AMP-Activated Protein Kinase $\alpha_1$ but Not $\alpha_2$ Catalytic Subunit Potentiates Myogenin Expression and Myogenesis

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The link between AMP-activated protein kinase (AMPK) and myogenesis remains poorly defined. AMPK has two catalytic α subunits, α1 and α2. We postulated that AMPK promotes myogenesis in an isoform-specific manner. Primary myoblasts were prepared from AMPK knockout (KO) mice and AMPK conditional KO mice, and knockout of the α1 but not the α2 subunit resulted in downregulation of myogenin and reduced myogenesis. Myogenin expression and myogenesis were nearly abolished in the absence of both AMPKα1 and AMPKα2, while enhanced AMPK activity promoted myogenesis and myotube formation. The AMPKα1-specific effect on myogenesis was likely due to the dominant expression of α1 in myoblasts. These results were confirmed in C2C12 cells. To further evaluate the necessity of the AMPKα1 subunit for myogenesis in vivo, we prepared both DsRed AMPKα1 knockout myoblasts and enhanced green fluorescent protein (EGFP) wild-type myoblasts, which were cotransplanted into tibialis anterior muscle. A number of green fluorescent muscle fibers were observed, showing the fusion of engrafted wild-type myoblasts with muscle fibers; on the other hand, very few or no red muscle fibers were observed, indicating the absence of myogenic capacity of AMPKα1 knockout myoblasts. In summary, these results indicate that AMPK activity promotes myogenesis through a mechanism mediated by AMPKα1.

Skeletal muscle, which comprises about 40% of the body mass of adults, is the main peripheral tissue responsive to insulin-stimulated uptake of glucose (1) and is critical in the development of type 2 diabetes (2). Proper myogenesis is critical for fetal muscle development (3–5) and postnatal muscle growth and regeneration, which relies heavily on the myogenic differentiation of satellite cells (6–8). In improper myogenesis and muscle regeneration, damaged muscle fibers are replaced with fibric tissue, leading to muscle atrophy and aging (9–11).

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme, composed of α, β, and γ subunits, which plays an important role in energy metabolism (12–14). In addition to its capacity to acutely regulate the activity of metabolic enzymes through phosphorylation, AMPK is increasingly recognized for its regulatory role in gene expression, cell differentiation, and tissue development (15, 16). The role of AMPK in muscle fiber atrophy has been well defined; a number of studies demonstrated that AMPK promotes muscle protein degradation and autophagy (17) and inhibits its protein synthesis (18). To date, however, the role of AMPK in myogenesis (formation of muscle fibers) has been sparingly studied. Our previous studies showed that low AMPK activity due to obesity is correlated with attenuated myogenin differentiation during fetal muscle development (19–21). We further observed that AMPK promotes myogenin expression and myogenesis through phosphorylation of histone deacetylase 5 (HDAC5) (22), suggesting that AMPK has a critical role in myogenesis.

The catalytic α subunit of AMPK has two isoforms, α1 and α2, which display differential expression during myogenic cell differentiation (23). Knockout (KO) of either the α1 or α2 subunit results in visually normal mice, but KO of both α1 and α2 subunits is lethal at around embryonic day 9.5 (E9.5) (24), showing that α1 and α2 subunits have compensatory roles in regulating fetal growth and development. We found that AMPKα1 KO but not AMPKα2 KO is associated with reduced muscle mass (22), which points to the isoform-specific role of AMPK in myogenesis. However, there is no direct evidence detailing such an effect. Here we demonstrate that AMPKα1 but not AMPKα2 has a major regulatory role in myogenic differentiation, and AMPKα1 KO results in a dramatic reduction in myogenesis.

