THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 37, pp. 31330–31341, September 7, 2012

Role of Phosphatidylinositol 3,4,5-Trisphosphate (PIP_3) 5-Phosphatase Skeletal Muscle- and Kidney-enriched Inositol Polyphosphate Phosphatase (SKIP) in Myoblast Differentiation*

Received for publication, June 6, 2012, and in revised form, July 18, 2012 Published, JBC Papers in Press, July 19, 2012, DOI 10.1074/jbc.M112.388785

Takeshi Ijuin and Tadaomi Takenawa[1]

From The Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

Background: SKIP is a PIP_3 5-phosphatase that negatively regulates insulin signaling in the skeletal muscle.
Results: Overexpression of SKIP inhibited IGF-II expression and the myoblast cell differentiation.
Conclusion: SKIP negatively regulated muscle cell differentiation through the attenuation of IGF-II-Akt-mTOR signaling pathway.
Significance: SKIP is a key regulator if muscle cell differentiation.

Insulin-like growth factors (IGFs) are essential for the development, regeneration, and hypertrophy of skeletal muscles. IGF-II promotes myoblast differentiation through phosphatidylinositol 3-kinase (PI 3-kinase), Akt, and mTOR signaling. Here, we report that skeletal muscle- and kidney-enriched inositol polyphosphate phosphatase (SKIP) negatively regulates myogenesis through inhibition of IGF-II production and attenuation of the IGF-II-Akt-mTOR signaling pathway. We also demonstrate that SKIP expression, which was markedly elevated during differentiation, was controlled by MyoD in C2C12 cells. Expression of SKIP inhibited IGF-II at the transcription level. These results indicate that SKIP regulates MyoD-mediated muscle differentiation. Silencing of SKIP increased IGF-II transcription and myoblast differentiation. Furthermore, knockdown of SKIP resulted in thick myotubes with a larger number of nuclei than that in control C2C12 cells. Taken together, these data indicate that SKIP controls the IGF-II-PI 3-kinase-Akt-mTOR auto-regulation loop during myogenesis. Our findings identify SKIP as a key regulator of muscle cell differentiation.

Skeletal muscle development is a multistep process that starts with the determination of myogenic precursor cells from mesodermal stem cells and ends with formation of multinucleated myotubes (1). Myogenesis of satellite cells is regulated by a number of muscle-specific transcription factors, including MyoD, Myf5, Mrf4, and myogenin (2, 3). MyoD is essential for myoblast specification during early embryonic development and for satellite cell activation during muscle regeneration in adults (4, 5). MyoD regulates genes expressed at different times during myogenesis (6). Mrf4 and Myf5 act upstream of MyoD to direct embryonic cells into the myogenic lineage (7). Myogenin directly controls the differentiation process, including the formation of myotubes (8, 9).

It has recently been reported that muscle-specific transcription factor MyoD controls SKIP[2] expression during muscle cell development (10). MyoD is required for expression of SKIP in skeletal muscles, partly via *cis*-acting elements in the *Skip* promoter, and the expression level of SKIP was up-regulated in early differentiated C2C12 myoblast cells. SKIP is a phosphatidylinositol 3,4,5-trisphosphate (PIP_3) 5-phosphatase that negatively regulates insulin-induced phosphatidylinositol 3-kinase (PI 3-kinase)-Akt signaling and glucose uptake among PIP_3 phosphatases in the skeletal muscle (11, 12). We previously reported that heterozygous SKIP knock-out mice exhibited increased systemic insulin sensitivity and increased insulin signaling in skeletal muscles (13). These mice had higher amounts of quadriceps muscle compared with the amounts in wild-type mice. Based on these data, we hypothesize that SKIP regulates insulin-like growth factor (IGF)-PI 3-kinase signaling because IGFs are essential for the development, regeneration, and hypertrophy of skeletal muscles.

The IGFs are required for the activation of satellite cells and differentiation through the expression of myogenic transcription factors (14); they are critical factors in the development and growth of skeletal muscles (15). In myoblast cells autocrine production of IGF-II is triggered by serum withdrawal, which is associated with differentiation (15). IGF-II interacts with the IGF-I receptor and then activates PI 3-kinase, which generates PIP_3. PIP_3 activates Akt (also referred to as PKB) through recruitment to the plasma membrane and subsequent transcription of myogenic genes. Several results have suggested that PI 3-kinase-Akt signaling is implicated in myogenic differentiation (16–18). Akt1 and Akt2 have been reported to be implicated in multiple steps during myogenesis, including myogenin

* This work was supported in part by grants from the Japan Diabetes Foundation and the Hyogo Science and Technology Association (to T. I.).
[1] To whom correspondence should be addressed: The Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan. Tel.: 81-78-382-5410; Fax: 81-78-382-6271; E-mail: takenawa@med.kobe-u.ac.jp.

[2] The abbreviations used are: SKIP, skeletal muscle- and kidney-enriched inositol polyphosphate phosphatase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI 3-kinase, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; IGF, insulin-like growth factor.

