ISSN : 1225-9918
Journal of Life Science **2012** Vol. 22. No. 4. 447~455 DOI : http://dx.doi.org/10.5352/JLS.2012.22.4.447

Regulation of Skeletal Muscle Differentiation by Akt

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Received January 6, 2012 / Revised March 6, 2012 / Accepted March 7, 2012

Akt plays an important role in a variety of cellular physiologies such as growth, proliferation, and differentiation. In skeletal muscle, Akt has been implicated in regulating regeneration, hypertrophy, and atrophy. In this study, the role of Akt has been examined during skeletal muscle differentiation. Culturing C2C12 myoblasts under low serum (1% horse serum) and high density converted cell morphology from a round shape to an elongated and multi-nucleated shape. Morphological changes were initiated from day 2 of differentiation. In addition, the expression of both myogenin G and myogenin D was elevated from day 2 of differentiation. Skeletal muscle differentiation was abolished by silencing Akt1 or Akt2, but was significantly enhanced by the over-expression of either Akt1 or Akt2. The activation of Akt was observed from day 2 of differentiation and disappeared after day 7. The expression of krüppel-like factor 4 was observed from day 6 of differentiation. Moreover, this expression was blocked in cells silencing either Akt1 or Akt2. In addition, the promoter activity of krüppel-like factor 4 was significantly reduced in cells silencing Akt1 or Akt2. These results suggest that Akt regulates skeletal muscle differentiation through the regulation of krüppel-like factor 4 expression.

Key words : Muscle, differentiation, Akt, KLF4, myogenin

Introduction

Myogenesis is necessary for normal development as well as the repair of damaged muscle fibers in postembryonic life [17]. Skeletal muscle differentiation is multistep process, in which mononucleated myoblasts withdraw from the cell cycle, initiate the expression of muscle-specific genes, and fuse with each other to form the multinucleated myotubes. The principal regulator of this process is MyoD family transcription factors [21]. Their stage-specific associations with different transcriptional co-regulators ultimately activate the differentiation program by inducing muscle-specific genes. However, the upstream signaling pathways that regulate muscle-specific gene expression are still unclear.

The Akt family of kinase consists of three different isoforms such as Akt1, Akt2, and Akt3. Three isoforms are encoded by three different genes but shares high sequence homology about 85% [26]. Despite their high sequence homology, each isoform of Akt seems to have distinctive function. For example, mice lacking Akt1 displays small organism size (~80% compared with wild type mice) but shows normal metabolism [5]. On the other hand, targeted mice of Akt2 show normal animal size but display type 2 diabetes-like syndrome [6]. Mice lacking Akt3 show normal animal size and metabolism except small brain size [7].

It has been reported that Akt plays an essential role in a variety of cellular physiologies such as growth [35], proliferation [18], survival/apoptosis [19], metabolism [16], migration [38], and in many types of differentiation [24]. Particularly, the isoform-specific function of Akt1 has been implicated in adipocyte differentiation [37], and cell migration [14]. Akt1 specifically phosphorylates forkhead transcriptional factor class O1 (FoxO1) to induce adipocyte differentiation [37]. It also has been reported that Akt1 specifically interacts with mammalian target of rapamycin complex 2 (mTORC2) and regulates cancer cell migration, invasion, and metastasis [15]. The role of Akt has been implicated in skeletal muscle differentiation during past decades. For instance, inhibition of phosphatidylinositol 3-kinase (PI3K) blocks myocytes differentiation [9]. In addition, expression of dominant-negative p85 subunit of PI3K significantly suppresses muscle differentiation [12]. Moreover, it has been reported that PI3K/Akt signaling pathway controls myogenin transcriptional activity during insulin-like growth factor-1 (IGF-1)-induced muscle differentiation [29].

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Krüppel-like factor (KLF) family of transcription factor binds GC-rich regions and related CACCC sequences in DNA [23]. KLF family of proteins acts as either transcriptional activator or repressor [30]. Some KLFs such as KLF3 and KLF8 are broadly expressed [31], but some KLF such as KLF1 is only expressed in erythrocytes [20]. KLF family of proteins has been shown to play a key role in multiple biological responses including erythropoiesis [3], adipogenesis [2], and the self-renewal of embryonic stem cells [11]. KLF4 regulates proliferation in a variety of cells. Forced expression of KLF4 suppresses DNA synthesis thereby inhibits cellular growth in both colon cancer and fibroblast cells [13]. In addition, KLF4 also influences growth in vascular smooth muscle cells [33]. Recently, the role of KLF4 in smooth muscle differentiation has been defined by using KLF4 knockout animal model. In this model, targeted-deletion of KLF4 in vascular smooth muscle accelerates smooth muscle differentiation [36]. However, the role of KLF4 in skeletal muscle differentiation is still unclear.

In the present study, we have shown that forced expression of Akt regulates the expression of KLF4 as well as skeletal muscle differentiation.

Materials and Methods

Materials

All culture media were purchased from Hyclone Laboratories Inc. (Logan, UT, USA). Rabbit polyclonal antibodies against Akt1 and Akt2 were obtained from Upstate Biotechnology Inc. (Lake placid, NY). Anti-pan Akt and anti-phospho-Akt (Ser473) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). The luciferase reporter gene assay system for the KLF4 promoter was a generous gift from Dr. Gary K. Owens at the University of Virginia. IRDye700- or IRDye800-conjugated rabbit or mouse secondary antibody was obtained from Li-COR Bioscience (Lincoln, NE, USA).

