Homozygous and heterozygous GH transgenesis alters fatty acid composition and content in the liver of Amago salmon (*Oncorhynchus masou ishikawae*)

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Biology Open 1, 1035–1042
doi: 10.1242/bio.20121263
Received 9th March 2012
Accepted 2nd July 2012

Summary

Growth hormone (GH) transgenic Amago (*Oncorhynchus masou ishikawae*), containing the sockeye GH1 gene fused with metallothionein-B promoter from the same species, were generated and the physiological condition through lipid metabolism compared among homozygous (Tg/Tg) and heterozygous GH transgenic (Tg/+) Amago and the wild type control (+/+). Previously, we have reported that the adipose tissue was generally smaller in GH transgenic fish compared to the control, and that the Δ-6 fatty acyl desaturase gene was down-regulated in the Tg/+ fish. However, fatty acid (FA) compositions have not been measured previously in these fish. In this study we compared the FAs composition and content in the liver using gas chromatography. Eleven kinds of FA were detected. The composition of saturated and monounsaturated fatty acids (SFA and MUFA) such as myristic acid (14:0), palmitoleic acid (16:1n-7), and cis-vaccenic acid (cis-18:1n-7) was significantly ($P<0.05$) decreased in GH transgenic Amago. On the other hand, the composition of polyunsaturated fatty acids (PUFAs) such as linoleic acid (18:2n-6), arachidonic acid (20:4n-6), and docosapentaenoic acid (22:5n-3) was significantly ($P<0.05$) increased. Levels of serum glucose and triacylglycerol were significantly ($P<0.05$) decreased in the GH transgenics compared with +/+ fish. Furthermore, 3′-tag digital gene expression profiling was performed using liver tissues from Tg/Tg and +/+ fish, and showed that Mid1 interacting protein 1 (Mid1ip1), which is an important factor to activate Acetyl-CoA carboxylase (ACC), was down-regulated in Tg/Tg fish, while genes involved in FA catabolism were up-regulated, including long-chain-fatty-acid–CoA ligase 1 (ACSL1) and acyl-coenzyme A oxidase 3 (ACOX3). These data suggest that liver tissue from GH transgenic Amago showed starvation by alteration in glucose and lipid metabolism due to GH overexpression. The decrease of serum glucose suppressed Mid1ip1, and caused a decrease of *de novo* FA synthesis, resulting in a decrease of SFA and MUFA. This induced expression of ACSL1 and ACOX3 to produce energy through β-oxidation in the GH transgenic Amago.

Key words: GH-transgenic fish, Homozygous, Heterozygous, Amago, *Oncorhynchus masou*, fatty acid composition, fatty acid contents, Illumina, Mid1 interacting protein 1

Introduction

Growth hormone (GH), well known as the main growth regulator in mammals (Isaksson et al., 1982), also influences directly or indirectly carbohydrate, lipid, nitrogen, and mineral metabolism (Eisemann et al., 1986; Davidson, 1987; Møller and Jørgensen, 2009), cell differentiation (Kopchick and Andry, 2000), immune system maintenance (Jeay et al., 2002), cardiac function (Lombardi et al., 1997), and the modulation of emotions, stress response, and behavior (Yoshizato et al., 1998). Experiments conducted on GH transgenic animals, in order to investigate the effects of GH on various physiological functions, have shown that the phenotypic effects in GH transgenic mammals have ranged from no effect to an approximate doubling of body size (Devlin, 1997). However, GH transgenic fish (e.g. salmonoids) showed dramatic increases in growth, typically 6- to 11-fold, and sometimes up to 40-fold or even higher (Devlin et al., 1994; Rahman et al., 1998). The physiological and endocrine systems in fish are adapted for aquatic life, and are likely to be quite different from the corresponding mammalian systems. GH transgenic fish have also shown some other peculiarities besides growth enhancement, such as morphological differences in the intestines (Stevens and Devlin, 2000a) and gills (Stevens and Devlin, 2000b), reduction in fertility (Rahman et al., 1998), down-regulation of expression of genes in the pituitary gland (Mori and Devlin, 1999), etc. Furthermore, there have been changes in metabolism as well, such as reduced expenditure of metabolized protein and faster utilization of ingested lipids (Krasnov et al., 1999), along with improved food conversion ratios (Rahman et al., 1998).

In order to provide energy for accelerated growth, GH transgenesis may, in general, increase the activity of enzymes

involved in the tricarboxylic acid (TCA) cycle as well as the catabolic reactions that break down carbohydrates, lipids and proteins for energy production via the TCA cycle (King et al., 2006). However, there have also been studies that showed no effect of transgenesis on the expression of TCA enzyme genes or glycolytic enzyme genes (Rise et al., 2006), and even a decrease in the potential for gluconeogenesis in the livers of GH transgenic fish (Martínez et al., 1996). These and other contradictions concerning protein and lipid metabolism in GH transgenic fish (which are reviewed by Leggatt et al., 2009) can be studied by comparing homozygous and heterozygous GH transgenic fish from various species with their respective controls.

