Effects of insulin, triiodothyronine and fat soluble vitamins on adipocyte differentiation and LPL gene expression in the stromal-vascular cells of red sea bream, *Pagrus major*

Hiromi Oku[a]*, Masaharu Tokuda[a], Takuji Okumura[a], Tetsuya Umino[b]

[a] National Research Institute of Aquaculture, Minami-ise, Mie, 516-0193, Japan

[b] Hiroshima University, Higashihiroshima, Hiroshima, 739-8528, Japan

*Corresponding author. Telephone: +81-0599-66-1830, Fax: +81-0599-66-1962, E-mail: hiromi@fra.affrc.go.jp

Running title: Fish adipocyte differentiation *in vitro*

Abstract

Various kinds of hormones including insulin, triiodothyronine (T_3) and fat-soluble vitamins have been proposed as mediators of adipocyte differentiation in mammals. To investigate the factors which are responsible for fish adipocyte differentiation, we developed a serum-free culture system of stromal-vascular cells of red sea bream adipose tissue and examined the effects of insulin (bovine), T_3, and fat-soluble vitamins (all-*trans* retinoic acid, retinyl acetate and 1,25-dihydroxyvitamin D_3) on the differentiation-linked expression of the lipoprotein lipase (LPL) gene. As assessed by the increase in LPL gene expression after 3 day cultivation, like in mammalian adipocytes, insulin enhanced the adipocyte differentiation in a concentration-dependent manner. During 2 week cultivation, bovine insulin promoted lipid accumulation in differentiating adipocytes concentration-dependently until the terminal differentiation. These results indicate that the differentiation of fish adipocytes is inducible by insulin alone. T_3 alone had no effect but enhanced the differentiation-linked LPL gene expression in the presence of insulin. Fat-soluble vitamins, unlike in mammalian adipocytes, did not show any significant effects. The method developed in this study should be of interest for the characterization of factors involved in fish adipocyte differentiation.

Key words: adipocyte, stromal-vascular cells, differentiation, lipoprotein lipase, LPL, teleost, red sea bream, *Pagrus major*

1. Introduction

Adipocyte differentiation is an important factor for fat accumulation in the body. Adipocytes are derived from fibroblast-like preadipocytes and grow in size by accumulation of lipids in the cytoplasms in association with the terminal differentiation (Hausman et al. 1980). In the early stage of adipocyte differentiation, preceding the terminal differentiation, many adipocyte-characteristic genes are sequentially activated and play established roles in promoting the differentiation process (Gaskins et al. 1989; Ntambi and Kim 2000). Lipoprotein lipase (LPL) gene is one of such genes and represents an early marker of adipocyte differentiation (Dani et al. 1990). LPL gene encodes a lypolytic enzyme responsible for lipid uptake in adipocytes (Nilsson-Ehle et al. 1980) and the gene expression and enzymatic activity are utilized to assess the degree of adipocyte differentiation *in vivo* and *in vitro* (Vu et al. 1996; McNeel et al. 2000; Ding and Mersmann 2001).

Adipocyte differentiation is mediated positively or negatively by various kinds of hormones (Gregoire et al. 1998; Boone et al. 2000). For examples, insulin is required for adipocyte differentiation in mammals and birds (Dani et al. 1986; Suryawan et al. 1997; Ramsay and Rosebrough 2003). Triiodothyronine (T_3) also stimulates adipocyte differentiation in the preadipocyte cell line Ob17 (Gharbi-Chihi et al. 1981).

Furthermore, it is well known that fat-soluble vitamins, especially metabolites of vitamin A and D, modulate the adipocyte differentiation in cultured cells (Kawada et al. 1990). All-*trans* retinoic acid (RA) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) inhibit the adipocyte differentiation in cultured cells at a superphysiological concentration (Sato and Hiragun 1988; Kawada et al. 1990; Suryawan and Hu 1997). Very low concentration (1pM-10nM) of RA stimulates the adipocyte differentiation (Safonova et al. 1994). Moreover, Vu et al. (Vu et al. 1996) have reported the stimulatory effects of $1,25(OH)_2D_3$ on the differentiation-linked LPL gene expression in 3T3-L1.

Adipocyte is a major site of lipid deposition in fish. The tissue distribution and number of adipocytes in fish body are critical factors in determining the body lipid content and the flesh quality of cultured species (Yamada and Nakamura, 1964; Yamada, 1981; Shindo et al. 1986; Zhou et al., 1996). An *in vitro* method for studying the proliferation and differentiation of Atlantic salmon preadipocytes has been developed (Vegusdal et al. 2004). Salmon preadipocytes proliferate *in vitro* as characterized by the expression of proliferating cell nuclear antigen (PCNA) (Vegusdal et al. 2004). And the supplementation of exogenous lipids to the culture medium was shown to induce salmon preadipocyte differentiation as characterized by lipid accumulation in the

cytoplasm and the appearance of adipocyte characteristic proteins including PPAR γ, C/EBP and leptin (Vegusdal et al. 2004). These findings have provided us with information on the molecular mechanisms of fish adipocyte proliferation and differentiation. However, knowledge about the extrinsic regulation of fish adipocyte differentiation is still limited.

