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Gαi2 Signaling Is Required for Skeletal Muscle Growth, Regeneration, and Satellite Cell Proliferation and Differentiation

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We have previously shown that activation of Gαi2, an α subunit of the heterotrimeric G protein complex, induces skeletal muscle hypertrophy and myoblast differentiation. To determine whether Gαi2 is required for skeletal muscle growth or regeneration, Gαi2-null mice were analyzed. Gαi2 knockout mice display decreased lean body mass, reduced muscle size, and impaired skeletal muscle regeneration after cardiotoxin-induced injury. Short hairpin RNA (shRNA)-mediated knockdown of Gαi2 in satellite cells (SCs) leads to defective satellite cell proliferation, fusion, and differentiation *ex vivo*. The impaired differentiation is consistent with the observation that the myogenic regulatory factors MyoD and Myf5 are downregulated upon knockdown of Gαi2. Interestingly, the expression of microRNA 1 (miR-1), miR-27b, and miR-206, three microRNAs that have been shown to regulate SC proliferation and differentiation, is increased by a constitutively active mutant of Gαi2 [Gαi2(Q205L)] and counterregulated by Gαi2 knockdown. As for the mechanism, this study demonstrates that Gαi2(Q205L) regulates satellite cell differentiation into myotubes in a protein kinase C (PKC)- and histone deacetylase (HDAC)-dependent manner.

Heterotrimeric G proteins are intracellular proteins and transduce external signals from a variety of cell surface receptors to intracellular effectors (1). G proteins are classified according to their α subunits into four subfamilies: Gαs, Gαi/o, Gαq/11, and $Gα_{12/13}$ (1). The Gαi subfamily is encoded by three genes, *GNAI1*, *GNAI2*, and *GNAI3*, and was originally identified by its ability to inhibit adenylyl cyclase activity. All three Gαi isoforms have been deleted by gene targeting in mice, and the resulting phenotypes indicate that they have both overlapping and distinct functions. Ablation of Gαi1 in mice modulates adenylyl cyclase activity in the hippocampus and impairs memory formation (2). Gαi2-deficient mice display growth retardation, develop ulcerative colitis (3), and present defects of the parasympathetic heart rate (4), while Gαi3 has been shown to modulate insulin regulation of autophagy in hepatocytes (5) and to be required for normal patterning of the axial skeleton (6) and for cytoskeleton-dependent control of cilium migration as an important step in establishing planar cell polarity in cochlear cells (7). However, the requirement for Gαi isoforms in skeletal muscle growth and regeneration has not been determined.

Mammalian skeletal muscle has the ability to regenerate and repair in response to exercise or injury. Regeneration of skeletal muscle is mainly executed by satellite cells (SCs) (8). SCs are a population of muscle stem cells that reside between the sarcolemma and the basal lamina. In neonatal muscle, growth is mainly achieved by addition of myoblasts derived from SCs to existing myofibers (9, 10). In the adult muscle, SCs—quiescent under normal physiological conditions—are activated in response to trauma and are able to self-renew, proliferate, and differentiate to fuse to damaged fibers or form new myofibers (8). We have previously demonstrated that expression of a constitutively active mutant of Gαi2 [Gαi2(Q205L)] promotes skeletal muscle hypertrophy *in vitro* and *in vivo*. Activated Gαi2 also accelerates muscle regeneration in the cardiotoxin (CTX) model of skeletal muscle injury (11). While that study demonstrated sufficiency, whether Gαi2 signaling is required or necessary for normal muscle homeostasis remained an open question and is the subject of the present study.

MATERIALS AND METHODS

Animal experiments. Animal experiments were performed in accordance with the Swiss ordinance on animal experimentation after approval by cantonal veterinarian authorities or by the local government's Committee on Animal Care and Welfare, Tübingen, Germany. Two male and two female NIE/Birnb/r129SvGαi2$^{+/-}$ mice (12), obtained from the Comparative Medicine Branch of the National Institute of Environmental Health Sciences, Research Triangle Park, NC, were shipped to Taconic Europe for breeding and generation of Gαi2 knockout (KO) mice or were maintained and bred in the animal facility of the Institute of Experimental and Clinical Pharmacology and Toxicology, Eberhard Karls University, Tübingen, Germany. To prolong the life expectancy of the Gαi2$^{-/-}$ animals on a C57BL/6 background, mice were bred and kept in individually ventilated cages under specific-pathogen-free conditions (13) and were used for the analysis of the number of paired box 7^{+} (Pax7^{+}) cells.