Our lab. has produced fast-growing GH transgenic Amago salmon, with reduced Δ-6 fatty acyl desaturase (Δ6FAD) gene expression, as revealed by functional microarray (Mori et al., 2007). Δ6FAD is important for the modification of polyunsaturated fatty acids (PUFAs) in many vertebrates (Zheng et al., 2004), and any reduction can have many downstream effects. PUFAs are synthesized from the essential fatty acid (FA) precursors, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). Production of γ-linoleic acid (18:3n-6) from 18:2n-6 is controlled by microsomal Δ6 FAD, and arachidonic acid (20:4n-6) is produced by Δ5FAD using the fatty acid produced by Δ6FAD (Zheng et al., 2004). Furthermore, the production of eicosapentaenoic (20:5n-3) and docosahexaenoic acid (22:6n-3) from 18:3n-3 also requires Δ6FAD (Cook, 1996). Therefore, a decrease in Δ6FAD gene expression, in turn, results in a decrease in the levels of various PUFAs, indicating that GH transgenesis should result in an alteration in PUFA levels. The effect of GH administration on the fatty acid composition in a mammal has been reported (Hougham and Cramer, 1980). However, although GH transgenic fish show reduced expression of the Δ6FAD gene, fatty acid compositions have not been measured previously using these fish. Furthermore, we have demonstrated quantitative changes of various pituitary hormones and down-regulation of fatty acid synthase (FAS) in the pituitary from GH transgenic Amago salmon by iTRAQ-MS/MS proteome and microarray analysis (Kurata et al., 2012). This analysis revealed that 61 unique proteins, differentially expressed in response to excess GH 1 treatment, were molecules related to endocrine systems, cell growth and proliferation, and metabolism, based on their biological function ontology. These molecules were functionally related directly or indirectly by pathway analysis. Meanwhile, we also revealed morphological changes in the liver of GH transgenic Amago (Sugiyama et al., 2010) without clear involvement with the down-regulation of FAS or Δ6FAD.

We report here the results of our investigation focusing on fatty acid metabolism in the GH transgenic Amago salmon. We produced GH transgenic Amago by fertilizing eggs from GH transgenic females with sperm from GH transgenic males, and obtained homozygous (Tg/Tg) and heterozygous (Tg/+) GH transgenics as well as the control (+/+) fish in this experiment. The fatty acid composition (%) of the eleven different fatty acids and their content (mg fatty acid per gram tissue) in the liver were measured using gas chromatography. We also performed a 3′-tag digital gene expression profiling of liver tissue from the Tg/Tg and +/+ fish in order to understand if changes in fatty acid composition or content, if any, were related to altered gene expression levels. Here we report the metabolic pathway of fatty acid biosynthesis and catabolism in GH transgenic Amago and the effects of GH transgenesis on fatty acid composition and content in the liver.

Materials and Methods

Experimental animals

GH transgenic Amago salmon were produced by injecting the gene construct of sockeye salmon GH1 gene fused with metallothionein-B promoter from the same species (OnMTGH1) (Devlin et al., 1994) into fertilized eggs of the fish. As a result, about 2% of the injected eggs yielded GH transgenic fish. In turn, eggs from these fish were fertilized with wild type milt, to produce the F1 generation of transgenic fish. Generation F5 Tg/+ Amago used by this experiment were produced by fertilizing domestic-type sperms with eggs collected from F4 transgenic fish containing the OnMTGH1. Tg/Tg fish used in this experiment were produced by mixing eggs and sperm obtained from F4 Tg/+ fish, and they (Tg/Tg) were separated from the Tg/+ and +/Tg fish by a perusal of the copy numbers of the GH transgene using real time PCR (LightCycler® LC480; Roche) with TaqMan probe. The transgene OnMTGH1 was detected using forward primer (5′-ATAAAAACGGTGCTTCGCCA-3′), reverse primer (5′-GCAGGCTTACCT-TGTCCCATT-3′) and double dye probe (5′-ATCGAAAAGGATCCCATCCT-TGGCA-3′) designed for OnMTGH1. Native GH1 intron of the Amago was used as the internal standard, and the forward primer (5′-CCTATTGTGT-CCCTTGGACAACTT-3′), reverse primer (5′-CAGGGAACATAGCCTTTACA-TTTCA-3′) and double dye probe (5′-CTGGATTCAATTCGTATCGCAGA-CGCTC-3′) were designed. PCR samples were prepared as follows: DNA was isolated from these transgenic fish using the DNA purification kit (QIAGEN) and diluted to 300 ng/μL in DNase-free water. One μL of the DNA was mixed with 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 1.0 μL of double dye probe (500 nM), 12.5 μL of 2× concentration of IQ super Mix (BIO-RAD), and 9.7 μL of distilled water. PCR cycling conditions were as follows: 95℃ for 30 sec, 40 cycles of 95℃ for 5 sec, 60℃ for 30 sec.

The fish were cultured in equal densities in circulating tanks under a natural light cycle, and fed to satiation with a stage-specific commercial diet for juvenile fish (1–4CDX and Masu 5–8p from Nippon Formula Feed Mfg Co., Ltd) until the end of the experiment (about 6 months). The mean weights of the Tg/Tg, Tg/+ (note that all the heterozygotes were produced using eggs from transgenic fish and sperm from wild type), and +/+ fish used in this experiment were 131 g, 109 g, and 85 g, respectively. All procedures used in this study were followed by the Nihon University Animal Care and Use Committee.

