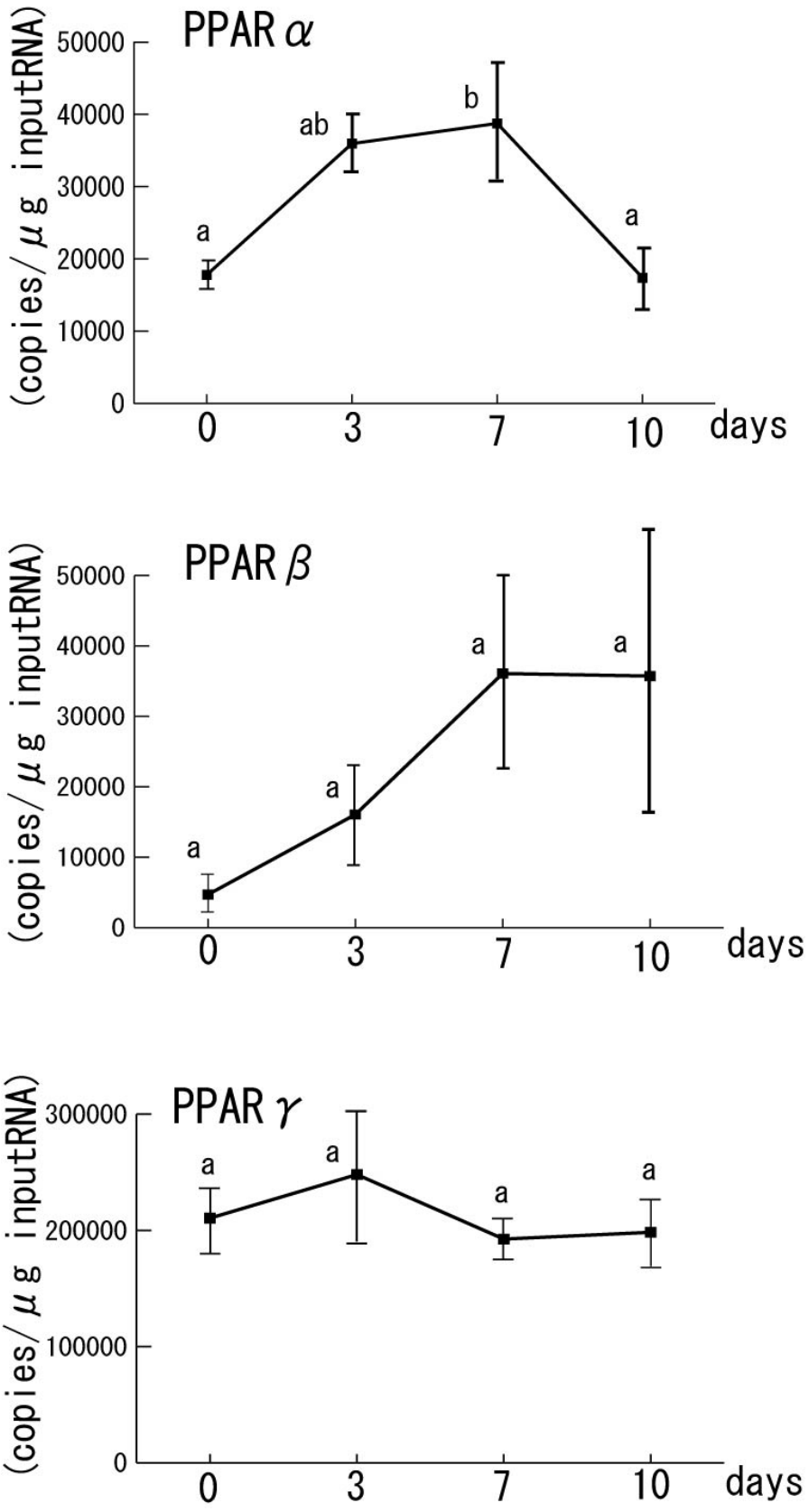
693 Fig.5


LPL1
LPL2
FAS
d6DES
SCDa
SCDb
Glut1
Glut3
βactin
(Copies/μg input RNA)
days
0
3
7
10
a
ab
b