MATERIALS AND METHODS

Mice. All animals were handled in accordance with protocols approved by the Animal Use and Care Committees of Washington State University and the University of Wyoming. Wild-type (WT) C57BL/6 mice and enhanced green fluorescent protein (EGFP) mice [B6-Tg(CAG-EGFP)131Osb/LeyS0pl, catalog number 006567], red fluorescent (DsRed) mice [B6.Cg-Tg(CAG-DsRed*MF1)Nagy/J, catalog number 006015], and B6:129-Gt(Rosa26)Sor[m1Cre/ERT2]Nagy/J mice (catalog number 004847), in which tamoxifen-inducible Cre recombinase is driven by the endogenous mouse Gt(Rosa26)Sor promoter, were obtained from the Jackson Laboratory (Bar Harbor, ME). AMPKα1−/− 129S2/SvPas (AMPKα1 KO) and AMPKα2−/− C57BL/6 (AMPKα2 KO) mice were generated as previously described (25, 26). Mice with AMPKα1 floxed, mice with AMPKα2 floxed, and mice with both AMPKα1 and AMPKα2 floxed were generated as previously described (26) and cross-bred with tamoxifen-inducible Cre mice to generate AMPK conditional KO mouse strains in which AMPKα1, AMPKα2, or both AMPKα1 and AMPKα2 can be deleted in response to tamoxifen. Red fluorescent (DsRed) mice [B6.Cg-Tg(CAG- DsRed*MF1)Nagy/J, catalog number 006015; Jackson Laboratory] were cross-bred with AMPKα1 conditional KO mice to generate DsRed-labeled AMPKα1 conditional KO mice.

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Antibodies and chemicals. Antibodies against AMPKα1 (catalog number 2523), phospho-AMPKα1 (Thr172 (number 2535), acetyl coenzyme A carboxylase (ACC) (catalog number 3662), phospho-ACC at Ser79 (catalog number 3661), tag (catalog number 2368), mouse IgG (catalog number 7076) and β-tubulin (catalog number 2146) and goat anti-mouse antibody–Alexa Fluor 555 (catalog number 4409), goat anti-rat antibody–Alexa Fluor 488 (catalog number 4416), and goat anti-rabbit antibody–Alexa Fluor 488 (catalog number 4413) were purchased from Cell Signaling (Danvers, MA). Antitrypsinogen (F5D), anti-embryonic myosin heavy chain (anti-EMH) (F1,652), and anti-myosin heavy chain (anti-MHC) (MF20) mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit antidesmin antibody (ab15200) and chick anti-GFP antibody (ab13970) were purchased from Abcam (Cambridge, MA). Rat antitrypsinogen antibody (4H8-2) was purchased from Enzo (Farmingdale, NY). Rabbit anti-DesRed antibody (catalog number 632496) was purchased from Clontech (Mountain View, CA). Goat anti-chick antibody–Alexa Fluor 488 (catalog number A1039) was purchased from Life Technologies (Grand Island, NY). IRDye 800CW–goat anti-rabbit secondary antibody and IRDye 680–goat anti-mouse secondary antibody were purchased from Li-Cor Biosciences (Lincoln, NE). Puromycin, compound C, cardiotitin (CTX), and 4-hydroxytamoxifen (TM) were purchased from Sigma (St. Louis, MO). MC1568 was purchased from Selleck (Houston, TX). AICAR (5-aminimidazole-4-carboxamide 1-beta-D-ribonucleoside) (5 mM) was purchased from Toronto Research Chemicals (Toronto, Canada). Lipo- fectamine was purchased from Invitrogen (Carlsbad, CA). Basic fibroblast growth factor (FGF2) (catalog number 233-FB-025) was purchased from R&D Systems (Minneapolis, MN).

Cell culture. Myogenic C2C12 cells were grown at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic mixture. Primary myo- blasts were extracted from neonatal mice according to a procedure de- scribed previously, with modifications (27). Briefly, muscle from hind limbs was minced and digested in DMEM with collagenase D and dispase II (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for about 30 min. The slurry was then passed through a 100-μm cell strainer. Cells were collected by centrifugation at 350×g for 5 min. The cell pellet was then resuspended in F-10 medium with 20% FBS, 1% antibiotic mixture, and 5 ng/ml FGF2. Primary myoblasts were seeded onto collagen-coated plates and enriched by preplating. The purity of enriched primary myoblasts was checked by fluorescence-activated cell sorter (FACS) analysis using an antidesmin antibody. When cells reached 80% confluence, cul- ture medium was switched to DMEM supplemented with 2% horse serum and a 1% antibiotic mixture to induce myogenic differentiation. AMPK conditional KO cells were supplemented with 1 μM 4-hydroxytamoxifen for 48 h to KO the AMPKα subunit.

Immunoblotting analyses. Immunoblotting analysis was performed as previously described, using an Odyssey infrared imaging system (Li- Cor Biosciences, Lincoln, NE) (28). Band density was normalized to β-tu- bulin content.