Isolation of total lipids and analysis of fatty acid composition and content using gas chromatography

Liver tissues were obtained from 5–6 Tg/Tg, Tg/+, and +/+ Amago, soaked in liquid nitrogen, and stored at −80℃ until further analysis. Total lipids were extracted and purified following the method of Folch et al. (Folch et al., 1957). Fatty acid methyl esters were prepared by transesterification with 15% boron trifluoride in methanol. Fatty acid composition was analyzed by detection on a 5890 series II GC (Agilent Technologies, Inc., Santa Clarita, CA, USA) equipped with a flexible fused-silica (FFS) capillary column (ULBON-HR-SS-10; 0.25 mm I.D. × 50 m, Shinwa Chemical Industries, Inc., Tokyo, Japan). The flame ionization detector (FID) and injector port were maintained at 250℃. The column temperature was programmed to ramp up from 150℃ to 210℃ at 2℃ per minute. The flow rate of helium carrier gas was 0.8 ml/min. Fatty acid methyl esters were identified by comparing retention times among the fatty acid methyl esters and the respective fatty acid methyl ester standards. Fatty acid contents (mg fatty acid per gram tissue) were calculated using an internal standard heptadecanoic acid (17:0). Composition (%) was calculated using peak area of detected signals.

Library construction and 3′-tag digital gene expression profiling

Total RNA was extracted from liver tissues from five different Tg/Tg and +/+ Amago after anesthetization, and these were soaked into RNAlater (QIAGEN), respectively. These tissues were cut into small pieces with scissors and, after keeping them for 24 hr at 4℃, stored at −80℃ until used in experiments. Tissues were lysed and total RNA was obtained using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Total RNA yield was measured by absorbency at 260 nm and electrophoresis performed. Total RNA samples (10 μg) were subjected to cDNA construction for Illumina sequencing using the libraries of 3′ region tags, according to the protocol described for DGE, Tag Profiling for *Nla III* Sample Prep Kit (Illumina, San Diego, CA). Briefly, mRNA was obtained by applying total RNA to oligo-dT magnetic beads. This mRNA was treated with reverse-transcriptase to provide first strand cDNA, and the second strand cDNA was obtained using DNA polymerase and RNase H. Then double strand cDNA was treated with *NlaIII* recognizing a CATG site, and the most 3′-region fragments attached to the beads were collected. These fragments were ligated to the Gex adapter 1. Adapter-ligated 3′-region fragments were digested with *MmeI* to yield 19 or 20 bp of the tag (including CATG) downstream of the *MmeI* site in the Gex adapter 1. After fragments of the mRNA-derived tag attached to the Gex adapter 1

were purified by ethanol precipitation, these fragments were ligated to the Gex adapter 2 at the site digested with *MmeI*. PCR was performed for the fragments ligated to the Gex adapters 1 and 2 and amplified products were purified with a PAGE gel. DNA from a band corresponding to almost 85 base pairs was collected and purified as a library. These DNA libraries were sequenced using an Illumina GA IIx sequencer and 38 bp reads were obtained from one lane per sample.

Computer analysis

CATG, which is the *NlaIII* site, was added to the 38 bp reads at the 5′ end. Sequences of 6 or more than 6 nucleotides at the 3′ end, which could be recognized as a part of the Gex 2 adapter sequence, were removed from those added reads. The length of those modified reads varied 4 (only containing the *NlaIII* site) to 36 nucleotides with the high peak of 21 and 22 nucleotides. These 21 and 22 nucleotides, which were the gene tags, were analyzed as queries for aligning them to reference genes in the database using the blast tool (BLAST 2.2.23). For aligning the gene tags, CATG at the 5′ end was mapped to genes without any sequence differences, while remaining sequences in the gene tags were mapped without any errors or with one mismatch. As references, *Salmo salar* and *Oncorhynchus mykiss* genes were obtained (March 22, 2010) from the nucleotide database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore). Although the gene tag is supposed to be derived only from the most 3′ end CATG site, some tags are from other CATG sites. Gene expression levels were calculated by summing all of those gene tags mapped to the same gene.

Analysis of 3-hydroxybutyric acid using Capillary Electrophoresis Time-of-Flight Mass Spectrometry (CE-TOFMS)

Five different liver tissues (500 mg) were obtained from Tg/Tg, Tg/+, and +/+ Amago. These tissues were frozen immediately in liquid nitrogen, and mixed according to each sample. Frozen liver tissues (300 mg) were immediately plunged into methanol (500 µL) containing internal standards (200 µM Internal Standard: H3304-1002, Human Metabolome Technologies, Tsuruoka, Japan). After freezing in liquid nitrogen to inactivate enzymes, liver tissue was homogenized 5 times at 4,000 rpm for 60 sec using cell disrupter beads MS-100R (TOMY, Japan). Then, 200 µL of Milli-Q water and 500 µL of chloroform were added to the samples, thoroughly mixed, and then centrifuged for 5 min at 2,300 × *g* and 4℃. The 400 µL of upper aqueous layer was centrifugally filtered through a Millipore 5 kDa cut-off filter to remove proteins. The filtrate was lyophilized and suspended in 50 µL of Milli-Q water and analyzed by CE-TOFMS (Soga et al., 2006). CE-TOFMS was carried out using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 Time of Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The system was controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies, Waldbronn, Germany).

Measurement of serum triacylglycerol and glucose

Sera were sampled from immature Tg/Tg (n=8), Tg/+ (n=7), and +/+ (n=7) Amago. Concentration of serum triacylglycerol (TAG) and glucose was measured using Fuji Dry Chem 3030 (Fujifilm). Samples (20 µL) were analyzed using TG-PIII (Fujifilm) for TAG and GLU-PIII (Fujifilm) for glucose, respectively. Data are expressed as means ± standard error (SE).

