ORIGINAL

ALK7 is a novel marker for adipocyte differentiation

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Abstract : Transforming growth factor-β (TGF-β) family members regulate a variety of cellular functions and play important roles in cell differentiation. Activin receptor-like kinase 7 (ALK7), a receptor for TGF-β family members, was initially cloned from rats as an orphan receptor and has been recently shown to be a type I receptor for nodal, activin B and activin AB. ALK7 is expressed not only in neurons, but also in insulin-producing islet β cells and white and brown adipose tissues ; however, the specific functions of ALK7 in these tissues are not known. In order to test whether ALK7 is involved in adipocyte differentiation, we analyzed its expression during adipocyte differentiation. ALK7 expression was detected in the late phase of adipocyte differentiation by reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting and immunofluorescence staining in 3T3-L1 cells. We also detected the expression of ALK7 by RT-PCR in stromal vascular fraction (SVF) cells. These results indicated that ALK7 is a novel marker specifically expressed during the late phase of adipocyte differentiation. Furthermore, our results suggest the possible involvement of nodal or activin B in adipocyte differentiation. J. Med. Invest. 53 : 238-245, August, 2006

Keywords **:** *ALK7, adipocyte differentiation, activin, adipogenesis, TGF-beta*

INTRODUCTION

TGF-β family members regulate a variety of cellular functions and play important roles in cell differentiation, adhesion, migration, and apoptosis (1). This superfamily consists of a large group of growth and differentiation factors, including TGF-βs, activins, inhibins, nodal, bone morphogenetic proteins (BMPs) and other related proteins (2-5). Activins signal through types I and II transmembrane serine/threonine kinase receptors (6), and bind to type II receptors independently (7). Upon ligand binding, type I receptors are recruited to type II receptors and produce activated receptor complexes. Activated type I receptors phosphorylate Smad proteins, which regulates the transcription of various genes in the nucleus (2, 8).

One of the type I receptors, ALK7, was initially cloned from rats as an orphan receptor (9-11). The ligand of this orphan receptor was recently identified as nodal, activin B and activin AB (12, 13). ALK7 mRNA was anatomically distributed in the central nervous system and the periphery (9-11). ALK7 is expressed not only in the neuron system, but also in insulin-producing islet β cells and white and brown adipose tissues (10, 11, 14) ; however, the specific function of ALK7 in adipose tissue is not known. Recent studies in adiposcience revealed that adipocytes play active roles in many physiologic and pathologic processes. In order to confirm whether ALK7 is involved in adipogenesis, we analyzed the expression of ALK7 during

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adipocyte differentiation using a preadipocyte cell line, 3T3-L1 and mouse primary cells of adipose tissue-derived SVF.

MATERIALS AND METHODS

Adipocyte cell culture

We used a preadipocyte cell line, 3T3-L1 and mouse primary culture of SVF cells. 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum, supplemented with penicillin and streptomycin (P/S) at 37℃ under humidified 5% CO_2 atmosphere. To prepare SVF cells, inguinal and visceral adipose tissues were excited from mice, and digested with 3.33 mg/ml type II collagenase with gentle rocking in Hanks' Balanced Salt Solution for 60 min at 37℃. After sequential filtration through 100 and 40 μm filters, the isolated SVF cells were suspended in fetal bovine serum (FBS), and cultured in DMEM with 10% FBS and P/S. Only SVF cells without passaging were used in this study.

After preadipocyte cells reached confluence, differentiation was induced by culturing cells in induction medium supplemented with adipogenic agents (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin in DMEM containing 10% FBS). Two days later, the induction medium was replaced by the maintenance medium (DMEM containing 10% FBS and P/S), and the medium was renewed every two days.