Real-time quantitative PCR. Total RNA was extracted by using TRIzol (Sigma, St. Louis, MO) followed by DNase (NEB, Ipswich, MA) treatment, and cDNA was synthesized by using a reverse transcription kit (Bio-Rad, Hercules, CA). Real-time PCR (RT-PCR) was carried out by using a CFX RT-PCR detection system (Bio-Rad) with a SYBR green RT-PCR kit from Bio-Rad (Hercules, CA). The following cycle param- eters were used: 34 three-step cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s. Primer sequences (with their respective PCR fragment lengths in parentheses) were as follows: forward primer 5'-GAGATCCTGGCGAC CGCCCAT-3' and reverse primer 5'-CCCCGGCTCTGTAGCGGAAAG-3' for myogenin (97 bp), forward primer 5'-TGGAGAAAGGGCGCTGAGAC-3' and reverse primer 5'-GTGAGACCTGGCTGTCGTC-3' for MRF (181 bp), forward primer 5'-TCTGGAGCCCTCTGTCGACC-3' and reverse primer 5'-CGGAGGGGAGGTTGAGGGG-3' for MyoD (100 bp), and forward primer 5'-AAACTCCGGAGTCGCCCT-3' and reverse primer 5'-GGACAGGCTGCATGCTC-3' for Myf-5 (125 bp). After amplification, a melting curve (0.01°C/s) was used to confirm product purity, and agarose gel electrophoresis was performed to confirm that only a single product of the right size was amplified. Relative mRNA content was normalized to 18S rRNA content.

Transfection. Plasmid transfection of C2C12 cells was performed by using Lipofectamine according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). Briefly, 12 h before transfection, cells were switched to medium without antibiotics. Transfections were carried out when cells reached 80% confluence, using a 1:3 ratio of DNA (μg) to Lipofectamine (μl); medium was switched to DMEM containing 10% FBS and 1% antibiotics 12 h following transfection.

For short hairpin RNA (shRNA) interference, AMPKα1 shRNA, AMPKα2 shRNA, and control shRNA (Santa Cruz Biotech, Santa Cruz, CA) were delivered into cells, and transfected cells were selected with puromycin (2 μg/ml).

Induction of muscle regeneration, fluorescent myogenic cell trans- plantation, and in vivo tracing. Myogenic cells were separated from neonatal mice. DesRed AMPKα1 conditional KO myoblasts were treated with tamoxifen for 48 h to induce AMPKα1 KO. WT EGFP myoblasts were treated simultaneously. Because in these mice, both DesRed and EGFP are under the control of a chicken β-actin promoter and the overall transgenic constructs between these two fluorescent mice are very similar and are in the same C57BL/6 background, DesRed and EGFP transgenesis does not affect the myogenic capacity of myo- genic cells. Three-month-old C57BL/6 mice were used as the recipient mice. Briefly, 1 day before cell transplantation, recipient mice were anesthetized by administration of a mixture of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg) (intraperitoneally [i.p.]). Next, 100 μl of 10 μM cardiotitin was injected into the tibialis anterior (TA) muscle to induce muscle regeneration (9). After 1 day, mice were ready for cell transplantation; 6×10^6 cells (3×10^6 WT green cells and 3×10^6 AMPKα1 KO red fluorescent cells, which were mixed before injec- tion) in 50 μl phosphate-buffered saline (PBS) were slowly injected into the TA muscle by using a 29-gauge needle (9, 29). Fourteen days after transplantation, mice were sacrificed, and TA muscle was col- lected, fixed in 4% paraformaldehyde, and frozen in isopentane cooled in liquid nitrogen. Frozen tissue was sectioned (5 to 10 μm thick). Sections were blocked in 5% goat serum in Tris-buffered saline (TBS) containing 0.3% Triton X-100 and stained with chick anti-GFP antibody (1:1,000), rabbit anti-DesRed antibody (1:500), and the corre- sponding fluorescent secondary antibodies. Green fluorescent muscle fibers and red fluorescent muscle fibers on sections at 100-μm inter- vals were counted.

Immunocytochemical staining. Cells grown on coverslips or multi- ple-well plates were fixed in 4% paraformaldehyde for 10 min, permeab- ilized with cold methanol for 5 min, quenched with 0.1% sodium borohydrate for 5 min, and incubated with anti-MHC or mouse IgG (1:100) at 4°C overnight. Fluorescent secondary antibody (1:1,000) was then added, and stained cells were incubated at room temperature for 1 h. Fluores- cence was examined by using a Leica inverted microscope (28). Fusion indexes of differentiating myoblasts were calculated by dividing the number of nuclei in MHC-positive myotubes by the total number of nuclei.