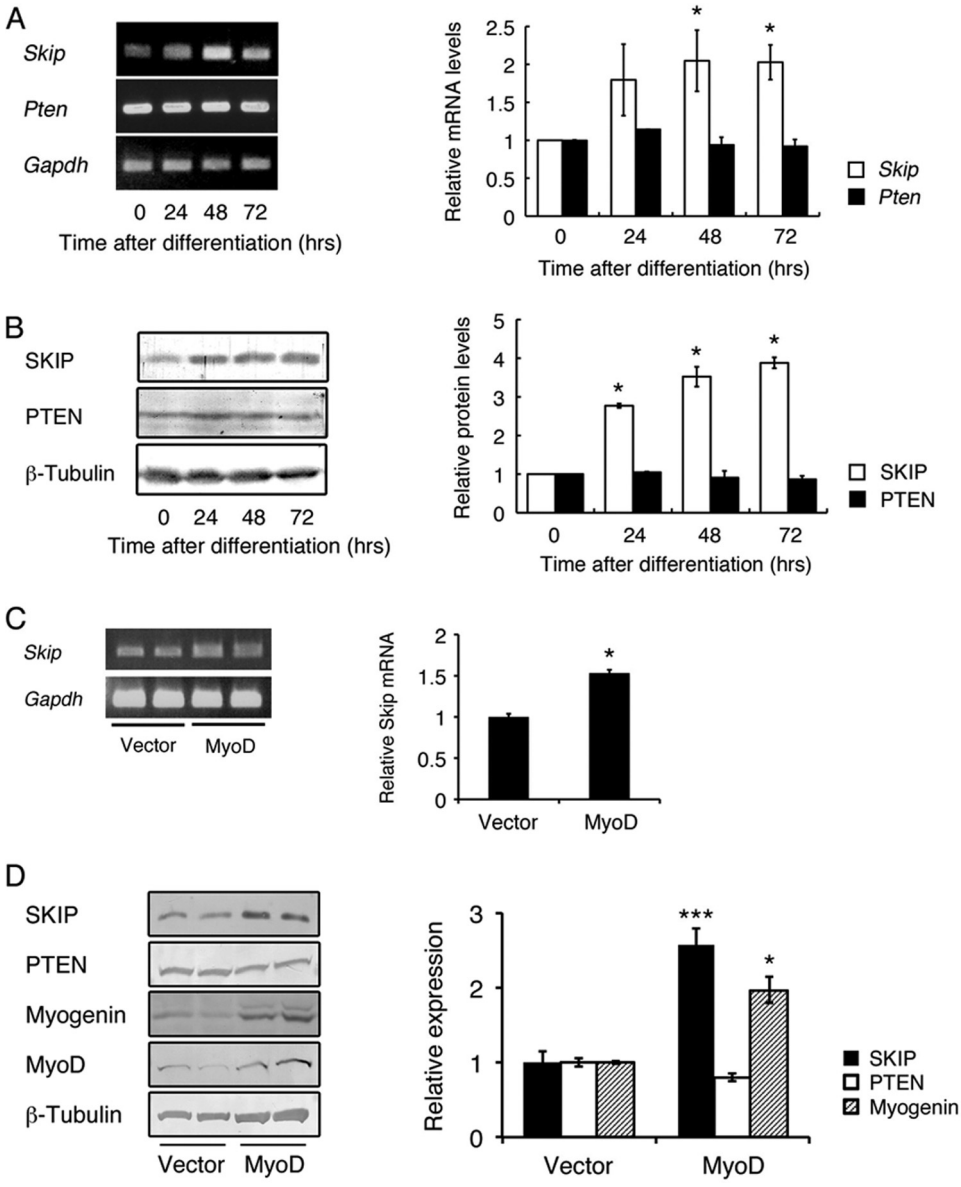


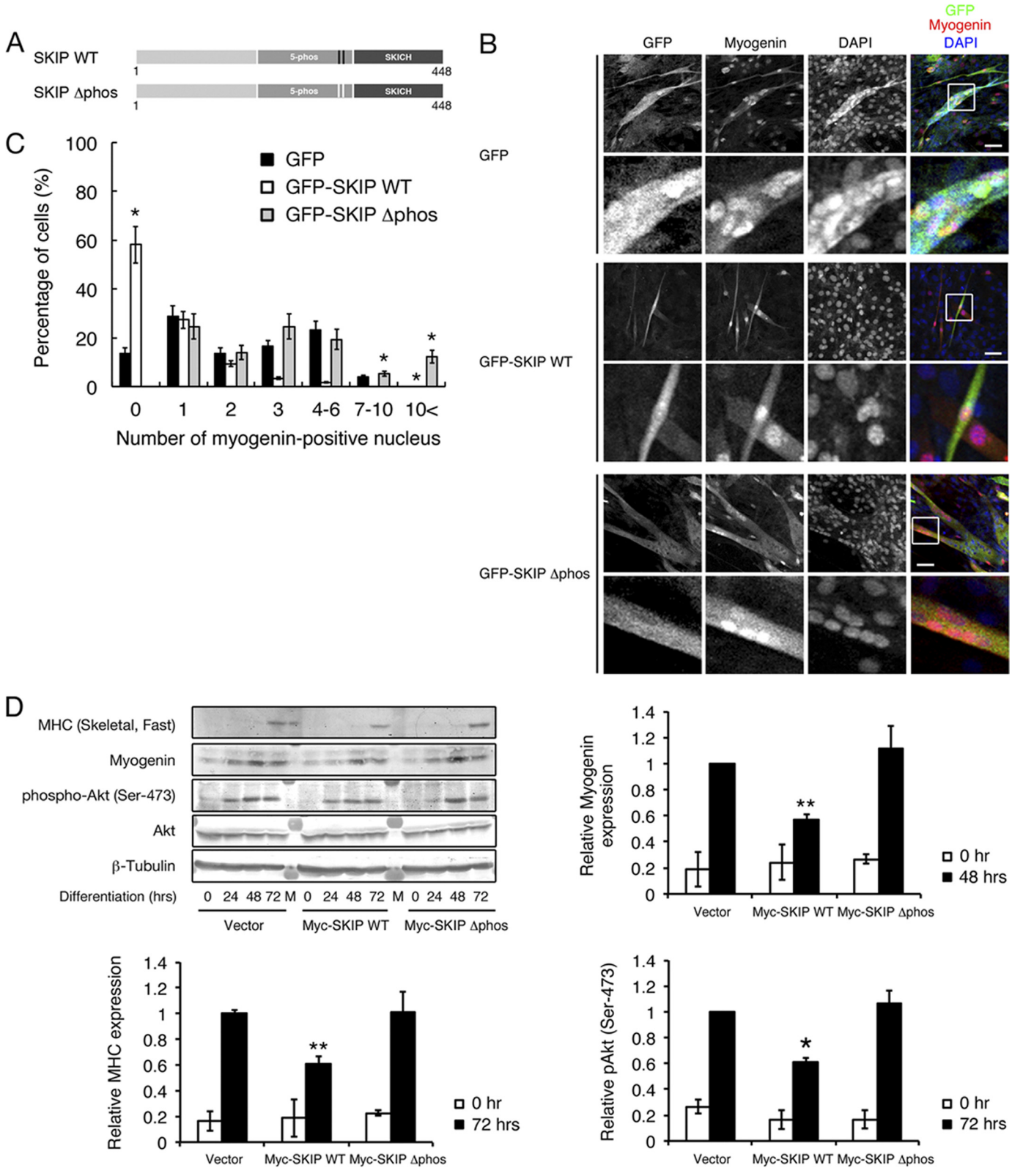
FIGURE 1. **Up-regulation of SKIP during myogenesis.** *A*, C2C12 myoblast cells were grown to 90% confluence in the growth medium containing 10% fetal bovine serum and then induced to differentiate by switching to the differentiation medium containing 2% horse serum. Total RNA was extracted at the indicated time after initiation of differentiation. *Skip* and *Pten* mRNA levels were measured by quantitative RT-PCR. Relative amounts of *Skip* and *Pten* mRNA were quantified by densitometry. Results are presented as the mean ± S.E. of five independent experiments (*, $p < 0.05$, *t* test, *versus* 0 h). *B*, measurement of SKIP and PTEN protein expression during myogenesis is shown. C2C12 cells were differentiated for the indicated times. Lysates were analyzed by Western blot analysis. The relative amount of SKIP and PTEN protein expression was quantified by densitometry. Results are presented as the mean ± S.E. of three independent experiments (*, $p < 0.05$, *t* test, *versus* 0 h). *C*, expression of MyoD increased *Skip* mRNA levels in C2C12 cells. Total RNA was extracted 72 h after initiation of differentiation. *Skip, Pten*, and *Gapdh* mRNA levels were measured by semiquantitative PCR. Samples were loaded in duplicate. *, $p < 0.05$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments. *D*, C2C12 cells were transfected with the pcDNA3.1 vector or pcDNA3.1-MyoD. Cells were cultured in the differentiation medium for 48 h, and expression of SKIP, PTEN, and myogenin was determined by Western blotting. Samples were loaded in duplicate. Relative amounts of SKIP, PTEN, and myogenin were quantified by densitometry. Results are presented as the mean ± S.E. of three independent experiments (*, $p < 0.05$, and ***, $p < 0.001$; *t* test *versus* vector).

expression at the early stages and the subsequent myotube maturation (19–22). Inhibition of IGF-II production led to loss of the induction of Akt phosphorylation and accumulation of myogenin and troponin-T (21). The mammalian target of rapamycin (mTOR), one of the downstream targets of Akt, is responsible for the initiation of myogenesis (17, 23). mTOR is also required for the autocrine production of IGF-II at the transcriptional level and its secretion (24–26). IGF-II-mediated signaling is necessary for the full transcriptional effect of MyoD at the myogenin promoter within the first few hours of initiation of differentiation (27); through this mechanism, cells maintain myoblast viability during the early stages of myogenesis (21, 28). Therefore, the PI 3-kinase-Akt-mTOR-IGF-II autocrine amplification loop is critically involved in skeletal myogenesis.

Despite the considerable amount of data available describing the regulation of this auto-regulation loop, the termination of this amplification remains to be elucidated. In this study we have examined the effect of SKIP during myogenesis to clarify its role in muscle cell differentiation.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—The antibody against skeletal myosin heavy chain (MY-32) was purchased from Sigma. Anti-Akt, phospho-Akt (Ser-473), mTOR, phospho-mTOR (Ser-2448), p70 S6 kinase, and phospho-p70 S6 kinase (Ser-421/Tyr-424) were purchased from Cell Signaling Technology (Beverly, MA). Anti-myogenin antibody was purchased from BD Pharmingen, and anti-β-tubulin and MyoD antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SKIP antibodies were generated as described previously (29).



Plasmids—Plasmids expressing Myc-tagged wild-type SKIP and a phosphatase-negative (Δphos) version of SKIP were generated as previously described (29). The SKIP expression vector was generated by cloning human SKIP cDNA into the pEGFP-C1 expression vector (BD Biosciences Clontech). Murine MyoD cDNAs were obtained by reverse transcription (RT)-PCR and subcloned into the pcDNA3.1(-) vector (Invitrogen). A DNA encoding a 5′-src myristoylation signal in-frame with a mouse Akt1 deleting PH domain (myr-Akt1) was obtained by RT-PCR.

Small Interfering RNAs (siRNAs)—Stealth™ siRNA oligonucleotides targeting mouse SKIP and IGF-II were designed and synthesized at Invitrogen. The targeted sequences of the SKIP Stealth™ siRNAs were as follows: SKIP (5′-UAAAGCUUCAGGCAGACGUUGACUC-3′ and 5′-GAGTCAACGTCTGCCTGAAGCTTTA-3′), IGF-II (5′-AACUGAAGCGUGUCAACAAGCUCCC-3′ and 5′-GGGAGCUUGUUGACACGCUUCAGUU-3′).