Statistical analysis

Values for fatty acid components (%) and contents (mg/g) were expressed as means ± SE. The statistical significance was determined by one-way analysis of variance followed by *a posteriori* comparison of the significant ANOVA results using Bonferroni or Dunnett's T3. Levene's multiple comparison test for variances indicated that the assumption of homogeneity of variances was rejected at the 5% significance level. For our statistical decisions, we used Dunnett's T3 multiple comparison test for means, which is robust for testing means under heterogeneous variance. Statistical significance was set at $P<0.05$.

Results

Analysis of fatty acid composition and content

Eleven kinds of major fatty acid were detected from the total lipids extracted from the liver of Amago, as shown in Fig. 1. The content of each fatty acid in the sample was calculated by means of adding an internal control. The fatty acid composition and content from Tg/Tg, Tg/+, and +/+ fish are presented in Fig. 2, where the patterns of composition (%) and content (mg/g) are indicated as Up and Down when compared to the +/+ fish. The colors red, blue, and green refer to Tg/Tg, Tg/+, and +/+ fish, respectively. The patterns of fatty acid composition and content

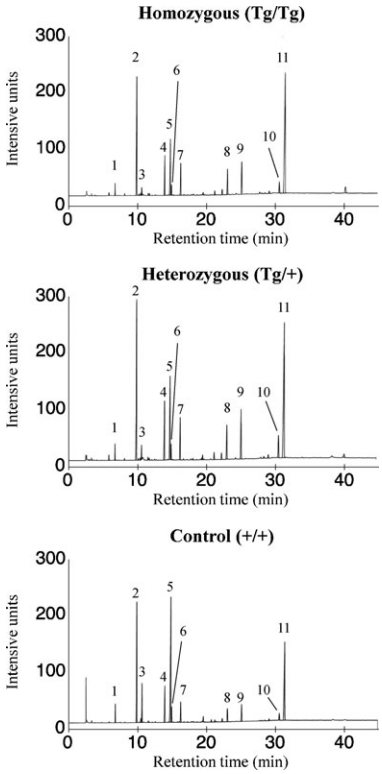

Fig. 1. Fatty acid analysis of liver extracted from homozygous (Tg/Tg) and heterozygous (Tg/+) GH transgenic Amago and the control (+/+) using Gas Chromatograhy. The signal peaks indicate 1: Myristic acid (14:0), 2: Palmitic acid (16:0), 3: Palmitoleic acid (16:1n-7), 4: Stearic acid (18:0), 5: Oleic acid (18:1n-9), 6: cis-Vaccenic acid (cis-18:1n-7), 7: Linoleic acid (18:2n-6), 8: Arachidonic acid (20:4n-6), 9: Eicosapentaenoic acid (20:5n-3), 10: Docosapentaenoic acid (22:5n-3), 11: Docosahexaenoic acid (22:6n-3).

were generally similar with some exceptions. The relative contents of saturated and monounsaturated fatty acid (SFA and MUFA) shown in Fig. 2 were trended higher in the +/+ fish than in the GH transgenic fish, particularly in the first six cases (myristic acid to cis-vaccenic acid) (Fig. 2), while for the rest of the fatty acids, the tendency was mostly the opposite. The +/+ fish showed significantly ($P<0.05$) higher compositions than the GH transgenics for myristic acid (14:0), palmitoleic acid (16:1n-7), and cis-vaccenic acid (cis-18:1n-7). In the case of content, 14:0 and 16:1n-7 were significantly ($P<0.05$) higher in the +/+ fish than in the GH transgenics.

For those between linoleic acid and docosapentaenoic acid in Fig. 2, the fatty acids content was generally higher in the GH transgenic fish than in the +/+ fish and, within the GH transgenics, mostly higher in the Tg/Tg than in the Tg/+. The compositions of linoleic acid (18:2n-6), arachidonic acid (20:4n-6), and docosapentaenoic acid (22:5n-3) were significantly ($P<0.05$) higher than those of +/+ fish, with the distribution in the descending order from the Tg/Tg, Tg/+ to +/+ fish. As to the content, only 22:5n-3 was significantly ($P<0.05$) higher in the Tg/Tg than in the +/+ fish. Contrary to the results of PUFAs, those of docosahexaenoic acid (22:6n-3) showed a decreased tendency in the GH transgenic fish. However, total fatty acid content of the liver from Tg/Tg was slightly decreased or not altered, compared with that of +/+ fish (Fig. 3).

