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Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*)

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ABSTRACT

A preadipocyte primary cell culture was established to gain knowledge about adipose tissue development in gilthead sea bream (*Sparus aurata*), one of the most extensively produced marine aquaculture species in the Mediterranean. The preadipocytes obtained from the stromal-vascular cell fraction of adipose tissue proliferated in culture, reaching confluence around day 8. At that time, the addition of an adipogenic medium promoted differentiation of the cells into mature adipocytes, which showed an enlarged cytoplasm filled with lipid droplets. First, cell proliferation and differentiation were analyzed under control and adipogenic conditions during culture development. Next, the effects of insulin, GH, and IGF-I on cell proliferation were evaluated at day 8. All peptides significantly stimulated proliferation of the cells after 48 h of incubation ($P < 0.002$ for GH and IGF-I and $P < 0.05$ for insulin), despite no differences were observed between the different doses tested. Subsequently, the effects of insulin and IGF-I maintaining differentiation when added to growth medium were studied at day 11, after 3 d of induction with adipogenic medium. The results showed that IGF-I is more potent than insulin enhancing differentiation ($P < 0.01$ for IGF-I compared with the control). In summary, a primary culture of gilthead sea bream preadipocytes has been characterized and the effects of several regulators of growth and development have been evaluated. This cellular system can be a good model to study the process of adipogenesis in fish, which may help improve the quality of the product in aquaculture.

1. Introduction

Global aquaculture production has increased in recent decades to meet an increasing human population's demand for fish and fishery products worldwide [1]. Some aquaculture practices, such as the use of hyperlipidic diets or new raw dietary materials, together with the low swimming activity of farmed fish, may have negative effects, such as reduced fish growth and animal welfare and increased visceral adiposity [2,3]. Fat increment can induce precocious sexual maturation, alterations in lipid metabolism, as well as a change in the fatty acid profile of the flesh [4,5].

Fat mass expansion can occur either by hypertrophy (increase in size of existing adipocytes) or by hyperplasia (increase in cell number by proliferation of precursor cells). At least in mammals, it is well known that mature adipocytes arise from multipotent mesenchymal stem cells (MSCs) after commitment and differentiation [6]. In fish, primary cultures of preadipocytes have been developed in the past years from Atlantic salmon [7], red sea bream [8], rainbow trout [9], grass carp [10], and the large yellow croaker [11]. Because in the Mediterranean area gilthead sea bream (*Sparus aurata*) is one of the most important commercialized species, we were interested in the development of an

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in vitro system to study adipogenesis in this marine species to help improve the quality of the aquaculture product.

In fish, as in mammals, GH is the main factor that regulates growth, as it has been clearly shown with GH acute injections or long-term treatments with implants, as well as with transgenic approaches [12–15]. However, most of the GH growth-related functions are mediated through IGF-I, which is produced primarily in the liver but also in other tissues in response to GH stimulation. In vivo, IGF-I plasma levels have been clearly correlated with BW and length in chinook and coho salmon [16,17]. Insulin-like growth factor-I is structurally and functionally related to insulin, a hormone also involved in many regulatory functions [18,19], which plasma levels had been also correlated with body mass in salmonids [20,21].

At the cellular level, in mammals IGF-I is considered to be the main regulator of proliferation and differentiation of preadipocytes [22]. In fish, several in vitro studies have also reported the effects of IGF-I on stimulating cell proliferation, including gilthead sea bream myocytes [23] and osteoblasts [24] and rainbow trout adipocytes [9]. However, the mitogenic effects of insulin are contradictory, because positive effects were observed in eel cartilage [25] and large yellow croaker preadipocytes [11], but no significant effects were observed in zebrafish cells [26] or rainbow trout myocytes [27]. Regarding differentiation, the positive role of IGF-I has been well reported in mammals, both in cell lines, such as the mouse 3T3L1 cells [28,29], as well as in primary cultures of preadipocytes of human, rat, and porcine origins [30,31].

The objective of the present study was to establish a primary culture of preadipocytes from gilthead sea bream and to analyze the roles of insulin, GH, and IGF-I in cell proliferation and differentiation to better understand the process of adipogenesis in this marine species, which is of high commercial value.

2. Materials and methods

2.1. Establishment of the preadipocyte primary culture

All plasticware for tissue culture was obtained from Nunc (Barcelona, Spain); and all reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) unless stated otherwise.

Gilthead sea bream (*Sparus aurata*) were obtained from the fish farm Tinamenor S.L. (Pesués, Cantabria, Spain) and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Fish were kept in 200-L fiberglass tanks under 12 h light/12 h dark photoperiod at 21°C ± 1°C and fed ad libitum twice daily with a commercial diet (Skretting España S.A., Burgos, Spain). All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the European Union, Spanish and Catalan government-established norms and procedures.