Cell culture and differentiation

C2C12 myoblasts were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 incubator. To initiate differentiation of C2C12 myoblasts into myotubes, cells were grown to 80-90% confluence and then replaced with differentiation medium (DM) containing DMEM with 1% horse serum (HS) for 10 days. Differentiation medium (DM) was changed every 24 hrs.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression of KLF in C2C12 myoblasts was quantified by RT-PCR analysis. GAPDH mRNA was included as an internal control. Total RNA in C2C12 myoblasts was isolated using Trizol reagent (Invitrogen, NY), as described in the user's manual. One microgram of total RNA was reverse transcribed into cDNA using ImProm-II reverse transcription systems (Promega, WI), which was then amplified by PCR using specific primers for MyoG (forward, 5′-aactaccttcctgtccacct-3′; reverse, 5′-taggcgctcaatgtactgga-3′) and MyoD (forward, 5′-aactgctctgatggcatgatg-3′; reverse, 5′-tggagatgcgctccactatg-3′). Equal amounts of RT-PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

Lentiviral knockdown

For gene silencing, HEK293-FT packaging cells were grown to ~70% confluence in 6-well plates. Cells were triple transfected with 4 μg of pLKO.1 lentiviral construct, 1 μg of Δ8.9, 1 μg of pVSV-G using a calcium phosphate method. Medium was replaced with fresh medium 8 hrs post-transfection. Lentiviral supernatants were harvested at 24 hrs post-transfection and passed through a 0.45 μm filter. Cell-free viral culture supernatants were used to infect C2C12 myoblasts in the presence of 8 μg/ml of polybrene. An additional round of infection was done at 48 hrs post-transfection. Infected cells were isolated by selection with 10 μg/ml puromycin for 2 days.

Gene expression by retroviral infection

To generate retroviral particles, ecotropic BOSC23 packaging cells were grown to ~70% confluence in 6-well dishes. Cells were triple transfected with 5 μg of pMIGR2 retroviral vector containing Akt1 and Akt2, 1 μg of pGag/Pol, and 1 μg of pVSV-G by calcium phosphate method. Medium was replaced with fresh medium 8 hrs post-transfection. Retroviral supernatants were harvested 24 hrs post-transfection and passed through a 0.45-μm filter. Cell-free viral culture supernatants were used to infect C2C12 myoblasts in the presence of 8 μg/ml of polybrene. An additional round of infection was performed at 48 hrs and 72 hrs post-transfection. Infection was validated by GFP expression

under fluorescent microscopy, and infected C2C12 myoblasts were grown in 15-cm dishes.

Promoter assay

pGL3 and pGL3-KLF4 are kindly provided by Dr. Gary K. Owens (Univ. of Virginia). For the measurement of promoter activity, dual-luciferase reporter assay system was employed. C2C12 myoblasts were plated in 12-well plates. Cells were co-transfected with the luciferase reporter constructs and the renilla luciferase plasmids using Lipofectamine 2000 (Invitrogen). Each well contained 0.88 μg of luciferase reporter plasmids, 0.8 μg of expression vectors, and 80 ng of renilla luciferase plasmids. Medium was replaced with fresh medium 7 hrs post-transfection. The cells were lysed and assayed for luciferase activity 12 hrs after post-transfection. Twenty microliters of protein extracts were analyzed in a Glomax^{TM} 20/20 luminometer (Promega, WI, USA).

Western blotting

Cells were lysed in 20 mM Tris-HCl, pH 7.3, 1 mM EGTA/EDTA, 1% triton X-100, 1 mM Na_3VO_4, 10% glycerol, 1 μg/ml leupeptin and 1 μg/ml aprotinin. After centrifugation at 12,000 rpm, cell lysates (30 μg) were subjected to 10% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were incubated with the indicated primary antibody and IRDye-conjugated secondary antibody. Protein bands were visualized by infrared image analyzer (Li-COR Bioscience).

Results

Differentiation of C2C12 myoblasts

To examine the effect of serum on the differentiation of C2C12 myoblasts, cells were grown on two different conditions. As shown in Fig. 1A, culturing cells under 10% FBS/DMEM condition for 10 days did not induce any morphological changes. However, culturing cells under 1% HS/DMEM initiated morphological changes from day 2 of incubation and finally acquired differentiated-phenotype of C2C12 myoblasts, for example, long-shaped, tube-like structure, and multinucleated morphologies. These results indicate that serum contents are major determinant for the differentiation of C2C12 myoblasts. Next, the expression of skeletal muscle marker gene expression was verified to further confirm the differentiation of C2C12 myoblasts. In correlation with morphological changes of C2C12 myoblasts under 1% HS/DMEM condition, expression of myogenin G (MyoG) and myogenin D (MyoD) was initiated on day 2 of incubation and lasted until day 10 (Fig. 1B). These results suggest that C2C12 myoblasts are able to be differentiated by culturing in the presence of 1% HS/DMEM condition.