Eight- to 10-week-old wild-type (WT) and KO mice were used for phenotyping and skeletal muscle isolation. The skeletal muscle phenotypes were the same in both strains. Before euthanasia, the mice were weighed, subjected to nuclear magnetic resonance (NMR) scans (EchoMRI), and then sacrificed with CO_2. Tissues and organs were har-

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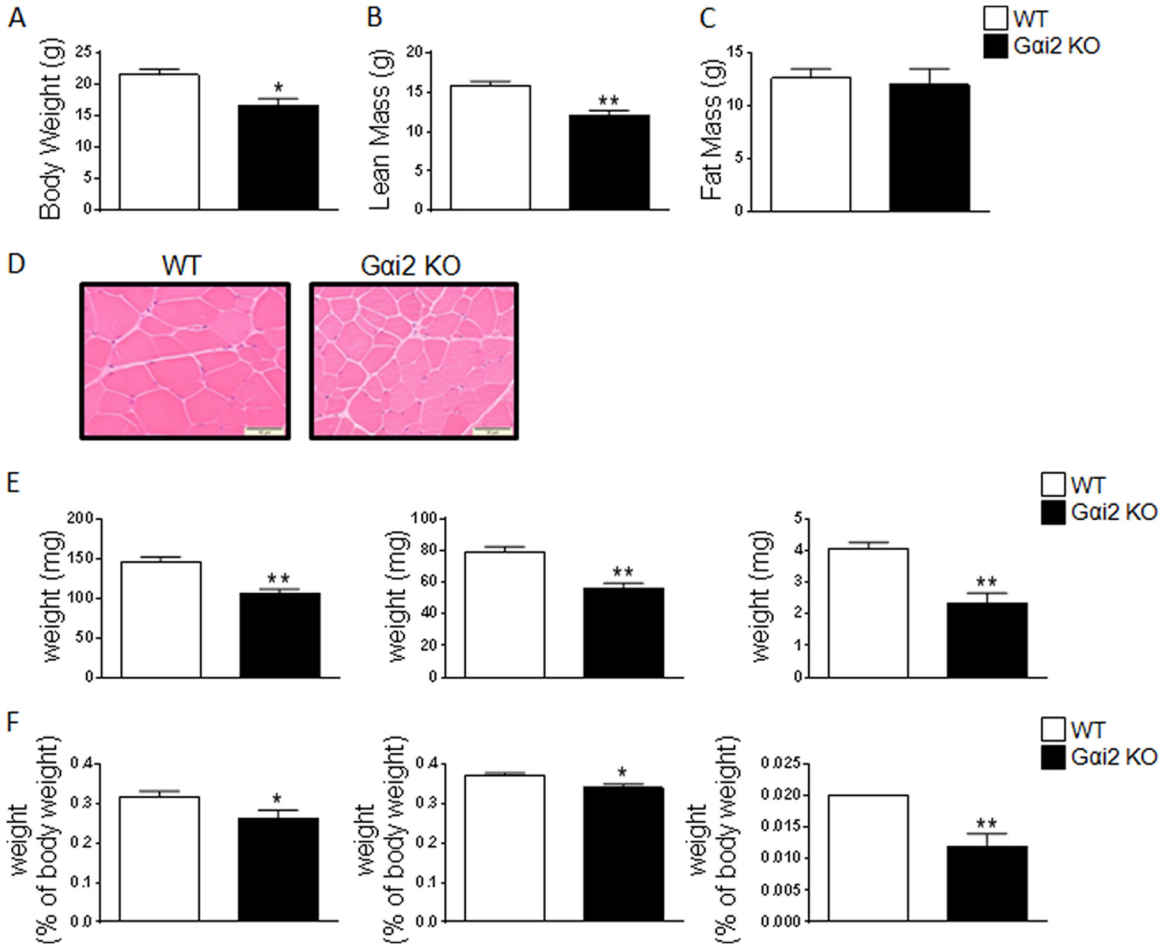

