Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Here, we describe optimal conditions for the culture of rainbow trout (*Oncorhynchus mykiss*) pre-adipocytes obtained from adipose tissue and their differentiation into mature adipocytes, in order to study the endocrine control of adipogenesis. Pre-adipocytes were isolated by collagenase digestion and cultured on laminin or 1% gelatin substrate. The expression of proliferating cell nuclear antigen was used as a marker of cell proliferation on various days of culture. Insulin growth factor-I stimulated cell proliferation especially on days 5 and 7 of culture. Tumor necrosis factor α (TNF α) slightly enhanced cell proliferation only at a low dose. We verified the differentiation of cells grown in specific medium into mature adipocytes by oil red O (ORO) staining. Quantification of ORO showed an increase in triglycerides throughout culture.

Immunofluorescence staining of cells at day 11 revealed the expression of CCAAT/enhancer-binding protein and peroxisome proliferator-activator receptor γ , suggesting that these transcriptional factors are involved in adipocyte differentiation in trout. We also examined the effect of TNF α on the differentiation of these adipocytes in primary culture. TNF α inhibited the differentiation of these cells, as indicated by a decrease in glycerol-3-phosphate dehydrogenase activity, an established marker of adipocyte differentiation. In conclusion, the culture system described here for trout pre-adipocytes is a powerful tool to study the endocrine regulation of adipogenesis in this species.

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Introduction

The high lipid content of diets used in aquaculture can induce an increase in fat depots that may affect animal health and decrease the productivity of this sector, especially in salmonids. In fish, like in mammals, the development of adipose tissue and accumulation of lipids is a continuous process that depends on genetic, hormonal, and dietary factors, which include the hypertrophy of existing adipocytes and the proliferation and differentiation of new ones. Therefore, in order to understand the development of adiposity, it is crucial to identify the factors that regulate the formation of adipocytes from precursor cells in the adipose tissue. In comparison with cell culture lines, primary cell cultures are more likely to represent the *in vivo* condition because cells are removed directly from the animal and grown in relatively simple culture medium (Hausman & Poulos 2005). In mammals, primary cell cultures have contributed to unraveling the major processes involved in adipogenesis, such as the proliferation of precursor cells and the differentiation of these cells into adipocytes. This type of culture system has been developed only in two fish species (Vegusdal *et al.* 2003, Oku *et al.* 2006). Nevertheless, from the data obtained in mammals, it can be concluded that factors that regulate adipogenesis differ considerably between species.

Furthermore, the use of a primary cell culture from a simple model, such as a vertebrate ectotherm, may allow study of the basic mechanisms of adipocyte biology and thereby contribute to clarifying pathological situations like obesity in humans.

Proliferation and differentiation are usually alternative and mutually exclusive pathways for cells (Freitag 1988, Freitag & Geddes 1992), and growth arrest is considered a requirement for adipocyte differentiation (Smyth *et al.* 1993). Among the multiple factors involved in this process in mammals, CCAAT/enhancer-binding protein (C/EBP) and peroxisome proliferator-activator receptor γ (PPAR γ) have been identified as transcriptional factors (MacDougald & Lane 1995) and are necessary for the transition of pre-adipocytes into adipocytes *in vitro*.

In mammals, insulin growth factor-I (IGF-I) affects adipocyte proliferation and differentiation *in vivo* and *in vitro* (Wright & Hausman 1995, Butterwith 1997, Gregoire *et al.* 1998, MacDougald & Mandrup 2002). This factor exerts its biological effects through a complex signaling pathway that involves the activation of specific cell surface receptors (Blüher *et al.* 2005). IGF-I is a mitogenic and differentiation factor for various mammalian cell lines, and primary stromal-vascular cell cultures, including pre-adipocytes (Smith *et al.* 1988, Schmidt *et al.* 1990, Richardson *et al.* 1998). IGF-I has

mitogenic and anabolic effects in fish myocytes in culture (Castillo *et al.* 2004). This growth factor induces proliferation, measured as thymidine incorporation, in primary cell cultures of trout myocytes (Castillo *et al.* 2004) as well as DNA synthesis in zebrafish embryonic cells (Pozios *et al.* 2001). However, to our knowledge, the effects of IGF-I in fish adipocyte lineage have not been studied, although IGF-I binding has been characterized in brown trout (*Salmo trutta*) adipose tissue (Planas *et al.* 2000).