Following the protocol previously described for rainbow trout by Bouraoui et al [9], we set up the conditions for culturing gilthead sea bream preadipocytes. We first tested multiple factors that could affect culture performance, including fish size (50 or 500 g), base medium (Dulbecco Modified Eagle Medium, DMEM, or Leibovitz, L15), NaCl concentration (30 or 60 mM), collagenase type (I or II), lysing of erythrocytes or not during the isolation procedure, cell density at the time of plating (2–5 × 10^4 cells/cm^2), and incubation temperature (18°C or 23°C) among others. Cells were routinely observed under the microscope, and some proliferation assays were run. For example, the number of cells recovered from 500-g fish was low, and they did not proliferate as well as those from 50-g fish. In addition, proliferation was higher in cells growing in DMEM than in L15, as well as at a temperature of 23°C than at 18°C. Results are not shown, except for the NaCl concentration effects as an example (see Fig. 2 in 3.2. Characterization of preadipocyte proliferation).

The final protocol consisted on extracting the visceral adipose tissue of fish that had a body weight of approximately 50 g. Ten fish on average were used for each culture. Anesthetized fish with ethyl 3-aminobenzoate methanesulfonate (MS-222, 0.1 g/L; Sigma A5040) were killed by a blow to the head and externally sterilized with 70% ethanol. Then, visceral adipose tissue was removed with autoclaved dissection material and collected in Krebs-HEPES buffer (pH 7.4) supplemented with 1% (vol/vol) antibiotic/antimycotic solution (A/A; Sigma A5955). Tissue was minced to small pieces by mechanical disruption with the use of 2 scalpels in sterile conditions inside a laminar air flow hood. Then, enzymatic digestion was performed by incubating the tissue with collagenase type II at 18°C with gentle agitation (250 rpm) for 1 h in a Movil-Rod agitator (JP Selecta, Abrera, Spain). Collagenase at 130 U/mL was prepared in Krebs-HEPES buffer supplemented with 1% bovine serum albumin. After that, the stromal-vascular cell fraction was recovered, which includes in addition to preadipocytes other cell types such as MSCs, endothelial cells, and macrophages. All these cells mostly disappear as the adipocytes progress in culture. Cells were then filtered through a 100-μm cell strainer to remove the large undigested tissue, washed with Krebs-HEPES buffer, and centrifuged 10 min at 2,000× *g* in an oscillating rotor to get rid of mature adipocytes. Differently from Bouraoui et al [9] cells at this point were not treated with an erythrocyte-lysing buffer, because they proliferated better without this treatment, and the erythrocytes were easily removed from the culture with the first medium change because they did not attach to the plate. Finally, the cells were resuspended in growth medium (GM) and plated in gelatin-treated 12-well plates at a density of 4.3 × 10^4 cells/cm^2 and incubated at 23°C with 2.5% CO_2. Gelatin was used according to Bouraoui et al [9] because it was established as a better substrate in terms of attachment and proliferation of the cells. The GM was composed of DMEM with 10% fetal bovine serum (FBS), 1% A/A, and 60 mM NaCl, because the cells proliferated better with this NaCl concentration (see Fig. 2 in 3.2. Characterization of preadipocyte proliferation), in addition to presenting a better appearance when observed under the microscope (data not shown).

2.2. Cell culture development characterization

Cells were maintained up to 21 d with fresh media replacement every 2 to 4 d. At day 8 after plating, once

confluence was reached, the GM was either maintained or changed to an adipogenic medium (AM) containing GM plus 10 μg/mL insulin, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 0.25 μM dexamethasone, and 5 μL/mL lipid mixture (that contained cholesterol and fatty acids from cod liver oil; Sigma L5146) to induce differentiation and to test the ability of the preadipocytes to become mature adipocytes. Development of the cells under both growing conditions (GM and AM) was followed, and images were taken at different times with the use of an Axiovert 40C inverted microscope (Carl Zeiss, Germany) coupled to a Canon digital camera. Specific assays to measure the degree of proliferation and differentiation of the cells were performed at different culture times as explained in 2.2.1 and 2.2.2 respectively.

2.2.1. Measurement of preadipocyte proliferation

Cell proliferation during culture development was analyzed with the assay based on the reduction by mitochondrial reductases of methylthiazolyldiphenyl-tetrazolium bromide (MTT) into formazan, as it was previously done for sea bream osteoblasts and pig primary adipocytes and mouse 3T3L1 cells [24,32,33] with minor modifications. The MTT solution (0.5 mg/mL final concentration; Sigma M5655) was added to the cells 18 h before the end of the experiment. Then, cells were washed with PBS, and the blue formazan crystals formed were resuspended in 150 μL of dimethyl sulfoxide per well and incubated for 3 h before spectrophotometric evaluation. Proliferation values were obtained by subtracting the background read at 650 nm from the absorbance measured at 570 nm. Cells with PBS instead of MTT were used as nonspecific, and the value from this reading was also subtracted from all the other data. Results are presented as fold change with the absorbance values normalized to day 4.

2.2.2. Measurement of adipocyte differentiation

To study the ability of the preadipocytes to differentiate into mature adipocytes, cells were induced to differentiate by the addition of AM. To evaluate differentiation, accumulation of neutral lipids into the cells was observed by oil red O (ORO) staining by following the protocol of Koopman et al [34] previously adapted for gilthead sea bream adipocytes by Capilla et al [24]. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm corresponding to cell protein content, which was obtained after Comassie blue staining for 1 h and dye extraction by incubation of the cells with 85% propylene glycol during 3 h at 60°C [24]. Data are presented in fold change with the values normalized to day 12 cells growing in GM.