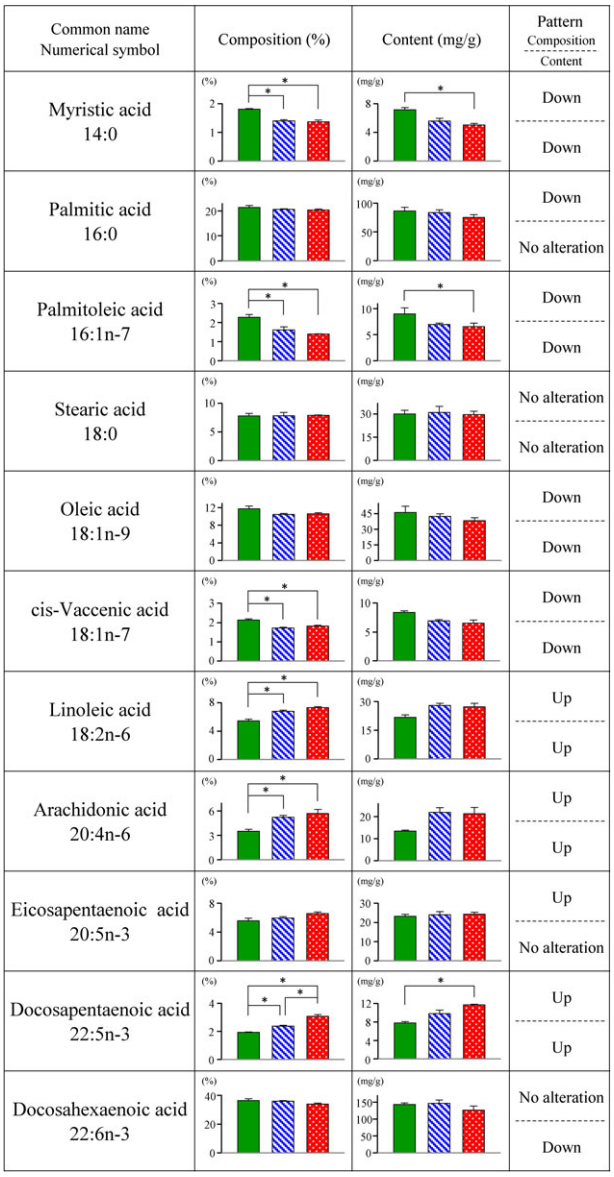

Fig. 2. Analysis of fatty acid compositions (%) and contents (mg/g) of liver from homozygous (red) and heterozygous (blue) GH transgenic Amago and the control (green). The percentage compositions of fatty acids were calculated, based on the peak areas from only eleven detected peaks. The contents of fatty acids were calculated using an internal standard heptadecanoic acid (17:0). Patterns of composition and content are shown as Up or Down in comparison with the control. Data are presented as means ± standard error. * indicates a significant difference ($P<0.05$).

3′-Tag digital gene expression profiling using liver tissue

We compared the transcriptomes from liver between the Tg/Tg and +/+ Amago by means of 3′-tag digital gene expression profiling. As shown in Table 1, 1.6 and 1.3 million reads (tags) were obtained from liver tissue of the Tg/Tg and +/+ fish, respectively. In these reads, multiple gene copies were eliminated and, therefore, the results represented the number of unique transcripts (genes).

These sequences were matched against the *O. mykiss* and *S. salar* databases. The numbers of registered genes in these databases were (at the time of our analysis) 8380 and 19116, respectively, and there were 8379 and 8532 unique matches for the +/+ and Tg/Tg fish, respectively. We then selected genes involved in fatty acid synthesis and catabolism, glucose starvation, inflammation, and hypoxia from these matches (Table 1), and summarized the expression profile on the FA metabolic pathway (Fig. 3). Concerning fatty acid synthesis, S-acyl fatty acid synthase thioesterase (TE-B), Mid1 interacting protein 1 (Mid1ip1), which is also known as glucose starvation response gene, acyl-CoA desaturase (Δ9FAD), Δ-6 fatty acyl desaturase (Δ6FAD), and elongation of long chain fatty acids 5b (ELOVL5b), were down-regulated in the Tg/Tg fish compared to the +/+ fish. On the other hand, genes concerning fatty acid catabolism, such as long-chain-fatty-acid–CoA ligase 1 (ACSL1) and acyl-coenzyme A oxidase 3 (ACOX3), were drastically up-regulated in the Tg/Tg fish when compared to the +/+ fish. Glucose-regulated protein 78 kDa (GRP78), which is known as glucose and hypoxia response gene, was about 10-fold greater in Tg/Tg than in +/+ fish. Genes involving inflammation, such as macrophage migration inhibitory factor (MIF), also known as hypoxia response gene, and inducible nitric oxide synthase (iNOS), were up-regulated, and thioredoxin (TRX) was down-regulated in the Tg/Tg fish.

3-Hydroxybutyric acid in liver tissue and serum triacylglycerol and glucose

The relative values of 3-hydroxybutyric acid were seen to be highest in the Tg/Tg fish and lowest in +/+ Amago (Fig. 3). On the other hand, the opposite trend was seen in the concentration of serum triacylglycerol and glucose, with the +/+ fish showing the highest values and Tg/Tg fish the lowest (Fig. 3). Furthermore, the differences here were statistically significant, even at $P<0.001$, for the comparison between the +/+ and Tg/Tg fish.

Discussion

MUFAs have been shown to be preferentially oxidized for energy when compared to long chain saturated fatty acids (Sidell et al., 1995). Therefore, when the rate of fatty acid decrease was used as an index of β-oxidation rate of fatty acid in carp dark muscle mitochondria, it was determined that the rate of decrease of 18:1 and 16:1 was high, and that it was distinctly lower in 16:0, 18:0, 18:2, 18:3, 20:4 and 22:6 when compared to MUFA (Murata and Higashi, 1979). These reports indicate that short and monounsaturated fatty acids such as 18:1or 16:1 are decreased faster than long chain PUFAs during energy production. This difference may be due to the structure of the fatty acid binding pocket in the ACSL1, an enzyme important for transporting fatty acids into mitochondria as fuel for β-oxidation (Grove and Sidell, 2004).