Knockdown of Akt prevents C2C12 myoblast differentiation

Akt plays an essential role in a variety of cellular differentiation. To examine the effect of Akt isoforms on the skeletal muscle differentiation, we have silenced each isoform of Akt in C2C12 myoblasts. As shown in Fig. 2A, C2C12 myoblasts expressed both Akt1 and Akt2. Akt1 shRNA effectively silenced Akt1 expression, without significantly affecting Akt2 expression, and *vice versa* for Akt2 shRNA. Silencing either Akt1 or Akt2 did not affect elongation of C2C12 myoblasts but abolished multi-nucleation and tube formation step during the differentiation of C2C12 myoblasts (Fig. 2B), indicating that both Akt1 and Akt2 are required for the differentiation of C2C12 myoblasts.

Over-expression of Akt1 or Akt2 enhances C2C12 myoblast differentiation

Since knockdown of Akt1 or Akt2 significantly blocked the skeletal muscle differentiation (Fig. 2), we examined the effect of Akt over-expression on the differentiation of C2C12 myoblasts. As shown in Fig. 3A, infection C2C12 myoblasts with retrovirus harboring each Akt isoform resulted in more than 100-fold increment of the expression in comparison with endogenous level. Multi-nucleated tube formation was achieved at day 6 of differentiation, however, tube formation was achieved as early as day 2 of differentiation after over-expression of either Akt1 or Akt2 (Fig. 3B). There was no big difference in between Akt1 and Akt2 over-expression. These results suggest that both Akt1 and Akt2 are required for the skeletal muscle differentiation.

Activation of Akt and induction of KLF4 during the differentiation of C2C12 myoblasts

Since our results showed that knockdown of Akt1 or Akt2 diminished skeletal muscle differentiation whereas over-expression of Akt1 or Akt2 facilitated skeletal muscle differentiation, it is possible that Akt activity is somehow involved in the mechanistic pathways of skeletal muscle differentiation. To examine the involvement of Akt in skel-

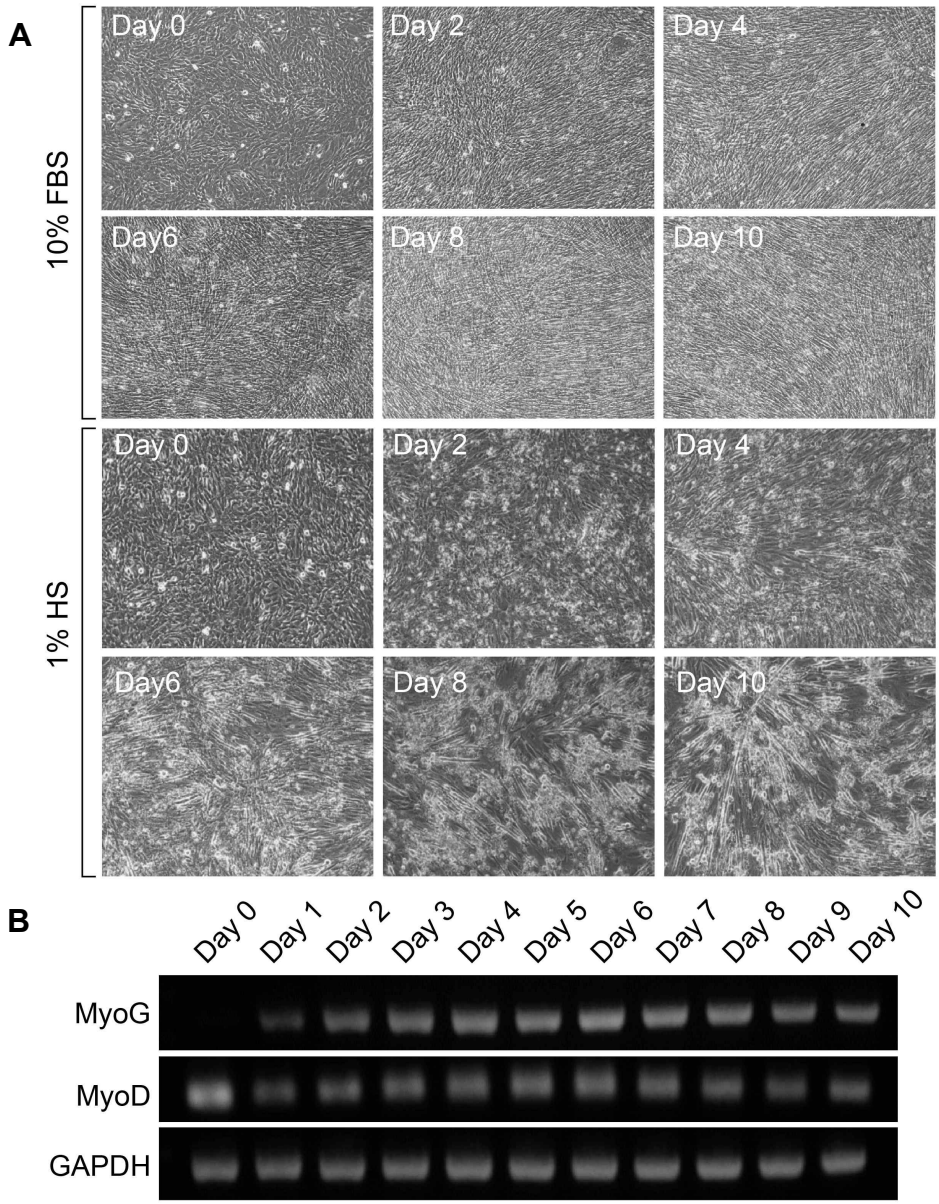

Fig. 1. Differentiation of C2C12 myoblast. (A) C2C12 myoblasts were confluently cultured and incubated in DMEM media containing either 10% FBS or 1% HS. Morphological changes were captured by optical microscope at the indicated times. Magnification is X10. (B) mRNA was isolated from C2C12 myoblasts at the indicated times of differentiation. Relative levels of mRNA of muscle-specific transcription factors (MyoD and MyoG) were determined by RT-PCR analysis. mRNA for GAPDH was included as internal control.