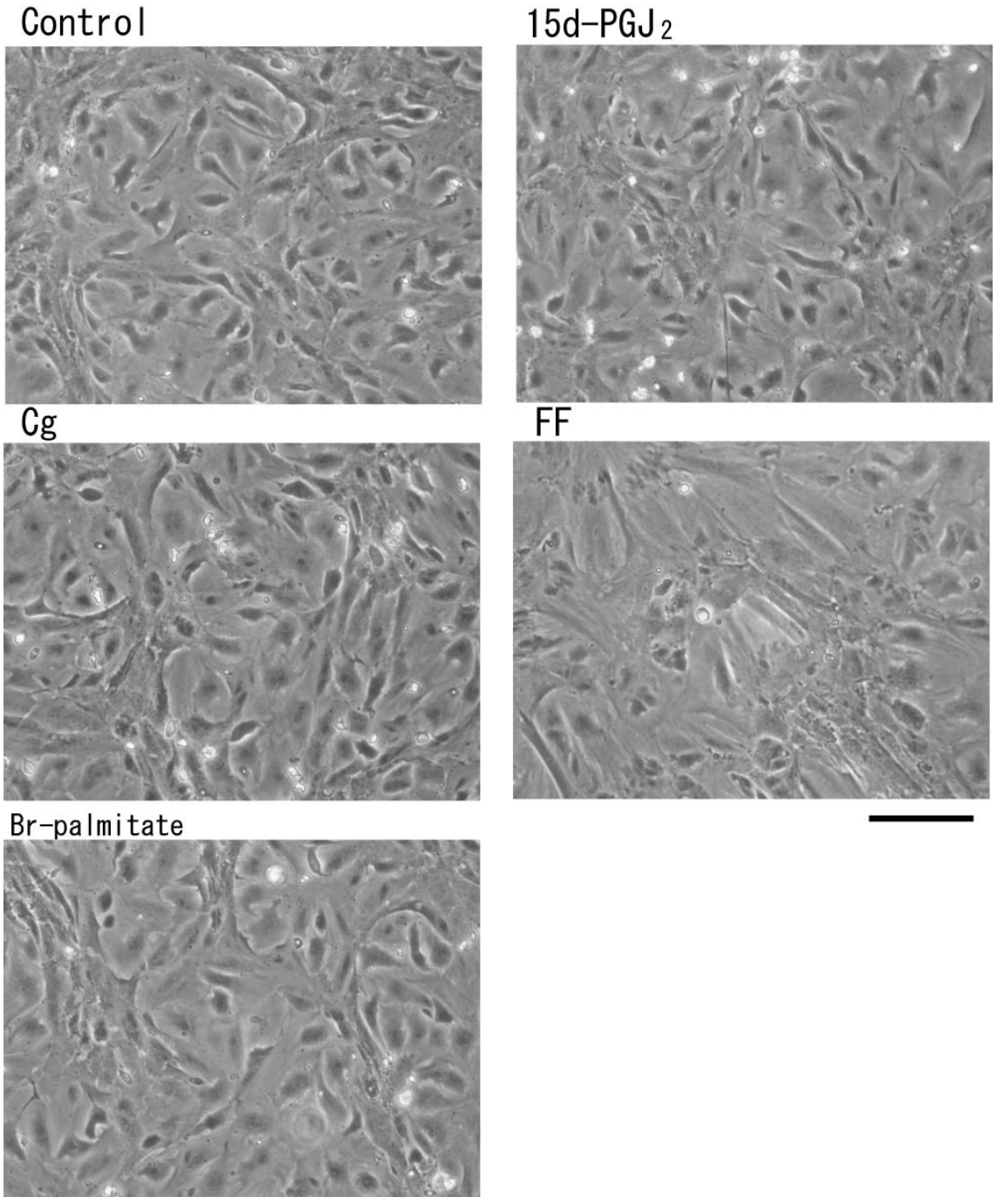
694

695 Fig.6


PPAR α
(copies/μg inputRNA)
50000
40000
30000
20000
10000
0
a
ab
b
a
0
3
7
10
days
PPAR β
(copies/μg inputRNA)
50000
40000
30000
20000
10000
0
a
a
a
a
0
3
7
10
days
PPAR γ
(copies/μg inputRNA)
300000
200000
100000
0
a
a
a
a
0
3
7
10
days

