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Characteristics of Proliferation and Differentiation-Dependent C/EBP, PPARγ and Leptin Gene Expression Patterns Intrinsic to Bovine Intramuscular, Perirenal and Subcutaneous Preadipocytes

[1]Makiko Yamamoto, [1]Yukio Taniguchi, [2]Koichiro Kano and [1,3]Takahisa Yamada
[1]Laboratory of Animal Breeding and Genetics, Graduate School of Agriculture,
Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
[2]Department of Animal Science, College of Bioresource Sciences,
Nihon University, Fujisawa, Kanagawa 252-8510, Japan
[3]Laboratory of Animal Genetics, Graduate School of Science and Technology,
Niigata University, Nishi-ku, Niigata 950-2181, Japan

Abstract: Adipose depotsite-specific variability in physiological characteristics of preadipocytes could be in part, intrinsic to the cells and become evident through *in vitro* proliferation and differentiation-dependent gene expression patterns in preadipocyte in the cell culture. This study was designed to characterize proliferation and differentiation-dependent gene expression patterns intrinsic to intramuscular, perirenal and subcutaneous preadipocytes. About 3 type preadipocytes are prepared using the ceiling culture method from bovine intramuscular, perirenal and are demonstrated subcutaneous adipose depots of a single Japanese Black steer. Adipose depot site-specific proliferation and differentiation-dependent gene expression patterns intrinsic to preadipocyte by comparing the mRNA levels of CCAAT enhancer binding protein C/EBPα, C/EBPβ, C/EBPδ, Peroxisome Proliferator-Activated Receptor γ (PPARγ) and leptin before and after the induction of differentiation among culture of the 3 type preadipocytes. These results may suggest the existence of the site-specific preadipocyte-intrinsic proliferation and differentiation program.

Key words: Adipose depotsite, C/EBP, differentiation, gene expression, leptin, PPARγ, preadipocyte, proliferation

INTRODUCTION

Certain aspects of preadipocyte function depend on the anatomic site of origin of the cells. Preadipocytes from rat perirenal adipose depot are capable of more extensive replication *in vitro* and *in vivo* than preadipocytes from epididymal adipose depot (Djian *et al*., 1983, 1985; Kirkland *et al*., 1990; Miller *et al*., 1984; Wang *et al*., 1989). Cloned preadipocytes from perirenal adipose depot are more likely to differentiate than epididymal preadipocytes (Djian *et al*., 1983; Kirkland *et al*., 1990; Wang *et al*., 1989). Perirenal preadipocytes cultured in differentiation-promoting media develop higher glycerol-3-phosphate dehydrogenase activity than preadipocytes from epididymal adipose depot (Djian *et al*., 1983). Differentiation of epididymal preadipocytes is less extensive than that of inguinal subcutaneous preadipocytes (Gregoire *et al*., 1990). There is evidence suggesting that preadipocytes from the omental adipose depot display greater replication rates than their subcutaneous counterparts (Pettersson *et al*., 1985) and that subcutaneous preadipocytes differentiate more readily in response to thiazolidinediones than their omental counterparts (Adams *et al*., 1997). Further, Kirkland *et al*. (1996) concluded that the adipose depot site-specific variability in preadipocyte function could be in part, a consequence of difference in the characteristics intrinsic to preadipocyte comprising the depot. The intrinsic characteristics become evident through studying *in vitro* proliferation and differentiation-dependent gene expression patterns in preadipocyte in cell culture. It has been reported that gene expression profiles differ extensively among undifferentiated preadipocytes isolated from human subcutaneous, mesenteric and omental adipose depots (Tchkonia *et al*., 2007) indicating the existence of adipose depot site-specific gene expression profiles intrinsic to preadipocyte. However, the adipose depot site-specific gene expression profiles intrinsic to preadipocyte are not detailed before (proliferation phase) and after the induction of

Corresponding Author: Takahisa Yamada, Laboratory of Animal Genetics, Graduate School of Science and Technology, Niigata University, Nishi-ku, Niigata 950-2181, Japan

differentiation (in differentiation phase). Further, intramuscular preadipocyte-specific intrinsic gene expression profiles are not studied.