etal muscle differentiation, we further determined the activation of Akt during C2C12 myoblast differentiation. As shown in Fig. 4, Akt was transiently activated during the differentiation of C2C12 myoblasts, for example, activation of Akt was initiated on day 2 and terminated on day 7 of differentiation. On the other hand, expression of KLF4 was initiated on day 6 and lasted until day 10 of differentiation. These results suggest that activation of Akt and expression of KLF4 are involved in the skeletal muscle differentiation, and temporal regulation of both Akt and KLF4 seems to be important for the process of skeletal muscle differentiation.

Knockdown of Akt prevents KLF4 expression

Since transient activation of Akt was followed by the expression of KLF4, it is possible that activation of Akt is required for the expression of KLF4. Hence, role of Akt on

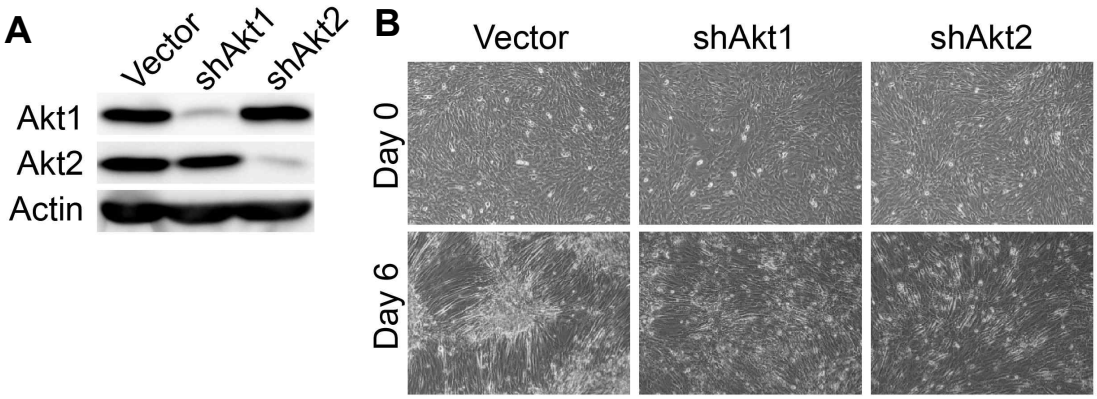

Fig. 2. Effect of Akt knockdown on the differentiation. (A) C2C12 myoblasts were infected with lentivirus harboring shAkt1 or shAkt2. Knockdown of Akt1 and Akt2 was verified by immunoblotting cell lysates with specific antibodies against Akt1 and Akt2. (B) C2C12 myoblasts infected with lentivirus harboring vector, shAkt1, and shAkt2. Cells were further incubated with differentiation medium containing 1% HS in DMEM. Optical images were taken at Day 0 and Day 6. Magnification is X10.