Statistics. For all studies, at least 3 independent experiments were conducted. All data are expressed as means ± standard errors of the mean (SEM). Data were analyzed by using the general linear model (GLM) of SAS (SAS Institute Inc., Cary, NC), and Tukey’s Studentized range test was used to determine the significance of differences among groups. A P value of <0.05 was considered significant.
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RESULTS

AMPKα1 inhibition attenuates myogenesis in C2C12 cells. The reduction of AMPK activity has significant physiological relevance, because obesity and its associated chronic low-grade inflammation reduce AMPK activity via elevating tumor necrosis factor alpha (TNF-α) levels (30, 31). To test if TNF-α inhibits AMPK in myoblasts, we treated C2C12 cells with TNF-α (10 ng/ml). TNF-α treatment reduced AMPK phosphorylation at Thr172, a marker of AMPK activity. We further analyzed phosphorylation of Ser79 in acetyl coenzyme A carboxylase (ACC), a site exclusively phosphorylated by AMPK, and found that, indeed, ACC Ser79 phosphorylation was reduced in cells treated with TNF-α (Fig. 1A).

To assess the relationship between decreased AMPK activity and myogenesis, we stably transfected C2C12 cells with plasmids expressing either AMPKα1 or AMPKα2 short hairpin RNA (shRNA). We found that about 40% of endogenous AMPKα1 and AMPKα2 were knocked down by their respective shRNAs (Fig. 1B); the activity of both subunits was also decreased, as indicated by decreased phosphorylation of ACC (Fig. 1C).

We further observed that AMPKα1 but not AMPKα2 knockdown was associated with decreased myogenin mRNA content (Fig. 1D). Consistently, myogenin protein content was decreased when AMPKα1 was knocked down (Fig. 1E). As expected, both myosin heavy chain (MHC) protein content and the number of mature myotubes were decreased only when AMPKα1 was knocked down (Fig. 1E and F).

AMPKα1 activity is necessary for myogenesis in primary myoblasts in vitro. Because only about a 40% knockdown of AMPKα subunits was achieved by shRNA, we assessed myogenin mRNA expression in differentiating primary myoblasts from neonatal WT, AMPKα1 KO, and AMPKα2 KO mice. Desmin staining showed that the primary myogenic cells which we prepared had very high purity, eliminating the possibility that the detected difference was due to an impurity of primary myogenic cells (Fig. 2A). We observed that myogenin mRNA expression was profoundly reduced in AMPKα1 KO myoblasts but not in AMPKα2 KO myoblasts (Fig. 2B). Consistently, AMPKα1 KO was associated with a dramatic decrease in myogenin protein content (Fig. 2C), whereas AMPKα2 KO showed no significant difference (Fig. 2D). We also found that AMPKα1 KO but not AMPKα2 KO was associated with a decreased MHC level (Fig. 2C and D). In addition, myotube formation in AMPKα1 KO myoblasts but not in AMPKα2 KO myoblasts was reduced (Fig. 2E and F). Two days after induction of myogenesis, the mRNA levels of additional myogenic regulatory factors (Myf5, MyoD, and MRF4) in WT myoblasts and AMPKα1 KO myoblasts were also tested. While no difference was seen in MyoD and MRF4 mRNA levels, the Myf5 mRNA level was higher in AMPKα1 KO myoblasts than in WT myoblasts, which suggests a specific regulatory effect of AMPK on myogenin expression (Fig. 2G). Myf5 is known to express prior to myogenin and is able to induce myogenin expression (32, 33), and thus, it is possible that the elevated level of Myf5 in AMPKα1 KO myoblasts was due to compensatory effects. Another possibility is that the halted fusion of AMPKα1 KO myoblasts into myotubes...
rendered more abundant myoblasts which express Myf5, resulting in higher Myf5 expression levels in AMPKα1 KO than in WT differentiating myoblasts.

