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Effects of Ectopic Expression of Transcription Factors on Adipogenic Transdifferentiation in Bovine Myoblasts

Yang Soo Moon*

Department of Animal Science & Biotechnology, Gyeongnam National University of Science and Technology, Jinju 660-758, Korea

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The present study was conducted to investigate whether myoblasts can be transdifferentiated into adipocytes by ectopic expression of adipogenic transcription factors, including peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein-α (C/EBPα), sterol regulatory element binding protein-1c (SREBP1c), and Krueppel-like factor 5 (KLF5), in primary bovine satellite cells. Transcription factors were transiently transfected into primary bovine myoblasts, and the cells were cultured with adipogenic differentiation medium for 2 days and then cultured on growth medium for an additional 8 days. Ectopic expression of PPARγ or C/EBPα alone was insufficient to induce adipogenesis in myoblasts. However, overexpression of both PPARγ and C/EBPα in myoblasts was able to induce adipogenic transdifferentiation as indicated by the appearance of mature adipocytes, the induction of adipogenic gene expressions, and the suppression of myogenic gene expressions. In addition, KLF5 and PPARγ co-transfected bovine myoblasts were converted to adipocytes but not in cells transfected with only KLF5 expression vector. Overexpression of SREBP1c alone was sufficient to induce transdifferentiation from myoblasts into adipocytes. These results demonstrate that primary bovine satellite cells can be transdifferentiated into adipocytes either by single ectopic expression or combined expression of adipogenic transcription factors in a culture system.

Key words : Myoblasts, adipogenesis, transdifferentiation, transcription factors, bovine

Introduction

The embryonic mesoderm of mammals gives rise to several specialized cell types, including adipocytes, myocytes, osteoblasts and chondrocytes. In response to specific conditions, these cells can switch into another cell type of different linage through transdifferentiation. Transdifferentiation is a postnatal change of differentiated cells to another type of cells [18]. This transdifferentiation process is thought to be controlled at transcriptional levels by several tissue-specific transcription factors [10]. Adipogenic transcription factors such as peroxisome proliferators-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein (C/EBP) family are considered to be very important genes which orchestra adipogenesis program [16]. Moreover, activated PPARγ in myogenic cells is known to inhibit myogenesis and transdifferentiate into adipocytes. Hence, compounds or agents that can activate PPARγ might act as potent transducers of myogenic cells to adipocytes [7]. Several *in vitro* studies demonstrate ectopic expression of adipogenic transcriptional factors in myoblast such as PPARγ and C/EBPα induce transdifferentiation [7,15]. Some studies show that expression of both PPARγ and C/EBPα is sufficient to induce transdifferentiation in goat primary myoblast cells and C2C12 mouse myoblast cell line [6,19]. But, the recent study has shown that *in vivo* gene transfer of PPARγ alone is insufficient to induce adipogenesis in skeletal muscle cells in a rat model [3]. This clearly indicates that along with PPAR γ, the co-expression of C/EBPα is necessary to induce transdifferentiation in *in vivo* system. Most of the studies of adipogenic transdifferentiation are conducted using myoblast cell lines. However, none of studies have been reported to examine whether primary bovine myoblasts can be transdifferentiated into adipocytes by ectopic expression of adipogenic transcription factors. In addition to PPARγ and C/EBPs, the role of adipocyte determination- and differentiation-dependent factor-1/sterol regulatory element binding protein-1 (ADD1/SREBP1) in adipocyte differentiation has been indicated [10]. ADD1/SREBP1 is induced very early during adipocyte differentiation and it also participates in adipocyte gene expression. ADD1/SREBP1 also stimulates the expression of many of the genes necessary for lipogenesis *in vivo* [5,10]. An additional transcriptional fac-

*Corresponding author
Tel : +82-55-751-3262, Fax : +82-55-751-3267
E-mail : ysmoon@gntech.ac.kr

tor, Krueppel-like factor 5 (KLF5), has been shown to function in adipogenesis [14]. In 3T3-L1 preadipocytes, KLF5 expression is induced at the early stage of adipocyte differentiation. Constitutive over-expression of dominant-negative KLF5 inhibits adipocyte differentiation, whereas over-expression of wild-type KLF5 induces differentiation even without hormonal stimulation. KLF5 also induces PPARγ expression during the early stage of differentiation of preadipocytes. However, the role of SREBP1c and KLF5 in transdifferentiation of myoblasts to adipocytes was not elucidated yet.