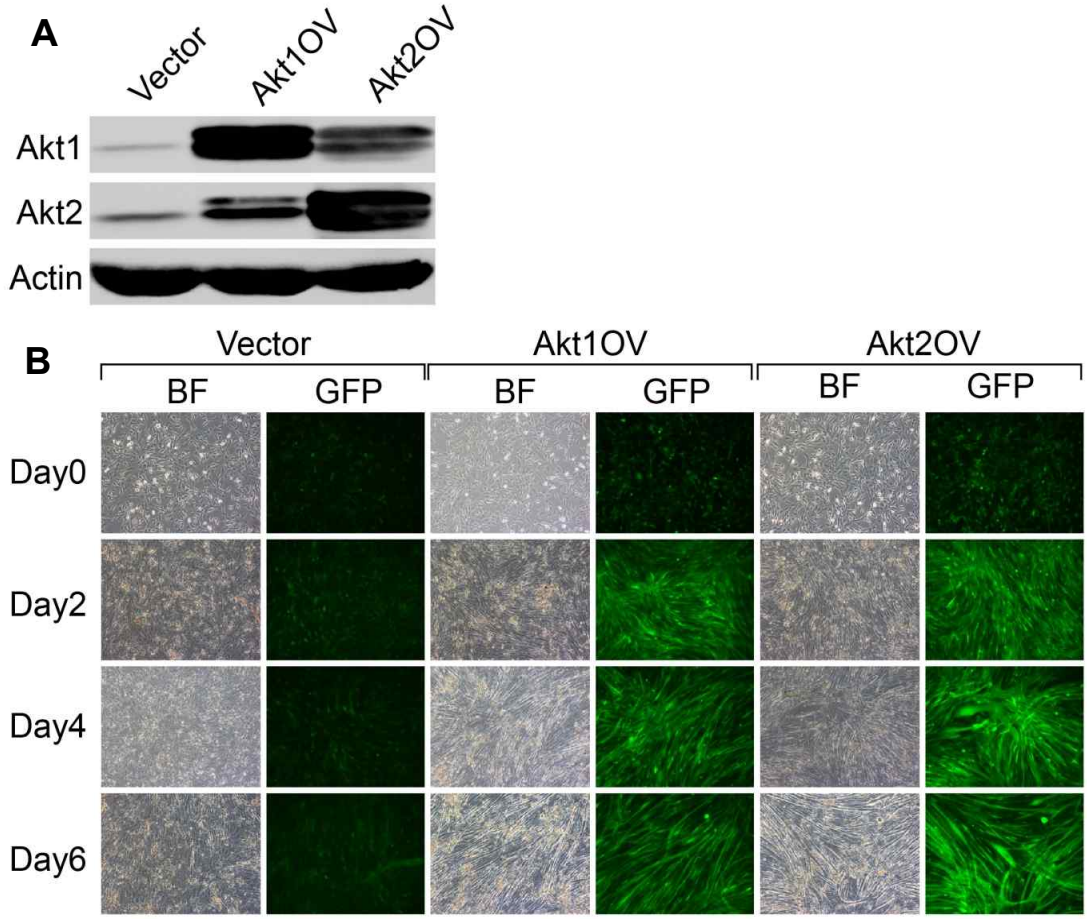

Fig. 3. Effect of Akt over-expression on the differentiation. (A) C2C12 myoblasts were infected with retrovirus containing Akt1 or Akt2 in pMIGR2 bicistronic vector. Over-expression of each Akt isoform was verified by western blotting with specific antibodies against Akt1 and Akt2. (B) After over-expression of Akt isoforms, C2C12 myoblasts were further incubated with differentiation medium containing 1% HS in DMEM. Morphological changes were captured under bright field (BF) or fluorescence filter (green fluorescent protein, GFP) at the indicated times.

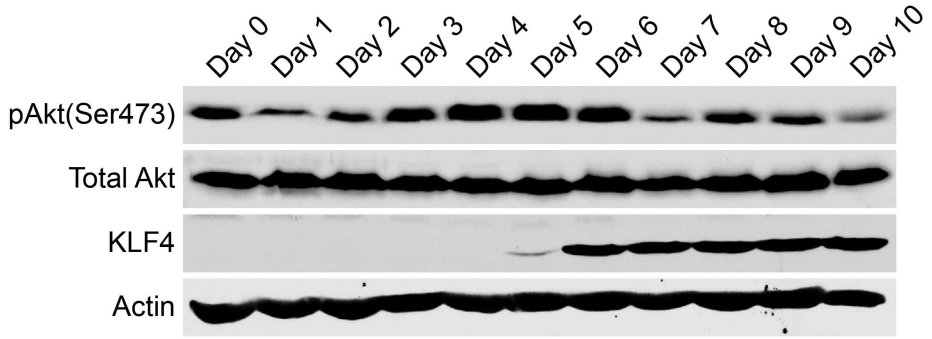

Fig. 4. Activation of Akt during C2C12 myoblast differentiation. Confluent stage of C2C12 myoblasts were incubated with differentiation medium containing 1% HS in DMEM for the indicated times. Cell lysates were analyzed for the activation of Akt and expression of KLF.

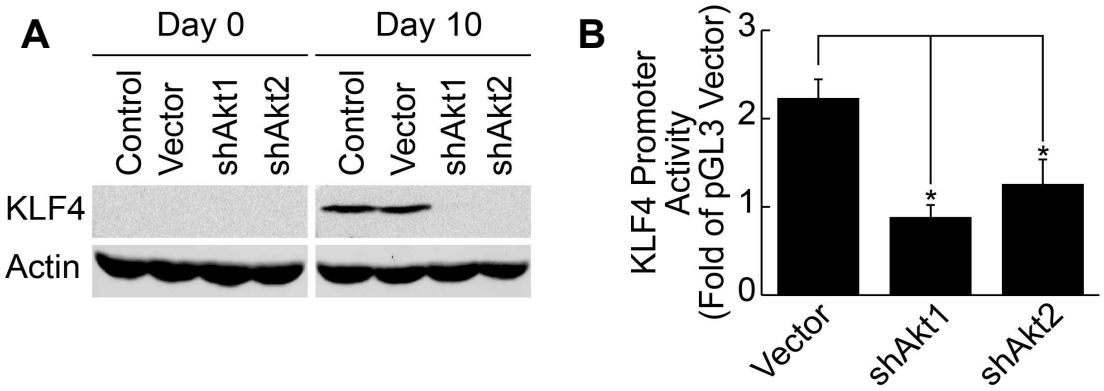

Fig. 5. Akt-dependent expression of KLF4. (A) Either Akt1 or Akt2 was silenced in C2C12 myoblasts using lentiviral shRNA delivery. Confluent stage of C2C12 myoblasts were incubated with differentiation medium containing 1% HS in DMEM. After ten days of differentiation, expression of KLF4 was verified by western blotting. (B) Either Akt1 or Akt2 was silenced in C2C12 myoblasts using lentiviral shRNA delivery. Cells were transfected with pGL3 vector or pGL3 containing promoter of KLF4. Promoter activity of KLF4 was expressed as a fold of pGL3 vector control. Values are expressed as ± mean of three independent experiments (n=3 for each experiment). p<0.05 was considered as significant and indicated as *.