FIG 1 Gαi2 KO mice display growth retardation and muscle hypotrophy. Eight- to 10-week-old mice were weighed, subjected to magnetic resonance imaging (MRI) scans, and then sacrificed. (A to C) Body weights (A) and lean (B) and fat (C) body masses of Gαi2 KO mice and WT gender-matched littermates. (D) Representative sections of quadriceps muscle stained with H&E. (E and F) Gαi2 KO mice display decreased quadriceps, gastrocnemius, and soleus muscle mass before (E) and after (F) normalization to final body weight ($n = 5$ to 8 8- to 10-week-old male mice/group). The data are expressed as means and SEM. *, $P < 0.05$; **, $P < 0.01$.

vested and weighed. With the exception of the gastrocnemius and tibialis muscles, all muscles and organs were first fixed in paraformaldehyde, embedded in paraffin, sectioned at a nominal 4 μm, stained with hematoxylin and eosin, and evaluated histopathologically. Gastrocnemius and tibialis anterior muscles were harvested and snap-frozen in liquid nitrogen, and a portion of the tibialis muscle was embedded in optimum cutting temperature (OCT) reagent and frozen in liquid nitrogen-cooled isopentane for histological analysis. For cardiotoxin-induced muscle injury, the tibialis muscles of 8-week-old mice were injected with 25 μl of 10 μM cardiotoxin (Sigma), and the mice were euthanized at the specified times. The tibialis muscle was dissected and fixed in 10% buffered formalin or embedded in OCT and frozen in liquid nitrogen-cooled isopentane and processed for histological analysis by the conventional hematoxylin-and-eosin (H&E) method or snap-frozen in liquid nitrogen for RNA and protein extraction.

For immunohistochemical detection of laminin and Pax7, dried cryosections were permeabilized with 3.2% paraformaldehyde for 10 min and blocked with Vector M.O.M blocking reagent (Vector Laboratories; MKB-2213) 1 to 10 diluted in PBTX (phosphate-buffered saline [PBS] plus 0.2% Triton X-100) containing 10% goat serum (PBTX-GS) for 1 h at room temperature. Sections were then incubated overnight with 0.025 mg/ml mouse monoclonal antibody to Pax7 produced internally from the mouse hybridoma (DHSB; Iowa University) in blocking solution at 4°C. After washing in PBTX two times for 3 min/wash, the sections were incubated with rat anti-mouse IgG antibody (Serotec; MCA336) and rabbit polyclonal antibody against laminin (Sigma; L9393) diluted 1:200 in PBTX-GS and incubated at room temperature for 3 h. After washing as described above, the sections were blocked in PBTX-GS for 30 min and then incubated for 1 h with Alexa Fluor 594–goat anti-rat and Alexa Fluor 488–goat anti-rabbit antibodies (Invitrogen; A11007 and A11070, respectively) diluted 1:100 and 1:1,000, respectively, in PBTX-GS. After washing in PBTX two times for 5 min each time, the slides were mounted with Prolong Gold antifade reagent plus DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen).

Satellite cell isolation and culture. SCs were isolated from 8- to 10-week-old male C57BL6/J mice and cultured as described previously (11).

Cell lines and reagents. C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC)/European Collection of Cell Cultures (ECACC). The cells were maintained in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Amimed), 1% penicillin-streptomycin (Invitrogen), and 1% glutamine (Invitrogen) at 37°C. Differentiation was initiated 72 h after seeding by changing to differentiation medium consisting of DMEM supplemented with 2% horse serum (HS; Cambrex), 1% penicillin-streptomycin, and 1% glutamine. The pro-