In the present study, we have examined whether adipogenic transdifferentiation of primary cultured bovine myoblasts could be induced by ectopic expression of adipogenic transcription factors, including PPARγ, C/EBPα, SREBP1c and KLF5.

Materials and Methods

Preparation of bovine satellite cells

Hind limb skeletal muscle tissues from male Hanwoo cattle were collected from a regional slaughter house. Skeletal muscle tissues were collected in air tight plastic bags. The slaughtered cattle were in the age of 24-26 months and with 550-600 kg body weight. Skeletal muscle was washed in PBS and the muscle tissue was minced into fine pieces using sterilized scissors and digested by trypsin-EDTA (Invitrogen, CA, USA) for 2 hours. Digested tissue was centrifuged at 900 g for 3 minutes and upper phase was filtered using 40 µm cell strainer. Filtrate was centrifuged at 900 g at room temperature for 20 minutes. Digestion media was removed leaving cell pellet in the tube. The collected cell pellet was washed three times by DMEM (Dulbecco's Modified Eagle's Medium; HyClone, HyClone Laboratories, Inc.UT, USA) containing 1% penicillin/streptomycin (Invitrogen) and cultured in 100 mm culture dish using DMEM/ 10% fetal bovine serum (FBS, HyClone)/ 1% penicillin/streptomycin/ 0.1% amphotericine by incubating at 37°C and 5% CO_2.

Plasmid gene construction

Several adipogenic genes such as PPARγ, SREBP1c, KLF5 and C/EBPα were PCR-amplified from bovine cDNA pool using specific primers and annealing temperature. The amplicon was first inserted in a cloning vector pCR2.1 (Invitrogen) and transfected into *E. coli* bacterial cells. The positive colonies were selected using colony PCR method. The DNA plasmid was isolated using mini prep kit following the standard protocol given by the manufacturer. The plasmids containing the gene for transcription factors were sent for sequencing (Macrogen Inc., Korea). Gene sequences were compared for homology with sequences in National Center for Biotechnology Information. Then, the plasmids containing genes were subjected to restriction digestion at *EcoRI* site and inserted into expression vector pCDNA3.1 (Invitrogen). These expression vectors were propagated in *E.coli* and were used for transfection studies.

Cell culture and transfection

Bovine satellite cells were plated at the density of 2×10^3 cells/cm^2 and grown in proliferation medium containing DMEM, 10% fetal bovine serum and 1% antibiotics. Post confluent cells (d 0) were further cultured in adipogenic differentiation medium (ADM) containing 17 µM pantathenate, 1 mM caprylic acid, 200 µM ascorbic acid, 33 µM biotin, 10 mM acetic acid, 10 ng/m insulin, 0.25 µM dexamethasone (DEX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in DMEM/FBS/antibiotics for 2 days [2]. Then the cells were cultured in growth medium containing 17 µM pantathenate, 1 mM caprylic acid, 200 µM ascorbic acid, 33 µM biotin, 10 mM acetic acid, 10 ng/ml insulin, in DMEM/FBS/antibiotics for 8 days. Media were changed every other day.