696

697 Fig.7



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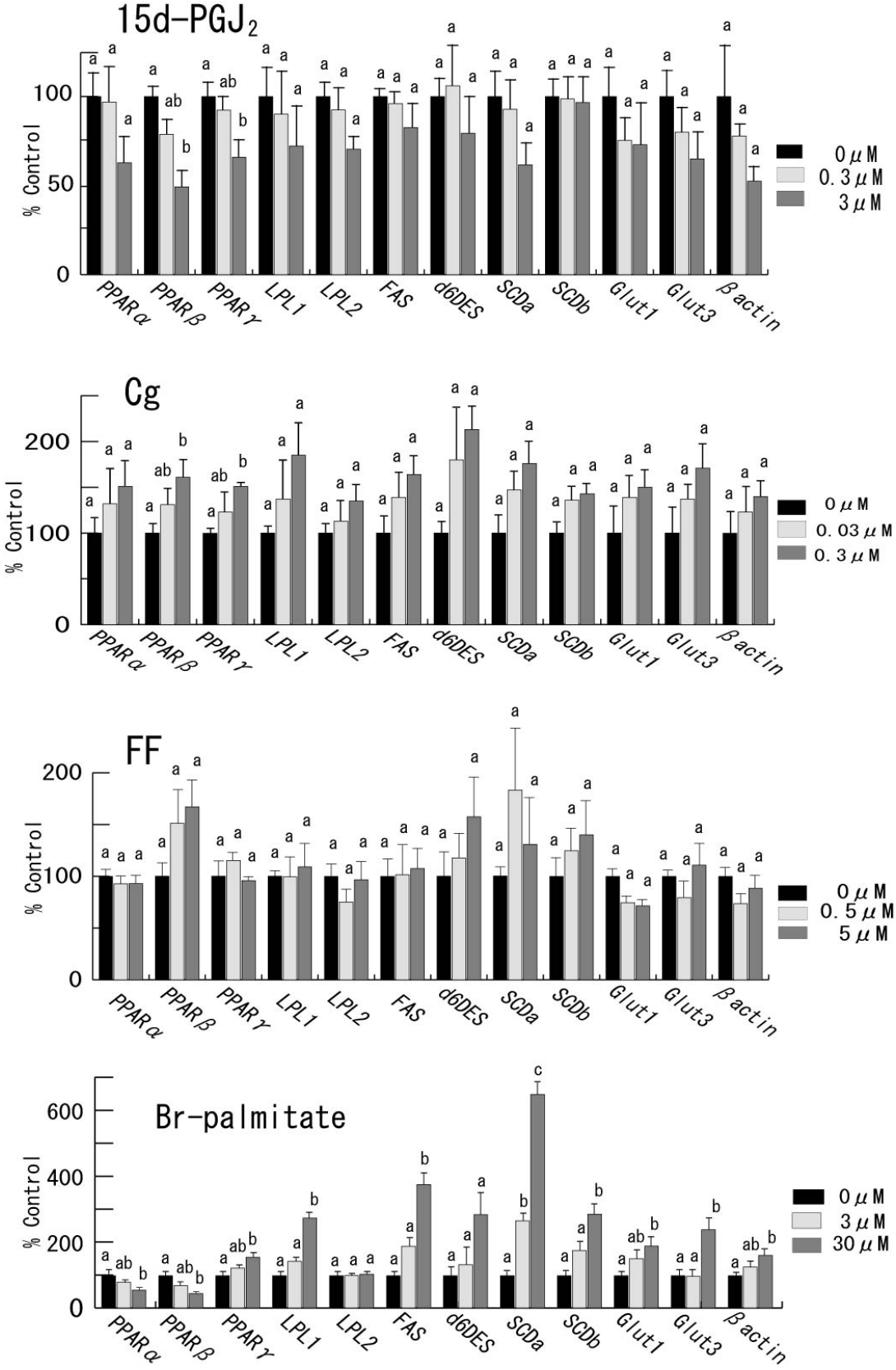
703 Fig.8


15d-PGJ2
% Control
0
50
100
0μM
0.3μM
3μM
PPARα
PPARβ
PPARγ
LPL1
LPL2
FAS
d6DES
SCDa
SCDb
Glut1
Glut3
βactin
Cg
% Control
0
100
200
0μM
0.03μM
0.3μM
PPARα
PPARβ
PPARγ
LPL1
LPL2
FAS
d6DES
SCDa
SCDb
Glut1
Glut3
βactin
FF
% Control
0
100
200
0μM
0.5μM
5μM
PPARα
PPARβ
PPARγ
LPL1
LPL2
FAS
d6DES
SCDa
SCDb
Glut1
Glut3
βactin
Br-palmitate
% Control
0
200
400
600
0μM
3μM
30μM
PPARα
PPARβ
PPARγ
LPL1
LPL2
FAS
d6DES
SCDa
SCDb
Glut1
Glut3
βactin