For the eventual control of adiposity in cultured species, the factors which modulate the adipocyte differentiation in fish need to be identified. In this study, to investigate the factors which may be responsible for fish adipocyte differentiation, we developed a primary culture system of adipose stromal-vascular cells of a marine teleost red sea bream (*Pagrus major*) and tested the effects of insulin, T_3, and fat-soluble vitamins on adipocyte differentiation *in vitro*. A preliminary step for defining the factors responsible for regulation of fish adipocyte differentiation is the development of a defined cell culture system. In the present study, we employed a serum-free culture system to investigate the hormonal effects on the adipocyte differentiation under strictly controlled conditions. The lipoprotein lipase (LPL) gene expression level was used as an early marker of the adipocyte differentiation and the lipid accumulation was used as a marker of the terminal differentiation process.

2. Materials and Methods

2.1 Experimental fish

Juveniles of red sea bream (*Pagrus major*) were purchased from a local hatchery station (Nissin Marine Tech, Aichi, Japan) and maintained in our institute (National research institute of aquaculture, minamiise, mie, Japan). After growing to 800-1200 g in body weight, the fish were used.

2.2 Culture media

The plating medium consisted of the DME / F12 (1:1) medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 65mM NaCl, 10% fetal bovine serum, 100μg/ml streptomycin and 100U/ml penicillin (Invitogen Corp.). The standard induction medium consisted of the DME / F12 (1:1) medium containing 0.042mg/l linoleic acid (Invitrogen Corp.). The standard induction medium was supplemented with 65mM NaCl, 100μg/ml streptomycin and 100U/ml penicillin (Invitrogen Corp.), 50μg/ml transferrin (Sigma, St. Louis, MO, US), 5ng/ml sodium selenite, and 50ng/ml hydrocortisone (Sigma). In *3.1 Experiment 1*, to eliminate the influences of any exogenous fatty acid (FA), the FA(-) induction medium was prepared with the 1:1 mixture of DME (Invitrogen Corp.) and Ham F12K nutrient (Invitrogen Corp.) instead of the DME / F12 medium in the standard induction medium. Bovine insulin (bIns), triiodothyronine (T_3), all-*trans* retinoic acid (RA), retinylacetate and 1,25

dihydroxyvitamin D_3 (1, $25(OH)_2D_3$) were purchased from Sigma. The fat soluble vitamins were prepared as ethanol solution. The addition of hormones to the culture media is indicated in the text or figure legends.

2.3 Preparation of stromal-vascular cells (SV cells) from red sea bream adipose tissue

Red sea bream SV cells were prepared by enzymatic digestion of visceral adipose tissue. The visceral adipose tissue was minced in PBS containing 5% bovine serum albumin, followed by the digestion with 1mg/ml type I collagenase (Invitrogen Corp.) for 1 hr at room temperature. The digested tissue suspension was filtered through 200μm nylon mesh and centrifuged at 800g for 3 min to separate the fat cake and SV fraction. The SV fraction was suspended in the plating medium and seeded in 35mm culture dishes. The cell density was approximately 4.3×10^4 / cm^2. The seeded cells were cultured in the plating medium at 25°C in 5% CO_2 in air. After 2 days, the attached SV cells were washed three times with the plating medium without fetal bovine serum, followed by an additional 2 days cultivation in the plating medium. Adipocyte differentiation was initiated by switching the medium to the FA(-) induction medium (3.1 Experiment 1) or standard induction medium (3.2 Experiment 2). The SV cells were maintained at 25°C in 5% CO_2 in air for the periods indicated in the text or figure legends. The culture medium was changed every 2 or 3 days.

2.4 Cytological observations

The cultured SV cells were fixed in 10% formaldehyde and subjected to Sudan black B and nuclear fast red staining.

2.5 RNA extraction, cDNA synthesis and quantification of LPL gene transcripts

The total RNA extraction and random primed cDNA synthesis were performed with SV total RNA isolation system (Promega, Madison, WI, USA) and Omniscript RT PCR kit (Qiagen, Hilden, Germany). Quantity of isolated RNA was determined by the absorbance at 260nm. The total RNA extraction and cDNA synthesis were carried out according to the manufacturers' instruction. Measurements of the LPL gene transcripts were made by means of a real time PCR with iCycler iQ real-time PCR detection system (BioRad, Hercules, CA, US). The 20μl of reaction mixture were prepared with iQ SYBR Green Supermix (BioRad) and the specific primers at a final concentration of 1μM. The oligonucleotide sequences designed for the amplification of 493 bp fragment of red sea bream LPL gene (GenBank accession no. **AB243791**) were as follows: 5'-CTCAAGACCCGCGAGAT-3' (sense) and 5'-AAGCGTCGCTCTGACC-3' (antisense). The real time PCR protocol consisted of 3min at 95°C followed by 40 cycles of 0.5min at 95°C, 0.5min at 50°C and 0.5min at 72°C. Reactions were carried out in triplicate and each transcript level was calculated as copies / μg RNA. Standard

curve was generated by amplification of serial dilutions of known quantities of cDNA fragment prepared from the cloned red sea bream LPL gene and the dynamic range of standard curves spanned four orders of magnitude. Fluorescent data were analyzed with the iCycler iQ Optical System Software Ver. 3.0a (Bio-Rad) and converted to the cycle threshold values. The efficiency of the PCR was more than 75% and the specificity of amplified products was verified by the analysis of melting curves.