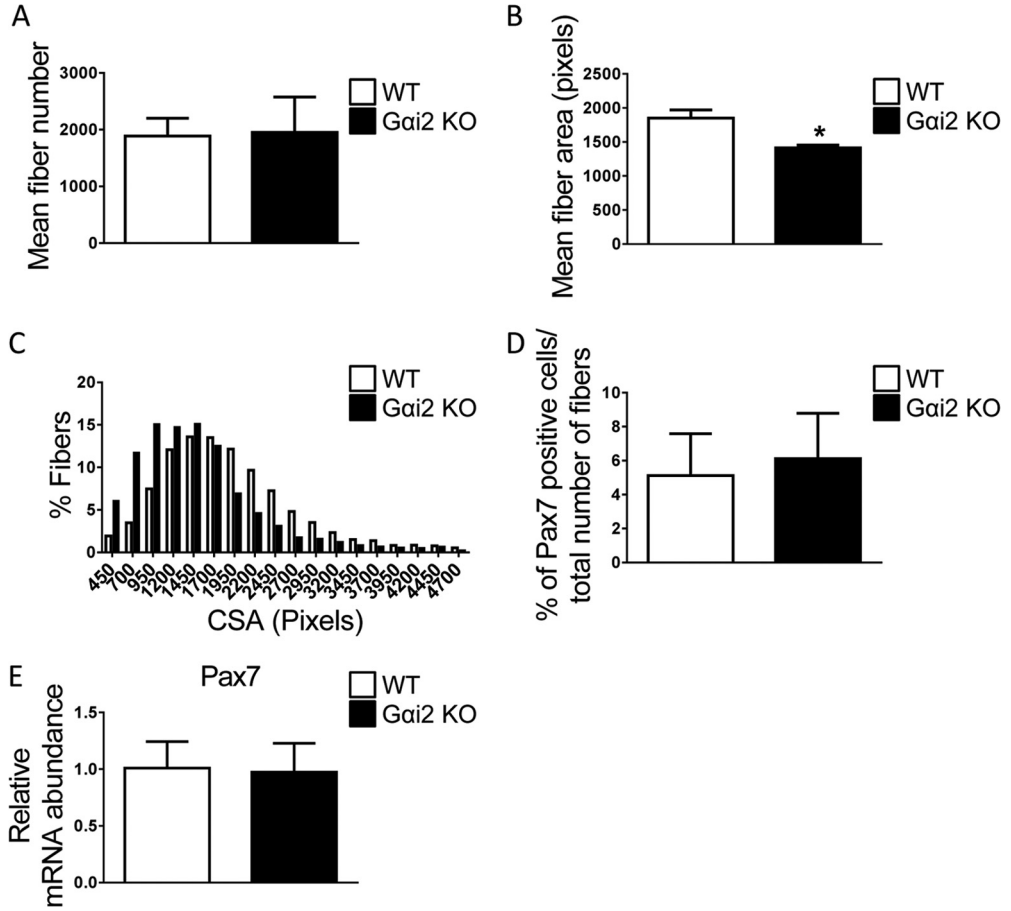

FIG 2 Reduced myofiber size but not number in Gαi2-null mice. (A and B) Tissue sections from WT and Gαi2 KO gastrocnemius muscles were stained with antilaminin antibody, and the number of fibers (A) and mean fiber area (B) were analyzed. The data are expressed as means and SEM. $n = 5$ to 8 8- to 10-week-old mice/group. *, $P < 0.05$. (C) Frequency histograms showing the distribution of myofiber CSA in WT and Gαi2 KO tibialis muscles. (D) Quantification of the percentages of Pax7$^+$ cells per total number of fibers, showing no difference between the tibialis muscles of WT and Gαi2 KO mice. Tissue sections were stained with antibodies to Pax7 and laminin. (E) Quantitative real-time PCR (qRT-PCR) analysis of the abundance of the transcript encoding Pax7 in tibialis muscles of WT and Gαi2 KO mice. The data were calculated as fold increase compared to WT ($n = 5$ to 8 8- to 10-week-old mice/group).

tein kinase C (PKC) inhibitor (PKCi) used in this study was synthesized at Novartis Pharma AG, Basel, Switzerland (11).

Reagents and adenoviral and lentiviral constructs. The adenoviral construct AdGαi2(Q205L) or the control empty vector (AdEV) was generated, amplified, and purified by Welgen Inc. as described previously (11). Lentiviral constructs for short hairpin RNA (shRNA) were from Sigma: shGαi2 (TRCN0000054733 NM_008138.2; 1×10^9 transducing units [TU]/ml) and turbo green fluorescent protein (GFP) control vector (SHC003H; 1×10^9 TU/ml) as a control. A small interfering RNA (siRNA) negative-control construct (On-Targetplus nontargeting control siRNA [D-001810]) and siGαi2 (sc-41753) were purchased from Thermo Scientific.