704

705

706 Table 1.

Target gene		Nucleotide sequence	Approximate position in human PPARγ*
Degenerated PCR			
PPARα	(Forward)	5' -GTGCACGCTTGCGAGGGNTGYAA-3'	(res. 124-132)
	(Reverse)	5' -AACCGCGAACTCGAATTTNGGCTC-3'	(res. 365-372)
PPARβ	(Forward)	5' -TGCCCCAAGCCGARAARGAGAA-3'	(res. 185-192)
	(Reverse)	5' -AACCGCGAACTCGAATTTNGGCTC-3'	(res. 365-372)
PPARγ	(Forward)	5' -AGCGGTTTTCACTAYGGGGT-3'	(res. 119-125)
	(Reverse)	5' -AACCGCGAACTCGAATTTNGGCTC-3'	(res. 365-372)
Gene specific primers for 3' RACE			
PPARα	(First)	5' -GACAAGTGTGAGCGCCG-3'	(res. 146-151)
	(Nested)	5' -AAGGCGGAGATGGTAACGGG-3'	(res. 194-199)
PPARβ	(First)	5' -GATCGCTGTGAGCGTT-3'	(res. 146-151)
	(Nested)	5' -CTTTGGGAATGTCCCATGA-3'	(res. 173-178)
PPARγ	(First)	5' -CGACATGGAGCACATGCA-3'	(res. 200-205)
	(Nested)	5' -CTGAAATACTTCCCCCTCAC-3'	(res. 223-229)
Gene specific primers for 5' RACE			
PPARα	(First)	5' -TTCTCTCACCGCTTCGGC-3'	(res. 290-295)
	(Nested)	5' -CAGCTCCACAGCGTCCAT-3'	(res. 274- 279)
PPARβ	(First)	5' -GATAGAAAACATGGACCCC-3'	(res. 248-253)
	(Nested)	5' -ACCGCTTTCTGCTTTCCA-3'	(res. 278-284)
PPARγ	(First)	5' -CATCTTTGCCACCAGGGT-3'	(res. 253-258)
	(Nested)	5' -AGCCTTGGCCTTGTTCATGTT-3'	(res. 227-233)

707

708

709

710 Table 2.

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Target gene	GenBank accession No.	(Foward) (Reverse) Sequences	Amplified fragment length
PPARα	AB298547	5' -GACAAGTGTGAGCGCCG-3' 5' -CATCTTTGCCACCAGGGT-3'	(362bp)
PPARβ	AB298548	5' -CTTTGGGAATGTCCCATGA-3' 5' -GATAGAAAACATGGACCCC-3'	(339bp)
PPARγ	AB298549	5' -CGACATGGAGCACATGCA-3' 5' -TTCTCTCACCGCTTCGGC-3'	(360bp)
Lipoprotein lipase 1 (LPL1)	AB243791	5' -CTCAAGACCCGCGAGAT-3' 5' -AAGCGTCGCTCTGACC-3'	(493bp)
Lipoprotein lipase 2 (LPL2)	AB054062	5' -ATTCATTCCTGCTGGTGAC-3' 5' -TCAGTGCTTCTCCAGAGTTAC-3'	(320bp)
Fatty acid synthetase (FAS)	AB298550	5' -AGCTGTTCATCTGGGGAT-3' 5' -CTGGGAAGAGGGCCATC-3'	(345bp)
Delta-6-desaturase (d6DES)	AB298553	5' -GCACTTCCAGCATCACGC-3' 5' -ACGAAGCTGATGAGCGC-3'	(330bp)
StearoylCoA desaturase a (SCDa)	AB298551	5' -CTTCGCCCACATTGGTTG-3' 5' -CACTCAAAGCAACCATTGC-3'	(342bp)
StearoylCoA desaturase b (SCDb)	AB298552	5' -CTTTGCTCACATCGGCTG-3' 5' -CGCTGAACGTGACAAACTT-3'	(342bp)
Glucose transporter 1 (Glut1)	AB298554	5' -TTGTCTTGGGCATCCTTATT-3' 5' -AGAGCTGCAGGACGACG-3'	(351bp)
Glucose transporter 3 (Glut3)	AB298555	5' -AGTTATTGGCATCCTGGTG-3' 5' -AGAGAGCTGGAGGATGATA-3'	(351bp)
βactin	AB252854	5' -GGCACTGCTGCCTCCTC-3' 5' -GCCAGGATGGAGCCTCC-3'	(309bp)

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