We reasoned that if AMPK was necessary for myogenesis, then knocking out both AMPKα1 and -α2 should prevent the formation of myotubes. Because the double knockout (DK) of AMPKα1 and -α2 subunits is embryonically lethal, we prepared primary myoblasts from tamoxifen-inducible AMPKα1 and AMPKα2 conditional KO mice (AMPK DK mice). AMPK DK primary myoblasts were treated with 1 μM 4-hydroxytamoxifen (TM) for 2 days to achieve complete depletion of AMPKα protein (Fig. 3A). As expected, the absence of AMPK activity was associated with dramatic reductions in myogenin mRNA expression levels (Fig. 3B), myogenin protein content (Fig. 3C), MHC levels (Fig. 3C), and myotube formation (Fig. 3D), clearly illustrating the critical role of AMPK in myogenesis.

Expression of AMPKα1 and AMPKα2 subunits during myogenesis. To dissect the effects of isoform-specific depletion of AMPKα1 and -α2 subunits on myogenesis, we tested whether the difference was caused by the differential expression of AMPKα1 and AMPKα2 in myoblasts. While the AMPKα1 mRNA level remained constant during 6 days of myogenic differentiation in C2C12 cells, the AMPKα2 mRNA level increased about 4-fold (Fig. 4A). In primary myoblasts, 2 days after myogenic induction, the AMPKα1 mRNA level decreased approximately 30%, while the AMPKα2 mRNA level increased 4-fold (Fig. 4B). The mRNA levels of AMPKα1 and AMPKα2 in the hind-limb muscles from neonatal (2 days old, with highly active myogenic differentiation) and adult (2 months old, with mature muscle fibers and only limited myogenesis) WT mice were further compared. The AMPKα1 mRNA level was about 9-fold higher in neonatal mice than in adult mice, whereas the AMPKα2 mRNA level was about 50% lower in neonatal mice than in adult mice (Fig. 4C). These data show that the AMPKα1 level decreases gradually during myogenic differentiation, while the AMPKα2 level increases during this process, indicating the importance of AMPKα1 in early myogenesis.

To further clarify AMPKα subunit expression, total AMPKα protein levels (α1 and α2 subunits were included because the antibody does not differentiate these two subunits) and activity, based on the phosphorylation of AMPKα Thr172, were measured in differentiating WT, AMPKα1 KO, and AMPKα2 KO myoblasts. Both AMPKα protein levels and activity were lower in AMPKα1 KO myoblasts than in WT myoblasts 3 days after induction of myogenesis, despite the finding that the AMPKα2 mRNA level increased dramatically 2 days after induction of myogenesis, which suggests an extremely low expression level of AMPKα2 in undifferentiated and differentiating myoblasts (Fig. 4B and D). There were no differences in AMPKα protein levels and activity between WT myoblasts and AMPKα2 KO myoblasts (Fig. 4D), further showing that AMPKα2 expression is minor in myoblasts.

AMPKα protein levels and activities in the hind-limb muscle from neonatal and adult WT and AMPKα KO mice were further measured. Both AMPKα protein levels and activities were lower in neonatal AMPKα1 KO mice than in neonatal WT mice, showing that AMPKα1 is the dominant form in neonatal muscle (Fig. 4E); however, the difference was reduced in adult mice (Fig. 4F). There was no significant difference in AMPKα protein levels and activities between neonatal AMPKα2 KO mice and neonatal WT mice (Fig. 4E), whereas the difference was significant in adult mice, which indicates that the α2 subunit was the dominant form in adult mice (Fig. 4F). These data suggest that AMPKα1 is abundantly expressed in myoblasts, while AMPKα2 is preferentially expressed in mature muscle fibers.

During muscle regeneration, satellite cells proliferate and then fuse to damaged muscle fibers or form new muscle fibers to replace damaged ones. To test the levels of AMPKα1 and AMPKα2 in regenerating muscle, we induced muscle regeneration in TA.
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muscle by cardiotoxin (CTX) injection. AMPKα1 and AMPKα2 mRNA levels at different days post-CTX injection were tested. Both AMPKα1 and AMPKα2 mRNA levels decreased at 1 day post-CTX injection, possibly due to the severe harmful effect of CTX on cell physiology. Surprisingly, a >30-fold increase in the AMPKα1 mRNA level was observed in muscle at 3 days post-CTX injection, which correlates with the expression of embryonic myosin heavy chain (EMH), a marker of muscle regeneration (9). However, AMPKα2 levels only returned to the level prior to damage at 3 days post-CTX injection (Fig. 4G and H). The increased AMPKα1 mRNA level in regenerating muscle strongly indicates its importance in myogenesis.