Gαi2 knockdown and proliferation assay. SCs isolated and cultured in expansion growth medium (GM2) for satellite cells (DMEM [high glucose] with sodium pyruvate [Invitrogen] supplemented with 20% FCS [Amimed], 10% HS [Cambrex], 1% chicken embryo extract [CEE; Fisher Scientific], and 1% penicillin-streptomycin [Invitrogen]) as described above were transfected with the siRNA negative-control construct (On-Targetplus nontargeting control siRNA (D-001810; Thermo Scientific) and siGαi2 (sc-41753; Thermo Scientific) using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer's instructions. The cells were cultured for 3 days in GM2 and labeled with 5-bromo-2′-deoxyuridine (BrdU) (EMD Biosciences) at a final concentration of 10 μM for 10 h. The cells were fixed with cold 70% ethanol for 5 min at room temperature, rinsed 3 times in PBS, incubated with 1.5 M HCl for 30 min at room temperature, and then washed two times in PBS. The cells were immunostained with an antibody to BrdU (Cell Signaling monoclonal antibody [MAb] 5292) and subsequently with Alexa Fluor 488 (Invitrogen; A11017) and DAPI (Promo Kine; PK-Ca707-40043) according to the manufacturer's instructions. The proliferation index was calculated as the percentage of BrdU-positive nuclei out of the total number of nuclei.

Adenovirus-mediated gene transfer and lentivirus-mediated gene silencing. SCs isolated as described above were either pretreated for 1 h with 1 μM PKCi or left unperturbed and then infected overnight with 3×10^8 particles of AdGαi2(Q205L) or control empty vector (AdEV) (multiplicity of infection = 300). The cultures were incubated for 48 h in differentiation medium (DM) for satellite cells, consisting of DMEM (high glucose) with sodium pyruvate (Invitrogen) supplemented with 2% HS (Cambrex), 1% CEE (Fisher Scientific), and 1% penicillin-streptomycin (Invitrogen), in either the absence or presence of 1 μM PKCi before immunostaining. For lentivirus-mediated gene silencing, SCs were infected overnight with a lentiviral construct for shGαi2 or with a control shGFP lentivirus (multiplicity of infection = 1) in GM2 or DM for analysis of satellite cell activation or differentiation markers, respectively. The cells