Cell Culture—C2C12 myoblast cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. For transfection, 5×10^4 cells were cultured in 3.5-cm-diameter dishes for 20 h, and then 2 mg of plasmid DNA or 100 nM siRNA was transfected into the cells. The cells were grown to 95% confluency, washed with serum-free medium, and incubated with differentiation medium containing 2% horse serum 24 h after transfection for the indicated times (24–96 h). For rescue experiments, 48 h after transfection, the cells were changed into differentiation medium supplemented with 50 ng/ml IGF-II (Merck).

Western Blot Analysis—C2C12 cells were lysed with ice-cold lysis buffer consisting of 40 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 0.1 mM PMSF, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1% Triton X-100. The lysates were solubilized in SDS sample buffer and boiled for 5 min, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Western blot analysis was carried out following the procedure recommended by the manufacturer. The results were analyzed and quantified by densitometry.

RT and Semi-quantitative RT-PCR—Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. After treating with DNase, RNA samples were subjected to reverse transcription by using Superscript II reverse transcription (Invitrogen). Specific primers used for semiquantitative RT-PCR were as follows: IGF-II, 5′-GACACGCTTCAGTTTGTCTGTT-3′ and 5′-GAAGTACGGCCTGAGAGGTAGA-3′; MyoD, 5′-AGCACTACAGTGGCG-ACTCA-3′ and 5′-CCTCCACTATGCTGGACAGG-3′; myogenin, 5′-CTACAGGCCTTGCTCAGCTC-3′ and 5′-AGATTGTGGGCGTCTGTAGG-3′; p21, 5′-CGGTGGAACTTTGACTTCGT-3′ and 5′-CAGTGCAAGACAGCGACAAG-3′; S26, 5′-AAGT-TTGTCATTCGGAACATTG-3′ and 5′-CATCGATTCCTAACAACCTTGC-3′; SKIP, 5′-TGGTCTTCAGATCACGATCATGCTC-3′ and 5′-GGCACT-GAGTCACTGTTGGCAGGA-3′; GAPDH, 5′-CGTAGACAAAATGGTGAAGG-3′ and 5′-GTTGTCATGGATGACCTTGG-3′. The results were analyzed and quantified by densitometry.

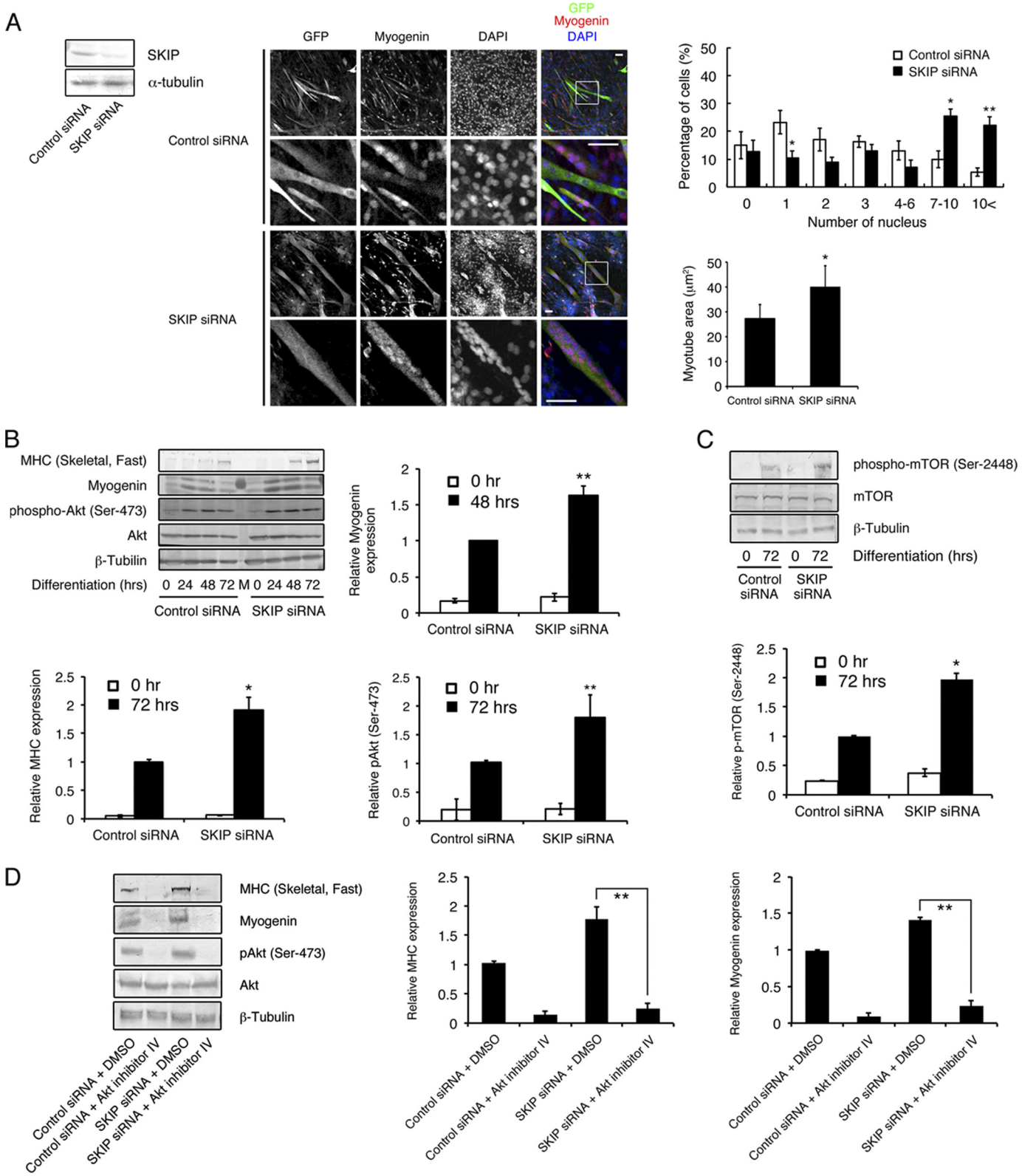
Immunohistochemistry—Cells cultured on coverslips were washed 3 times with phosphate-buffered saline (PBS) before fixation in 3.7% formaldehyde for 15 min. Cells were permeabilized for 5 min in PBS containing 0.2% Triton X-100, washed 3 times with PBS, and incubated with myogenin antibodies for 1 h at room temperature. The cells were then washed with PBS 3 times and with PBS before incubation with Alexa Fluor 568-conjugated anti-mouse antibodies and DAPI. Cells were imaged using a confocal microscope (Olympus Co., Tokyo, Japan).

RESULTS

Induction of SKIP Expression during C2C12 Cell Differentiation—SKIP is a PIP_3 5-phosphatase abundantly expressed in skeletal muscles; the transcription of SKIP increased within 72 h of induction of differentiation in C2C12 cells (10). To examine the role of SKIP in myoblast cell differentiation, we monitored SKIP expression in C2C12 cells during differentiation. C2C12 cells underwent growth arrest; differentiation was induced after switching the cells to the differentiation medium containing 2% horse serum. Typically, cell fusion was observed 72 h after induction, and myotubes were formed at 96 h. The mRNA level of *Skip* was also enhanced within 24 h of the induction of differentiation, and then it reached the maximum level at 48 h (Fig. 1*A*). Protein expression levels of SKIP were significantly elevated within 24 h (Fig. 1*B*). In contrast, the expression levels of PTEN, a PIP_3 3-phosphatase, remained constant throughout the differentiation (Fig. 1, *A* and *B*). During the development of pig skeletal muscles, MyoD has been shown to control SKIP expression (10). In C2C12 cells, expression of MyoD resulted in higher expression of SKIP after the initiation of differentiation for 48 h (Fig. 1, *C* and *D*). In contrast, expression of PTEN was not altered by the expression of MyoD (Fig. 1, *C* and *D*). These data suggest that MyoD is a potent regulator of SKIP transcription during myogenesis, and increased expression of SKIP might contribute to the termination of muscle cell differentiation.