Clearly, higher energy production is required to sustain the greatly accelerated growth of GH transgenic Amago, compared to the +/+ fish. In this experiment, the average weight of Tg/Tg fish was greater than that of Tg/+ or +/+ fish. Therefore, higher levels of oxidation of fatty acids is expected in the GH transgenic fish compared to the +/+ fish, and it is possible that the composition and contents of fatty acids 14:0 to 18:1n-7 were generally higher in the +/+ fish than in the GH transgenics (Fig. 2). Our speculation about energy shortage in GH transgenic fish is also consistent with the higher expression of the ACSL1 (934-fold) and ACOX3 (14.3-fold). ACSL1 has an important role for β-oxidation (Grove and Sidell, 2004), as described in the previous paragraph. ACOX3, also called pristanoyl-CoA oxidase, is known to contribute to the first, and presumed rate-limiting,

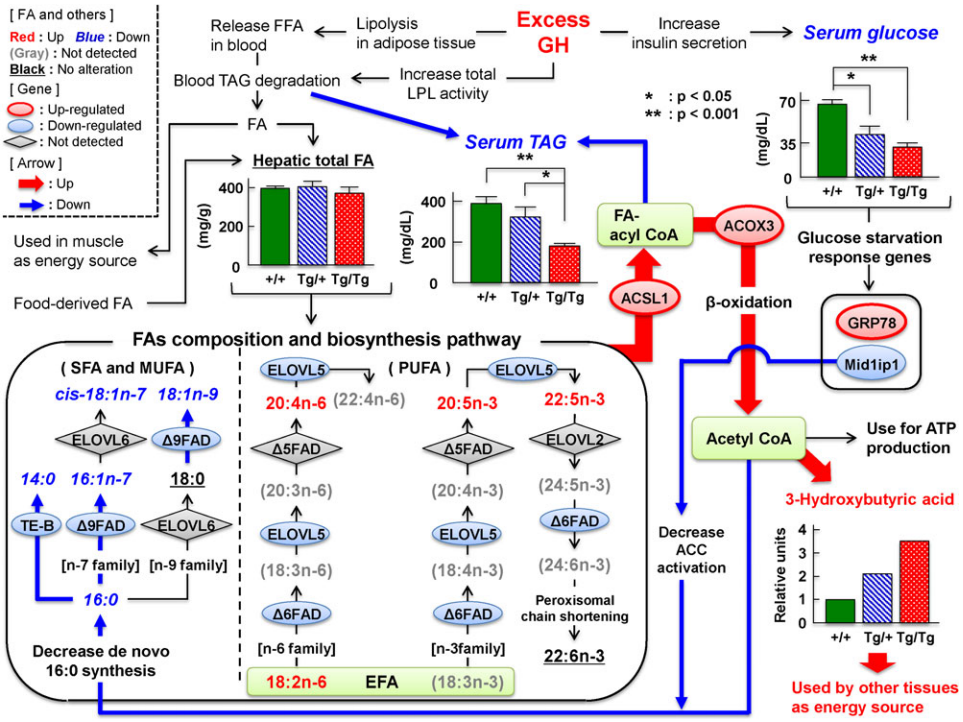

Fig. 3. Flow chart depicting the metabolic pathway of fatty acids in the liver of GH transgenic Amago based on 3′-tag digital gene expression profiling and comparison of serum triacylglycerol and glucose, and 3-hydroxybutyric acid. Values for serum triacylglycerol and glucose were expressed as means ± standard error. The statistical significance was determined by one-way analysis of variance followed by *a posteriori* comparison of the significant ANOVA results using Dunnet's T3 test. Abbreviations: ACC, acetyl-CoA carboxylase; ACOX3, acyl-CoA oxidase 3; ACSL1, long-chain-fatty-acid–CoA ligase 1; ATP, adenosine triphosphate; ELOVL2, elongation of long chain fatty acids 2; ELOVL5, elongation of long chain fatty acids 5; ELOVL6, elongation of long chain fatty acids 6; FA, fatty acid; EFA, essential fatty acid; FFA, free fatty acid; GRP78, glucose-regulated protein 78 kDa; LPL, lipoprotein lipase; Mid1ip1, Mid1 interacting protein 1; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol; TE-B, S-acyl fatty acid synthase thioesterase; Δ5FAD, Δ-5 desaturase; Δ6FAD, Δ-6 fatty acyl desaturase; Δ9FAD, acyl-CoA desaturase; Tg/Tg, homozygous GH transgenic Amago; Tg/+, heterozygous GH transgenic Amago; +/+, control Amago.

Table 1. A summary of the data from 3′-tag digital gene expression profiling. 3′-tag digital gene expression profiling was done using total RNA extracted from liver tissue from five different homozygous GH transgenic Amago (Tg/Tg) and the control (+/+). The gene tags sequenced using an Illumina GA IIx sequencer were used to query reference genes using BLAST, which yielded *Oncothynchus mykiss* and *Salmo salar* genes from GenBank of NCBI.

Mapping results		
Parameters	+/+	Tg/Tg
Reads	1,654,609	1,327,349
Kind of reads	207,801	166,839
Number of Gene ID		
O. mykiss	2,341	2,387
S. salar	6,038	6,145
Total	8,379	8,532

List of genes involved in fatty acid biosynthesis and catabolism, glucose starvation, inflammation, and hypoxia obtained by 3′-tag digital gene expression profiling

	Gene Name	Abbreviation	Accession No.	Counts +/+	Tg/Tg	-fold difference
Fatty acid biosynthesis and catabolism						
(Up)	Long-chain-fatty-acid–CoA ligase 1	ACSL1	BT058879	1	934	934
	Acyl-coenzyme A oxidase 3	ACOX3	BT058841	3	43	14.3
	Elongation of long chain fatty acids 5a	ELOVL5a	GU238431	92	141	1.5
(Down)	S-acyl fatty acid synthase thioesterase	TE-B	BT073300	259	1	259
	Mid1-interacting protein 1*	Mid1ip1	BT074222	215	2	107.5
	Acyl-CoA desaturase	Δ9FAD	BT059328	1,640	36	45.6
	Δ-6 fatty acyl desaturase	Δ6FAD	NM_001123575	3,558	341	10.4
	Elongation of long chain fatty acids 5b	ELOVL5b	GU324549	347	52	6.7
Glucose starvation						
(Up)	Glucose-regulated protein 78 kDa**	GRP78	AB196459	8	82	10.3
Inflammation						
(Up)	Macrophage migration inhibitory factor**	MIF	BT073684	13	171	13.2
	Inducible nitric oxide synthase	iNOS	AJ295231	268	749	2.8
(Down)	Thioredoxin	TRX	BT074362	206	8	25.8