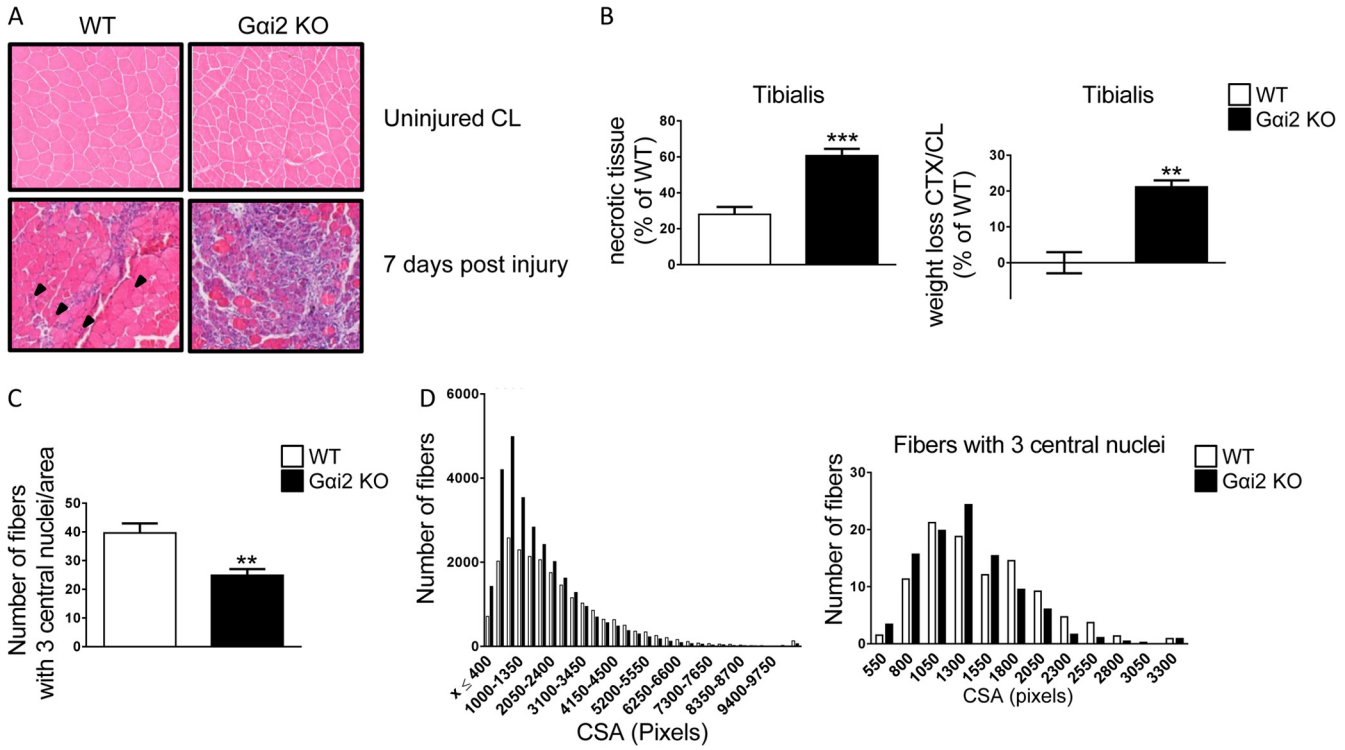

FIG 3 Gαi2 KO mice display aberrant muscle regeneration in the CTX model of skeletal muscle injury. (A) The tibialis muscles of WT or Gαi2 KO mice were injected with CTX, and the mice were sacrificed at the indicated time point. Representative images of tibialis muscle sections stained with H&E are shown. The wild-type muscle shows regenerating myofibers with central nuclei (arrowheads) and heterogeneous sizes, while the Gαi2 KO tibialis is composed mostly of necrotic fibers and inflammatory cells. CL, contralateral. (B) Quantitation of necrotic tissue and muscle weight loss. The results are expressed as percentages and SEM of the WT weight in the cardiotoxin-injected leg (CTX) and CL leg. $n = 6$ to 12 mice/group; **, $P < 0.01$; ***, $P < 0.001$. (C) Quantitative analysis of regenerating myofibers at day 14 postinjury. Tibialis muscle sections were stained with antilaminin antibodies and DAPI, and the number of fibers with central nuclei was evaluated. The data are expressed as mean numbers and SEM of fibers with 3 central nuclei/area. **, $P < 0.01$. (D) Frequency histograms showing the distribution of myofiber CSA in WT and Gαi2 KO tibialis muscles at day 14 postinjury (left) and CSA of myofibers with 3 central nuclei (right).

were cultured for an additional 72 h to achieve a high degree GFP expression.

Myotube size analysis and fusion assay. To analyze changes in myotube size, myotubes infected as described above were washed with cytoskeleton-stabilizing buffer (CSB) consisting of 80 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 5 mM EGTA, 1 mM $MgCl_2$, polyethylene glycol (PEG) 35000 (40 g/liter; molecular weight, 35,000) in distilled water (pH 7.4) and fixed with 4% paraformaldehyde in CSB for 15 min at room temperature. The cells were then permeabilized with 0.2% Triton in CSB. Nonspecific binding was blocked with 10% goat serum (Gibco; 16210), followed by incubation of the fixed myotubes with an anti-myosin heavy chain (anti-MyHC) antibody (Upstate; 05-716) diluted in PBS and subsequently with Alexa Fluor 488 (Invitrogen; A11017) and diaminopimelic acid (DAP) diluted in PBS. Diameters were measured using CellInsight Technology. The data are expressed as means ± standard errors of the means (SEM). To analyze fusion, SCs were infected and immunostained as described above. The fusion index was calculated as the average number of nuclei per myotube. The nuclei within 80 to 100 individual myotubes were counted.