FIGURE 2. **Expression of SKIP impairs myogenic differentiation.** *A*, shown is the domain structure of SKIP WT and SKIP Δphos phosphatase-negative mutant used in this study. SKIP possesses an N-terminal inositol polyphosphate 5-phosphatase catalytic domain (*5-phos*) and a C-terminal SKIP C-terminal homology (*SKICH*) domain. Amino acids necessary for 5-phosphatase activity of SKIP (*black lines*) are deleted in the Δphos mutant. *B*, expression of SKIP inhibited myotube formation is shown. C2C12 cells were transfected with the pEGFP-C1 vector, pEGFP-C1 SKIP WT, or pEGFP-C1 SKIP Δphos mutant. Cells were induced to differentiate for 72 h and immunostained with anti-myogenin antibody and DAPI. Cells were visualized by confocal microscopy. Enlarged images of *boxed areas* are shown in the *lower panels*. *Purple* indicates regions of co-localization of myogenin and DAPI. *Scale bar*, 80 nm. *C*, quantification of myotube formation shown in *B* is shown. The percentage of cells with indicated number of myogenin-positive nuclei is shown. The number of myogenin-positive nuclei was counted in 200 cells. Results are presented as the mean ± S.E. of three independent experiments (*, $p < 0.05$; *t* test *versus* GFP). *D*, C2C12 cells expressing the pcDNA3.1-myc vector, pcDNA3.1-myc-SKIP WT, or pcDNA3-myc-SKIP Δphos were induced to differentiate for the indicated times. Cell lysates were collected and subjected to Western blotting. Relative amounts of phospho-Akt, myogenin and MHC at 72 h are shown in the graphs. Expression levels were quantified by densitometry. *M* indicates the lane loaded with protein molecular marker. Results are presented as the mean ± S.E. of three independent experiments (*, $p < 0.05$ and **, $p < 0.01$; *t* test *versus* Vector).

Negative Regulation of C2C12 Cell Differentiation by SKIP through the PI 3-Kinase-Akt-mTOR Signaling Pathway—To address the role of SKIP in myogenesis, the effects of the expression of SKIP wild type (SKIP WT) and the phosphatase-negative mutant of SKIP (SKIP Δphos) on the differentiation of C2C12 cells was examined (Fig. 2*A*). Expression of GFP-SKIP WT significantly inhibited the formation of myotubes and the number of myogenin-positive nuclei (Fig. 2, *B* and *C*). These factors were unaltered by the expression of GFP-SKIP Δphos compared with the control cells (Fig. 2, *B* and *C*). Differentia-



tion of C2C12 cells is characterized by the expression of myogenic genes, namely, myogenin and myosin heavy chain (MHC). Expression of myogenin reaches its maximum after the initiation of differentiation for 48 h, and MHC expression is markedly increased after 72 h. Expression of SKIP WT resulted in a slight decrease in myogenin expression and Akt phosphorylation after 48 h, with a marked decrease in MHC expression after 72 h (Fig. 2*D*), neither of which was affected by the expression of the SKIP Δphos mutant. Based on these results, we hypothesized that SKIP negatively regulates myogenesis through the regulation of the PI 3-kinase-Akt signaling pathway.

Next we examined the role of endogenous SKIP during myogenesis. Silencing of SKIP increased the number of myogenin-positive nuclei in individual cells (Fig. 3*A*). In these cells, myotubes appeared to be more robust than in the control siRNA-transfected cells (Fig. 3*A*). The myotubes from SKIP siRNA-transfected cells had an increased area (Fig. 3*A*). In these cells the expression of myogenin and MHC also increased after 48 h of initiation of differentiation (Fig. 3*B*). Next, the effect of endogenous SKIP on Akt and mTOR phosphorylation was examined during myogenesis. As shown in Fig. 1, *A* and *B*, expression of endogenous SKIP is markedly increased after the initiation of differentiation for 48 h. Silencing of SKIP increased Akt phosphorylation and mTOR phosphorylation at Ser-2448 (Fig. 3, *B* and *C*). Treatment of these cells with Akt inhibitor VI abolished the expression of myogenin and MHC (Fig. 3*D*). Taken together, these data indicate that endogenous SKIP likely regulates myogenesis through the PI 3-kinase-Akt-mTOR signaling pathway.

SKIP Expression Suppressed IGF-II Production during Myogenesis—It has been reported that mTOR regulates the early stage of myogenesis by controlling autocrine IGF-II production at the transcriptional level, which is critical for myogenesis (25, 26, 30). To establish the role of SKIP in myogenic IGF-II production, the effects of SKIP expression and knockdown on IGF-II expression were examined. The effect of SKIP expression on *IGF-II mRNA* production was examined by semiquantitative PCR. *IGF-II* mRNA levels were significantly lower in the Myc-SKIP WT-expressing cells than in the control cells (Fig. 4*A*). In contrast, knockdown of SKIP caused a significant increase in *IGF-II* mRNA production after 72 h of initiation of differentiation (Fig. 4*B*). Myogenin is a muscle-specific transcription factor whose mRNA expression is dependent on Akt activity, which is also stimulated by the IGF-II auto-regulation loop (31). As expected, SKIP expression significantly inhibited myogenin mRNA expression, whereas SKIP knockdown enhanced the expression of myogenin mRNA (Fig. 4, *A* and *B*). Expression of IGF-binding protein-5 (*IGFBP-5*) mRNA, which is induced in an early stage of myogenesis and promotes muscle cell differentiation (31), was not affected by the expression or silencing of SKIP (Fig. 4, *A* and *B*). The expressions of the cell cycle inhibitor p21 and the ribosomal protein S26 are also induced during the early stage of myogenesis because it is accompanied by the termination of cell cycle progression and promotion of protein synthesis. However, their expression was not altered by the overexpression or silencing of SKIP (Fig. 4, *A* and *B*). These data indicate that SKIP negatively regulates *IGF-II* and myogenin mRNA levels during myogenesis.

SKIP Inhibited Myogenic Differentiation by Switching Off the IGF-II Auto-regulation Loop—Secreted IGF-II up-regulates its own gene expression through the PI 3-kinase-Akt signaling pathway; this positive auto-regulatory loop by IGF-II contributes to the expression of myogenin and MHC (31). The negative regulation of IGF-II transcription by SKIP led us to the hypothesis that SKIP might also regulate this auto-regulation loop by controlling PI 3-kinase signaling. Therefore, we examined the effect of SKIP overexpression on IGF-II signaling. IGF-II-dependent Akt phosphorylation was markedly decreased by the expression of SKIP WT, whereas it was not altered by the expression of the Δphos mutant (Fig. 5*A*). Exogenous IGF-II also mediates the expression of myogenin and MHC, which was not altered in the SKIP WT-expressing cells (Fig. 5*B*). These results suggest that SKIP regulates differentiation by acting on IGF-II expression. Knockdown of IGF-II expression significantly inhibited myogenic Akt phosphorylation after 72 h of induction of differentiation, which was not induced by transfection of SKIP siRNA in combination with IGF-II siRNA (Fig. 6, *A* and *B*). These data indicate that negative regulation of myogenesis by SKIP requires IGF-II. Cells co-transfected with SKIP and IGF-II siRNA did not undergo differentiation, and these cells had a decreased number of multinucleated cells and decreased expression of myogenin and MHC in comparison with the control cells (Fig. 6*B*). To confirm these data, we examined whether the exogenous addition of IGF-II in the SKIP-expressing C2C12 cells would trigger differentiation. Supplementing with IGF-II induced the expression of myogenin and MHC in C2C12 cells. Under these conditions, the number of multinucleated cells was significantly increased to a comparable level of cells lacking SKIP expression (Fig. 6*C*). These results

FIGURE 3. **Knockdown of SKIP increased PI 3-kinase signaling during myogenesis.** *A*, silencing of SKIP increased myogenic differentiation. C2C12 cells were transfected with control siRNA and SKIP siRNA in combination with GFP. Expression of SKIP was confirmed by Western blot analysis (*left panel*). Cells were induced to differentiate for 72 h and immunostained with anti-myogenin antibody and DAPI (*middle panels*). Enlarged images of *boxed areas* are shown in the *lower panels*. *Purple* indicates the region of co-localization of myogenin and DAPI. *Scale bar*, 80 μm. The percentage of cells with the indicated number of myogenin-positive nuclei and myotube area is shown in the graph (*right panels*). The number of myogenin-positive nuclei was counted in 200 cells. *, $p < 0.05$ and **, $p < 0.01$ (*t* test). *B*, silencing of SKIP increased expression of myogenin and MHC. C2C12 cells transfected with control siRNA or SKIP siRNA were induced to differentiate for 24–72 h. Lysates were subjected to Western blot analysis for phosphorylated Akt at Ser-473, myogenin, and MHC. Protein levels after 72 h of differentiation were quantified by densitometry. *M* indicates the lane loaded with protein molecular marker. *, $p < 0.05$ and **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments. *C*, C2C12 cells transfected with control siRNA or SKIP siRNA were induced to differentiate for the indicated times. Lysates were subjected to Western blot analysis for mTOR, phospho-mTOR at Ser-2448, and β-tubulin. *, $p < 0.05$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments. *D*, silencing of SKIP increased expression of myogenin and MHC. C2C12 cells transfected with control siRNA or SKIP siRNA were induced to differentiate for 72 h in the presence of an Akt inhibitor. Lysates were subjected to Western blot analysis for phosphorylated Akt at Ser-473, myogenin, and MHC. Protein levels after 72 h of differentiation were quantified by densitometry. **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments.