2.6 Analyses of triglyceride, protein and DNA amounts

The cultured SV cells were scraped and homogenized in 25mM Tris-HCl - 1mM EDTA and subjected to the analyses of soluble protein, DNA and trigriceride amounts. The soluble protein amount was determined by the method of Lowry (Lowry et al. 1951) with DC protein assay (BioRad) and DNA amount was determined with Hoechst 33258 by the method of Labarca and Paigen (1980). Total cellular lipids were extracted with chloroform-methanol (2:1), followed by the analysis of triglyceride amounts. The triglyceride amount was determined by the enzymatic method with TG Test WAKO (WAKO, Osaka, Japan).

2.7 Statistical analyses

Statistical analyses of differences among treatment means were carried out by ANOVA

and Fisher's Protected Least Significant Difference (StatView 4.51, Abacus Concepts, Berkeley, CA, USA). Differences were considered significant if $P < 0.05$.

3. Results

3.1 Experiment 1: The effects of insulin on the terminal differentiation of red sea bream adipocytes.

To examine the effects of insulin on the terminal differentiation of fish adipocytes, the relationship between insulin concentration and the lipid synthesis during adipocyte differentiation in red sea bream SV cells was investigated. In experiment 1, to eliminate the influence of exogenous lipid, the serum-free medium without any exogenous lipid (see *2. Materials and Methods*) was used and therefore the triglycerides accumulated in the red sea bream SV cells should be dependent exclusively on the *de novo* lipid synthesis from glucose or acetate. Since the red sea bream SV cells could not survive in the serum-free medium without hydrocortisone supplementation, all the tests were conducted in the presence of 50ng/ml hydrocortisone.

When cultured in the serum-free medium supplemented with 5 μ g/ml bovine insulin, the SV cells of red sea bream visceral adipose tissue showed phenotypic features compatible with the terminal differentiation of mammalian adipocytes. During the 2

week cultivation in the presence of 5 μ g/ml bovine insulin, lipid droplets were accumulated in the cytoplasm as characterized by sudan black B staining (Fig. 1a) and the amount of triglyceride (μg TG / μg DNA) increased by 3.8 fold (Fig. 1b). In response to increasing concentrations of bovine insulin, there was a trend for increased cellular TG contents (Fig. 2), indicating that the lipid synthesis in differentiating adipocytes was enhanced by insulin in a concentration-dependent manner.

3.2 Experiment 2: The hormonal effects on the differentiation-linked LPL gene expression in red sea bream SV cells.

During the adipocyte differentiation of red sea bream SV cells, the expression level of the LPL gene showed a 3.6 fold increase at day3 and then plateued (Fig. 3), indicating that LPL gene expression is linked to early stage of adipocyte differentiation as in mammals. By using the cultured SV cells of red sea bream, the effects of insulin, T_3, RA, retinyl acetate and $1,25(OH)_2D_3$ on the differentiation-linked LPL gene expression were examined. Insulin showed a stimulatory effect on LPL gene expression. The LPL gene expression level increased 4.7 fold by the supplement of 50μg/ml bovine insulin in comparison with that of the control (0μg/ml) ($P<0.05$, Fig. 4). T_3 alone had no effect (Fig. 4) but the concomitant addition with insulin enhanced LPL gene expression (Fig.

5). In the presence of 5 μ g/ml bovine insulin, the LPL gene expression level at 20nM T_3 was 3.3 fold higher than that of the control ($P<0.05$, Fig. 4). With or without bovine insulin supplementation, the addition of RA, retinyl acetate and $1,25(OH)_2D_3$ did not show any significant effects on LPL gene expression (Fig. 4 and Fig. 5).

3. Discussion

In this study, we described a culture system of SV cells of red sea bream adipose tissue in a serum-free medium as a potential model system for studies of fish adipocyte differentiation. As reported in many studies conducted on using mammalian cell lines, many chronological events including growth arrest, clonal expansion, adipocyte-characteristic gene expression and lipid accumulation in the cytoplasm take place during the adipocyte differentiation (Ailhaud 1996; Gregoire 1998). In our experimental condition, red sea bream SV cells were able to undergo adipocyte differentiation with respect to a functional gene expression and lipid accumulation in the cytoplasm (Fig. 1 and Fig. 3). Different from the method previously reported by others (Vegusdal et al., 2004), we developed a chemically defined serum-free culture system which allowed fish adipocyte differentiation and thereby the process of fish adipocyte differentiation can be analyzed in the strictly controlled conditions. The method

developed in this study should be of interest for the mechanisms of fish adipocyte differentiation, especially for the characterization of factors involved in the modulation of the differentiation process.