Tumor necrosis factor α (TNF α) is a multifunctional cytokine that is synthesized and secreted from macrophages and adipose tissue in mammals (Hotamisligil *et al.* 1994), and its expression and production increases with augmented adipocyte size (Morin *et al.* 1997). The catabolic and anti-adipogenic effects of TNF α decrease adipose conversion in various cell lines as well as in rat, porcine, and human primary cells (Grégoire *et al.* 1992, Hotamisligil *et al.* 1994, Vassaux *et al.* 1994, Boney *et al.* 1996, Boone *et al.* 2000). TNF α acts directly on lipid metabolism by decreasing parameters related with fatty acid uptake (decrease in lipoprotein lipase (LPL), fatty acid transport (FAT), and fatty acid transport protein (FATP) expressions) and lipogenesis (decrease in fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) expression, and glycerol-3-phosphate dehydrogenase (GPDH) activity), and by increasing lipolysis (Dani *et al.* 1989, Sethi & Hotamisligil 1999). TNF α mRNA expression has been reported in primary rainbow trout monocytes (Zou *et al.* 2002) and in adipose tissue of sea bream (Saera-Vila *et al.* 2007). In rainbow trout (*Oncorhynchus mykiss*), TNF α contributes to lipid metabolism by stimulating lipolysis *in vivo* and *in vitro* (Albalat *et al.* 2005). The interaction of TNF α and IGF-I on adipocyte development has also been examined. The specific responses of developing pre-adipocytes to TNF α may be influenced by the presence of IGF-I (Kras *et al.* 2000).

Very few studies on the culture of pre-adipocytes *in vitro* have been attempted in fish (Vegusdal *et al.* 2003, Oku *et al.* 2006). A protocol for studying the proliferation and differentiation of these cells in Atlantic salmon adipose tissue has been described (Vegusdal *et al.* 2003). These authors observed that PPAR γ , C/EBP, and leptin were expressed in mature adipocytes grown in culture, as shown for mammals, and the supplementation of 10% of fetal bovine serum (FBS) to the culture medium did not affect pre-adipocyte differentiation. Furthermore, the effects of insulin, tri-iodothyronine (T₃), and fat-soluble vitamins (all-*trans* retinoic acid, retinyl acetate, and 1,25 dihydroxyvitamin D₃) on adipocyte differentiation and LPL gene expression have been analyzed in cultures derived from the stromal-vascular cells of red sea bream (*Pagrus major*), using serum-free culture conditions (Oku *et al.* 2006).

Nevertheless, information on the regulation of adipogenesis is still scarce and the role of IGF-I and TNF α has not been analyzed in fish adipocytes. Rainbow trout is one of the most common cultured fresh water fish species. The final product can be affected by excess visceral fat and therefore a greater understanding of adipogenesis in this species is of value to

aquaculture industries. The establishment of culture conditions that favor the proliferation and differentiation of trout pre-adipocytes is required to gain a better understanding of molecular and physiological mechanisms of adipose tissue development and accumulation. Here, we define the optimal conditions for the culture of rainbow trout pre-adipocytes and their differentiation into mature adipocytes, in order to study the control of these processes by endocrine and adipokinetic factors. Specifically, we analyzed the effect of serum, IGF-I, and TNF α on pre-adipocyte proliferation and the action of TNF α on adipocyte differentiation. The presence of differentiating transcription factors, such as PPAR γ and C/EBP, was also demonstrated.

Materials and Methods

Cell preparation and culture conditions

Rainbow trout (*O. mykiss*) between 200 and 250 g were obtained from the 'Truchas del Segre' fish farm (Lleida, Spain). Fish were acclimatized to environmental conditions at 18 $\pm$ 1 °C and natural photoperiod in facilities at the University of Barcelona (Faculty of Biology) in closed circuit flow systems. They were fed daily *ad libitum* with a commercial diet (DIBAQ AQUATEX, Segovia, Spain). Fish were killed by a sharp blow to the head. They were weighed and perivisceral adipose tissue was excised by sterile dissection. This tissue was minced into sections of $\sim$ 1 mm², and after one wash with Krebs-HEPES buffer (pH 7.4), the fragments were incubated with digestion medium Krebs-HEPES buffer containing 1% BSA (Sigma-Aldrich) and 130 UI/ml collagenase (type II, Sigma-Aldrich). The minced tissue was incubated for 1 h with gentle shaking at a speed of 50 shakes/min in a water bath at 18 °C. The cell suspension was passed through a 100 μ m filter to remove large undigested tissues. The suspension was then centrifuged at $\sim$ 700 g for 10 min. Floating mature adipocytes and medium were removed. The cell pellet was treated with erythrocyte lysing buffer (0.154 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM sodium EDTA) for 10 min at room temperature. Cells were washed with Krebs-HEPES buffer containing 1% BSA followed by centrifugation at 700 g for 10 min. The sedimentary cells were resuspended in growth medium: Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, and 9 mM bicarbonate antibiotics. Cells were counted, diluted, and plated into 24-well plates treated with laminin/poly-D-lysine (LP) or 1% gelatin at a density of 2×10^4 cells/cm² in the medium described above. Cells were maintained at 18 °C in humidity of 3% CO₂ atmosphere. After 24 h, unattached cells were removed by extensive washing with DMEM. The adherent cells were subsequently maintained in the growth medium until reaching confluence after 1 week. They were then induced to differentiate by means of growth medium supplemented with 10 μ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μ M dexamethasone, and

10 µl/ml lipid mixture, which contained 45 µg/ml cholesterol, 100 µg/ml cod liver oil FA, 250 µg/ml polyoxyethylene sorbitan monooleate, and 20 µg/ml D- α -tocopherol acetate (Sigma-Aldrich; referred to as differentiation medium). Cells in differentiation and growth medium were grown in parallel. Media were replaced every 2 days.