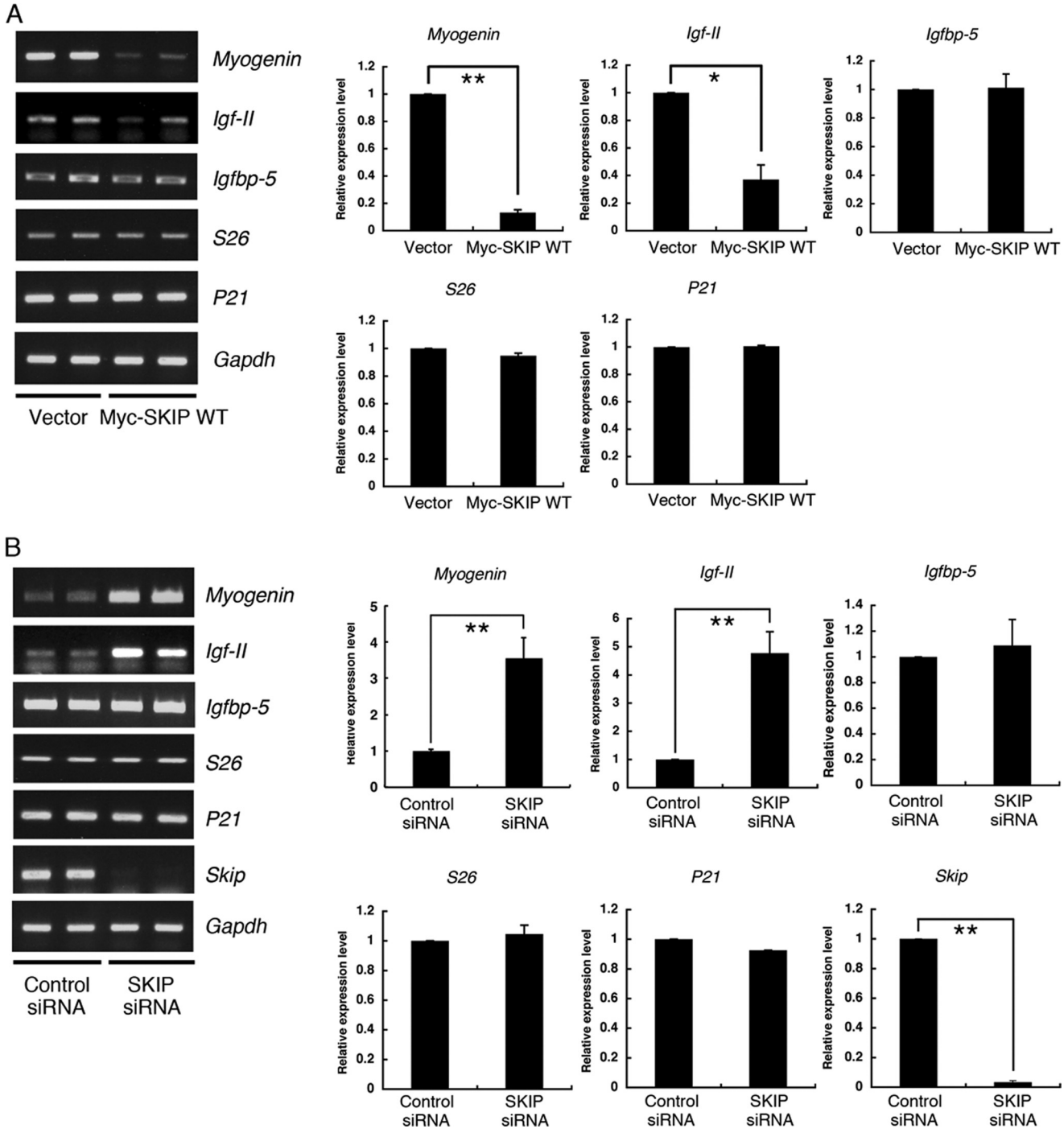


FIGURE 4. **SKIP regulates *IGF-II* gene expression during muscle cell differentiation.** *A*, C2C12 cells transfected with the pcDNA3.1-myc-vector (*Vector*) or pcDNA3.1-Myc-SKIP WT (*Myc-SKIP WT*) were induced to differentiate for 72 h. Total RNAs were extracted from the cells, and myogenin, *MyoD*, *Igf-II*, *Igfbp-5*, *S26*, and *P21* mRNA levels were measured by semiquantitative RT-PCR. Samples were loaded in duplicate. Relative amounts of these mRNAs were quantified by densitometry, which were normalized by the amount of *Gapdh* mRNA. *, $p < 0.05$ and **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of four independent experiments. *B*, C2C12 cells transfected with control siRNA or SKIP siRNA were cultured in differentiation medium for 72 h. Myogenin, *MyoD*, *Igf-II*, *Igfbp-5*, *S26*, *P21*, and *Skip* mRNA levels were measured by quantitative RT-PCR. The average results of three independent experiments are shown. mRNA levels were measured by semiquantitative RT-PCR. Samples were loaded in duplicate. Relative amounts of these mRNAs were quantified by densitometry, which were normalized by the amount of *Gapdh* mRNA. **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of four independent experiments.

demonstrate that the effect of SKIP on muscle cell differentiation is almost fully explained by the regulation of *IGF-II* mRNA expression.

mTOR is a downstream regulator of Akt that is required for IGF-II production and myogenesis. Treatment with rapamycin, a specific mTOR inhibitor, completely inhibited the expression of myogenin and MHC, which was not restored by silencing of SKIP (Fig. 7). Further supplementation with IGF-II did not induce the expression of these myogenic proteins (Fig. 7). These results suggest that SKIP negatively regulates the IGF-II-PI 3-kinase-Akt-mTOR auto-regulation loop and the subsequent IGF-II expression.