RT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was dissolved in diethyl pyrocarbonate-treated water and the concentration of RNA was determined by absorbance at 260 nm. We analyzed the expression of ALK7, other activin receptors and adipogenic markers including CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ, adipocyte fatty acid-binding protein (aP2) and peroxisome proliferator-activated receptor γ (PPARγ) by RT-PCR. Single-strand cDNA was synthesized using a SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. PCR was conducted using MyCycler Thermal Cycler (BIO-RAD). In the case of ALK7, 5% dimethyl sulfoxide (DMSO) was added to the reaction mixture to improve PCR amplification.

The primer sets used in this study are shown in Table 1. Most of those primers were described previously (15). PCR conditions were optimized for detection within a linear range. For the amplification of ALK7, PCR was performed as follows : an initial denaturing step of 94℃ for 2 min, followed by 40 cycles of 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 1 min. All other genes were amplified by the following programs including 'stepdown' steps: 10 initial cycles of 30 sec denaturation at 94℃, 15 sec annealing at 74-54℃, 30 sec elongation at 72℃ where the annealing temperature was decreased by 4℃ per 2 cycles, followed by 28 cycles of 30 sec denaturation at 94℃, 15 sec annealing at 54℃, 30 sec elongation at 72℃, and a final extension step at 72℃ for 10 min. To amplify C/EBPα in SVF cells, the first 10 initial stepdown cycles were followed by 30 cycles of 30 sec denaturation at 94℃, 15 sec annealing at 54℃, 30 sec elongation at 72℃, and a final extension step at 72℃ for 10 min. To

Table 1. Sequences of the primer sets used for PCR

Primers	Forward	Reverse	GenBank accession number
mActRI	5'-ATGACTACCTTCAGCTCACT-3'	5'-CTTCGCCAGAGAAGTTAATG-3'	BC058718
mActRIB	5'-ACCGCTACACAGTGACCATT-3'	5'-TCTTCACATCTTCCTGCACG-3'	NM007395
mActRII	5'-CTTAAGGCTAATGTGGTCTC-3'	5'-GACTAGATTCTTTGGGAGGA-3'	M65287
mActRIIB	5'-ATCGTCATCGGAAACCTCCC-3'	5'-CAGCCAGTGATCCTTAATC-3'	M84120
mC/EBPα	5'-AGTCGGCCGACTTCTACGAG-3'	5'-GATGTAGGCGCTGATGT-3'	M62362
mC/EBPβ	5'-GCAAGAGCCGCGACAAG-3'	5'-GGCTCGGGCAGCTGCTT-3'	X62600
mPPARγ	5'-AGGCCGAGAAGGAGAAGCTGTTG-3'	5'-TGGCCACCTCTTTGCTCTGCTC-3'	U01664
maP2	5'-TCTCACCTGGAAGACAGCTCCTCCTCG-3'	5'-TTCCATCCAGGCCTCTTCCTTTGGCTC-3'	K02109
mALK7	5'-GGCTGTGAAAATATTCTCCTCC-3'	5'-GGATCTGAAGGCACCATGTCAT-3'	AY127050
m18S	5'-GGGAGCCTGAGAAACGGC-3'	5'-CCGCTCCCAAGATCCAACTAC-3'	X00686

amplify C/EBPβ and PPARγ in 3T3-L1, the first 10 initial stepdown cycles were followed by 25 cycles of the same amplification as C/EBPα in SVF cells. To amplify aP2 and 18S, similar PCR reactions were performed with 23 and 21 cycles of amplification in 3T3-L1 and SVF cells, respectively. PCR products were separated on 2% agarose gel and visualized with ethidium bromide.

Western blotting and immunofluorescence

Cells were harvested and lysed in 50 mM Tris-HCl (pH 7.5), 1% NP-40, 150 mM NaCl, 1% aprotinin and 1 mM PMSF by sonication. Protein concentrations of cell lysates were determined using BioRad Protein assay kit. 10 μg of protein was subjected to 12% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, MA, USA). After blocking with 5% skim milk in PBS, the membrane was incubated with the primary antibody, rabbit anti-ALK7 (13) (diluted 1 : 500), followed by incubation with the secondary antibody conjugated to horseradish peroxidase, anti-rabbit IgG (Amersham Biosciences ; diluted 1 : 2500) for 3 hours. ALK7 was detected by chemiluminescence with the ECL Western blotting detection system (Amersham Biosciences).