Cell proliferation and IGF-I effects

Cell proliferation was evaluated by immunostaining with a primary mouse monoclonal antibody directed against the proliferating cell nuclear antigen (PCNA; clone PC10; Zymed Laboratories, Inc., Barcelona, Spain). We compared cell proliferation in plates pre-coated with LP and 1% gelatin at 10% FBS (days 3, 5, 7, 9, and 11).

To test the effects of IGF-I on pre-adipocyte proliferation, the plates were coated with 1% gelatin. Cells were incubated with or without recombinant human IGF-I at 100 nM (Reutlingen, Germany), in a medium containing 2 or 5% FBS (days 3, 5, 7, and 9).

Cells from both the experiments were immunostained for PCNA on days 3, 5, 7, and 9. The procedure was followed simultaneously in cells with and without IGF-I treatment. Cells were washed in PBS and fixed in ethanol for 20 min at 4 °C. Cells were then treated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity. After three washes, primary antibody raised against PCNA was applied for 1 h, the goat anti-mouse biotin-labeled secondary antibody was added for 10 min; and the avidin-biotin peroxidase complex (ABC; Dako) was applied for 10 min. 3,3'-diaminobenzidine was used as the chromogen. Cells were counterstained with Mayer's hematoxylin for 2 min and washed in water. Finally, cells were dehydrated in a graded alcohol series and mounted with histomount. The PCNA-labeling index was expressed as the percentage of the number of PCNA-labeled nuclei divided by the total number of nuclei examined under a microscope.

Combined effect of IGF-I and TNF α on pre-adipocyte proliferation

Another experiment was performed to study the combined effect of IGF-I and TNF α on cell proliferation. Twenty-four hours after seeding on 1% gelatin, cells were cultured in DMEM containing 2 mM L-glutamine, 10 mM HEPES, 9 mM bicarbonate antibiotics, and 2% FBS. Cells were then exposed to recombinant human IGF-I (Mediagnost; 100 nM) or human recombinant TNF α at 10 and 100 ng/ml (Sigma-Aldrich) in separate wells. Besides, IGF-I (100 nM) was added together with TNF α at different concentrations (10 and 100 ng/ml). Cells cultured in growth medium in the absence of both hormones were used as control.

On day 5, cells were immunostained with a primary mouse monoclonal antibody directed against the PCNA (clone PC10; Zymed Laboratories, Inc.) to evaluate proliferative cells as described above.

Pre-adipocyte differentiation

Oil red O (ORO) staining of cell culture To visualize cell morphology and lipid accumulation, on day 11, cells cultured in the two media (growth and differentiation medium) were stained with ORO following Ramírez-Zacarias *et al.* (1992). Media from three culture wells per condition were aspirated. After two washes, cells were fixed with cold 10% neutral buffered formalin for 30 min and then rinsed with water. They were then stained with ORO (0.35 g, 60% isopropanol) for 2 h. Cells were exhaustively rinsed with water and the nuclei were counterstained using Mayer's hematoxylin for 2 min. Finally, stained cells were rinsed with running tap water.

Quantification of lipid accumulation

Estimation of cell accumulation of lipid droplets as triglycerides was based on the protocol described by Laughton (1986) with slight modifications. The procedure is based on ORO staining of neutral lipids and Coomassie brilliant blue staining of cellular protein. Cells from three wells from three separate experiments were fixed with perchloric acid and after washing were incubated with ORO dissolved in propylene glycol (2 mg/ml) for 2 h at room temperature. Cells were first washed thrice with 85% propylene glycol and twice with water. The ORO within the lipid droplets was extracted using isopropanol for 10 min at room temperature. Sample (100 µl) was measured using a microplate reader at 490 nm and blanked to cell-free wells. Next, the wells were emptied and washed for protein determination. Cells were incubated with Coomassie brilliant blue for 1 h at room temperature. After washing the cells twice with water, they were incubated with propylene glycol for 3 h at 60 °C to extract Coomassie stain. Sample (100 µl) was measured using a microplate reader at 630 nm and blanked to cell-free wells. The specific lipid content was calculated as the ratio of absorbance value obtained for ORO and Coomassie blue staining.