the expression of KLF4 was examined. As shown in Fig. 5A, KLF4 was not expressed in undifferentiated C2C12 myoblasts, however, incubation of C2C12 myoblasts with 1% HS/DMEM significantly induced KLF4 in both control and vector cells. Expression of KLF4 was completely blocked in C2C12 myoblasts silencing either Akt1 or Akt2. In addition, the promoter activity of KLF4 was significantly reduced in C2C12 myoblasts silencing either Akt1 or Akt2 (Fig. 5B). These results suggest that either Akt1 or Akt2 affects the promoter activity KLF4 and regulates the expression of KLF4 during the skeletal muscle differentiation.

Discussion

In the present study, it has been demonstrated that skeletal muscle differentiation is regulated by Akt signaling and possibly through the expression of KLF4. It has been reported that C2C12 myoblasts form myotubes under certain condition such as low serum (2%) and high cell density [27]. Likewise, C2C12 myoblasts underwent myotube formation even at 1% horse serum (Fig. 1). MyoG and MyoD are transcriptional regulator for skeletal differentiation [34], and expression of MyoG and MyoD was significantly induced by the medium containing 1% HS in DMEM (Fig. 1). Therefore, it is likely that incubation of C2C12 myoblasts under low serum contents and high cell density induces differentiation by induction of transcriptional factors such as MyoG and MyoD. However, the mechanism by which low serum and high cell density induce the expression of MyoG and MyoD seems to be different from that of Akt activation since activa-

tion of Akt is observed from day 6 of differentiation.

It has been reported that Akt regulates a variety of cellular differentiation such as adipocyte differentiation, cardiomyoctye differentiation, and phenotypic change of vascular smooth muscle cells [4,9,10,37]. Likewise, our results showed that activation of Akt was observed during skeletal muscle differentiation (Fig. 2). Currently, how Akt is activated under low serum contents and high cell density is not clear. One plausible explanation would be cell-to-cell contact-induced activation of Akt. Indeed, it has been reported that Akt is activated by a variety of extracellular matrix proteins which are ligands for integrin receptors [28]. It is also notable that activation of Akt is secondary effect since confluent stage was not immediately followed by the phosphorylation of Akt (Figs. 1 and 3). In contrast, activation of Akt was achieved 4 days after confluent stage of C2C12 myoblasts. In these regards, activation of Akt seems to be acquired by low serum contents- and high cell density-induced autocrine factor(s). Therefore, temporal regulation of Akt activation seems to be important factor for the skeletal muscle differentiation.

Several lines of evidence support that Akt is important regulator for the skeletal muscle differentiation. First, activation of PI3K/Akt signaling pathway seems to be involved in the skeletal muscle differentiation. For instance, variety of pharmacological inhibitors blocking PI3K activity suppresses skeletal muscle differentiation [25]. In addition, our results also showed that Akt is activated during the progression of differentiation (Fig. 2). Second, forced control of Akt expression affects the skeletal muscle differentiation. It has been reported that blocking Akt activation by over-expressing dominant negative form of Akt suppresses skeletal muscle differentiation [25]. Likewise, our results also demonstrated that silencing either Akt1 or Akt2 significantly attenuated the differentiation of C2C12 myoblasts (Fig. 2). By contract, over-expression of either Akt1 or Akt2 significantly enhanced the differentiation of C2C12 myoblasts (Fig. 3). Therefore, attainment of kinase activity of Akt seems to be required for skeletal muscle differentiation. Especially, both Akt1 and Akt2 might be important regulators of skeletal muscle differentiation.

Recently, it has been reported that KLF family transcriptional factors regulates versatile type of differentiation [22]. Among the KLFs, KLF4 has been implicated in many types of differentiation such as vascular smooth muscle, adipocyte, inflammatory monocytes, and testicular Sertoli cells [1,8,32,36]. Recently, it has been reported that KLF4 is also involved in the regulation of skeletal muscle differentiation. Although this report suggests that ERK5 is an important mediator for KLF4 expression, our results also suggest that Akt also seems to be an important factor for the induction of KLF4 expression. For example, activation of Akt was followed by the expression of KLF4 (Fig. 4). In addition, silencing either Akt1 or Akt2 completely blocked the expression of KLF4 (Fig. 5A). More importantly, promoter activity of KLF4 was significantly reduced in C2C12 cells silencing either Akt1 or Akt2 (Fig. 5B). Therefore, it is possible that transient activation of Akt is required for sustained expression of KLF4 which leads to myogenic fusion of individual myocytes.

In summary, C2C12 myoblasts underwent morphological changes by the differentiation condition. Differentiation of C2C12 myoblast was significantly attenuated by silencing Akt1 or Akt2 whereas significantly enhanced by forced expression of Akt1 or Akt2. Akt was transiently activated during the progression of C2C12 myoblast differentiation. Expression of KLF4 as well as promoter activity of KLF4 was markedly reduced in C2C12 myoblasts silencing Akt1 or Akt2. Therefore, it is likely that Akt regulates skeletal muscle differentiation through the expression of KLF4.

Acknowledgement

This study was supported by Medical Research Institute grant (2005-30) of Pusan National University.