Oil Red O staining was performed to visualize oil droplets in the differentiated cells. Cells were washed once with PBS, fixed with 3.7% formaldehyde in PBS, and incubated in 0.3% Oil Red O solution to visualize oil droplets. We also used Sudan III to investigate oil drops when the cells were analyzed by immunofluorescence at the same time. For co-imaging of Sudan III fluorescence and ALK7 immunofluorescence, cells were fixed for 30 min in 2% paraformaldehyde at room temperature, permeabilized with 0.1% Triton X-100, and blocked with 1.5% BSA prior to incubation with polyclonal rabbit anti-ALK7 antibody (diluted at 1 : 100). FITC-labeled anti-rabbit IgG was used as the secondary antibody.

RESULTS

ALK7 expression during adipocyte differentiation

As a model system for adipogenesis, we used the 3T3-L1 preadipocyte cell line. On reaching confluence, 3T3-L1 preadipocytes were growth arrested by contact inhibition. These growth-arrested cells reentered the cell cycle after hormonal induction, proliferation arrested again, and finally underwent terminal adipocyte differentiation over several days. Under these conditions, we investigated ALK7 expression with known adipogenic markers by RT-PCR. Transcription of the C/EBPβ gene has been shown to be expressed shortly after exposure of the cells to differentiation inducers (16). C/EBPβ in turn transactivates the expression of C/EBPα and PPARγ genes, the two transcription factors responsible for establishing and maintaining the adipocyte phenotype. PPARγ is a major regulator of terminal adipocyte differentiation and drives the expression of several adipocyte-specific genes, such as the fatty acid binding protein, aP2 (17).

In our experiment, 3T3-L1 cells started to express C/EBPβ mRNA immediately after induction (day 1), as shown in Fig. 1A. The expressions of PPARγ and aP2 were detected from day 2. Under these conditions, all activin receptors except ALK7 were expressed constantly before and after adipocyte differentiation in 3T3-L1 cells (Fig. 1B). In contrast, ALK7 was not expressed in 3T3-L1 preadipocytes but was significantly induced in the late phase of adipocyte differentiation (day 4-day 6), as shown in Fig. 1B.

In order to confirm the expression of ALK7, we next tested the expression of ALK7 by Western blotting. We prepared cell extracts from samples from day 4 to day 8 after adipogenic induction, and performed Western blotting with the anti-ALK7 antibody. Distinct 55kDa bands corresponding to ALK7 protein were detected weakly from day 4. On days 6 and 8, the expression of ALK7 protein gradually increased (Fig. 2).

We further analyzed the expression of ALK7 by immunofluorescence during adipogenesis and immunostained 3T3-L1 cells with anti-ALK7 antibody from day 4 after adipogenic induction. Oil droplets were visualized by Sudan III staining. On day 4, the expression of ALK7 was easily detected (Fig. 3). As expected, cells that expressed ALK7 at a high level accumulated oil droplets, indicating that they were differentiated into mature adipocytes. In contrast, no accumulation of oil droplets was observed in ALK7-negative cells.