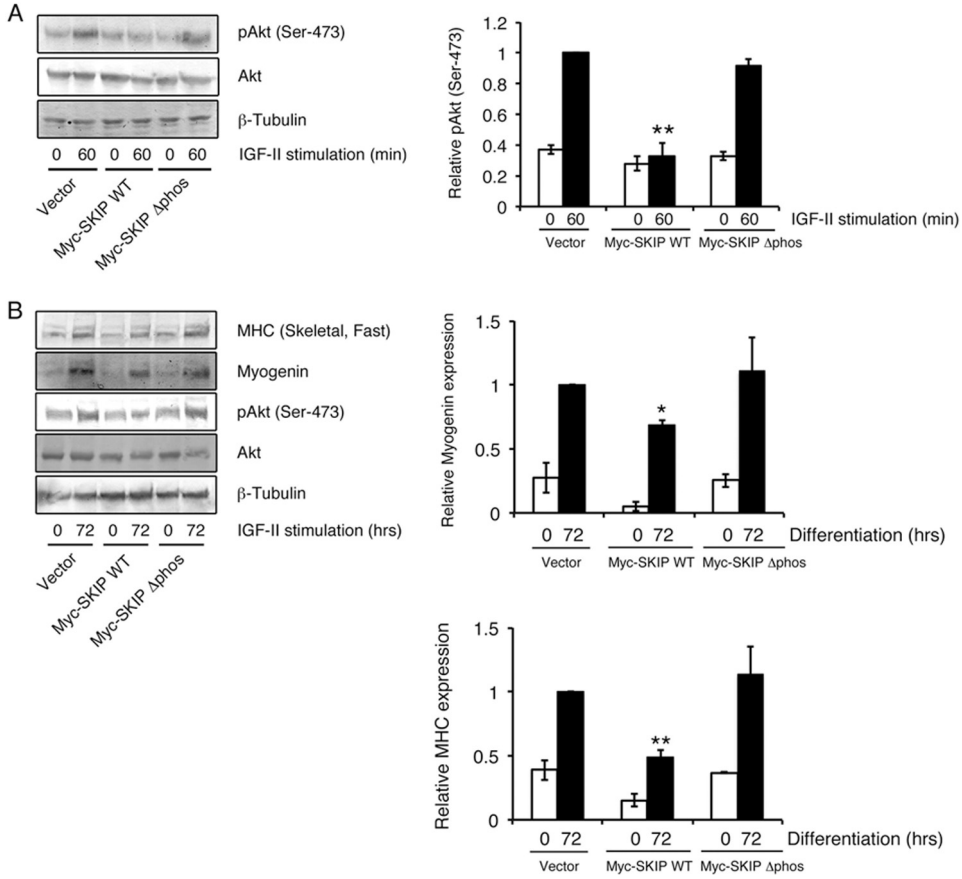


FIGURE 5. **SKIP negatively regulates IGF-II signaling during myogenesis.** *A*, C2C12 cells expressing pcDNA3.1-myc-SKIP WT or SKIP Δphos was stimulated with IGF-II (50 ng/ml) for 60 min. Cell lysates were collected and subjected to Western blot analysis. Expression levels were quantified by densitometry. *, $p < 0.05$ and **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments. *B*, C2C12 cells expressing pcDNA3.1-myc SKIP WT or SKIP Δphos were induced to differentiate for 72 h in the presence of IGF-II (50 ng/ml). Cell lysates were collected and subjected to Western blot analysis. Relative amounts of myogenin and MHC are shown in the graphs. Expression levels were quantified by densitometry. *, $p < 0.05$ and **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments.

DISCUSSION

SKIP is a PIP_3 5-phosphatase abundantly expressed in the skeletal muscles; it negatively regulates the PI 3-kinase-Akt signaling pathway induced by insulin stimulation (11, 29). It has been reported that activation of the PI 3-kinase-Akt signaling pathway is necessary for the early onset of myogenesis. C2C12 cells treated with the PI 3-kinase inhibitor LY294002 did not form myotubes (24). In this study we found that the expression level of SKIP was markedly elevated within 24 h of myogenesis. The overexpression of MyoD in C2C12 cells triggered increased SKIP expression during differentiation. These results indicate that SKIP is one of the muscle-specific genes that is controlled by MyoD transcriptional factors. Although PTEN has also been implicated in myogenesis (32), its expression remained constant during differentiation. Our results provide the first evidence that MyoD plays negative role in the regulation of muscle cell differentiation.

C2C12 cells undergo cell fusion and myotube formation, which are accompanied by the expression of myogenin and MHC during differentiation and all of which are suppressed by the expression of wild-type SKIP. These suppressions are dependent on the PIP_3 5-phosphatase activity of SKIP, suggesting that this activity is implicated in the regulation of myogenesis in C2C12 cells. The number of myogenin-positive nuclei in differentiated myocytes was significantly increased by the silencing of SKIP, which indicates that endogenous SKIP regulates myocyte hypertrophy and/or maturation. MyoD activates p21 and induces the withdrawal of myoblasts from the cell cycle (33). P21 regulates cell cycle arrest, which is essential for muscle cell differentiation during the early stages (34). Because SKIP expression levels were low during the early stages of differentiation, induction of p21 was not likely to be altered by the expression or silencing SKIP.

SKIP is a regulator of insulin-dependent Akt activation in skeletal muscle cells (11, 12). Among the Akt species, Akt1 and Akt2 have been implicated in muscle cell differentiation. During muscle cell differentiation, knockdown of Akt1 blocked the expression of myogenin and the expression of the constitutively active form of Akt1 triggered the expression of myogenin and MHC during differentiation (21). In contrast, silencing of Akt2 resulted in a normal differentiation although the myotubes were thinner with fewer nuclei (22). In C2C12 cells, silencing of SKIP slightly increased myogenin expression and markedly increased the number of nuclei and the myotube width in C2C12 myotubes, which was completely inhibited by treatment with Akt inhibitor IV. Thus, these data indicate Akt mediates the negative regulation of muscle cell differentiation by SKIP.

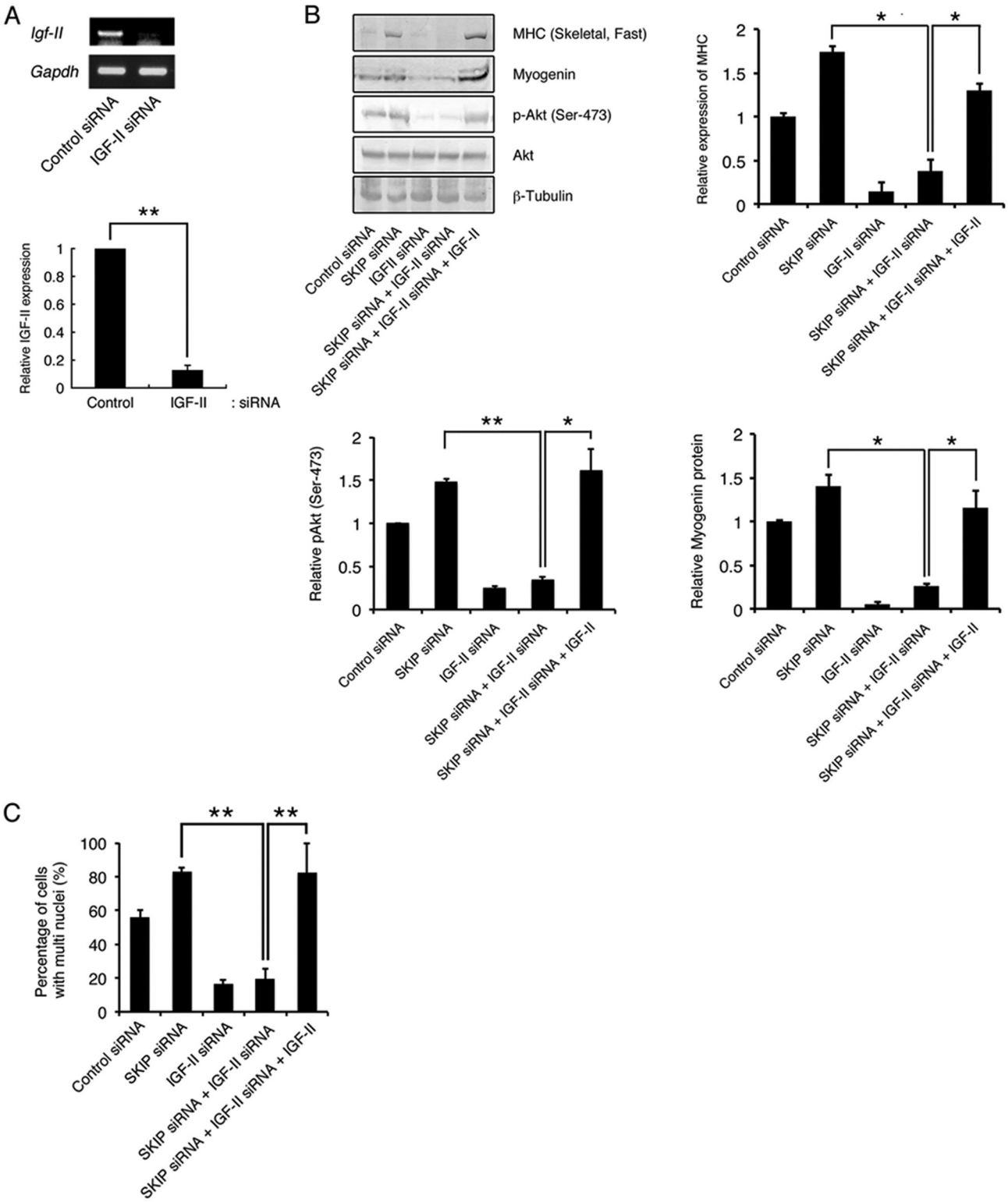


FIGURE 6. **SKIP regulates myoblast differentiation by controlling IGF-II expression.** *A*, C2C12 cells were transfected with control siRNA or IGF-II siRNA for 72 h and then subjected to semiquantitative RT-PCR to measure the levels of *IGF-II* mRNA. Expression levels were quantified by densitometry. **, $p < 0.01$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments. *B*, supplementation with IGF-II rescued the negative regulation of muscle cell differentiation by SKIP. C2C12 cells transfected with the indicated siRNAs were induced to differentiate for the indicated times. Phosphorylation of Akt at Ser-473, expression of MHC, and myogenin after the initiation of differentiation for 72 h were analyzed by Western blotting. Expression levels were quantified by densitometry. *, $p < 0.05$ (*t* test). Represented results are the mean ± S.E. of three independent experiments. *C*, silencing of IGF-II suppressed the increased muscle cell fusion induced by SKIP. The percentage of cells with >10 myogenin-positive nuclei were determined. Approximately 200 cells were counted in every experiment. *, $p < 0.05$ (*t* test). Results are presented as the mean ± S.E. of three independent experiments.