References

1. Alder, J. K., 3rd Georgantas, R. W., Hildreth, R. L., Kaplan, I. M., Morisot, S., Yu, X., McDevitt, M. and Civin, C. I. 2008. Kruppel-like factor 4 is essential for inflammatory monocyte differentiation *in vivo*. *J. Immunol.* **180**, 5645-5652.
2. Banerjee, S. S., Feinberg, M. W., Watanabe, M., Gray, S., Haspel, R. L., Denkinger, D. J., Kawahara, R., Hauner, H. and Jain, M. K. 2003. The Kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-gamma expression and adipogenesis. *J. Biol. Chem.* **278**, 2581-2584.
3. Basu, P., Morris, P. E., Haar, J. L., Wani, M. A., Lingrel, J. B., Gaensler, K. M. and Lloyd, J. A. 2005. KLF2 is essential for primitive erythropoiesis and regulates the human and murine embryonic β-like globin genes *in vivo*. *Blood* **106**, 2566-2571.
4. Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J. and Yancopoulos, G. D. 2001.

Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. *Nat. Cell Biol.* **3**, 1014-1019.

5. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., Kadowaki, T. and Hay, N. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev.* **15**, 2203-2208.
6. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., 3rd Crenshaw, E. B., Kaestner, K. H., Bartolomei, M. S., Shulman, G. I. and Birnbaum, M. J. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ). *Science* **292**, 1728-1731.
7. Easton, R. M., Cho, H., Roovers, K., Shineman, D. W., Mizrahi, M., Forman, M. S., Lee, V. M., Szabolcs, M., de Jong, R., Oltersdorf, T., Ludwig, T., Efstratiadis, A. and Birnbaum, M. J. 2005. Role for Akt3/protein kinase Bγ in attainment of normal brain size. *Mol. Cell. Biol.* **25**, 1869-1878.
8. Godmann, M., Katz, J. P., Guillou, F., Simoni, M., Kaestner, K. H. and Behr, R. 2008. Kruppel-like factor 4 is involved in functional differentiation of testicular Sertoli cells. *Dev. Biol.* **315**, 552-566.
9. Halayko, A. J., Kartha, S., Stelmack, G. L., McConville, J., Tam, J., Camoretti-Mercado, B., Forsythe, S. M., Hershenson, M. B. and Solway, J. 2004. Phophatidylinositol-3 kinase/mammalian target of rapamycin/p70S6K regulates contractile protein accumulation in airway myocyte differentiation. *Am. J. Respir. Cell Mol. Biol.* **31**, 266-275.
10. Hayashi, K., Takahashi, M., Kimura, K., Nishida, W., Saga, H. and Sobue, K. 1999. Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells. *J. Cell. Biol.* **145**, 727-740.
11. Jiang, J., Chan, Y. S., Loh, Y. H., Cai, J., Tong, G. Q., Lim, C. A., Robson, P., Zhong, S. and Ng, H. H. 2008. A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat. Cell Biol.* **10**, 353-360.
12. Kaliman, P., Canicio, J., Shepherd, P. R., Beeton, C. A., Testar, X., Palacin, M. and Zorzano, A. 1998. Insulin-like growth factors require phosphatidylinositol 3-kinase to signal myogenesis: dominant negative p85 expression blocks differentiation of L6E9 muscle cells. *Mol. Endocrinol.* **12**, 66-77.
13. Katz, J. P., Perreault, N., Goldstein, B. G., Lee, C. S., Labosky, P. A., Yang, V. W. and Kaestner, K. H. 2002. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development* **129**, 2619-2628.
14. Kim, E. K., Yun, S. J., Do, K. H., Kim, M. S., Cho, M., Suh, D. S., Kim, C. D., Kim, J. H., Birnbaum, M. J. and Bae, S. S. 2008. Lysophosphatidic acid induces cell migration through the selective activation of Akt1. *Exp. Mol. Med.* **40**, 445-452.
15. Kim, E. K., Yun, S. J., Ha, J. M., Kim, Y. W., Jin, I. H., Yun, J., Shin, H. K., Song, S. H., Kim, J. H., Lee, J. S., Kim, C. D. and Bae, S. S. 2011. Selective activation of Akt1 by mammalian target of rapamycin complex 2 regulates cancer cell migration, invasion, and metastasis. *Oncogene* **30**, 2954-2963.
16. Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.* **271**, 31372-31378.
17. Le Grand, F. and Rudnicki, M. A. 2007. Skeletal muscle satellite cells and adult myogenesis. *Curr. Opin. Cell Biol.* **19**, 628-633.
18. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M. K., Han, K., Lee, J. H., Ciarallo, S., Catzavelos, C., Beniston, R., Franssen, E. and Slingerland, J. M. 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat. Med.* **8**, 1153-1160.
19. Marte, B. M. and Downward, J. 1997. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem. Sci.* **22**, 355-358.
20. Miller, I. J. and Bieker, J. J. 1993. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. *Mol. Cell. Biol.* **13**, 2776-2786.
21. Miner, J. H. and Wold, B. 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA* **87**, 1089-1093.
22. Nemer, M. and Horb, M. E. 2007. The KLF family of transcriptional regulators in cardiomyocyte proliferation and differentiation. *Cell Cycle* **6**, 117-121.
23. Pearson, R., Fleetwood, J., Eaton, S., Crossley, M. and Bao, S. 2008. Kruppel-like transcription factors: a functional family. *Int. J. Biochem. Cell Biol.* **40**, 1996-2001.
24. Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D. and Glass, D. J. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* **286**, 1738-1741.
25. Sarker, K. P. and Lee, K. Y. 2004. L6 myoblast differentiation is modulated by Cdk5 via the PI3K-AKT-p70S6K signaling pathway. *Oncogene* **23**, 6064-6070.
26. Scheid, M. P. and Woodgett, J. R. 2001. PKB/AKT: functional insights from genetic models. *Nat. Rev. Mol. Cell Biol.* **2**, 760-768.
27. Toma, C., Pittenger, M. F., Cahill, K. S., Byrne, B. J. and Kessler, P. D. 2002. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**, 93-98.
28. Troussard, A. A., Mawji, N. M., Ong, C., Mui, A., St - Arnaud, R. and Dedhar, S. 2003. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. *J. Biol. Chem.* **278**, 22374-22378.
29. Tureckova, J., Wilson, E. M., Cappalonga, J. L. and Rotwein, P. 2001. Insulin-like growth factor-mediated muscle differentiation: collaboration between phosphatidylinositol 3-kinase-Akt-signaling pathways and myogenin. *J. Biol. Chem.*