The expression vectors of various transcription factors such as PPARγ, SREBP1c, and C/EBPα were transiently transfected to bovine satellite cells using lipofectamine plus reagents (Invitrogen) according to the instruction from the manufacturer. Briefly, the cells were plated in 60 mm dishes (2×10^3 cells /cm^2 in DMEM with 10% FBS without antibiotics) and allowed to attach overnight. Two solutions were prepared under the clean bench, (a) 2 µg of the vector construct was diluted in 100 µl of Opti-MEM medium (b) 6 ul of lipofectamine in 100 ul of Opti-MEM medium. These two solutions were mixed gently and incubated at room temperature for 30 mins to form DNA-liposome complex. Further, the complex was laid over the cells throughout the plate. The plates were mixed gently by rocking the plate back and forth. The medium was changed after 4 hours and was incubated for 48 hours prior to adipogenic treatment to induce transdifferentiation. After transfection cells were further cultured in ADM containing 17 µM pantathenate, 1 mM caprylic acid, 200 µM ascorbic acid, 33 µM biotin, 10 mM acetic acid, 10 ng insulin, 0.25 µM DEX, 0.5 mM IBMX in

DMEM/FBS/antibiotics for 2 days. Then the cells were cultured in adipogenic proliferation medium containing 17 µM pantathenate, 1 mM caprylic acid, 200 µM ascorbic acid, 33 µM biotin, 10 mM acetic acid, 10 ng/ml insulin, in DMEM/FBS/antibiotics for 8 days. For the positive control, post-confluent cells (day 0) were cultured in ADM containing troglitazone (TGZ, 10 µM) for 8 days. Control cells (empty vector transfected cells) were cultured only in adipogenic induction medium. The medium was changed every other day.

RNA isolation and RT-PCR analysis

Trizol reagent (Invitrogen) was used to extract total RNA from cultured cells, according to the manufacturer's protocol, and RNA was stored in DEPC-treated water at -80°C until further use. Concentrations of the extracted RNA samples were measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE, USA).

RNA was reverse-transcribed into the first strand cDNA using Superscript-II reverse transcriptase (Invitrogen). Total RNA (1.5 µg in 20 µl total volume) was primed with oligo $(dT)_{20}$ primers (Bioneer Co., Daejeon, Korea), and reverse transcription was carried out to obtain cDNA at 42°C for 50 min and 72°C for 15 min. Subsequently, 1 µl of the cDNA product and 10 pmoles of each gene-specific primer (Table 1) were used to check the levels of several adipogenic and myogenic genes. The PCR of the specific gene was performed for denaturation at 94°C for 30 sec, annealing at specific gene primer's annealing temperature (Table 1) for 30 sec and extension at 72°C for 1 min. The DNA was visualized by ethidium bromide staining and analyzed using gel viewer software and EasyDoc Gel documentation system (EasyDoc, Korea). The mRNA levels were corrected using the transcription level of the beta-actin gene as an internal standard.

Oil Red O staining

Oil red O (Sigma, MO, USA) stock solution was prepared by mixing 5 mg/ml of Oil red O in 60% tri-ethyl phosphate (Sigma). Oil red O Stock solution was diluted into working Oil red O solutions (6:4 Oil red O: autoclaved de-ionized water). The diluted working solution was filtered by Whatman paper (Whatman International Ltd., Maidstone, England) before use. The cells cultured in 6-well plates were fixed by 10% formalin for 10 minutes after removing the culture media. Then, the formalin fixed cells were washed three times by autoclaved de-ionized water. The working solutions of Oil red O (1 ml/well) were added in the wells. Fifteen minutes later, the staining solution was removed and washed the wells with autoclaved de-ionized water. After air drying, wells were mounted with de-ionized water and observed under microscope.

Table 1. Primers used for reverse transcription polymerase chain reaction (RT-PCR)