In order to detect endogenous ALK7 expressed on the cell surface, we performed similar immunostaining without the permeabilization of plasma membranes with Triton X-100. As shown in Fig. 4, cells expressing ALK7 on the cell surface accumulated oil droplets on day 4. On day 6 after induction, we observed a remarkable increase of oil droplets com-

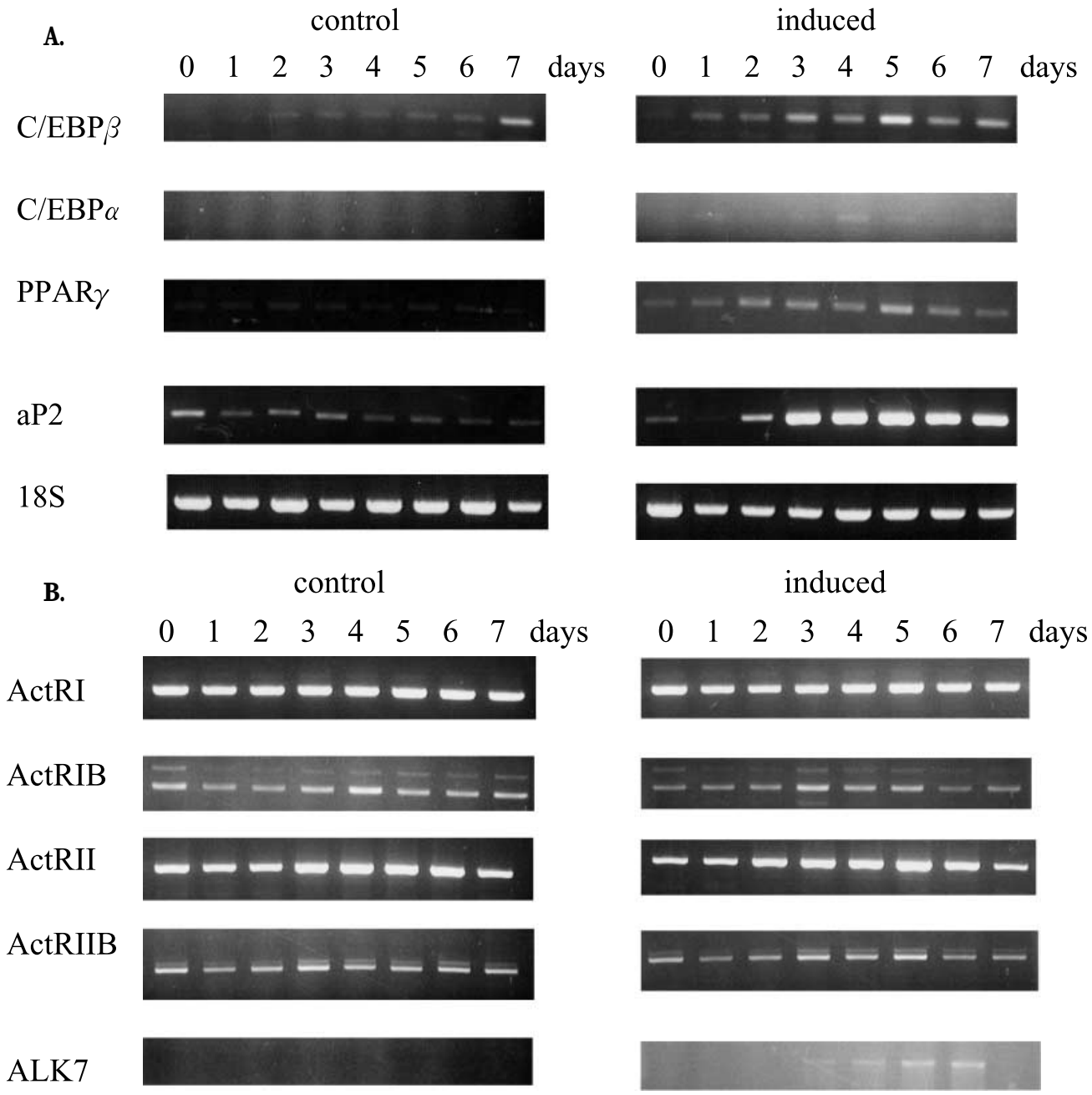

Fig. 1. Gene expressions during the differentiation of 3T3-L1 cells.
(A) Expressions of adipogenic markers.
After 3T3-L1 preadipocytes reached confluence (day 0), differentiation was induced by treating the cells with the adipogenic medium. Two days later (day 2), the medium was replaced by the maintenance medium and cultured as previously described in MATERIALS AND METHODS. Total RNA was isolated on the indicated days after the induction of differentiation. The specific primers listed on Table 1 were used for RT-PCR analyses.
(B) Expressions of activin receptors.
Total RNA preparation and RT-PCR assays were performed as described in Fig. 1A.