2.3. Endocrine regulation of preadipocyte proliferation

The effects of insulin, GH, and IGF-I on cell proliferation were analyzed. On day 8, the cells were starved by changing the GM to a medium consisting on DMEM with 1% A/A and only 0.02% FBS for 5 h. After that, the medium was changed again to a DMEM containing 1% A/A, 2% FBS, and the corresponding dose of peptide. Growth hormone was tested at 1 and 10 nM; IGF-I at 10 and 100 nM; and insulin at 10, 100, and 1,000 nM final concentrations. Porcine insulin was obtained from Sigma–Aldrich, recombinant human IGF-I was purchased from Bachem (Weil am Rhein, Germany), and recombinant human GH from Genway Biotech Inc (San Diego, CA, USA). We used mammalian peptides because of availability and because their mitogenic effects in fish cells have been shown to be similar than those caused by their homologous counterparts [23,35]. Proliferation was measured by the MTT assay by adding the MTT solution to the medium 30 h later, to end the experiments exactly after 48 h of peptide stimulation (counting the last 18 h in the presence of MTT). Data were obtained from the absorbance readings as described in 2.2.1. Values are presented in fold change respect to control condition without peptide.

2.4. Endocrine regulation of adipocyte differentiation

To analyze the endocrine regulation of cell differentiation, cells at day 8 were first induced to differentiate with AM for 3 d. At day 11, the medium was changed again to GM that contained either insulin (1,000 nM), IGF-I (100 nM), or lipid mixture (5 μL/mL), which was used as a positive control condition, or a combination of lipid mixture with insulin or IGF-I. Lipid accumulation in the cells was assayed 5 or 10 d later, using the protocol of ORO staining and extraction. Data were obtained from the absorbance readings as described in 2.2.2 and presented in fold change respect to control cells with only GM.

2.5. Statistical analysis

Results are presented as mean ± SEM. To perform the statistical analyses, first the data were log-transformed. Then, it was confirmed that the data were normally distributed according to the Shapiro–Wilk test and that presented homogeneity in the variance according to Levene test. Finally, statistical differences were analyzed by one-way ANOVA followed by Tukey post hoc test. When the data did not follow the ANOVA presumptions, the nonparametric Kruskal–Wallis followed by Mann–Whitney tests were performed. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Structural characterization of cell culture development

In the present study we have established a primary culture of preadipocytes from gilthead sea bream as a model of study. Once the culture conditions were set up, we proceeded to characterize cell growth and development. First, we observed the cells routinely under a microscope to determine structure.

Figure 1 shows representative images of the cells at different culture days growing in GM or AM. One day after plating, the cells attached corresponding to preadipocytes presented the characteristic small triangular appearance of MSCs (Fig. 1 a). Most other cell types present in the stromal-vascular cell fraction, including erythrocytes, macrophages, or endothelial cells, did not attach and were washed with the medium change. From there, preadipocytes in GM

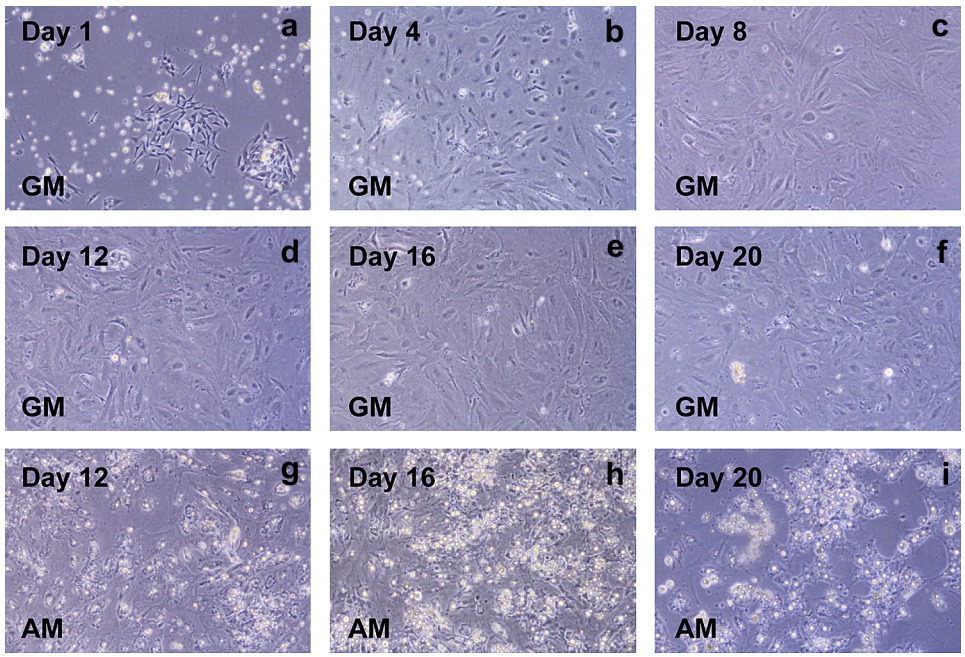

Fig. 1. Representative phase-contrast images of gilthead sea bream preadipocyte cells at different days of culture (1, 4, 8, 12, 16, and 20). The cells were growing in control (GM; panels a–f) or adipogenic conditions (AM; panels g–i) as described in 2. Materials and methods. Magnification, ×10. AM, adipogenic medium; GM, growth medium.