Genes	Primers	Sequences(5'-3')	Tm (°C)
tSREBP1c	Forward	AGTTCTGTGTCCATCCTCTTTAGTCCACTT	63
	Reverse	TTTCTCTCTCAGAGTAGGGTATCGGTAAGG	
C/EBPα	Forward	ATGGAGTCGGCCGACTTCTAC	63
	Reverse	CGCGCAGTTGCCCATGGCCTT	
C/EBPβ	Forward	ATGCAACGCCTGGTGGTCTGG	64
	Reverse	GCAGTGGCCGGAGGAGGCGAG	
PPARγ	Forward	ATGGGTGAAACCCTGGGAGAT	50
	Reverse	ATACAAGTCCTTGTAGATTTC	
GPDH	Forward	ATGACCGGCAAGAAAGTCTGC	56
	Reverse	GATCTTGCCGATGAACTGATG	
FABP4	Forward	ATGTGTGATGCATTTGTAGGT	46
	Reverse	TTATGCTCTCTCATAAACTCT	
Adipogenin	Forward	ATGAAGTACCCTCTGGTGCCA	59
	Reverse	GCAGCAGGGCCTCTCCTCCTC	
Myf5	Forward	ATGGACATGATGGACGGCTGC	55
	Reverse	TAGCACATGATAGATGAGCCT	
MyoD	Forward	ATGGAGTTGACTGTCGCCGCCG	63
	Reverse	GAGCACCTGGTAAATCGGGTT	
Actin	Forward	GCCACGAGGCCCAGAGCAAG	63
	Reverse	GGGGCCACACGCAGCTCATT	

Statistical Analysis

The graphed data were analyzed by ANOVA using the GLM procedure of Satistical Analysis System (SAS Inst, NC, USA). Significant differences were detected ($p<0.05$) by Duncan's multiple range test using SAS.

Results and Discussion

Effects of PPARγ and C/EBPα on adipogenic transdifferentiation in bovine myoblasts

Recent advances in the understanding of the transcriptional basis of adipogenesis allow a direct examination of the ability of myogenic cells to enter the adipogenic pathway. Satellite cells originated from skeletal muscle are considered to be stem-like cells to proliferate and self-renew [4]. Even though satellite cells are able to produce only cells of the myogenic linage, recent studies have shown that satellite cells also have multipotential [1,6,12,19,21]. Thus, we determined whether bovine skeletal satellite cells transfected with PPARγ and C/EBPα can be transdifferentiated into adipocytes. Cells transfected with empty vector or PPARγ or C/EBPα expression vector alone did not show any accumulation of lipid droplets in the cells (Fig. 1A). On the other hand, Oil red O stained mature adipocytes were observed in the cells transfected with both PPARγ and C/EBPα (Fig. 1A). In the PPARγ or C/EBPα transfected cells, the expressions of adipogenic genes, fatty acid binding protein 4 (FABP4), glycerophosphate dehydrogenase (GPDH), and adipogenin were not detected. However, troglitazone (TGZ, 10 μM) treated cells and co-transfected (PPARγ and C/EBP α) cells were expressed adipogenic genes and inhibited expression of myogenic genes such as myf5 and myo-D. These results indicate that two adipogenic transcription factors, PPARγ and C/EBPα, are necessary to induce transdifferentiation of bovine myoblasts into adipocytes. We also demonstrated here that PPARγ and C/EBPα have profound effects on the process of myogenesis. Their expression was sufficient to block muscle differentiation, thereby inducing a transdifferentiation of myoblasts to adipocyte cells. Recent report has shown that satellite cells isolated from Hanwoo or porcine are transdifferentiated into adipocytes by PPARγ agonist [12,16]. According to these reports, PPARγ agonists are able to induce transdifferentiation of satellite cells to adipocytes. Our results are similar to the report of study on mouse G8 myoblasts [7]. Ectopic expressions of PPARγ and C/EBPα in fibroblastic cell lines are induced transdifferentiation into adipocytes [6,9]. Some studies show that expression of either PPARγ or C/EBPα is sufficient to induce transdifferentiation in goat primary myoblast cells and C2C12 mouse myoblast cell line [6,19]. But, the other report indicates that mouse G8 myoblasts need the ectopic expression of both PPARγ and C/EBPα to induce transdifferentiation [7]. Though, the G8 and C2C12 cell lines are originated from the mouse species, the reason for the difference in transdifferentiation by ectopic expression of genes is to be explained in the future.