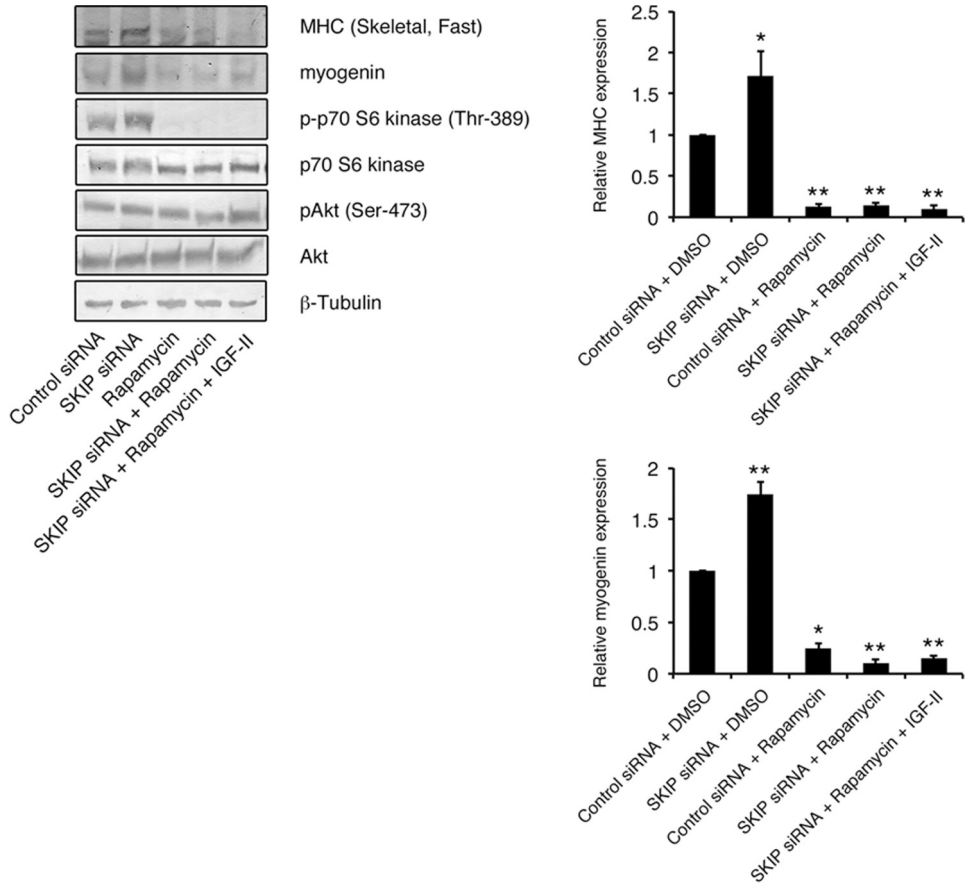


FIGURE 7. **SKIP negatively regulated mTOR activity during C2C12 cell differentiation.** Supplementing with rapamycin (100 nM) suppressed the muscle cell differentiation mediated by the silencing of SKIP. C2C12 cells transfected with the indicated siRNAs were induced to differentiate for 72 h. Expression of MHC and myogenin as well as phosphorylation of Akt at Ser-473 and p70 S6 kinase at The-389 were analyzed by Western blotting. Expression levels were quantified by densitometry. Results are presented as the mean ± S.E. of three independent experiments (*, $p < 0.05$ and **, $p < 0.01$, *t* test; *versus* control siRNA + DMSO).

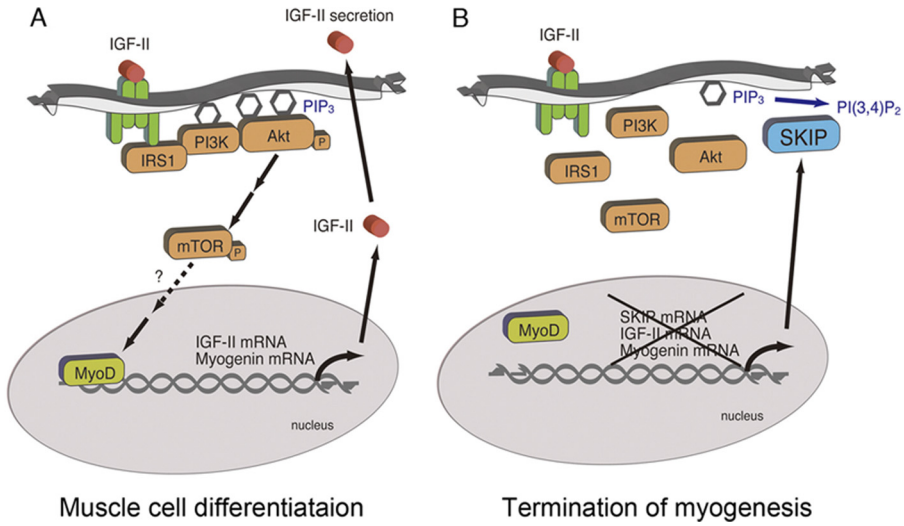


FIGURE 8. **Proposed model of SKIP-regulated skeletal muscle myogenesis.** *A*, SKIP expression is relatively low during the early stages of myogenesis, thereby enhancing PI 3-kinase signaling and subsequent MyoD-dependent IGF-II expression. IGF-II signaling is enhanced during differentiation by the IGF-II auto-regulation loop, resulting in the expression of the myogenic proteins, namely, myogenin and myosin heavy chain. *B*, induction of SKIP expression by MyoD mediates the hydrolysis of PIP_3 to phosphatidylinositol 3,4-diphosphate (*PI(3,4)P*$_2$), which results in the inhibition of Akt phosphorylation. SKIP negatively regulates IGF-II signaling and the subsequent expression of myogenin and MHC through the hydrolysis of PIP_3. Excess expression of SKIP leads to termination of differentiation.

During myogenic differentiation, MyoD increases the expression of IGF-II (30, 35). IGF-II is a growth factor whose expression is required for myogenic differentiation. In C2C12 cells, the expression of SKIP WT significantly inhibited IGF-II-mediated Akt phosphorylation. Therefore, SKIP is likely to dephosphorylate PIP_3 pools that are generated by IGF-II stim-

ulation that activate Akt. In addition, IGF-II up-regulates its own gene expression through the PI 3-kinase-Akt signaling pathway and promotes muscle cell differentiation by switching on the IGF-II auto-regulation loop (31). Our results show that endogenous SKIP negatively regulates Akt phosphorylation and the subsequent expression of myogenin and *IGF-II* mRNA levels during myogenesis. Induction of the expression of myogenin and MHC by the attenuation of SKIP was completely suppressed by the silencing of IGF-II. These expressions were restored by further supplementing with recombinant IGF-II. These results suggest a role for SKIP in switching off the IGF-II auto-regulation loop.

IGF-II transcription during myogenesis is controlled by mTOR, which is located downstream of the PI 3-kinase-Akt signaling pathway (26). Knockdown of SKIP resulted in increased IGF-II-dependent phosphorylation of mTOR at Ser-2448 and the subsequent expression of myogenin, which were completely inhibited by the treatment with the mTOR inhibitor rapamycin. These results demonstrate that SKIP predominantly regulates the IGF-II-PI 3-kinase-Akt-mTOR auto-regulation loop during myogenesis. It should be noted that the expression of both IGF-II and SKIP is initially triggered by MyoD at the early onset of myogenesis; furthermore, SKIP functions as a negative regulator of MyoD functions such as IGF-II expression.