Both AMPKα1 and AMPKα2 are able to promote myogenesis. The question that remains is whether both AMPKα1 and AMPKα2 subunits are able to promote myogenin expression and, thus, myogenesis. To address this question, we treated C2C12 cells and primary myoblasts with 5-aminimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR) and an AMPK inhibitor, compound C. Because high levels of AMPK activity are known to inhibit universal protein synthesis (by attenuating mTOR signaling and inhibiting myogenin and MHC expression), we sought to minimize such possible side effects by using a low concentration of AICAR (0.125 mM) to induce a low level of activation; similarly, a low concentration of compound C (1 μM) was used. AMPK activity was assessed by measuring phosphorylation levels of AMPKα Thr172 and ACC Ser79 following AICAR and compound C treatments in C2C12 cells (Fig. 5A). AMPK activation by AICAR resulted in an increase in the myogenin mRNA level and protein content and an increase in the MHC protein content in C2C12 cells (Fig. 5B and C). In agreement with this finding, myotube formation was enhanced in AICAR-treated C2C12 cells, which indicates that a low level of AMPK activation promotes myogenesis (Fig. 5D). A reduction in myogenin mRNA (Fig. 5B) and protein (Fig. 5C) levels was observed in C2C12 cells treated with compound C. The MHC level was also decreased following compound C treatment (Fig. 5C).

The same treatment regimens were applied to primary myoblasts. AICAR treatment resulted in increased myogenin mRNA and protein levels, while compound C treatment resulted in reduced myogenin mRNA and protein levels in WT, AMPKα1 KO, and AMPKα2 KO differentiating myoblasts (Fig. 5E and G to I). Immunofluorescence analysis showed corresponding changes in myotube formation (Fig. 5F) (only images of AMPKα2 KO cells are shown)). AICAR treatment increased the MHC protein levels in WT, AMPKα1 KO, and AMPKα2 KO differentiating myoblasts. Compound C treatment resulted in decreased MHC levels in WT differentiating myoblasts (Fig. 5G) and AMPKα2 KO differentiating myoblasts (Fig. 5I) but resulted in an increase in MHC levels in AMPKα1 KO differentiating myoblasts (Fig. 5H). Although further analysis will be necessary, the increased MHC level in AMPKα1 KO differentiating myoblasts (AMPKα2 remaining) following compound C treatment may be related to the importance of the AMPKα2 subunit in governing protein synthesis, including MHC synthesis.

AMPKα1 potentiates myogenic differentiation of engrafted myoblasts. To test the influence of AMPK KO on myogenic differentiation in vivo, myoblasts were isolated from EGFP WT mice and DsRed mice with an AMPKα1 conditional KO. DsRed-expressing AMPKα1 conditional KO myoblasts were treated with tamoxifen for 48 h to delete AMPKα1. CTX was injected into TA

FIG 3 Double-knockout AMPKα subunits dramatically reduce myogenesis. WT and AMPKα1 and AMPKα2 double-KO (DK) primary myoblasts were prepared, purified, and induced for myogenesis. (A) AMPKα subunit contents in AMPKα DK myoblasts following 4-hydroxytamoxifen (TM) treatment. (B) Myogenin mRNA expression in AMPKα DK myoblasts following TM treatment (2 days after induction of myogenesis). (C) Myogenin and MHC protein contents in AMPKα DK myoblasts following TM treatment (3 days and 4 days after induction of myogenesis, respectively). (D) Immunocytochemical staining using an anti-MHC antibody and fusion index 4 days after induction of myogenesis following TM treatment. Data shown are means ± SEM (n ≥ 3). *P, P < 0.05 versus the WT; ***, P < 0.0001 versus the WT.
muscle of WT mice to induce muscle regeneration. Twenty-four hours after CTX injection, $3 \times 10^4$ EGFP myoblasts and $3 \times 10^4$ DsRed AMPK KO myoblasts were mixed and injected together into each damaged TA muscle. Cotransplantation of WT EGFP myoblasts and AMPK KO DsRed myoblasts eliminated the variations due to the transplantation procedure and physiological conditions. Fourteen days after myoblast transplantation, a number of EGFP-positive (EGFP) muscle fibers was observed in transplanted TA muscle, whereas a much smaller number of DsRed muscle fibers was observed (Fig. 6). These data clearly indicate that WT myoblasts had a greater potential to differentiate into muscle fibers in vivo than AMPK KO myoblasts, showing the promoting effect of AMPK on myogenic differentiation.