Effects of KLF5 on adipogenic transdifferentiation in myoblasts

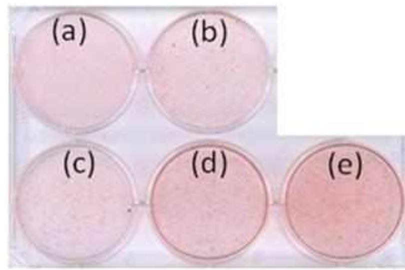
Members of the KLF family of transcription factors are important regulators of development, cellular differentiation and growth in tumor development [9]. Recent studies also suggest a potential role for KLFs in adipogenesis [9,14]. KLF5 expression is induced at an early stage of differentiation and this is followed by expression of PPARγ in 3T3-L1 [13]. As KLF5 is a molecule on the upper stream of PPARγ in adipogenesis, it could be also act as a potential regulator to transdifferentiate myoblast into adipocyte. To test this idea, we examined whether ectopic expression of KLF5 could induce adipogenic transdifferentiation in myoblasts. Cells transfected with KLF5 did show a little accumulation of lipid droplets (Fig. 2A). However, the intensity of the staining of Oil red O was less than those of cells co-transfected with KLF5 and PPARγ. Even though, Oil red O stained cells were observed in KLF5 transfected cells, over-expression of KLF5 alone did not induce the expression of adopogenic genes such as adipogenin and PPAR γ compared with those in control cells transfected with pcDNA3.1 vector (Fig. 2B). The expression of myogenic gene, myf5 also was not inhibited in the cells transfected by KLF5. When the cells were co-transfected with KLF5 and PPARγ, the expression of adipogenic genes were stimulated and down regulated gene expression of the myf5. These results suggest that KLF5 alone is insufficient to induce transdifferentiation, but co-transfecion of KLF5 and PPARγ on to myoblast is able to induce the adipogenic transdifferentiation from myoblast.

Effect of SREBP1c on adipogenic transdifferentiation in myoblasts

We also examined the effect of another adipogenic transcription factor such as; SREBP1c on transdifferentiation of

myoblast to adipocytes. SREBP1c is expressed in the early phase of adipogenic differentiation, so we were very keen to know if SREBP1c alone brought about the trans-differentiation or not. In order to determine whether ectopic expression of tSREBP1c (truncated SREBP1c: N-terminal domain of protein, amino acids 1-436, which is the active form of SREBP1c) could involve in the regulation of trans-differentiation of myocytes, tSREBP1c was transfected into cells with different concentration. The over-expression of tSREBP1c stimulated the adipogenic transcription factors