proliferated, showing an elongated appearance already observed at day 4 (Fig. 1b). This shape was maintained in the cells growing in GM up to day 20 (Fig. 1c–f). At day 8, when AM was added to some cells to induce the formation of adipocytes, these cells changed to a more rounded shape with an enlarged cytoplasm that became filled with lipid droplets (Fig. 1g–i), characteristic of mature cultured adipocytes.

3.2. Characterization of preadipocyte proliferation

Next, to investigate proliferation of the cells during the culture, MTT assays were performed in parallel to morphologic studies. Previously, during the set up process, several culture conditions were tested to obtain the best-performing results. Figure 2 shows the proliferation profiles obtained with cells growing at 2 different NaCl concentrations (30 or 60 mM). The cells presented similar profiles, but proliferation was higher in cells growing with 60 mM NaCl. In comparison with their own day 4 (corresponding to each NaCl concentration), significant differences were observed at days 12 ($P < 0.001$) and 16 ($P < 0.001$) for the cells in 60 mM NaCl, whereas with 30 mM NaCl these differences were only significant at day 16 ($P < 0.004$) (Fig. 2).

Once the culture conditions were established, and after the characterization of the cells, MTT assays were run for cells growing in GM or AM up to day 20. Cells in GM showed increased proliferation from day 4 to day 12, with significant differences with respect to day 4, both at day 8 ($P < 0.02$) and at day 12 ($P < 0.001$), and then reached a plateau (Fig. 3). From days 12 to 20, no significant differences were observed with time within the incubation media (GM or AM); however, at day 20 a significantly higher number of cells were seen in AM than in GM ($P < 0.01$) (Fig. 3), suggesting that some component in this medium may be stimulating cell proliferation.

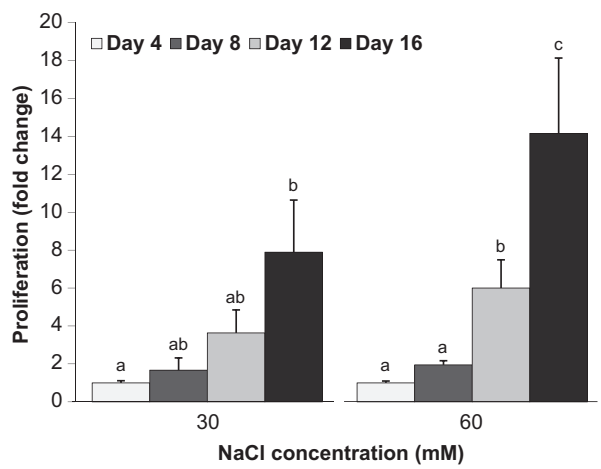

Fig. 2. Proliferation profile of gilthead sea bream preadipocyte cells at different days of culture in media with 2 different NaCl concentrations (30 and 60 mM). Proliferation of cells growing in control (GM; days 4 and 8) or adipogenic conditions (AM; days 12 and 16) was measured with the MTT assay as described in 2. Materials and methods. Data are means ± SEMs of 3 to 4 independent experiments with wells run in duplicate. Means with different superscript letters differ ($P < 0.05$), with each NaCl concentration analyzed separately. AM, adipogenic medium; GM, growth medium; MTT, methylthiazolyldiphenyl-tetrazolium bromide.

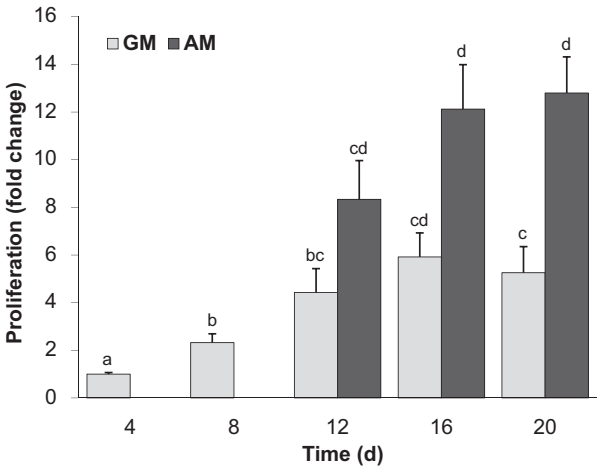

Fig. 3. Proliferation profile of gilthead sea bream preadipocyte cells at different days of culture (4, 8, 12, 16, and 20). Proliferation of cells growing in control (GM) or adipogenic conditions (AM) was measured with the MTT assay as described in 2. Materials and methods. Data are means ± SEMs of 3 to 4 independent experiments with wells run in duplicate. Means with different superscript letters differ ($P < 0.05$). AM, adipogenic medium; GM, growth medium; MTT, methylthiazolyldiphenyl-tetrazolium bromide.