By using the culture system developed in this study, we investigated the hormonal effects on fish adipocyte differentiation *in vitro*. In red sea bream SV cells, insulin and T_3 showed stimulatory effects on adipocyte differentiation *in vitro*. Bovine insulin stimulated the differentiation-linked LPL gene expression (Fig. 4) and the lipid synthesis during the terminal differentiation of adipocytes (Fig. 2) in cultured red sea bream SV cells. T_3 alone had no effect (Fig. 4) but the concomitant addition of T_3 and insulin stimulated the differentiation-linked LPL gene expression (Fig. 5). Both insulin and T_3 are known as inducers of adipocyte differentiation in mammals (Dani et al. 1986; Gharbi-Chihi et al. 1981). The regulatory mechanisms of fish adipocyte differentiation by insulin and T_3 would be similar to those of mammals.

It is well established in mammals that fat-soluble vitamins also mediate the adipocyte differentiation. The all-*trans* retinoic acid (RA), a metabolite of vitamin A, suppresses the mammalian adipocyte differentiation at a superphysiological concentration (0.1-10μM) whereas it shows stimulatory effects at very low concentration (1pM-10nM) (Safonova et al. 1994). $1,25(OH)_2D_3$, an active metabolite of vitamin D_3, inhibits the

adipocyte differentiation at 6.4-20 x 10^{-10} M in ST 13 and 3T3-L1 preadipocyte cell lines (Sato and Hiragun 1988) while Vu et al. (1996) have reported the stimulatory effects of 1,25$(OH)_2D_3$ on the differentiation-linked LPL gene expression in 3T3-L1 at 10^{-9}-10^{-7} M. In our results, RA and 1,25$(OH)_2D_3$ did not show any significant effects on the differentiation-linked LPL gene expression in red sea bream adipocytes, suggesting the regulation of fish adipocyte differentiation by fat-soluble vitamins in fish may differ from that of mammals. However, it remains to be determined if these fat-soluble vitamins actually maintained their biological activity in our experimental conditions. Further analyses including the investigation of appropriate culture conditions are required to determine the roles of fat-soluble vitamins in fish adipocyte differentiation.

Our previous study indicated that dietary retinoic acid and retinyl acetate up-regulate the development of visceral adipose tissue in red sea bream (Ogata and Oku, 2001), although the present study indicated that neither retinoic acid nor retinyl acetate affect the red sea bream adipocyte differentiation as assessed by the differentiation-linked LPL gene expression (Fig. 4 and Fig. 5). Adipose tissue growth involves not only adipocyte differentiation from precursor cells but the cell proliferation and an increase in the adipocyte size. The development of visceral adipose tissue by dietary retinoic acid and retinyl acetate in red sea bream (Ogata and Oku, 2001) may result from the adipocyte

proliferation and/or increase in adipocyte size.

LPL gene has been identified in fish species and utilized as a molecular probe in physiological studies (Liang et al. 2002, Oku et al. 2002, Saera-Vila et al. 2005). Our research group has identified several lipase species including LPL and investigated the nutritional regulation in red sea bream (Oku, unpublished data). In our results, the red sea bream LPL gene showed differentiation-linked expression (Fig. 3), suggesting LPL gene will be a useful model for investigation of the molecular mechanisms of fish adipocyte differentiation.

In summary, we developed a serum-free culture system of red sea bream SV cells as a potential model for the study of fish adipocyte differentiation and investigated the effects of insulin, T_3 and fat-soluble vitamins on the adipocyte differentiation *in vitro*. The results indicated that insulin is required for the lipid synthesis during the terminal differentiation of red sea bream adipocytes. Furthermore, in red sea bream SV cells, like in mammalian adipocytes, insulin and T_3 showed stimulatory effects on the adipocyte differentiation as assessed by the increase in the differentiation-linked LPL gene expression levels, whereas fat-soluble vitamins, unlike in mammalian adipocytes, did not show any significant effects. The method developed in this study will be useful for further investigation of the regulatory mechanisms of fish adipocyte differentiation.

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Figure legends

Fig.1 The lipid accumulation in the cytoplasm (a) and changes in the amounts of triglyceride, protein and DNA (b) during the terminal differentiation of red sea bream SV cells.

The red sea bream SV cells were cultured for 2 weeks in the FA(-) induction medium containing 5μg/ml bovine insulin. (a) The cells were fixed in 10% formaldehyde and stained with Sudan black B and Nuclear fast red. The bar indicates 50μm. (b) The experiment was repeated six times with isolated cell cultures derived from six different fish. Means (mean ± SEM) not sharing a common superscript are significantly different ($P<0.05$).

Fig.2 Effects of insulin on the lipid accumulation in the cytoplasm (a) and the amounts of triglyceride, protein and DNA (b) in the differentiated red sea bream SV cells.

The red sea bream SV cells were cultured for 2 weeks in the FA(-) induction medium containing various concentrations of bovine insulin (bIns). (a) The cells were fixed in 10% formaldehyde and stained with Sudan black B and Nuclear fast red. The bar indicates 50μm. (b) The experiment was repeated six times with isolated cell cultures

derived from six different fish. Means not sharing a common superscript are significantly different ($P<0.05$).