**DISCUSSION**

To date, the relationship between AMPK and myogenesis has been controversial. A previous study suggested that AMPK activation blocked myogenesis through a PGC-1α-dependent mechanism involving SIRT1 in C2C12 cells (34). However, a high dose of AICAR (1 mM) was used in this study. Because AMPK is known to inhibit protein synthesis, highly activated AMPK would be expected to inhibit protein synthesis globally and would likely inhibit cell proliferation. Moreover, pathophysiological changes such as obesity and chronic inflammation are known to inhibit, rather than activate, AMPK (31), suggesting that inhibition, rather than activation, is more relevant to the pathophysiological roles of AMPK (35–37). AMPK is phosphorylated by a constitutively active kinase, LKB1, which suggests that dephosphorylation rather than phosphorylation may be the main mechanism in the regulation of AMPK activity (37–39). In our previous studies, we observed that low AMPK activity correlated with impaired myogenesis (19). Moreover, we recently reported that AMPK positively regulates myogenesis through phosphorylation of HDAC5 and that AMPK KO but not AMPK KO was associated with reduced muscle mass (22).

To dissect the relationship between decreased AMPK activity and myogenesis, we used shRNA to selectively knock down either AMPK KO or AMPK KO. Our results indicated that AMPK KO but not AMPK KO knockdown was associated with decreased myogenesis, as shown by decreased expression levels of myogenin and MHC and less formation of myotubes. Our data were further strengthened by using primary myoblasts isolated from neonatal WT, AMPK KO, and...
AMPKα2 KO mice. During myogenic differentiation, AMPKγ1 KO myoblasts showed reduced expression levels of myogenin and MHC and less formation of myotubes than WT myoblasts. Also, the absence of both AMPKα1 and AMPKα2 was associated with further reductions in myogenesis, which indicates that AMPK activity is necessary for optimal myogenic differentiation.

We questioned why AMPKα2 depletion failed to reduce myogenin and MHC levels. We postulated that it may be caused, in

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FIG 6 AMPKα1 is critical for myogenic differentiation of engrafted myoblasts. (A to E) Immunostaining of muscle fibers formed by transplanted WT EGFP myoblasts and DsRed AMPKα1 KO myoblasts. (E') Magnified image of a selected area in panel E. (F) Numbers of muscle fibers formed by transplanted myoblasts in three independent experiments. BF, bright field.
part, by the differential expression of AMPKα1 and AMPKα2 in myoblasts. Indeed, AMPKα1 is expressed during the early stages of myogenesis; however, the AMPKα2 level increases during myogenic differentiation and becomes the dominant isoform in mature muscle, as indicated by previous reports showing that AMPKα2 is the major form in skeletal muscle (40, 41). When treated with low levels of AICAR, WT, AMPKα1 KO, and AMPKα2 KO myoblasts all showed increased myogenesis, which indicates that both AMPKα1 and AMPKα2 promote myogenesis. As the dominant AMPK isoform in early differentiating myoblasts, it was not unexpected that AMPKα1 KO inhibited myogenin expression and myogenesis, while the depletion of AMPKα2 did not affect myogenesis. This notion is further supported by the profound upregulation of AMPKα1 but not AMPKα2 mRNA expression during muscle regeneration, indicating that AMPKα1 has a critical role in muscle fiber regeneration.

Finally, consistent with our in vitro data, AMPKα1 KO dramatically reduced myogenic differentiation of myoblasts transplanted into mouse TA muscle in vivo. Surprisingly, very few or no AMPKα1 KO myoblasts fused with muscle fibers, while WT myoblasts did. These data show that AMPKα1 KO significantly impaired the ability of myoblasts to participate in muscular regeneration, indicating that AMPKα1 has a critical role in muscle fiber regeneration. Considering that proper muscle fiber regeneration is essential for maintaining muscle function (8), improper muscular regeneration due to attenuated AMPK activity is expected to accelerate muscle atrophy and aging (11).

Our observation that AMPK activity is necessary to maintain normal myogenesis has important physiological implications, because improper muscle regeneration is largely responsible for the muscle wasting and loss of contractile function during aging and muscular atrophy (42). AMPK likely provides a convenient molecular target to facilitate muscle development and muscle regeneration.

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