3.3. Characterization of adipocyte differentiation

To further characterize the cell culture, the ability of the cells to differentiate into mature adipocytes was studied with ORO to stain accumulated intracellular lipids at different days during the culture. A representative image of day 20 cells growing in GM or AM and stained with ORO is shown in Figure 4A. The difference in shape between cells growing on each media can be observed and, specially, the presence of several lipid droplets stained in red, in the cytoplasm of cells growing in AM, which have differentiated into adipocytes. The ORO quantification showed as expected, significantly higher lipid content in the cells growing in AM than in GM at all times ($P < 0.001$), with significant differences within control cells growing in GM at day 20 of culture ($P < 0.02$) (Fig. 4B).

3.4. Insulin, GH, and IGF-I effects on preadipocyte proliferation

The endocrine regulation of proliferation in the newly established preadipocyte cell culture of gilthead sea bream was also studied. We tested the effects of different doses of insulin, GH, and IGF-I after 48 h of incubation in day 8 cells. All 3 peptides significantly increased proliferation (measured with the MTT assay) compared with the control situation ($P < 0.002$ for GH and IGF-I; $P < 0.05$ for insulin), but no concentration dependence was observed for any of the hormones (Fig. 5).

3.5. Insulin and IGF-I effects on adipocyte differentiation

Finally, we studied the endocrine regulation of cell differentiation. The effects of insulin and IGF-I alone or in combination with lipid mixture increasing the differentiation of day 11 cells, previously induced to mature by the addition of AM for 3 d, were analyzed by means of ORO staining at 2 different time points. As expected, at day 16 the lipid mixture (used as a positive control condition) stimulated differentiation compared with control cells (with GM only, $P < 0.001$), and in a similar degree also it did IGF-I alone ($P < 0.01$) (Fig. 6A). By contrast, insulin by itself was not able to increase lipid accumulation in the cells in comparison with the control cells with only GM at this time. The combination of lipid mixture with insulin or with IGF-I gave results similar to those obtained with lipid mixture alone ($P < 0.001$ and $P < 0.01$, respectively, compared with the control); thus, no additive effects of lipids and peptides were observed. In day 21 cells, the effects of lipid, alone or combined with either peptide, were similar to those observed at day 16 (Fig. 6B). For the peptides, no significant differences were observed after 10 d of incubation either with insulin or with IGF-I.

4. Discussion

Adipose tissue accumulation in fish can induce some problems in aquaculture, including a decrease in efficiency production because of an increase in the proportion of tissue that is finally discarded, reducing the quality of the final product. In farmed gilthead sea bream, the quantity of visceral adipose tissue is notably higher than the content found in wild fish [36]. Visceral fat in gilthead sea bream is positively correlated with whole body fat content, according to a preferential perivisceral fat deposition [37]. Thus, increases in visceral fat depots can even result in metabolic alterations that potentially may affect fish welfare [2–4]. Therefore, a better knowledge on the regulatory factors that affect adipose tissue growth and development in fish is of great importance nowadays, especially in species such as gilthead sea bream with notable accumulation of visceral fat [36].

For the first time in gilthead sea bream, we have set up cell culture conditions to obtain differentiated adipocytes in vitro from this Mediterranean species. The difficulties to achieve the optimal conditions of incubation temperature, medium salinity, cell density, or fish size to obtain good growth and development of preadipocytes showed that species-specific culture characteristics are crucial. Specifically in gilthead sea bream, higher content of NaCl (60 mM) and higher temperature of culture (23°C) than in rainbow trout (no NaCl added to the DMEM and 18°C) [9] were needed, whereas more similar NaCl concentration and incubation temperature values (65 mM and 25°C) were used to culture red sea bream preadipocytes [8]. These adjustments are in agreement with the natural living conditions of these species; besides as a euryhaline species, gilthead sea bream has been shown to adequately adapt to live in variable environmental salinities [38,39].

In a similar way, differences in culture conditions and medium composition to induce cells to differentiate have been observed in different mammalian species, chicken, and in the few fish species in which primary adipocyte cultures have been established. In mammalian preadipose cells, including cell lines, it is well known that the use of a cocktail composed of insulin, dexamethasone, and IBMX induces mature adipocyte differentiation [40–42].