Fig.3 Time-course of the LPL gene expression in red sea bream SV cells during adipocyte differentiation.

The red sea bream SV cells were cultured for 10 days in the standard induction medium containing 5μg/ml bovine insulin. The experiment was repeated six times with isolated cell cultures derived from six different fish. Values (mean ± SEM) are reported as percentage to the mean value of the control (0day) and taken as 100 %. Means not sharing a common superscript are significantly different ($P<0.05$).

Fig.4 Effects of various concentrations of hormones on the differentiation-linked LPL gene expression in red sea bream SV cells.

The red sea bream SV cells were cultured for 3 days in the presence of various concentrations of bovine insulin (bIns), triiodothyronine (T_3), all-*trans*-retionoic acid (RA), retinyl acetate, or 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The hormones were added to the standard induction medium. The experiment was repeated six times with isolated cell cultures derived from six different fish. Values (mean ± SEM) are reported as percentage to the mean value of the control taken as 100 %. Means not

sharing a common superscript are significantly different ($P<0.05$).

Fig.5 Effects of concomitant addition of insulin and various concentrations of hormones on the differentiation-linked LPL gene expression in red sea bream SV cells.

The red sea bream SV cells were cultured for 3 days in the presence of 5μg/ml of bovine insulin (bIns) and various concentrations of triiodothyronine (T_3), all-*trans*-retionoic acid (RA), retinyl acetate, or 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The hormones were added to the standard induction medium. The experiment was repeated six times with isolated cell cultures derived from six different fish. Values (mean ± SEM) are reported as percentage of the mean value obtained for the control and taken as 100 %. Means not sharing a common superscript are significantly different ($P<0.05$).

Fig. 1a

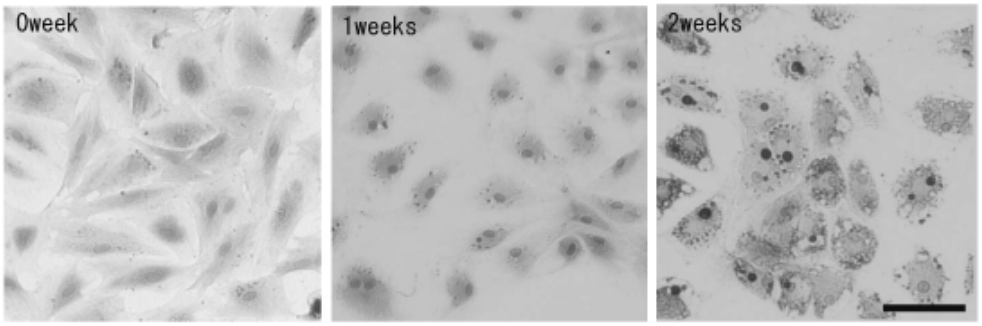

Fig.1b

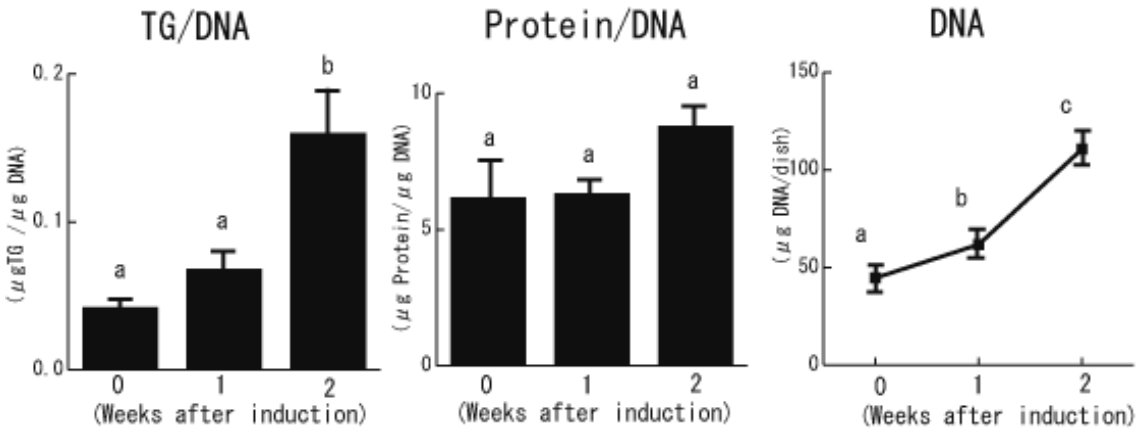
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Fig.2a