In the last decade, some of the molecular controls regulating preadipocyte proliferation and differentiation have been established (Brun *et al*., 1996; Fajas *et al*., 1998; Gregoire *et al*., 1998; Loftus and Lane, 1997). Most of the evidence is from experiments using clonal cell lines such as 3T3-L1 or 3T3-F442A. The sequential transcription and translation of individual transcription factors control its proliferation and differentiation. After the induction of differentiation, the earliest-appearing transcription factors are CCAAT enhancer binding protein C/EBPβ and C/EBPδ. Increased C/EBPβ (and perhaps C/EBPδ) stimulates the production of C/EBPα and Peroxisome Proliferator-Activated Receptor γ (PPARγ) which are the major transcription factors in adipocyte differentiation. Several genes for adipocyte-specific proteins such as leptin appearing after adipose conversion of preadipocyte have response elements for C/EBPα, PPARγ or both.

To characterize adipose depot site-specific proliferation and differentiation-dependent gene expression patterns intrinsic to preadipocyte, Expression levels of C/EBPα, C/EBPβ, C/EBPδ, PPARγ and leptin are before and after the induction of differentiation among culture of 3 type preadipocytes prepared from intramuscular, perirenal and subcutaneous adipose depots of a single Japanese Black steer. Adipose depot site-specific intrinsic preadipocyte function can partly contribute to the site-specific feature of the adipose depot development, through their intrinsic programs to proliferate and differentiate and then to convert into adipocytes. Thus, the present study will lead to the understanding of the site-specific molecular mechanisms underlying the development of adipose depots including economically important intramuscular one.

MATERIALS AND METHODS

Preadipocyte preparation: Intramuscular, perirenal and subcutaneous adipose depot samples from a single 30 months old Japanese Black steer were used for cell culture. The sample of subcutaneous adipose depot was removed from the section between 10-12th ribs and intramuscular adipose depot sample was dissected from the muscle core of the *Musculus longissimus* in this section and then the surrounding muscle fibers were separated from the intramuscular adipose tissue. The adipose tissue was sliced into pieces and digested in a 0.1% collagenase solution. By filtration and centrifugation following enzymatic dispersion, the adipocytes were obtained as a thin white layer floating in the centrifuge tube. The pipetting, washing and centrifugation of the liberated adipocytes were repeated 3 times. The adipocytes were cultured according to the ceiling culture method described previously (Matsumoto *et al*., 2008; Nobusue *et al*., 2008; Sugihara *et al*., 1987; Yagi *et al*., 2004) with slight modifications. Briefly, the cells were transferred to 75 cm^2 cell culture flasks which were completely filled with Dulbecco's Modified Eagle's Medium (DMEM) containing 20% Fetal Calf Serum (FCS). The flasks were inverted and incubated at 37°C in 5% CO_2 to allow attachment of the adipocytes to the upper surface of the flasks. Following the attachment of cells which took approximately 1 week, the flask was placed upside down and the medium was changed every 4 days. After a normal position culture for 14 days, the cells were used as preadipocytes for the following experiment.

This study conformed to the guidelines for animal experimentation of the Graduate School of Agriculture, Kyoto University (Kyoto, Japan).

Cell culture: The preadipocytes were seeded into 6-well culture plates at a density of 2×10^3 cells cm^{-2} and allowed to grow into confluent monolayer in the growth medium (DMEM containing 20% FCS) in a humidified atmosphere of 5% CO_2 at 37°C. At confluence to induce adipocyte conversion, the medium was replaced by the differentiation medium (the growth medium supplemented with 5 μg mL^{-1} insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 μM dexamethasone). After 4 days, the differentiation medium was removed and the cells were maintained in the growth medium until a maximum of 12 days.

RNA preparation and quantification of target mRNA levels: Total RNA was prepared from cultured cells at time points of 6 (-6 day), 4 (-4 day) and 2 days (-2 day) and immediately before the induction of differentiation (0 day) and of 2 (2 day), 4 (4 day), 6 (6 day), 8 (8 day), 10 (10 day) and 12 days after the induction (12 day) with the TRIZOL reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 μg of total RNA with oligo $(dT)_{12\text{-}18}$ primers in a 25 μL reverse transcription reaction by using First-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, UK) as recommended by the manufacturer. The resulting cDNA was used directly for PCR amplification without further purification.