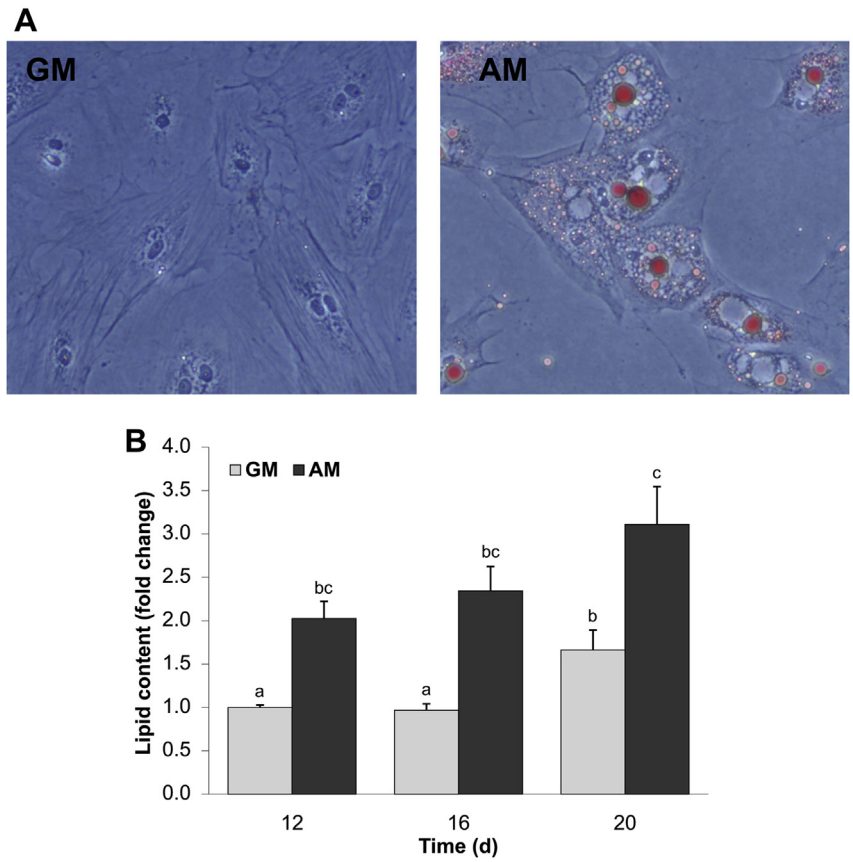

Fig. 4. (A) Representative phase-contrast images of gilthead sea bream preadipocyte cells stained with oil red O at day 20 of culture growing in control (GM) or adipogenic conditions (AM). Magnification, ×20. (B) Differentiation profile of gilthead sea bream preadipocyte cells at different days of culture (12, 16, and 20). Adipogenic differentiation of the cells growing under control (GM) or adipogenic conditions (AM) was measured by extraction of the oil red O stain as described in 2. Materials and methods. Data are means ± SEMs of 6 to 7 independent experiments with wells run in duplicate. Means with different superscript letters differ ($P < 0.05$). AM, adipogenic medium; GM, growth medium.

A common trait in fish is that lipids, especially high concentrations of fatty acids, play an essential role in adipocyte differentiation [7–11], whereas the presence of lipids is not so crucial for the differentiation of preadipocyte cell lines, such as the 3T3L1 cells [40,41], or primary preadipocytes derived from distinct fat depots of humans, rodents, rabbits, or pigs [42–44]. However, activated peroxisome proliferator-activated receptor γ (PPARγ) by fatty acids and other specific ligands has been described to be involved in the differentiation-linked adipogenic gene expression in mammals [42]. In fish, the mechanism involved in the activation of cell differentiation by fatty acids has not been elucidated, and contradictory results have been found. Gene expression of PPARγ was activated by 2-bromopalmitate (a PPAR agonist) in red sea bream, but its expression level was not linked to adipocyte differentiation during cell culture [45], whereas PPARγ gene or protein expression was increased with adipocyte differentiation in trout and salmon [7,8,46]. Further studies are necessary to better understand the role of fatty acids in piscine adipocyte differentiation.

The observed profile of proliferation during the culture again was similar to that of red sea bream [8] but appeared to be slower than in rainbow trout [9], because the plateau for maximum proliferation was reached after 7 to 9 d in rainbow trout, whereas in gilthead sea bream 16 d were needed to achieve maximum levels of proliferation. Moreover, the increased level of proliferation observed after the addition of differentiation medium indicated that some cells may continue proliferating during the differentiation phase in adipocyte culture of gilthead sea bream contrary to what has been well described for mammalian cells [6,42]. Nevertheless, structural changes throughout gilthead sea bream culture were similar to those previously described for other cultured fish preadipocytes [7–11], changing from a fibroblast appearance to the more rounded shape with an enlarged cytoplasm with lipid droplets accumulation, characteristic of differentiated cells.

Furthermore, despite a certain percentage of cells was able to differentiate spontaneously into adipocytes, evidenced by the increase in lipid content in cells maintained in only GM by day 20, the AM used clearly enhanced lipid accumulation in the cells and induced the structural change to mature adipocytes. This is a characteristic of this type of primary culture and also of adipocyte cell lines such as the 3T3L1 cells [40,41], whereas other cell models differentiate