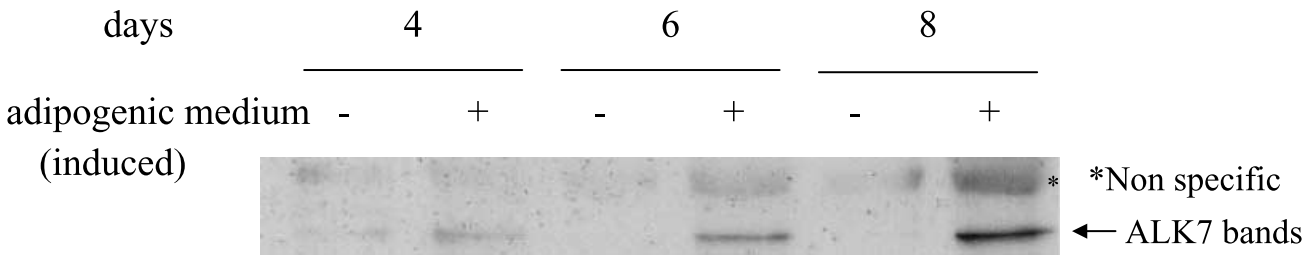

Fig. 2. Expression of ALK7 protein during 3T3-L1 adipocyte differentiation. Western blotting analysis was performed using anti-ALK7 antibody and the protein band was detected by enhanced chemiluminescence (ECL).