Quantification of the levels of C/EBPα, C/EBPβ, C/EBPδ, PPARγ and leptin mRNAs was performed by competitive PCR using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA as an internal control as

Table 1: Sequences of the primers used for the competitive PCR assay

mRNA species	Sense primers	Antisense primers
C/EBPα	5'-TCGCCATGCCGGGAGGACT-3'	5'-GTGTGCGATCTGGAACTGC-3'
C/EBPβ	5'-ACGTCCTCGTCGTCCA-3'	5'-TTGCTCCACCTTCTTCTG-3'
C/EBPδ	5'-AGAGCGCCATCGACTTCA-3'	5'-TTGAAGAACCGCCGCAGTC-3'
PPARγ	5'-TATGACCTGAAGCTCC-3'	5'-CTTTGGTCAGCGGGAA-3'
Leptin	5'-ATGACACCAAAACCCTCA-3'	5'-TCGGTGGAGTAGAGGGAA-3'
GAPDH	5'-GAGGGACTTATGACCACTGT-3'	5'-TCATTGTCGTACCAGGAAATG-3'

previously described (Zhao *et al*., 1995) with minor modifications. The competitors PCR-amplified competitively with the target cDNA were constructed using the corresponding cDNA clones for C/EBPα (Taniguchi and Sasaki, 1996), C/EBPβ (Yamaoka *et al*., 1997), C/EBPδ (Taniguchi and Sasaki, 1997) and leptin (Taniguchi *et al*., 2002), λ phage DNA for PPARγ and a template DNA included in Competitive DNA construction kit (Takara Co. Ltd., Shiga, Japan) for GAPDH as template, as described previously (Dusserre *et al*., 2000). The primer sequences used for the competitive PCR were shown in Table 1. The 0.1 μL reverse transcription product was coamplified with the defined amount of the competitor in a 20 μL PCR reaction as previously described (Tu *et al*., 1997). About 35 cycles of PCR amplification were carried out with denaturation at 94°C for 30 sec, annealing at 45°C (PPARγ), at 50°C (leptin), at 55°C (C/EBPβ, C/EBPδ and GAPDH) or at 58°C (C/EBPα) for 30 sec and extension at 72°C for 1 min. The PCR products were then separated on 3% agarose gels, stained with ethidium bromide and recorded using the FAS-III image analysis system (Toyobo, Kyoto, Japan). The signal intensities of the products from the target cDNA and competitor were quantified using the NIH image program and compared. The amount of the competitor yielding equal intensity to product from the target cDNA was determined for the levels of the corresponding mRNA in the RNA preparation. Then corrected the levels of C/EBPα, C/EBPβ, C/EBPδ, PPARγ and leptin mRNAs for the corresponding levels of GAPDH mRNA to obtain mRNA expression levels in the RNA preparation.

Statistical analysis: A single experiment was performed in triplicate RNA preparations and mean of triplicate RNA preparations was represented as mRNA expression levels in a preadipocyte sample in a single experiment. Since each maximum of the levels of C/EBPα, C/EBPβ, C/EBPδ, PPARγ and leptin mRNAs was indistinguishable among the 3 type preadipocytes, their mRNA levels in a preadipocyte sample were expressed as a percent of each maximal mRNA level in the sample. About 4 independent experiments are performed. Comparisons of mRNA levels among the 3 type preadipocytes at the same time point and among the 10 time points in the same preadipocyte were performed by an analysis of variance with Duncan's new multiple range testing as a post-hoc test using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Morphology: The intramuscular, perirenal and subcutaneous preadipocytes prepared in this study, irrespective of their origin in anatomical depot sites were morphologically fibroblast like cells and possessed no visible lipid droplets based on oil red O staining. By the exposure to the differentiation medium for 4 days, the 3 type preadipocytes, irrespective of their origin in depot sites were in large part converted to morphologically spherical and lipid staining-positive cells which are characteristic of adipocyte at 12th day. Further, overconfluent and time-matched preadipocytes did not show lipid accum ulation over a period of 12 days.