RNA isolation and analysis. RNA was isolated using TRIzol reagent according to the manufacturer's instructions. cDNA synthesis was performed with SuperScript first-strand synthesis (Invitrogen). Real-time PCR was performed with an Applied Biosystems 7500 Fast PCR machine.

TaqMan probe sets. The TaqMan probe sets for mouse Myh4 (Mm01332541_m1), Myh1 (Mm01332489_m1), Myh2 (Mm00454991_m1), Myh7 (Mm00600555_m1), Myf5 (Mm00435125_m1), myogenin (Mm00446194_m1), Pax3 (Mm00435491_m1), Pax7 (Mm01354484_m1), Myh3 (Mm01332463_m1), Gαi2 (Mm00492379_m1), Myh8 (Mm01329494_m1), microRNA 1 (miR-1) (002222), miR-27b (000409), miR-133a (002246), miR-133b (002247), and miR-206 (000510) were purchased from Applied Biosystems.

Protein phosphorylation. Quantitative determination of phosphorylated p70S6K (Thr389), S6RP (Ser240/244), glycogen synthase kinase 3β (GSK3β) (Ser9), and Akt (Ser473) in whole-cell lysate samples was performed using the Akt Signaling Panel II whole-cell lysate kit (K15177D-1) and normalized on an Akt Signaling Panel II base kit (K15177A-3) from MesoScaleDiscovery (MSD), using an MSD reader according to the manufacturer's instructions.

Nuclear-cytosol fractionation and HDAC activity. Cell fractionation for histone deacetylase (HDAC) activity was achieved using the CelLytic NuClear Extraction Kit from Sigma (P8340) according to the manufacturer's instructions. Briefly, cytoplasmic lysis buffer (including phosphatase and protease inhibitors from Thermo Scientific [78440]) was added to the cells and incubated on ice for 15 min. Whole-cell lysates were centrifuged, the supernatant was collected (cytoplasmic fraction), and the crude nuclear pellet was resuspended in the provided nuclear extraction buffer containing phosphatase and protease inhibitors. The tubes were mounted on a vortex mixer and agitated at 4°C at medium to high speed for 15 to 30 min. Samples were centrifuged for 5 min at 20,000 to 21,000 × *g*. HDAC activity assays were performed using the colorimetric HDAC activity assay from Millipore (17-374) according to the manufacturer's instructions. Briefly, nuclear extracts were diluted in 20 μl of double-distilled H_2O; then, 10 μl of 10× HDAC assay buffer was added, followed by addition of 10 μl of the colorimetric substrate, and the samples were incubated at 37°C for 1 h. Subsequently, the reaction was stopped by adding 10 μl of activator solution and left for an additional 20 min at