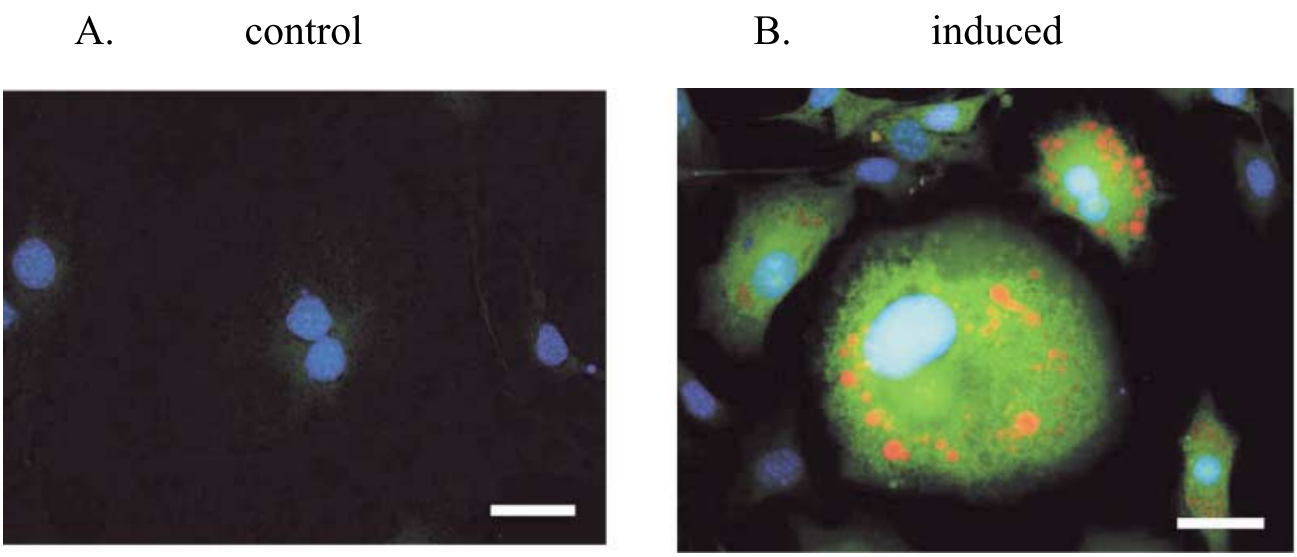

Fig. 3. Immunostaining of ALK7 in 3T3-L1 cells. The cells were treated with the maintenance medium (A) or the induction medium (B) for 2 days. After culturing the cells for 2 days in the maintenance medium, the cells were fixed with 2% paraformaldehyde, and ALK7 protein was probed with a polyclonal anti-ALK7 followed by FITC-conjugated secondary antibody (green). Oil drops were detected with Sudan III staining (red). The nuclei were stained with DAPI (blue). The scale bar represents 30 μm.

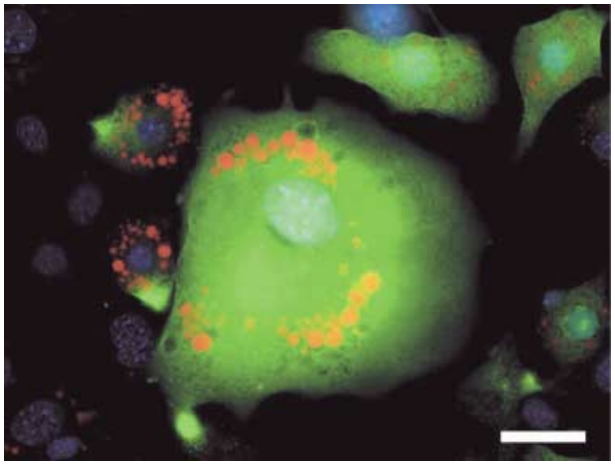

Fig. 4. Immunostaining of ALK7 expressed on the cell surface on day 4 after adipogenic induction. Cells were treated as in Fig. 3 except that permeabilization with Triton X-100 was omitted. The localization of ALK7 (green) was detected by anti-ALK7 antibody and FITC-conjugated secondary antibody. Oil drops and nuclei were detected with Sudan III (red) and DAPI (blue), respectively. The scale bar represents 30 μm.

pared to on day 4 (data not shown). In particular, large oil droplets were detected on day 6, and ALK7 was expressed on those cells. These data suggested that signaling through ALK7 expressed on the cell surface may be involved in the late phase of adipocyte maturation.

ALK7 expression during primary adipocyte differentiation

In order to further confirm the involvement of ALK7 in adipogenesis, we used a mouse primary culture of SVF cells. SVF cells have also been particularly useful for validating results obtained from preadipose cell lines such as 3T3-L1 (18) and they represent the physiological system compared with 3T3-L1 cells. To evaluate whether these fibroblast-like cells can differentiate into mature adipocytes, we examined their differentiation using Oil Red O staining. As shown in Fig. 5, SVF cells cultured in the adipogenic medium showed a remarkable increase of oil droplets in contrast to the control cells. Oil droplets appeared after 3 or 4 days of induction with dynamic morphological changes of the cell shape. Fibroblast-like cells differentiated into round-shaped cells, which is characteristic of oil droplet accumulation. On the other hand, we did not observe spontaneous cell differentiation without treatment with the induction medium.

We examined the expression of activin receptors by RT-PCR during adipocyte differentiation.