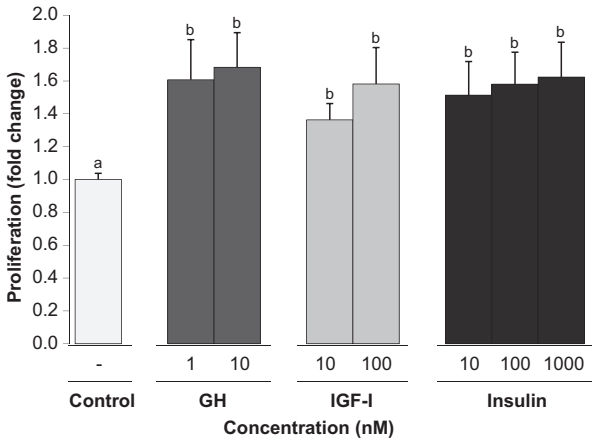

Fig. 5. Effects of GH (1 and 10 nM), IGF-I (10 and 100 nM), and insulin (10, 100, and 1,000 nM) on the proliferation of gilthead sea bream preadipocyte cells. On day 8, after 5 h of starvation (DMEM + 0.02% FBS), stimulation with the different doses of peptides was performed in DMEM with 2% FBS for 48 h. Proliferation of the cells was measured with the MTT assay as described in 2. Materials and methods. Data are means ± SEMs of 4 to 5 independent experiments with wells run in duplicate. Means with different superscript letters differ ($P < 0.05$), with each peptide analyzed separately but with the control in all cases. DMEM, Dulbecco Modified Eagle Medium; FBS, fetal bovine serum; MTT, methylthiazolyldiphenyl-tetrazolium bromide.

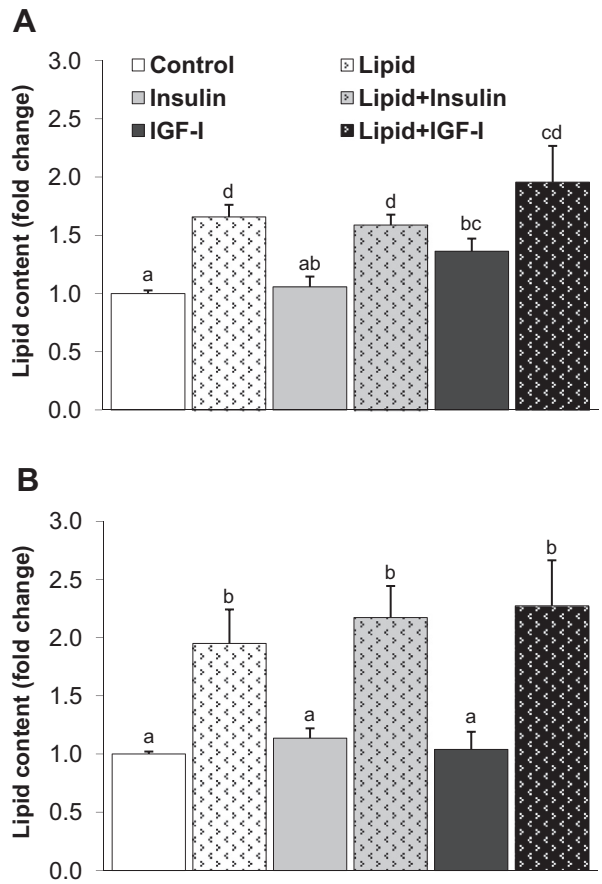

Fig. 6. Effects of insulin (1,000 nM), IGF-I (100 nM), lipid mixture (5 μL/mL), and combinations of lipid with insulin or IGF-I on the differentiation of gilthead sea bream preadipocyte cells. On day 8, the cells were induced to differentiate with complete AM medium (10 μL/mL lipid mixture) for 3 d. Then, stimulation of the differentiation with the lipid mixture or the peptides was performed in GM medium for 5 d (A) or 10 d (B). Adipogenic differentiation was measured by extraction of the oil red O stain as described in 2. Materials and methods. Data are means ± SEMs of 4 independent experiments with wells run in duplicate. Means with different superscript letters differ ($P < 0.05$). AM, adipogenic medium; GM, growth medium.

spontaneously in vitro, such as the cultured myocytes derived from muscle isolated–enriched satellite cells [23,27,35,47]. Regardless of the species analyzed, proliferated cells from stromal-vascular fraction need the addition of a cocktail or medium of differentiation to completely differentiate in a relatively short time in culture. This makes the preadipocyte culture an interesting model to study the effects of hormones on proliferation and differentiation into mature adipocytes. Besides, this is not only interesting from a point of view of in vitro studies, because new adipocytes form constantly to replace lost adipocytes such that approximately 10% of total human adipocytes are renewed every year [48]. In addition, it is well established that changes in circulating hormone concentrations can result in marked differences in adipose tissue growth [49,50], which is also important in fish production. Therefore, in vitro studies such as the present one can be the base to study the effects of other factors such as nutrients in adipose tissue development.

In the present study, stimulation of cell proliferation with GH incubation was similar to the increase in proliferating cells observed after treatment with insulin or IGF-I. Stimulatory effects of GH on preadipocyte proliferation have been described in mammals [42], but the effects were contradictory, depending on cell model and species. In primary culture, GH has been shown to stimulate or to not affect preadipocyte proliferation in humans and rats but to reduce proliferation in pigs [51]. In addition, GH was reported to be mitogenic in gilthead sea bream myocytes [23]. This hormone has been commonly shown species-specificity effects, and in the study of Rius-Francino et al [23] sea bream GH resulted more potent than human GH stimulating proliferation. Nevertheless, in our study even using human GH the effects on proliferation were clear.