Assay of GPDH activity

Cell differentiation was assessed by quantifying GPDH activity. The measurement was performed following Sottile & Seuwen (2001) with slight modifications. Cells were grown and induced to differentiate in 24-well plates. At day 16, cells were washed with PBS and an ice-cold homogenization solution was added (20 mM Tris-HCl, 1 mM EDTA, and 1 mM β -mercaptoethanol, pH 7.3) and cells were then stored at -20 °C for 1 h. Frozen plates were allowed to thaw at room temperature. After mechanical processing of samples with a gauge needle, the assay mixture was added to each well (0.1 M triethanolamine, 2.5 mM EDTA, 0.1 mM β -mercaptoethanol, and 334 µM NADH, pH 7.7) and plates were incubated for 10 min at 30 °C. The reaction was started by adding 4 mM dihydroxyacetone phosphate. GPDH

activity was measured spectrophotometrically at 340 nm. The protein content of cell cultures was determined by the Bradford method. Results were expressed as mU/mg protein (1 U = 1 μ mol NADH/min).

Immunofluorescence staining

Cells cultured on glass cover slips until day 11 were fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. After washing with PBS–glycine (PBS–Gly), cells were permeabilized with 0.05% Triton X-100 in PBS–Gly for 10 min at room temperature. After being washed with PBS–Gly, cells were blocked with 1% BSA in PBS–Gly for 20 min at room temperature. They were then incubated with primary antibody PPAR γ (1:50) or C/EBP (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS–Gly containing 1% BSA for 1 h at room temperature, washed for 10 min in PBS–Gly, and incubated with an Alexa Fluor488-conjugated goat anti-rabbit antibody (1:500), as a secondary antibody, in combination with Hoechst (1:1000). After washing with PBS, cells were mounted with Mowiol, and immunofluorescence was captured with a confocal microscope (Confocal Olympus Fluoview 500).

Immunoprecipitation

Cells at day 11 were treated with lysis buffer (10 mM Tris–HCl, 140 mM NaCl, 5 mM EDTA, 1 mM phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride, 50 mM sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1% Nonidet P-40, pH 7.6). The cell suspension was centrifuged for 15 min at 16 000 *g* and the supernatant was stored at -80°C until analysis. Lysates were pre-cleaned by the addition of 50 μ l Protein A-Sepharose (GE Healthcare Bio-Sciences AB) in immunoprecipitate buffer (10 mM Tris–HCl, 140 mM NaCl, and 5 mM EDTA). After 1 h at 4°C , samples were centrifuged for 30 s at 16 000 *g*, the supernatant was transferred to a fresh tube, and incubated with 2 μ g anti-PPAR γ antibody for 1 h. Subsequently, 50 μ l Protein A-Sepharose was added and incubated overnight at 4°C . Samples were washed three times in immunoprecipitate buffer and analyzed by SDS-PAGE.

Western blot

The presence of PPAR γ in cell cultures was evaluated by western blot analysis. On days 5 and 11, cells grown in the differentiation medium were lysed. After one wash in PBS, cells were harvested in lysis buffer and frozen at -80°C until analysis. Homogenates of adipose tissue from trout were used as a control to confirm the specificity of PPAR γ immunodetection. Protein content was determined using the Bradford method (Braddford 1976). Proteins (50 μ g) were separated by 10% SDS-polyacrylamide gel and transferred on PVDF membrane for 2 h at 100 V. After 3×5 min washes with buffer (10 mM Tris–HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.5), the membrane was blocked with washing

buffer containing 5% dry milk for 2 h at room temperature. The blot was probed with anti-PPAR γ antibody (Santa Cruz Biotechnology) diluted 1:500 overnight at 4°C . The following day, the membrane was incubated for 1 h at room temperature with secondary antibody conjugated to horseradish peroxidase. Antibody band was visualized by chemiluminescence (ECL).

Effect of TNF α on cell differentiation

To evaluate the effect of TNF α on adipocyte differentiation, cells were grown to confluence (day 7) and exposed to recombinant human TNF α (Sigma–Aldrich). At confluence, growth medium was replaced by differentiation medium containing DMEM, glutamine, and antibiotics as listed previously but without lipid mixture, and supplemented with insulin (10 μ g/ml), IBMX (0.5 mM), dexamethasone (0.25 μ M), and several concentrations of TNF α (1, 10, and 100 μ g/ml). Control 1 cells were cultured in growth medium, control 2 contained differentiation medium without lipid mixture while control 3 cells were cultured with differentiation medium with lipid mixture. Media were replaced every 2 days.

Statistical analysis

Data are given as mean $\pm$ S.E.M. Results were analyzed by one-way ANOVA followed by Tukey's test. The effect of substrate on proliferation was analyzed by paired *t*-test. Differences were considered significant when $P < 0.05$.