A



B

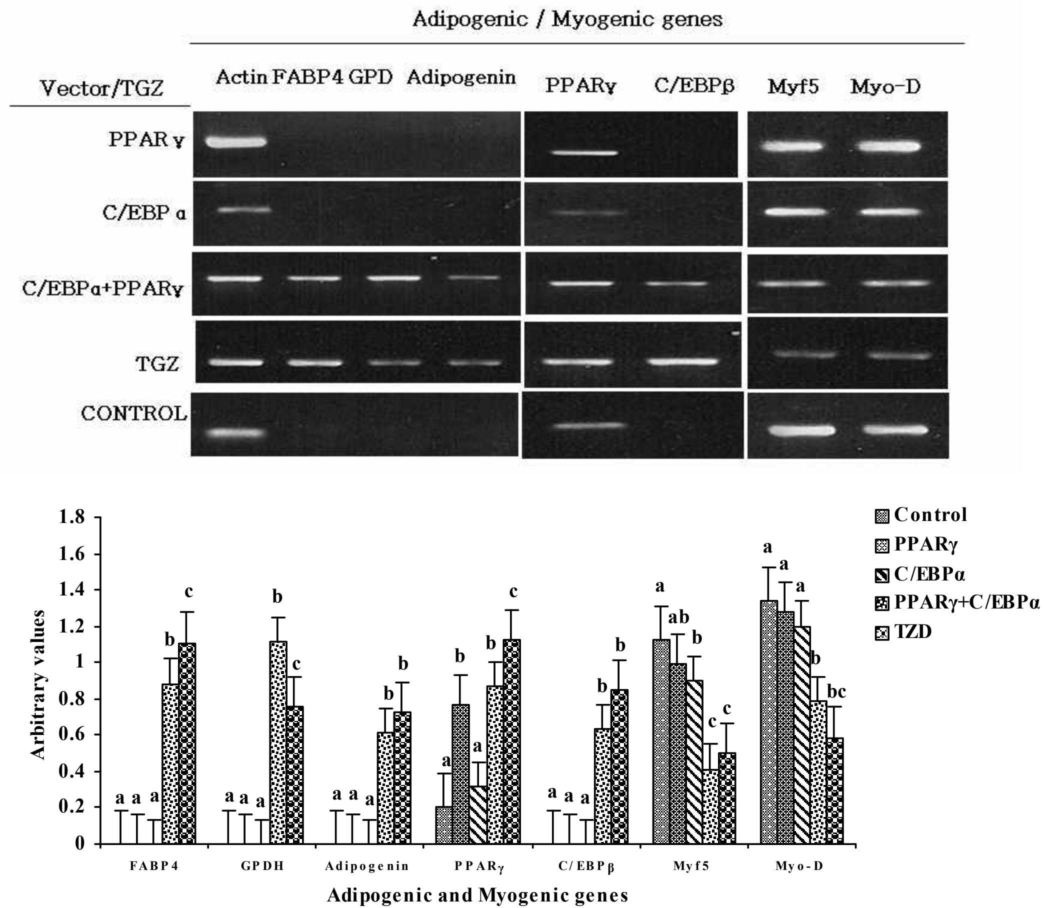


Fig. 1. Ectopic expression of adipogenic transcription factors PPARγ, C/EBPα or both PPARγ and C/EBPα in bovine satellite cells. A. Transfected cells with appropriated plasmid vectors were cultured under adipogenic medium for 10 days and stained with Oil red O. (a) pcDNA3.1, (b) PPARγ, (c) C/EBPα, (d) PPARγ+C/EBPα, (e) Troglitazone (TGZ). TGZ was treated on the cells as a positive control. B. Representative ethidium bromide-stained agarose gel showing amplified adipogenic and myogenic marker genes (top). The RT-PCR results were the representative of three separate experiments with the same protocol. Data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments (bottom). a-c, Mean values with different superscripts are significantly different p<0.05.