*also known as glucose starvation response gene.
**also known as hypoxia response gene.

step in the branched-chain β-oxidation pathway (Westin et al., 2007). These suggest the enhancement of fatty acid catabolism in the GH transgenic fish.

Interestingly, although the fish were fed to satiation in the study, the pattern of fatty acids of GH transgenic fish shown in Fig. 2 appears to indicate some energy shortage. Undernourishment might explain decreased serum triacylglycerol (TAG) and serum glucose, as well as the trend towards increased 3-hydroxybutyic acid, a ketone body (Fig. 3). Both are normal vertebrate responses to fasting/low feeding rate. Changes in fatty acid distribution in polar lipids and TAG from liver tissue of rainbow trout have been reported during starvation (Satoh et al., 1984). Specifically, fatty acid 18:1 drastically decreased, while 16:0, 16:1, 18:2n-6 and 22:5n-3 increased, during starvation.

There is a decrease in SFA and MUFA synthesis in GH transgenic Amago. Carbohydrates are processed by the liver and, when there is excess, the energy from carbohydrates is transformed into fat (triacylglycerol). Because GH transgenic fish are already somewhat starved, there will be little excess carbohydrate, because carbohydrates are used directly by starved tissues. Therefore, less *de novo* FA synthesis occurs.

De novo 16:0 was generated by fatty acid synthase (FAS) for the sequential 2-carbon elongation reactions using malonyl-CoA produced by Acetyl-CoA carboxylase (ACC), a rate-limiting enzyme of *de novo* FA synthesis (Kim et al., 2010). Mid1 interacting protein 1 (Mid1ip1, also known as MIG12, S14-R) is an important factor in enhancing ACC activity (Kim et al., 2010; Inoue et al., 2011), and it was a glucose-responsive target of carbohydrate regulatory element binding protein (ChREBP) (Tsatsos et al., 2008). The mRNA and protein of Mid1ip1 were reduced in the fasted state (Tsatsos et al., 2008; Kim et al., 2010). The serum level of glucose in the GH transgenic Amago was significantly decreased ($P<0.05$) compared with that of +/+ fish, and the Mid1ip1 mRNA level was down-regulated (Fig. 3; Table 1). These data suggest that low blood glucose caused a down-regulation of Mid1ip1 mediating ChREBP, which reduced ACC activation, resulting in attenuation of *de novo* FA synthesis, observed as a decrease of 16:0 in the GH transgenic fish.

This low blood glucose down-regulated *de novo* synthesis of 16:0 is caused by two factors. First is the increase of insulin secretion to suppress the elevated blood glucose level by GH-mediated glycolysis. In GH transgenic mice, the serum insulin level was higher and the serum level of glucose was lower compared to control (Frick et al., 2001). Second is starvation. In the state of starvation, Mid1ip1 expression in +/+ fish was decreased to almost the same level as in the GH transgenics (unpublished). These data indicate that Mid1ip1 expression was decreased by starvation in non-transgenic control Amago.

Glucose-regulated protein 78 kDa (GRP78), also known as glucose response gene, was 10.3-fold up-regulated in the Tg/Tg fish (Table 1), and its expression is induced by glucose starvation (Lee et al., 1983). Microarray analysis of zebrafish exposed to starvation showed Δ9FAD, Δ6FAD and ELOVL5 were down-regulated (Drew et al., 2008), and these genes were also down-regulated in Tg/Tg fish (Table 1). Therefore, these data further support the GH transgenic Amago being in a state of starvation, as described in the previous paragraph.

If there would be less need for *de novo* FA synthesis, the genes involved in FA synthesis will be down-regulated as shown in Fig. 3.

When there is less *de novo* synthesis of FAs (or even simply less *de novo* synthesis relative to food intake), there will be less 16:0 (%) entering the fatty acid pool. The fatty acids 14:0, 16:0, 16:1n-7, 18:0, 18:1n-7 and 18:1n-9 can all be produced *de novo* from carbohydrate precursors. But if *de novo* FA synthesis is decreased (or even decreased relative to total energy intake), then all of these fatty acids (which are rooted at 16:0, the main product of *de novo* FA synthesis) will likely be decreased as shown in Fig. 3.

If *de novo* FA synthesis is decreased, then the percentage of essential fatty acids will increase (this must occur percentagewise). This explains the increase in 18:2n-6 in the composition (%) as shown in Fig. 2. This also explains an increase in 20:4n-6 in spite of decreased ELOVL5 and Δ6FAD activity. Similarly, this explains the increased percentage of 20:5n-3.