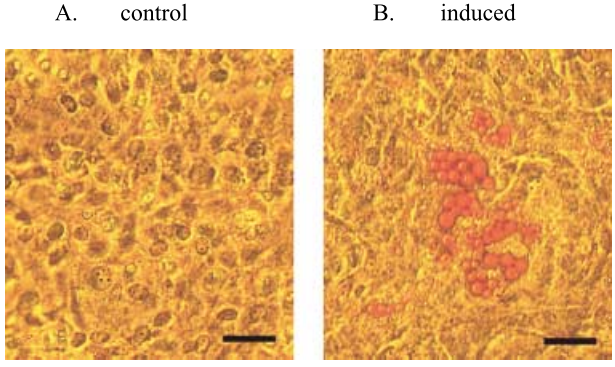

Fig. 5. Oil red O staining of SVF cells. The cells were treated with the maintenance medium (A) or the induction medium (B). Following 2 days' induction, the cells were cultured for 4 days in the maintenance medium, fixed with 3.7% formaldehyde, and stained with 0.3% Oil Red O solution. Representative photomicrographs (magnification, ×200) are shown. The scale bar represents 30 μm.

We prepared RNA from SVF cells on day 0 to day 5 after adipocyte induction. As is the case with 3T3-L1 cells, ALK7 was induced in the late phase of adipocyte differentiation in SVF cells, whereas the expression levels of all activin receptors except ALK7 were not changed during adipogenesis (Fig. 6 A, B). These results confirmed that ALK7 is a marker specifically expressed in the late phase of adipocyte differentiation.