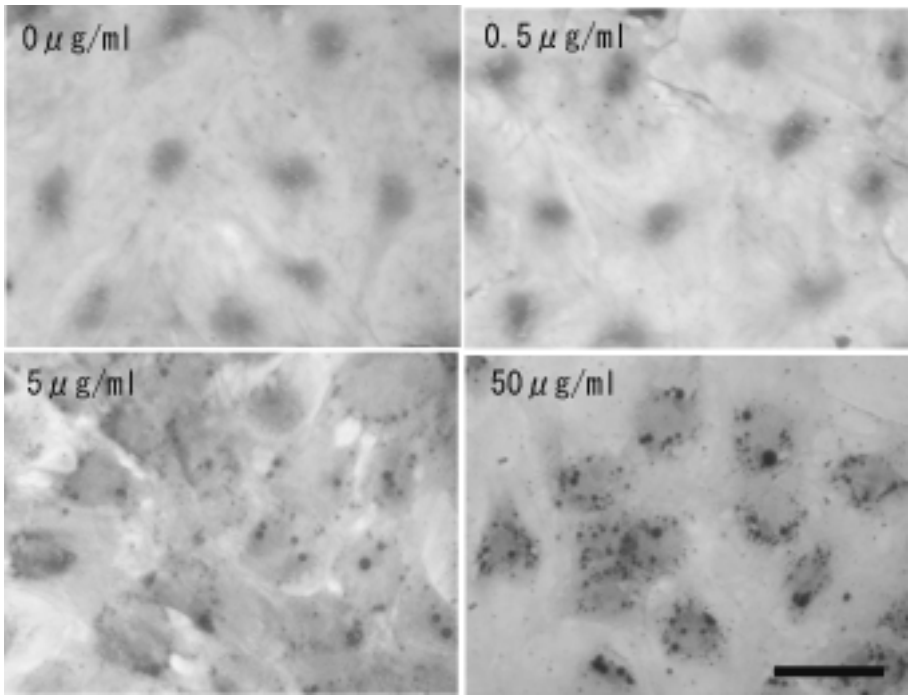
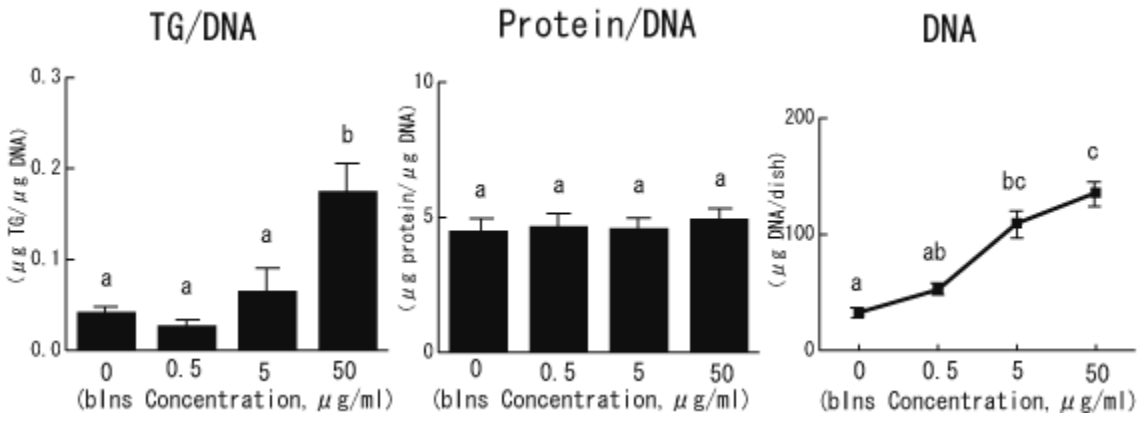


Fig.2b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(bIns Concentration, μg/ml)