Results

Cell morphology and proliferation

Here, we provide the first description of conditions to maintain primary trout pre-adipocyte cultures. Phase contrast microscopy at day 1 showed that most cells were attached and small (Fig. 1A). Throughout their development, the cells cultured in growth medium maintained an elongated and fibroblast-like phenotype with an extended cytoplasm, devoid of large lipid droplets (Fig. 1B and C), and very few started spontaneous differentiation.

We compared the proliferation of cells grown on LP and 1% gelatin substrate in the presence of 10% FBS. Cells grown on gelatin proliferated to a slightly greater extent than those on LP at days 3 and 5 in the presence of growth medium (Fig. 2). Cells still continued to proliferate after confluence in the presence of differentiation medium, although at a slower rate, reaching the maximum and similar level of proliferation at day 9 in both LP and gelatin. At day 11, a decrease in PCNA staining was detected in both substrates, indicating diminished proliferative capacity after differentiation. Cell density increased in a similar way in the two substrates and no differences were observed in cell attachment, spread, or

Figure 1 Photomicrographs of trout pre-adipocyte cells isolated from perivisceral adipose tissue at different days of growth. Cells were grown on 1% gelatin substrate until confluence at day 7 in the presence of 10% FBS. Cells on (A) day 1, (B) day 5, and (C) day 7 ($\times 20$).

growth. We used 1% gelatin as a substrate for the subsequent experiments.

Effect of IGF-I on proliferative activity

To evaluate the effect of IGF-I on pre-adipocyte proliferation, on day 1, cells were incubated in growth medium

Figure 2 Percentage of PCNA-labeled primary culture of pre-adipocytes from rainbow trout. Data expressed as mean $\pm$ S.E.M. for days 3, 5, 7, 9, and 11, $n=3$ from three independent cultures. Where error bars are not visible, they are contained within the symbol. Different letters indicate differences between groups ($P \leq 0.05$).

supplemented with 100 nM IGF-I, at a range of FBS concentrations (2–5%) in order to minimize the effects of serum factors. IGF-I induced proliferation to a similar degree at 5 and 2% FBS (Fig. 3). A comparison of the distinct conditions at day 5 showed that proliferation was significantly lower in the absence of IGF-I at 2% FBS. At day 7 (confluence), both the decrease in serum concentration and the absence of IGF-I induced significant decreases in

Figure 3 Effect of exposure to recombinant human IGF-I on the proliferative capacity of trout pre-adipocytes. Cells were isolated and seeded at 2×10^4 cells/cm² on plates pre-coated with 1% gelatin. On day 1, cells were incubated with IGF-I (+) at 100 nM or without (-), in growth medium containing 2 or 5% fetal bovine serum (FBS), for 3, 5, 7, and 9 days. $n=3$ from three independent cultures. Data are expressed as mean $\pm$ S.E.M., and the results from the same day were compared between different groups (2 and 5% FBS, with and without IGF-I). Different letters indicate differences between groups ($P \leq 0.05$).

proliferation. At day 9, no clear effect of IGF-I was observed on the proliferative activity of differentiated cells.

Combined effect of IGF-I and TNF α on pre-adipocyte proliferation

The effect of simultaneous incubation with IGF-I and TNF α on cell proliferation was also examined. Cells were cultured in 2% FBS and exposed to several treatments: control, IGF-I (100 nM), TNF α (10 and 100 ng/ml), IGF-I (100 nM) + TNF α (10 ng/ml), and IGF-I (100 nM) + TNF α (100 ng/ml). IGF-I alone stimulated cell proliferation at day 5 (Fig. 4). The addition of TNF α at low dose (10 ng/ml) showed a tendency to stimulate cell proliferation. At 100 ng/ml, TNF α had no effect on pre-adipocyte proliferation. However, when applied together, TNF α and IGF-I promoted cell proliferation. This promotion was only significant at a low concentration of TNF α , but no synergistic effect was observed.

Cell differentiation

Identification and quantification of lipid accumulation At confluence, pre-adipocytes were induced to differentiate into adipocytes in the presence of diverse hormone/factors (10 μ g/ml insulin, 0.25 μ M dexamethasone, and 0.5 mM IBMX) and lipid mixture (see Materials and Methods). Under these conditions, typical changes in cell morphology during differentiation were observed (Fig. 5). Cells lost their fibroblast-like appearance and became larger and spherical. Cytoplasm gradually accumulated small lipid droplets. The detection of these droplets was visualized by ORO staining (Fig. 5B2). By contrast, cells incubated in

Figure 4 Effect of TNF α and IGF-I on pre-adipocyte proliferation. Cells at day 1 were cultured in growth medium and 2% of FBS, and exposed to several conditions: control (without IGF-I), 100 nM IGF-I, 10 and 100 ng/ml TNF α , IGF-I + 10 ng/ml TNF α , IGF-I + 100 ng/ml TNF α . Cells were immunostained with PCNA antibody on day 5.