However, it is difficult to explain the increase of PUFA content (mg fatty acid/gram tissue) such as 20:4n-6 and 22:5n-3 and the decrease of that of 22:6n-3 using only this hypothesis, since the increase of the total amount of PUFAs compensated decreased the total amount of SFA and MUFA. Therefore, the total amount of hepatic FA in the GH transgenic fish reached almost the same level as that of the +/+ fish (Fig. 3). 18:2n-6 is a kind of essential fatty acid which cannot be synthesized in animals, including mammals and fish, due to a lack of Δ12-desaturase (Tvrzicka et al., 2011). For this reason 18:2n-6 must be taken from the diet. 18:2n-6 comprises 24% of the total FA in the food (supplementary material Table S1) and, furthermore, GH transgenic salmon have a larger appetite than control, and have an enhanced feed conversion efficiency (Devlin et al., 1999; Cook et al., 2000; Fredrik Sundström et al., 2004). We suggest that GH transgenic Amago were eating more food than +/+ fish, and this may lead to the increase of 18:2n-6.

On the other hand, the reason for the increase of 20:4n-6 (1.0% in the food) and 22:5n-3 (1.1% in the food) was difficult to attribute to being derived from their food since the composition of these PUFA in food is lower than 18:2n-6 (24.3% in the food). Furthermore, the activity of n-3 and n-6 PUFA synthesis pathways seems to be lower in GH transgenic fish than in +/+ fish due to a down-regulation of the Δ6FAD and ELOVL5 genes, which are important for desaturation and elongation of essential fatty acids of the n-3 and n-6 families (Table 1; Fig. 3). Meanwhile, the liver from GH transgenic Amago showed vasodilation and presumably angiogenesis (Sugiyama et al., 2010). The activation of transcription factor hypoxia inducible factor (HIF) is important for angiogenesis (Ke and Costa, 2006), and GRP78 and macrophage migration inhibitory factor (MIF) gene expression are known to induced by hypoxia (Table 1) (Song et al., 2001; Fu et al., 2010). These data suggest that the liver from GH transgenic Amago was exposed to hypoxia, and there is a report that n-3 and n-6 FAs were increased in the hypoxic liver (Bruder et al., 2005). This might be connected to the result of an increase in PUFA such as 18:2n-6 in the liver from GH transgenic Amago.

However, content of 20:5n-3 and 22:6n-3 in the liver from GH transgenics showed no alteration or decrease, regardless of the content of 22:6n-3 in the feed being about 10% of the total fatty acid (supplementary material Table S1). This means that there is a possibility that 20:5n-3 and 22:6n-3 had been metabolized into other substances. 22:6n-3 (DHA) supplementation led to increased formation of DHA-derived lipid mediators such as 17-HDHA and protectin D1, which were able to protect the liver from CCL4-induced

necroinflammatory damage (González-Périz et al., 2006). Similarly, 20:5n-3 is metabolized into 18-hydroxyeicosapentaenoic acid and it also effectively suppresses lipopolysaccharide-induced TNF-α secretion from macrophages (Weylandt et al., 2011). On the other hand, morphological changes such as abnormal fissures and vasodilation were observed in the liver from the GH transgenic Amago (Sugiyama et al., 2010), and decrease of some immunologically-relevant gene and a significant lower ($P<0.05$) activity of serum lysozyme involved in natural immunity in GH transgenic fish were reported in our previous paper (Mori et al., 2007). Therefore, we have speculated that the liver from the GH transgenic Amago was inflamed and might have some disorder. Furthermore, 3′-tag digital expression profiling showed that macrophage migration inhibitory factor (MIF) was 13.2-fold up-regulated, thioredoxin (TRX), a redox-active protein with antioxidative property, was 25.8- fold down-regulated, and inducible nitric oxide synthase (iNOS) was 2.8-fold up-regulated in the Tg/Tg fish compared with +/+ fish (Table 1). MIF has been well known as a potent pro-inflammatory mediator (Baugh and Donnelly, 2003). TRX has a specific affinity with MIF, and suppresses airway inflammation by inhibiting MIF production (Son et al., 2009; Torii et al., 2010). Because of up-regulation of MIF and down-regulation of TRX in the Tg/Tg fish, it is likely that the liver in the GH transgenic Amago is inflamed.

Furthermore, iNOS gene expression is induced by pro-inflammatory mediators such as cytokines and endotoxins (Wahl et al., 2003; Lee et al., 2004). Moreover, nitric oxide (NO) is known to exert vasodilator activity (Moncada et al., 1988), and this is consistent with the vasodilation observed in the liver from GH transgenic Amago (Sugiyama et al., 2010). NO is a free oxygen radical and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (Aktan, 2004). Furthermore, it has been reported that NO productivity is increased according to increase of iNOS (Hallemeesch et al., 2003). These results suggest that the liver from Tg/Tg fish may have higher NO concentration than those of +/+ fish, and this may lead to vasodilation and inflammation. Therefore, 20:5n-3 and 22:6n-3 may have been metabolized to repress the liver inflammation in the Tg/Tg fish.

In this experiment, we depicted overall metabolism occurred in the liver from GH transgenic Amago by changes in fatty acid content and composition. A decrease in serum glucose suppressed Mid1ip1, and caused a decrease of *de novo* 16:0 synthesis, and then followed decreases in SFA and MUFA. This induced expression of ACSL1 and ACOX3 to produce energy through β-oxidation. This β-oxidation also produced 3-hydroxybutyric acid. On the other hand, this research also implied a connection between these changes of PUFA content and the physical condition predicted by morphological changes in the liver of GH transgenic Amago.

Acknowledgements

This work was funded in part by Nihon University College of Bioresource Sciences Research Grant for 2011 and Japanese grants from the Educational Ministry (23380117).

Competing Interests

The authors have no competing interests to declare.

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