Gene expression: The levels of C/EBPα, C/EBPβ, C/EBPδ, PPARγ and leptin mRNAs were analyzed every other day over a period from 6-12th days and proliferation and differentiation-dependent expression levels of them were compared among intramuscular, perirenal and subcutaneous preadipocytes (Fig. 1).

C/EBPβ: C/EBPβ mRNA levels were higher in perirenal preadipocyte than in intramuscular and subcutaneous preadipocytes in proliferation phase at -6 and -4th day. The perirenal mRNA levels were decreased at -2 day and no difference was detected among the 3 type preadipocytes in proliferation phase at -2 and 0 day. The mRNA levels in the 3 type preadipocytes were increased at 2nd and 4th days except for the perirenal mRNA levels at 2nd days and there was a maximum at 2nd and 4th days, respectively for subcutaneous preadipocyte (the preceding type) and for intramuscular and perirenal preadipocytes. Thereafter, the mRNA levels in the 3 type preadipocytes were decreased at 6 day and no difference was detected among them except for the subcutaneous mRNA levels at 12th day.

C/EBPδ: C/EBPδ mRNA levels were higher in subcutaneous preadipocyte than in intramuscular and perirenal preadipocytes in proliferation phase at 6-4 days. The subcutaneous mRNA levels tended to be decreased