Figure 5 Photomicrographs of trout adipose stromal-vascular cells and adipocytes in primary culture stained with oil red O on day 11. (A) Undifferentiated cells cultured in growth medium. (B) Differentiated cells in the presence of 10 μ g/ml insulin, 0.25 μ M dexamethasone, and 0.5 mM IBMX supplemented with lipid mixture ($\times 40$).

growth medium alone during the same period of culture preserved their fibroblast-like morphology (Fig. 5B1). Quantification of the accumulation of triglyceride containing lipid droplets throughout the culture (labeled with ORO in cells and extracted with isopropanol) is shown in Fig. 6. ORO staining on days 3–7 demonstrated that cells accumulated significantly less lipid, especially at day 3. The replacement of proliferation to differentiation medium at day 7 increased lipid accumulation, and the highest level of triglycerides was observed at day 11 of culture.

Presence of PPAR γ and C/EBP in trout adipocytes

To identify the factors involved in adipocyte differentiation, we performed immunofluorescence staining of PPAR γ and C/EBP. These two transcription factors were localized in the nuclei, as revealed by Hoechst staining (Fig. 7). Hormonal cocktail and lipid mixture contained in the differentiation

Figure 6 Specific lipid content expressed spectrophotometrically as the ratio of absorbance value obtained for ORO and Coomassie blue staining. Triglyceride accumulation in trout adipocytes cultured in growth medium (days 3, 5, and 7). On day 7, medium was replaced by the differentiation medium containing 10 μ g/ml insulin, 0.25 μ M dexamethasone, and 0.5 mM IBMX supplemented with lipid mixture, as described in Materials and Methods. Data from five experiments (four wells per experiment) are shown as mean $\pm$ S.E.M. Different letters indicate differences between groups ($P \leq 0.05$).

Figure 7 Adipocyte immunofluorescence using PPAR γ and C/EBP antibodies. All groups were incubated with secondary Ab PE-conjugated goat anti-rabbit F(ab')₂. Cell nuclei were counterstained with Hoechst. Hoechst staining is shown in the first and third panels in blue, PPAR γ staining in the second panel in green, and C/EBP α in the fourth panel in red. (A) Cells incubated with PBS, (B) proliferating cells with primary antibodies (PPAR γ , C/EBP α), and (C) differentiating cells with PPAR γ and C/EBP α primary antibodies. Bar, 10 μ m.

medium added from days 7 to 11 induced the expression of PPAR γ and C/EBP (Fig. 7C). By contrast, cells grown in growth medium showed very low protein expression (Fig. 7B). No signal was detected in differentiated cells incubated with PBS instead of PPAR γ or C/EBP antibodies (Fig. 7A).

The specificity of the band for PPAR γ detected by western blot was checked by immunoprecipitation (Fig. 8). Western blot revealed the presence of one specific protein band corresponding to PPAR γ (54 kDa) with higher intensity in differentiated cells (day 11) than in proliferated cells (day 5). These results suggest that PPAR γ and C/EBP participate as transcription factors in the early stages of adipogenesis.

Effect of TNF α on the differentiation of trout adipocytes

We evaluated the degree of differentiation in trout adipocyte culture at day 16 using GPDH activity as an indicator of this

cellular process (Fig. 9). The activity of this enzyme increased from 54.35 ± 4.28 mU/mg protein before differentiation of cells cultured in the absence of any adipogenic stimuli and in the presence of 10% FBS (control 1) up to 86.27 ± 15.26 mU/mg protein in cells maintained in differentiation medium containing 10 μ g/ml insulin, 0.25 μ M dexamethasone, and 0.5 mM IBMX but without lipid mixture (control 2). The highest level of GPDH was observed when we supplemented the differentiation medium with lipid mixture (145.17 ± 10.58 mU/mg protein; control 3). The addition of increasing doses of TNF α (1, 10, and 100 ng/ml) to the differentiation medium (without lipid mixture) inhibited differentiation. GPDH activity was reduced significantly at 100 ng/ml TNF α (Fig. 9). The suppression reached almost 50% of control values (control 2). The reduction of GPDH was accompanied by similar decrease in lipid accumulation and no effect of TNF α was observed on cell number (data not shown).

Figure 8 Protein band of PPAR γ expressed in trout adipocyte at days 5 and 11. Cells at day 11 were immunoprecipitated with 2 μ g Ab anti-PPAR γ (IP) and a homogenate of adipose tissue from trout (AT) was used as a control.