Besides the well-known direct effects of GH in lipid metabolism [52], the action of GH in proliferation appeared to be indirect, because GH markedly stimulated IGF-I production in rat preadipocytes, which in turn promoted cell proliferation [42]. Furthermore, additional studies have reported that adipocytes recently differentiated from precursor cells in response to incubation with GH are much more sensitive to the mitogenic effect of IGF-I than precursor cells, resulting mainly in proliferation of young differentiated cells rather than in proliferation of preadipocytes [22]. In the present study we have not measured the production of IGF-I in cultured cells or its possible contribution to GH effects, neither in precursor cells nor early differentiated adipocytes, but we have clearly observed a mitogenic effect with the addition of IGF-I in gilthead sea bream preadipocytes. This finding is in agreement with the literature in which IGF-I was shown to

promote proliferation in primary culture of pig preadipocytes [51] and rainbow trout cells [9]. In addition, IGF-I has been reported to stimulate proliferation of 3T3L1 preadipocytes [22] and bone marrow–derived human MSCs during differentiation into adipocytes [53]. The same effect has been reported in other piscine cell models such as zebrafish embryonic cells [26], rainbow trout [27] and gilthead sea bream [23] myoblasts, and gilthead sea bream osteoblasts [24]. Moreover, human or trout IGF-I has been previously reported to be equally effective in fish cells [35].

In addition to GH and IGF-I, insulin has been reported to be mitogenic on mammalian [54,55] and piscine [8,11] cultured preadipocytes, and this mitogenic effect has now been confirmed in our gilthead sea bream preadipoctes. We have previously demonstrated the presence of both insulin and IGF-I receptors in trout adipose tissue [56], in trout preadipocytes in culture [57], and in sea bream muscle [58]. Although it has been suggested that insulin receptors are more important in metabolism, whereas IGF-I receptors mediate growth, this dichotomy is not so clear in fish [18,19,58]. Nevertheless, insulin at high concentrations could mimic the effects of IGF-I in preadipocyte proliferation of gilthead sea bream as reported in mammals [22]. A possible synergic effect between insulin and dexamethasone on cell proliferation cannot be discarded, as described in rat preadipocytes [59]. From the 2 main signaling transduction pathways shared by insulin and IGF-I receptors, the mitogen-activated protein kinase pathway is preferentially stimulated by both peptides at early stages of cell culture in rainbow trout and gilthead sea bream myocytes [47,60] and rainbow trout adipocytes [57], which has been associated with increases in cell proliferation. Further studies should be done to understand the mechanisms of action that mediate the mitogenic effects observed in cultured preadipocytes of gilthead sea bream.

For the endocrine regulation of differentiation, the observed positive effects of IGF-I on preadipocyte maturation of gilthead sea bream, estimated as lipid accumulation, corroborated the evidence previously shown that IGF-I enhances the maturation of preadipocytes into adipocytes in many mammalian adipocyte models and species [28,29,31,61,62]. The fact that IGF-I was more effective than insulin to enhance gilthead sea bream preadipocyte differentiation at early stages (5 d of stimulation) does not mean that insulin is not necessary for the differentiation of these cells, because insulin was present in the adipogenic medium first used to induce differentiation. It has been previously shown that the presence of insulin plus troglitazone (a PPARγ agonist of the drug class of thiazolidinediones known as an antidiabetic agent) significantly increased lipid content in rainbow trout adipocytes maintained in GM and that insulin alone was able to stimulate lipoprotein lipase expression, which is associated with the adipocyte phenotype, already at early stages of differentiation [8,63]. In red sea bream and the large yellow croaker, insulin was also able to increase lipid accumulation and glycerol-3-phosphate dehydrogenase activity, and to stimulate lipoprotein lipase expression [8,11]. Therefore, it is possible that insulin stimulates gilthead sea bream adipocyte differentiation to potentiate the effects of other adipogenic compounds present in the differentiation medium. However, only the addition of lipid mixture and not the presence of hormones enhanced lipid accumulation after 10 d, which suggested that hormones can trigger the process of differentiation, but, once it is already switched on, the presence of hormones does not further induce more lipid synthesis and accumulation. Nevertheless, as mentioned before, the dependence on the presence of lipids or high concentration of fatty acids for differentiation appears to be crucial in fish and other species such as chicken, but not so much in mammals. The molecular bases for those species differences are unknown; among others, different sensibility of fatty acids to PPARs or distinct rates of lipogenesis could partially explain variations in the dependency of exogenous lipid supply for differentiation.

In conclusion, we have established a cell culture system to study the processes of proliferation and differentiation in gilthead sea bream adipocytes, and we have investigated the possible endocrine regulators of adipogenesis in this species. These results contribute to the knowledge on the hormonal control of adipose tissue growth and development in gilthead sea bream and potentially in the improvement of quality of aquaculture products.

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