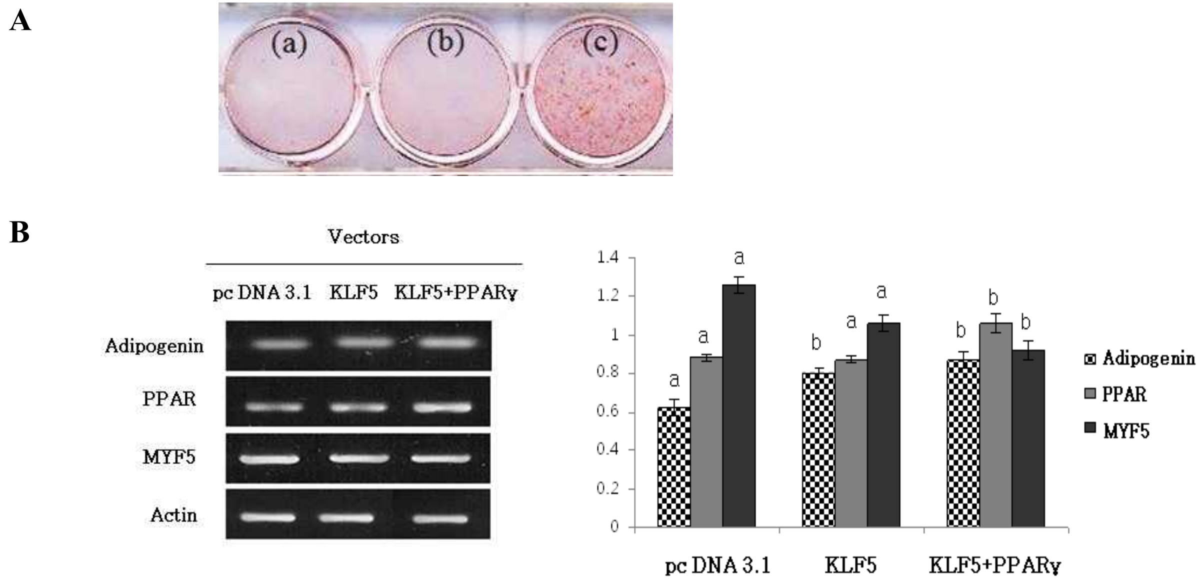


Fig. 2. Ectopic expression of adipogenic transcription factor KLF5 or both KLF5 and PPARγ in bovine satellite cells. A. Oil red O stained cells transfected with appropriated plasmid vectors. (a) pcDNA3.1, (b) KLF5, (c) KLF5+ PPARγ. B. Representative ethidium bromide-stained agarose gel showing amplified adipogenic and myogenic marker genes (left). The RT-PCR results were the representative of three separate experiments with the same protocol. Data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments (right). a-b, Mean values with different superscripts are significantly different $p<0.05$.

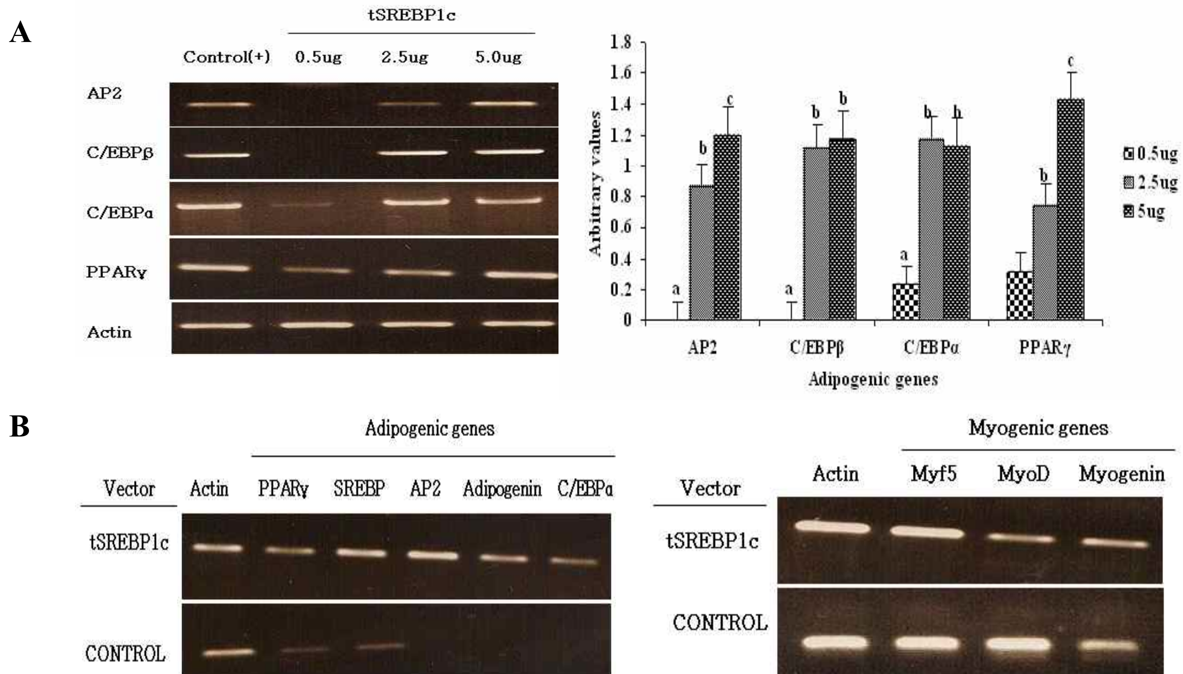


Fig. 3. Ectopic expression of adipogenic transcription factor tSREBP1c in bovine satellite cells. A. Adipogenic gene expressions in cells transfected with different concentration of tSREBP1c (left). The TGZ was used as a positive control. The RT-PCR data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments (right). a-c, Mean values with different superscripts are significantly different $p<0.05$. Expression of adipogenic (B) and myogenic (C) genes in cells transfected with tSREBP1c. The control cells were treated with empty vector (pcDNA3.1) without tSREBP1c.