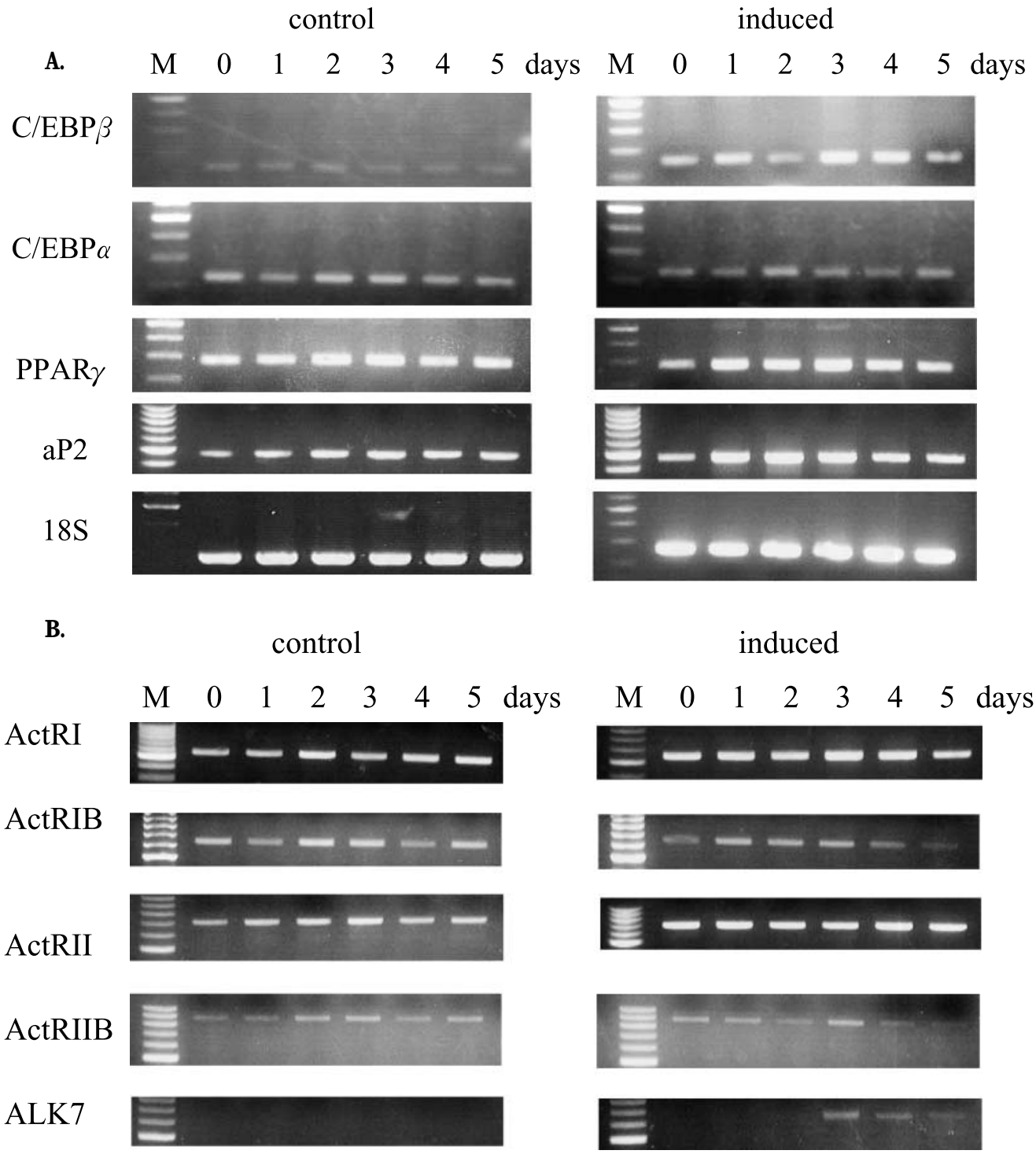

Fig. 6. Gene expressions during the differentiation of SVF cells.
(A) Expressions of adipogenic markers.
Total RNA preparation and RT-PCR assay were performed as described in Fig. 1A. Lane M denotes the DNA size marker, 100bp DNA ladder (Promega).
(B) Expressions of activin receptors.
Total RNA preparation and RT-PCR assay were performed as described in Fig. 1A.

DISCUSSION

Obesity is a nutritional disorder most widespread in humans. It serves as a significant risk factor for many diseases such as diabetes, cancer, heart disease, and hypertension (19, 20). Increased adipose tissue mass can result from the multiplication of new fat cells through adipogenesis and/or from increased deposition of cytoplasmic triglycerides in fat cells (21). To study the process of adipocyte differentiation, 3T3-L1 preadipocyte cells have been widely used as an *in vitro* model system.

TGF-β superfamily members have been shown to regulate the differentiation of a variety of cell types, including adipocytes (22, 23). For example, TGF-β inhibits adipocyte differentiation through Smad2- and Smad3-dependent pathways (24). Smad proteins interact with C/EBPβ and decrease gene expression of PPARγ. Myostatin, a TGF-β family member, is a potent negative regulator of skeletal muscle growth. Myostatin binds ActRIIB and ALK4 or ALK5, and activates a TGF-β-like signaling pathway via Smad2 and Smad3 (25). Myostatin also inhibits preadipocyte differentiation in 3T3-L1 cells, which is mediated, in part, by the altered regulation of C/EBPα and PPARγ (26). Recently, activin A has been shown to inhibit adipogenesis in the early phase via Smad3-mediated impairment of the transactivation function of C/EBP (15).

In this study, we found that ALK7 is specifically induced during the late phase of adipogenesis, although all the other activin receptors are expressed constantly in both 3T3-L1 and SVF cells. The specific expression of ALK7 on the cell surface of oil droplet-accumulating cells suggests that ALK7 may function in those cells. Recently, ligands for ALK7 were identified as nodal, activin B and activin AB (12, 13). ALK7 is highly expressed not only in the nervous system, but also in adult pancreatic islets, duodenum, prostate, fetal testis and ovary, where activins play significant roles (27). The recent finding that activin B can augment glucose-dependent insulin secretion in the mouse pancreatic β cell line (13) raises the possibility that ALK7-dependent activin B signaling may be involved in the regulation of adipogenesis ; however, further study will be necessary to clarify the specific functions of ALK7 in adipocytes.

In summary, we found that ALK7 is induced in the later phase of adipocyte differentiation in 3T3-L1 cells and SVF cells. ALK7 could be a useful marker for the analysis of adipogenesis.

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