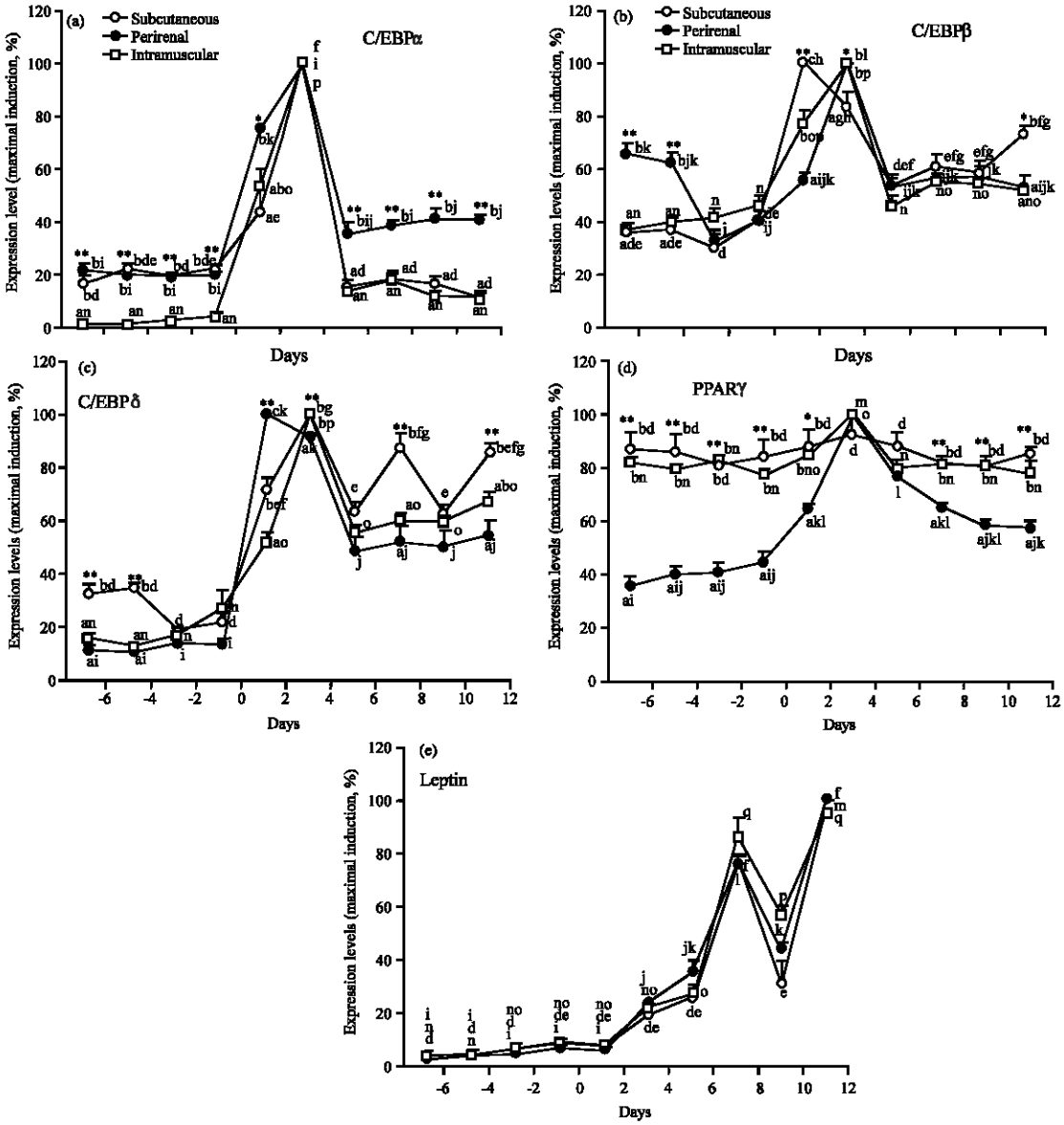

Fig. 1: Expression levels of (a) C/EBPα, (b) C/EBPβ, (c) C/EBPδ, (d) PPARγ and (e) leptin mRNAs during proliferation and differentiation of intramuscular, perirenal and subcutaneous preadipocytes prepared from a single Japanese Black steer. Quantification of the expression levels was performed by competitive PCR using GAPDH mRNA as an internal control. Each point represents the mean of 4 independent experiments with the standard error. Open circles, closed circles and open squares represent the data from subcutaneous, perirenal and intramuscular preadipocytes, respectively. Means of the 3 type preadipocytes at the same time point (*$p<0.05$; **$p<0.01$) and of the 10 time points in the same preadipocyte ($p<0.05$) were compared with Duncan's new multiple range testing as a post hoc test. [a, b] and [c]: Means of different preadipocytes at a time point without a common letter in their superscripts significantly differ; [d, e, f, g] and [h]: Means of different time points in subcutaneous preadipocyte without a common letter in their superscripts significantly differ; [i, j, k, l] and [m]: Means of different time points in perirenal preadipocyte without a common letter in their superscripts significantly differ; [n, o, p] and [q]: Means of different time points in intramuscular preadipocyte without a common letter in their superscripts significantly differ

at -2 day and no difference was detected among the 3 type preadipocytes in proliferation phase at -2 and 0 day. The mRNA levels in the 3 type preadipocytes were increased at 2 and 4 days and there was a maximal level at 2nd and

4th day, respectively for perirenal preadipocyte (the preceding type) and for intramuscular and subcutaneous preadipocytes. Thereafter, the mRNA levels in the 3 type preadipocytes were decreased at 6 day and no difference was detected among them except for the subcutaneous mRNA levels at 8 and 12th day.

C/EBPα: C/EBPα mRNA levels were higher in perirenal and subcutaneous preadipocytes than in intramuscular preadipocyte at all time points in proliferation phase. The mRNA levels in the 3 type preadipocytes were increased at 2nd and 4th day except for the subcutaneous mRNA levels at 2nd day and there was a maximum at 4th day. Thereafter, the mRNA levels in the 3 type preadipotytes were decreased at 6th day but the mRNA levels were higher in perirenal preadipocyte than in intramuscular and subcutaneous preadipocytes.

PPARγ: Unexpectedly, PPARγ mRNA expression was detected at higher levels for intramuscular and subcutaneous preadipocytes at all time points in proliferation phase (particularly, the subcutaneous mRNA levels were indistinguishable from a maximal level at 4th day). Thus, the difference in the mRNA levels in proliferation phase was detected between intramuscular and subcutaneous preadipocytes and perirenal preadipocyte. The mRNA levels were increased after the induction except for the subcutaneous mRNA levels and reached a maximal level at 4th day. Thereafter, the mRNA levels were decreased at 6th day except for the subcutaneous mRNA levels. In differentiation phase at 8, 10 and 12th day, the mRNA levels were higher in intramuscular and subcutaneous preadipocytes than in perirenal preadipocyte.

Leptin: In the 3 type preadipocytes, leptin mRNA expression was observed at markedly low levels in proliferation phase, increased gradually after the induction and reached a maximal level at 12th day while the mRNA levels transiently fell at 10th day from an unknown reason. Thus, no difference in leptin mRNA levels was detected among the 3 type preadipocytes at all time points in proliferation and differentiation phases.