C/EBPα, β and PPARγ in a dose-dependent manner (Fig. 3A). tSREBP1c-transfected cells induced adipogenic gene markers (Fig. 3B) which demonstrated that myoblast was induced to transdifferentiate into adipocytes. The over-expression of tSREBP1c in myoblast also down regulated the myogenic genes MyoD, myf5 and myogenin (Fig. 3C). These results demonstrate that the over-expression of truncated SREBP1c could induce the transdifferentiation of myoblasts to adipocytes under the adipogenic conditions.

In summary, we have examined whether adipogenic transdifferentiation of primary cultured bovine myoblasts could be induced by ectopic expression of adipogenic transcription factors. Our study has shown that both PPARγ and C/EBPα were necessary to induce transdifferentiation of bovine myoblasts into adipocytes. Co-transfecion of KLF5 and PPARγ on to myoblast has shown the adipogenic transdifferentiation from myoblast. Over expression of tSREBP1c alone induced transdifferentiation from myoblasts into adipocytes. Our findings are relevant to understanding the molecular regulation of the transdifferentiation of myoblasts into adipocytes and they are useful to improve meat quality in animal industry. Our results suggest that adipogenic transcription factors can be applied for the regulation of transdifferentiation from myoblasts to adipocytes through co-transfection(C/EBPα and PPARγ, KLF5 and PPARγ) or single over-expression (tSREBP1c) in bovine primary myoblasts.

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초록 : 한우(Bos taurus coreanae) 유래 myoblast에서 전사인자 과발현에 의한 지방세포로의 교차분화 유도

문양수*
(경남과학기술대학교 동물생명과학과)

본 연구는 한우유래 myoblast에서 지방세포분화 유도 전사인자들을 과발현시켜 지방세포로의 교차분화를 유도하기 위하여 실시하였다. 한우 유래 satellite cell을 배양한 후 adipogenic transcription factor인 PPARγ, C/EBPα, SREBP1c, KLF5등을 단독 또는 co-transfection을 실시하여 세포에 과발현을 유도하였다. 이들 세포들은 adipogenic differentiation medium에서 2일간 배양한 후growth medium에서 8일간 추가로 배양하였다. 지방세포로의 교차분화 유무는 Oil-red O염색과 지방세포 마커 유전자들의 발현으로 확인하였다. PPARγ과 C/EBPα를 각각 단독으로 과발현을 유도한 경우myoblast에서 지방세포로의 교차분화를 유도하기에는 충분하지 못하였다. 그러나 PPARγ와 C/EBPα을 co-transfection을 실시한 경우 지방세포로의 교차분화가 유도되었고, 세포내지방구 형성, 지방세포 마커유전자의 발현, 근세포 마커유전자의 발현 감소 등이 확인되었다. KLF5 와 PPARγ를 동시에 과발현할 경우 지방세포로의 교차분화를 볼 수 있었지만 KLF단독의 경우는 교차분화를 유도하지 못하였다. 활성형SREBP1c (tSREBP1c)의 경우, 단독으로 myoblast에 과발현을 처리한 경우만으로 지방세포로의 교차분화를 유도할 수 있었다. 이들 결과는 한우유래 satellite cell을 이용하여 지방세포분화 전사인자를 단독 혹은 조합하여 이들 세포에 과발현 시킬 경우 지방세포로의 교차분화를 유도할 수 있음을 보여 주었다.