Fig.3

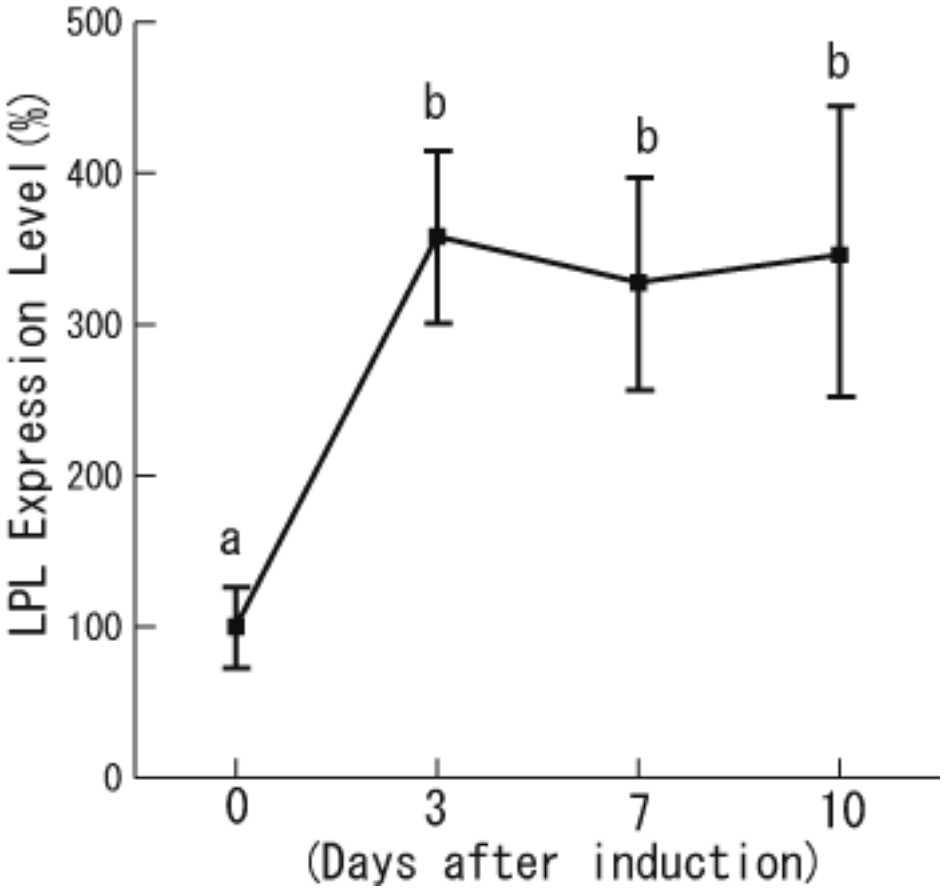

LPL Expression Level (%)
500
400
300
200
100
0
a
b
b
b
0
3
7
10
(Days after induction)

Fig. 4

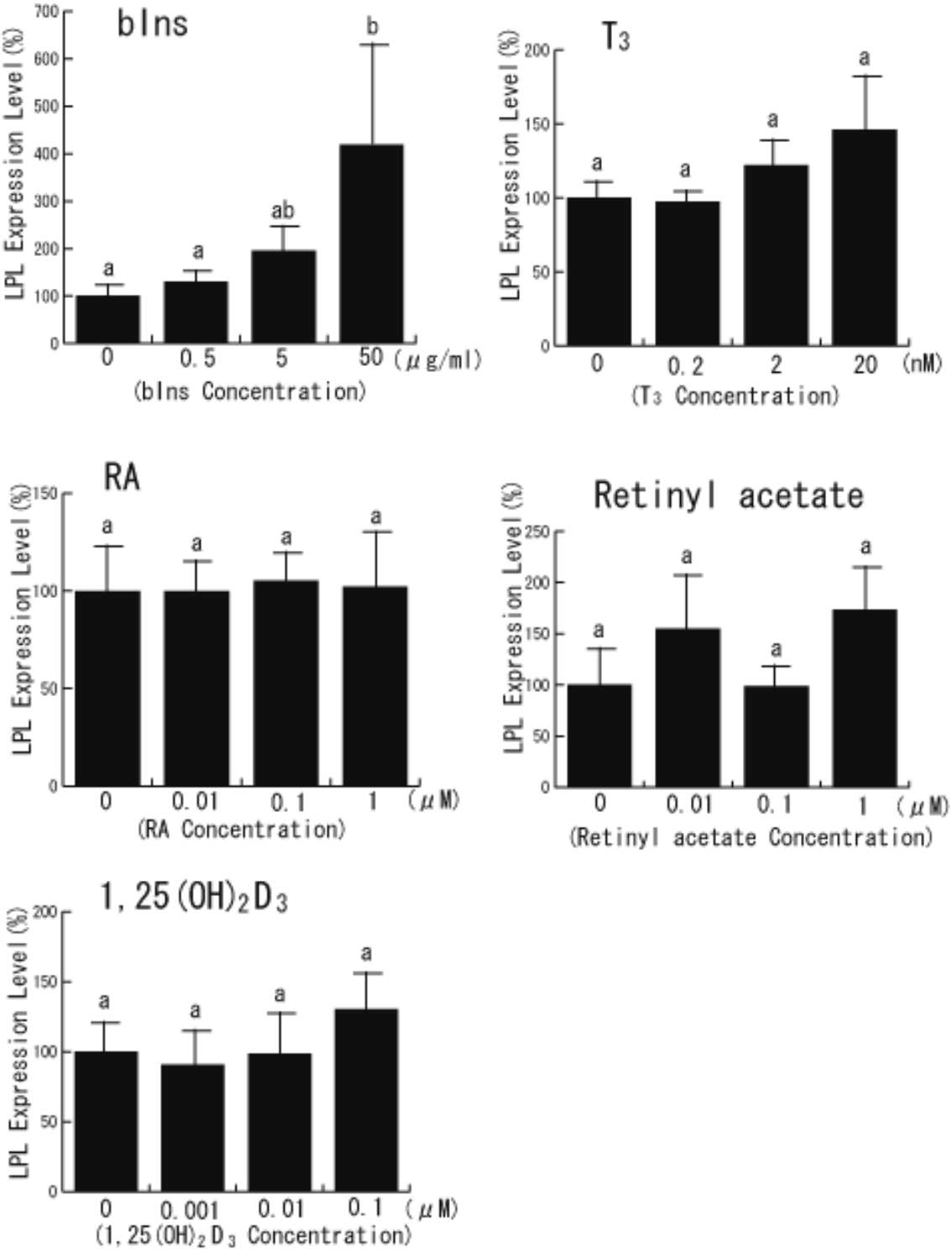
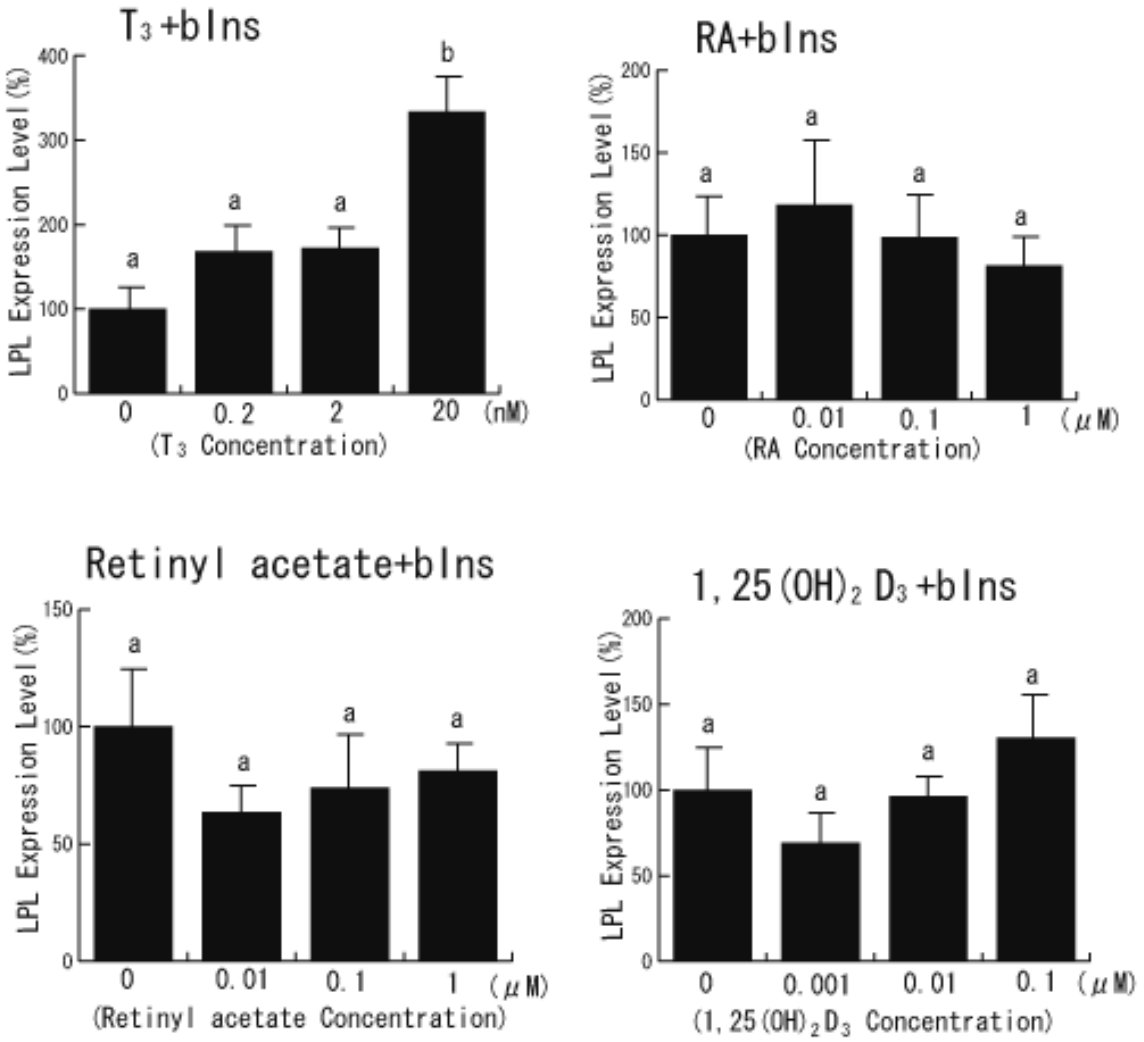