DISCUSSION

Expression levels of C/EBPα, C/EBPβ, C/EBPδ, PPARγ and leptin was compared before and after the induction of differentiation among intramuscular, perirenal and subcutaneous preadipocytes. Based on this comparison, it observed that adipose depot site-specific proliferation and differentiation-dependent gene expression patterns intrinsic to preadipocyte as shown below. First, in proliferation phase, Characteristics of perirenal preadipocyte-specific increase of C/EBPβ mRNA levels at -6 and -4th day of subcutaneous preadipocyte-specific increase of C/EBPδ mRNA levels at -6 and -4th day and of perirenal and subcutaneous preadipocyte-specific increase of C/EBPα mRNA levels at all time points. These characteristics may reflect that C/EBPβ and C/EBPδ expression are increased specifically to perirenal and subcutaneous preadipocytes, respectively and promote C/EBPα expression in proliferation phase. On the other hand, characteristics of intramuscular and subcutaneous preadipocyte-specific increase of PPARγ mRNA levels at all time points in proliferation phase were detected unexpectedly. This characteristic remains unexplained. Second at 2nd and 4th day in differentiation phase, Characteristics of the subcutaneous preadipocyte-specific preceding type of C/EBPβ mRNA expression and of the perirenal preadipocyte-specific preceding type of C/EBPδ mRNA expression. These characteristics may reflect subcutaneous and perirenal preadipocyte-specific enhancement of response of C/EBPβ and C/EBPδ expression, respectively to the induction of differentiation.

Third, at 6-12th day in differentiation phase, characteristics of perirenal preadipocyte-specific increase of C/EBPα mRNA levels were detected at 6-12th days and of perirenal preadipocyte-specific decrease of PPARγ mRNA levels at 8-12th days. These characteristics, together with the fact that no difference in C/EBPβ and C/EBPδ mRNA levels was observed among the 3 type preadipocytes at 6-12th days with the exception of subcutaneous C/EBPβ at 12th days and subcutaneous C/EBPδ at 8 and 12th days may reflect perirenal preadipocyte-specific preference of C/EBPα expression to PPARγ expression at 6-12th days in differentiation phase. Further, the results of adipose depot site-specific proliferation and differentiation-dependent gene expression patterns intrinsic to preadipocyte may suggest the existence of the site-specific preadipocyte-intrinsic proliferation and differentiation program. C/EBPβ, C/EBPδ, C/EBPα and PPARγ expression were dramatically decreased from 4-6th days in all the 3 type preadipocytes except for subcutaneous PPARγ. It believed that this decrease is one of common differentiation-dependent gene expression patterns intrinsic to preadipocyte. However, discard the possibility that this decrease may be attributed to exclusion of inducing agent could not discard.

It has been reported that the adipocyte size is significantly different among intramuscular, perirenal and subcutaneous adipose depots in cattle and human

(Cianzio *et al*., 1985; Hood, 1982; Van Harmelen *et al*., 1998). A variety of studies have suggested that lipogenic and/or lipolytic features are different among adipose depots (Bolinder *et al*., 1983; Bouchard *et al*., 1993; Kather *et al*., 1977; Lafontan *et al*., 1979; Marin *et al*., 1996; Martin and Jensen, 1991; Vikman *et al*., 1995, 1996). Some of these features may result from hormonal, paracrine, nutritional, circulatory and neurological milieux as well as anatomical constraints in the adipose depots but some could be a consequence of mechanism intrinsic and unique to cells themselves comprising the adipose depots such as the site-specific preadipocyte-intrinsic proliferation and differentiation program.

Therefore, it should note that the present study will provide an useful information for the understanding of the site-specific molecular mechanisms of development of bovine adipose tissue, including intramuscular one which improves the palatability and acceptability of beef for the consumer and is involved in economically important factor, marbling, for high quality beef (JMGA, 1998) and that the 3 type bovine preadipocytes used in this study appear to be an appropriate model for investigating the development of bovine adipose tissue *in vivo*.

CONCLUSION

From findings, it is said that the present study will provide an useful information for the understanding of adipose depot site-specific molecular mechanisms underlying the development of adipose depots including economically important intramuscular one.

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