Discussion

Here, we provide the first description of a cell culture system to grow and differentiate trout pre-adipocytes into adipocytes. In mammals, many factors are involved in the proliferation and differentiation of these cells in primary culture (Hausman

Figure 9 GPDH activity of adipocytes used as a marker of adipogenesis. (A) At confluence (day 7), cells were incubated in growth media (control 1), differentiation media without lipid mixture (control 2), and differentiation media supplemented with lipid mixture (control 3). (B) Cells were exposed to differentiation media in the presence of hormones without lipid mixture (as described above), supplemented with 1, 10, and 100 ng/ml TNF α . Data from two experiments (three to four wells per experiment) are shown as mean $\pm$ S.E.M. Different letters indicate differences between groups ($P \leq 0.05$).

et al. 2001). However, little information is available on the processes regulating proliferation in fish. The use of either LP or 1% gelatin as substrate resulted in pre-adipocyte proliferation. Differences between the two substrates were observed on days 3 and 5, with greater proliferation in cells cultured in gelatin. Cells were attached, spread, and developed well on both substrates and although proliferation was fastest between days 5 and 7 of culture in 1% gelatin, the two substrates reached a similar final level of proliferation on day 9. Our study is the first to use gelatin for pre-adipocyte attachment and proliferation in fish. Most studies in mammals have used laminin as substrate in primary cell culture, as in

porcine stromal-vascular cultures, in which laminin increases the number, size, and proportion of adipocytes (Hausman *et al.* 1996). Other studies have described that differentiating pre-adipocytes show greater adherence to laminin than non-pre-adipocytes. The poly-D-lysine was also used as a substrate as it contributes to the attachment of cells and the formation of a uniform monolayer, but this substrate impaired porcine pre-adipocyte development (Yu & Hausman 1998). To date, few studies have applied gelatin as a substrate in pre-adipocyte cultures. Plates coated with gelatin increase human adipostromal cell number and proliferation (Inoue *et al.* 2005). Furthermore, gelatin provides an extracellular matrix that maintains proliferation and increases viability, both of which may be desirable for long-term cultures (Aidan *et al.* 2007).

Here, we tested the effect of IGF-I and TNF α on proliferation in primary cultures of trout pre-adipocytes. Mammalian IGF-I and TNF peptides are effective in fish systems *in vitro*, and numerous examples have been reported (Duan 1998, Albalat *et al.* 2005, Saera-Vila *et al.* 2007, Codina *et al.* 2008). Fish and mammalian IGF-I also give similar results in binding studies (Leibush *et al.* 1996, Navarro *et al.* 1999, Castillo *et al.* 2002). Comparison of the sequences of fish genes and peptides with those of mammals indicates that these factors have been conserved throughout vertebrate evolution, (Duan 1998, Laing *et al.* 2001, García-Castillo *et al.* 2002), which is consistent with the conservation of their biological potency.

IGF-I is a potent paracrine/autocrine adipogenic and mitogenic factors in mammals. This hormone is considered to be the major regulator of cell proliferation and differentiation of pre-adipocytes into adipocytes (Blüher *et al.* 2005). The addition of IGF-I to pre-adipocyte cell lines and primary cultures induces both mitogenic and differentiation responses in rat and pig (Ramsay *et al.* 1989, Schmidt *et al.* 1990, Ramsay 2000). Although our study did not examine the potential action of IGF-I on differentiation, we found that recombinant human IGF-I stimulated the proliferation of rainbow trout pre-adipocytes. This observation is consistent with the findings of studies on other species, in which IGF-I effectively triggers this process (Nougues *et al.* 1993, Wright & Hausman 1995, Butterwith 1997). In mammals, pre-adipocytes express IGF-I receptors (Blüher *et al.* 2005). Furthermore, the adipose tissue of brown trout expresses IGF-I receptors (Planas *et al.* 2000). On the basis of findings from studies on mammalian stromal-vascular cells, pre-adipocytes, and adipocytes (Shimizu *et al.* 1986, Smith *et al.* 1988, Ramsay 2000), we propose that trout pre-adipocytes also present these receptors. The signaling pathway by which IGF-I could stimulate cell proliferation is through activation of MAPK as shown in 3T3-L3 pre-adipocytes (Blüher *et al.* 2005). In our study, the proliferative action of IGF-I on cells was improved by the presence of factors or nutrients in the serum, like the mitogenic competence factor, which enhances IGF-I-induced proliferation (McWade *et al.* 1995).

IGF-I and TNF α are both known as paracrine factors, with opposing effects on adipose cell differentiation. IGF-I induces

both mitogenic and differentiation responses in pre-adipocyte cultures (Wabitsch *et al.* 1995), whereas TNF α inhibits the expression of adipogenic enzymes (Boone *et al.* 2000). Nevertheless, TNF α can also act as a stimulatory factor of cell proliferation at low doses in rat stromal-vascular cell cultures (Kras *et al.* 2000) or fibroblast cell lines including BALB/3T3 and 3T-L1 (Palombella & Vilcek 1989, Cornelius *et al.* 1990). In the present study, trout cell culture was shown to be slightly responsive to the proliferative action of TNF α .