276, 39264-39270.
30. Turner, J. and Crossley, M. 1999. Mammalian Kruppel-like transcription factors: more than just a pretty finger. *Trends Biochem. Sci.* **24**, 236-240.
31. van Vliet, J., Turner, J. and Crossley, M. 2000. Human Kruppel-like factor 8: a CACCC-box binding protein that associates with CtBP and represses transcription. *Nucleic Acids Res.* **28**, 1955-1962.
32. Wang, C., Han, M., Zhao, X. M. and Wen, J. K. 2008. Kruppel-like factor 4 is required for the expression of vascular smooth muscle cell differentiation marker genes induced by all-trans retinoic acid. *J. Biochem.* **144**, 313-321.
33. Wassmann, S., Wassmann, K., Jung, A., Velten, M., Knuefermann, P., Petoumenos, V., Becher, U., Werner, C., Mueller, C. and Nickenig, G. 2007. Induction of p53 by GKLF is essential for inhibition of proliferation of vascular smooth muscle cells. *J. Mol. Cell. Cardiol.* **43**, 301-307.
34. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S. and et al. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* **251**, 761-766.
35. Wullschleger, S., Loewith, R. and Hall, M. N. 2006. TOR signaling in growth and metabolism. *Cell* **124**, 471-484.
36. Yoshida, T., Kaestner, K. H. and Owens, G. K. 2008. Conditional deletion of Kruppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. *Circ. Res.* **102**, 1548-1557.
37. Yun, S. J., Kim, E. K., Tucker, D. F., Kim, C. D., Birnbaum, M. J. and Bae, S. S. 2008. Isoform-specific regulation of adipocyte differentiation by Akt/protein kinase Bα. *Biochem. Biophys. Res. Commun.* **371**, 138-143.
38. Zhou, G. L., Tucker, D. F., Bae, S. S., Bhatheja, K., Birnbaum, M. J. and Field, J. 2006. Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration. *J. Biol. Chem.* **281**, 36443-36453.

초록 : Akt에 의한 근육세포의 분화 조절

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Akt는 다양한 세포에서 성장, 발달, 증식, 분화와 같은 생리적 활성에 중요한 역할을 하고 골격근 세포에서 Akt는 재생 및 비대와 위축을 조절한다고 알려져 있다. 골격근 세포의 분화에 있어서 Akt의 역할을 밝히고자 본 연구를 수행하였다. 골격근 세포를 분화 시키기 위해 고밀도 및 저농도의 serum 상태에서 배양하며, 분화된 C2C12 근아세포는 둥근 모양에서 다핵을 가진 긴 모양으로 바뀐다. 이러한 형태학적 변화는 분화 시킨 후 2일부터 일어났다. 또한, 골격근 분화 표지인자인 myogenin D와 myogenin G의 발현은 2일 후 관찰되었다. C2C12 세포주에 Akt1 또는 Akt2의 발현을 저하시키면 이와 더불어 골격근으로의 분화도 저해됨을 확인하였고, 이와는 반대로 Akt1 또는 Akt2를 과발현 시키면 골격근으로 분화가 촉진됨을 알 수 있었다. 이와 더불어 Akt의 활성은 분화유도 2일 후부터 관찰되었고 7일 이후로는 감소하였다. Kruppel-like factor 4의 발현은 6일부터 증가하는 것이 관찰이 되었다. Kruppel-like factor 4의 발현 또한 Akt1 또는 Akt2의 발현양이 감소된 C2C12 근아세포에서 줄어들어 있는 것을 확인하였다. 또한 Kruppel-like factor 4의 프로모터 부위에 대한 전사조절능력이 Akt1 또는 Akt2의 발현을 저하시켰을 때 같이 떨어짐을 확인하였다. 이러한 결과들로 보아 Akt가 골격근 분화를 조절하는 데 있어 중요하며, Kruppel-like factor 4 발현이 이를 조절하는 데 있어 중요한 역할을 할 것이라 판단된다.