bIns
LPL Expression Level (%)
a
a
ab
b
0
0.5
5
50 (μg/ml)
(bIns Concentration)
T3
LPL Expression Level (%)
a
a
a
a
0
0.2
2
20 (nM)
(T3 Concentration)
RA
LPL Expression Level (%)
a
a
a
a
0
0.01
0.1
1 (μM)
(RA Concentration)
Retinyl acetate
LPL Expression Level (%)
a
a
a
a
0
0.01
0.1
1 (μM)
(Retinyl acetate Concentration)
1,25(OH)2D3
LPL Expression Level (%)
a
a
a
a
0
0.001
0.01
0.1 (μM)
(1,25(OH)2D3 Concentration)

Fig.5


T3 +bIns
LPL Expression Level (%)
400
300
200
100
0
a
a
a
b
0
0.2
2
20 (nM)
(T3 Concentration)
RA+bIns
LPL Expression Level (%)
200
150
100
50
0
a
a
a
a
0
0.01
0.1
1 (μM)
(RA Concentration)
Retinyl acetate+bIns
LPL Expression Level (%)
150
100
50
0
a
a
a
a
0
0.01
0.1
1 (μM)
(Retinyl acetate Concentration)
1,25(OH)2 D3 +bIns
LPL Expression Level (%)
200
150
100
50
0
a
a
a
a
0
0.001
0.01
0.1 (μM)
(1,25(OH)2D3 Concentration)