In conclusion, we have found a novel mechanism by which the expression of SKIP regulates the myogenic action of autocrine IGF-II production. During the early stage of muscle cell differentiation, the activation of the PI 3-kinase signaling pathway leads to MyoD-dependent production of IGF-II. An increase in this IGF-II auto-regulation loop further stimulates muscle cell differentiation and myotube formation (Fig. 8*A*). Activated MyoD function, in turn, increased with increasing SKIP expression (Fig. 8*B*), leading to the inactivation of IGF-II-dependent PI 3-kinase activity and subsequent muscle cell differentiation (Fig. 8*B*). In addition to muscle cell differentiation, IGF-II has important roles in muscle development, satellite cell proliferation and differentiation, and injury-induced muscle regeneration. The importance of IGF-II in muscle development is characterized by poor muscle hypertrophy and the dystrophic phenotype of *Igf1r* knock-out mice (36). In SKIP heterozygous knock-out mice, the weight of the quadriceps muscle was increased (13). SKIP might be implicated in muscle hypertrophy through regulation of IGF-II production. The targeted therapeutic use of a SKIP inhibitor to promote muscle regeneration and muscle cell hypertrophy will require understanding the precise mechanisms by which SKIP regulates muscle cell differentiation.

REFERENCES

1. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) Control of muscle development by dueling HATs and HDACs. *Curr. Opin. Genet. Dev.* **11,** 497–504
2. Cserjesi, P., and Olson, E. N. (1991) Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol. Cell. Biol.* **11,** 4854–4862
3. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., and Hollenberg, S. (1991) The myoD gene family. Nodal point during specification of the muscle cell lineage. *Science* **251,** 761–766
4. Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H., and Jaenisch, R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75,** 1351–1359
5. Sabourin, L. A., Girgis-Gabardo, A., Seale, P., Asakura, A., and Rudnicki, M. A. (1999) Reduced differentiation potential of primary MyoD$^{-/-}$ myogenic cells derived from adult skeletal muscle. *J. Cell Biol.* **144,** 631–643
6. Bergstrom, D. A., and Tapscott, S. J. (2001) Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family. *Mol. Cell. Biol.* **21,** 2404–2412
7. Kassar-Duchossoy, L., Gayraud-Morel, B., Gomès, D., Rocancourt, D., Buckingham, M., Shinin, V., and Tajbakhsh, S. (2004) Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* **431,** 466–471
8. Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H. (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364,** 501–506
9. Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., and Nonaka, I. (1993) Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364,** 532–535
10. Xiong, Q., Chai, J., Zhang, P. P., Wu, J., Jiang, S. W., Zheng, R., and Deng, C. Y. (2011) MyoD control of SKIP expression during pig skeletal muscle development. *Mol. Biol. Rep.* **38,** 267–274
11. Ijuin, T., and Takenawa, T. (2003) SKIP negatively regulates insulin-induced GLUT4 translocation and membrane ruffle formation. *Mol. Cell. Biol.* **23,** 1209–1220
12. Ijuin, T., and Takenawa, T. (2012) Regulation of insulin signaling and glucose transporter 4 (GLUT4) exocytosis by phosphatidylinositol 3,4,5-trisphosphate (PIP3) phosphatase, skeletal muscle, and kidney-enriched inositol polyphosphate phosphatase (SKIP). *J. Biol. Chem.* **287,** 6991–6999
13. Ijuin, T., Yu, Y. E., Mizutani, K., Pao, A., Tateya, S., Tamori, Y., Bradley, A., and Takenawa, T. (2008) Increased insulin action in SKIP heterozygous knockout mice. *Mol. Cell. Biol.* **28,** 5184–5195
14. Philippou, A., Halapas, A., Maridaki, M., and Koutsilieris, M. (2007) Type I insulin-like growth factor receptor signaling in skeletal muscle regeneration and hypertrophy. *J. Musculoskelet. Neuronal Interact.* **7,** 208–218
15. Florini, J. R., Ewton, D. Z., and Magri, K. A. (1991) Hormones, growth factors, and myogenic differentiation. *Annu. Rev. Physiol.* **53,** 201–216
16. Jiang, B. H., Zheng, J. Z., and Vogt, P. K. (1998) An essential role of phosphatidylinositol 3-kinase in myogenic differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14179–14183
17. Hribal, M. L., Nakae, J., Kitamura, T., Shutter, J. R., and Accili, D. (2003) Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J. Cell Biol.* **162,** 535–541
18. Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., Yancopoulos, G. D., and Glass, D. J. (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* **3,** 1009–1013
19. Gonzalez, I., Tripathi, G., Carter, E. J., Cobb, L. J., Salih, D. A., Lovett, F. A., Holding, C., and Pell, J. M. (2004) Akt2, a novel functional link between p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways in myogenesis. *Mol. Cell. Biol.* **24,** 3607–3622
20. Héron-Milhavet, L., Mamaeva, D., Rochat, A., Lamb, N. J., and Fernandez, A. (2008) Akt2 is implicated in skeletal muscle differentiation and specifically binds prohibitin2/REA. *J. Cell. Physiol.* **214,** 158–165
21. Wilson, E. M., and Rotwein, P. (2007) Selective control of skeletal muscle differentiation by Akt1. *J. Biol. Chem.* **282,** 5106–5110
22. Rotwein, P., and Wilson, E. M. (2009) Distinct actions of Akt1 and Akt2 in skeletal muscle differentiation. *J. Cell. Physiol.* **219,** 503–511
23. Cuenda, A., and Cohen, P. (1999) Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J. Biol. Chem.* **274,** 4341–4346
24. Conejo, R., Valverde, A. M., Benito, M., and Lorenzo, M. (2001) Insulin produces myogenesis in C2C12 myoblasts by induction of NF-κB and down-regulation of AP-1 activities. *J. Cell. Physiol.* **186,** 82–94
25. Erbay, E., and Chen, J. (2001) The mammalian target of rapamycin regu-

lates C2C12 myogenesis via a kinase-independent mechanism. *J. Biol. Chem.* **276,** 36079–36082
26. Erbay, E., Park, I. H., Nuzzi, P. D., Schoenherr, C. J., and Chen, J. (2003) IGF-II transcription in skeletal myogenesis is controlled by mTOR and nutrients. *J. Cell Biol.* **163,** 931–936
27. Bergstrom, D. A., Penn, B. H., Strand, A., Perry, R. L., Rudnicki, M. A., and Tapscott, S. J. (2002) Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. *Mol. Cell* **9,** 587–600
28. Lawlor, M. A., and Rotwein, P. (2000) Insulin-like growth factor-mediated muscle cell survival. Central roles for Akt and cyclin-dependent kinase inhibitor p21. *Mol. Cell. Biol.* **20,** 8983–8995
29. Ijuin, T., Mochizuki, Y., Fukami, K., Funaki, M., Asano, T., and Takenawa, T. (2000) Identification and characterization of a novel inositol polyphosphate 5-phosphatase. *J. Biol. Chem.* **275,** 10870–10875
30. Wilson, E. M., Hsieh, M. M., and Rotwein, P. (2003) Autocrine growth factor signaling by insulin-like growth factor-II mediates MyoD-stimulated myocyte maturation. *J. Biol. Chem.* **278,** 41109–41113
31. Ren, H., Yin, P., and Duan, C. (2008) IGFBP-5 regulates muscle cell differentiation by binding to IGF-II and switching on the IGF-II auto-regulation loop. *J. Cell Biol.* **182,** 979–991
32. Mandl, A., Sarkes, D., Carricaburu, V., Jung, V., and Rameh, L. (2007) Serum withdrawal-induced accumulation of phosphoinositide 3-kinase lipids in differentiating 3T3-L6 myoblasts. Distinct roles for Ship2 and PTEN. *Mol. Cell. Biol.* **27,** 8098–8112
33. Sorrentino, V., Pepperkok, R., Davis, R. L., Ansorge, W., and Philipson, L. (1990) Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature* **345,** 813–815
34. Odelberg, S. J., Kollhoff, A., and Keating, M. T. (2000) Dedifferentiation of mammalian myotubes induced by msx1. *Cell* **103,** 1099–1109
35. Wilson, E. M., and Rotwein, P. (2006) Control of MyoD function during initiation of muscle differentiation by an autocrine signaling pathway activated by insulin-like growth factor-II. *J. Biol. Chem.* **281,** 29962–29971
36. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* **75,** 59–72