Our study focuses on the possible effects of co-incubation of IGF-I with TNF α on the growth of pre-adipocytes. The combination of both hormones promoted proliferation but without a synergistic effect. However, in stromal-vascular cell culture of rat (Kras *et al.* 2000), low levels of TNF α stimulated proliferation and acted synergistically with IGF-I to further enhance proliferation. The net result of this interaction would be an increase in the uncommitted cell pool from which these cells might then be mostly recruited to become adipocytes or other cell types supporting adipose tissue growth (Kras *et al.* 2000).

The culture of trout pre-adipocytes in differentiation medium containing insulin, dexamethasone, IBMX, and lipid mixture, in the presence of 10% FBS, resulted in increased lipid accumulation, as evidenced by ORO staining, and this medium was essential to initiate the differentiation of these cells into mature adipocytes. Nevertheless, the lipid mixture is important in trout adipocyte differentiation. Salmon pre-adipocytes differentiate only in the presence of lipid mixture (Vegusdal *et al.* 2003). However, the differentiation of red sea bream stromal-vascular cells is induced when linoleic acid, in the presence of insulin and T₃, is applied (Oku *et al.* 2006). Fatty acids play a pivotal role in cell differentiation in fish. By contrast, the presence of lipids is not a requisite for the differentiation of pre-adipocyte cell lines and primary pre-adipocytes derived from distinct fat depots in mammals, including rodents, rabbits, and pigs (Gregoirie *et al.* 1998, Van Harmelen *et al.* 2004, Niemela *et al.* 2007), in which hormones such as insulin, dexamethasone, and IBMX exert a greater effect on adipocyte differentiation.

GPDH is a lipogenic enzyme involved in cell differentiation. In our study, the medium containing insulin, dexamethasone, and IBMX stimulated GPDH activity, which is used as a differentiation parameter in many species (Ramsay & Rosebrough 2003, Van Harmelen *et al.* 2004). This combination of hormones induced approximately a twofold increase in GPDH activity when we supplemented cultures with lipid mixture. Again, this observation indicates the importance of the presence of fatty acids in trout pre-adipocyte differentiation. GPDH activity also increases during the differentiation of salmon pre-adipocytes (Vegusdal *et al.* 2003) and in primary culture of bovine adipocytes in the presence of oleate (Sato *et al.* 1996).

Our study is the first to demonstrate the effect of TNF α on fish adipogenesis. This cytokine is a negative regulator of adipogenesis in several adipocyte models (Smyth *et al.* 1993). In our study, the treatment of cells with TNF α in culture

decreased GPDH activity. Similar results have been described in rat adipocytes and 3T3-L1 (Smas & Sul 1995, Xing *et al.* 1997, Kras *et al.* 2000). TNF α can act on lipid metabolism by decreasing the expression of lipoprotein lipase and by increasing lipolysis in 3T3-L1 adipocytes (Morin *et al.* 1995, Holden & Pakula 1996). The effects observed in trout pre-adipocyte culture are consistent with the inhibition of lipoprotein lipase activity by TNF α in isolated trout adipose tissue (Albalat *et al.* 2006) and the increase in lipolysis in isolated trout adipocytes (Albalat *et al.* 2005).

Adipocyte differentiation is induced by a series of programed changes in gene expression in mammals (Hwang *et al.* 1997). PPAR γ and C/EBP α are key adipogenic transcription factors (Tchkonia *et al.* 2002). In the current study, the expression of PPAR γ and C/EBP α in trout adipocyte was detected by immunofluorescence, and PPAR γ protein levels detected by western blot increased with differentiation of cells. This is in agreement with results found by Vegusdal *et al.* (2003) who reported the presence of PPAR γ and C/EBP α in salmon adipocytes by western blot and immunocytochemistry, suggesting an adipogenic function of these factors also in fish. Other studies have shown the expression of PPAR γ and C/EBP α in porcine adipose precursor cells (Hausman 2000, Kim & Moustaid-Moussa 2000). Studies in transgenic animal models have shown that adipogenesis is blocked when these factors are absent (El Jack *et al.* 1999). Furthermore, PPAR γ and C/EBP α activate adipocyte-specific genes and are involved in the growth arrest required for adipocyte differentiation in humans (Niemela *et al.* 2007). The adipocyte model offers a valuable tool to study the role of these factors during the regulation of adipose tissue growth.

In conclusion, we have established culture conditions for supporting proliferation and differentiation of pre-adipocyte cells isolated from trout adipose tissue and to study the endocrine regulation of these processes. This *in vitro* model will be very helpful to understand the pattern of gene expression involved in the control of adipogenesis and to obtain markers for the proliferation and differentiation capacity of the adipose tissue.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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