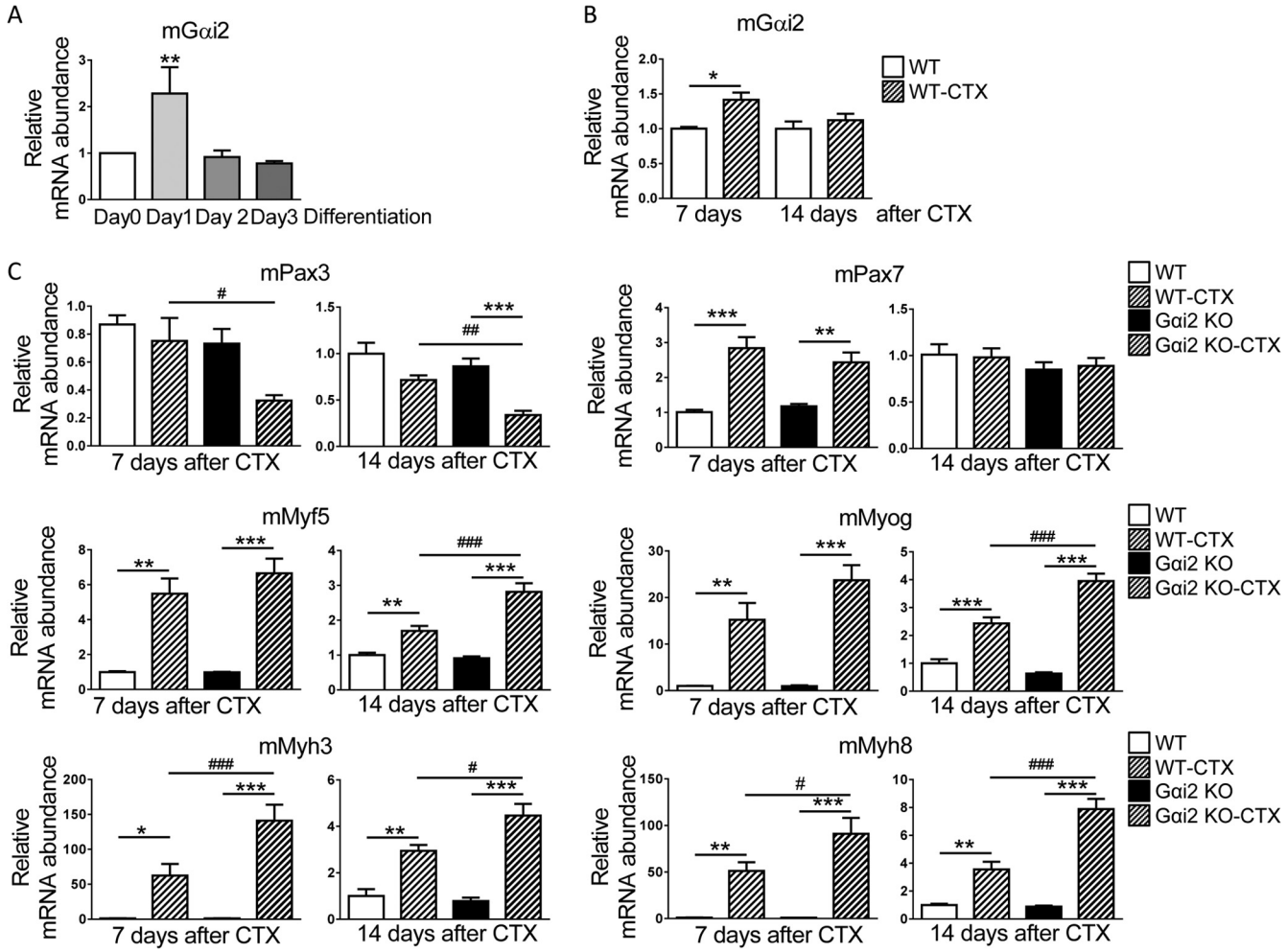

FIG 4 RNA expression profile of Gαi2 during C2C12 differentiation and regeneration and of markers of regeneration in the tibialis muscle following CTX injection (-CTX). (A) C2C12 cells were cultured in growth medium and then differentiated for 72 h. RNA was extracted at the indicated time points, and Gαi2 transcript abundance was analyzed by qRT-PCR. $n = 3$; **, $P < 0.01$. (B and C) Mice were subjected to CTX injection as described in the legend to Fig. 3. Shown is qRT-PCR analysis of the abundance of transcripts encoding Gαi2 (B) and various markers of differentiation and regeneration (C) in Gαi2 KO mice compared to WT mice. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ versus contralateral leg; #, $P < 0.05$, ##, $P < 0.01$, and ###, $P < 0.001$ versus WT-CTX. The error bars indicate SEM. The prefix "m" indicates mouse-specific probes for RT-PCR.

room temperature. Samples were then read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 405 nm. HDAC activity was expressed as relative optical density (OD) values per microgram of protein sample. The kit contains negative and positive controls that consist of nuclear extract of HeLa cells treated or not with trichostatin A, respectively.

Statistical analysis. Statistical significance was evaluated with one-way analysis of variance (ANOVA) for multiple groups, followed by Bonferroni's or Tukey's *post hoc* test to evaluate differences among groups. A Mann-Whitney-Wilcoxon test was used to compare two groups.

RESULTS

Gαi2 is required for postnatal skeletal muscle growth and regeneration. Gαi2 KO mice exhibit growth retardation and die prematurely (12). In the subset of viable Gαi2 KO mice that survived postnatally, growth retardation was evident as early as the end of the first week after birth and persisted throughout adulthood compared to WT mice (Fig. 1A). When body composition was analyzed by Echo-MRI, Gαi2 KO mice displayed a relative decrease in lean mass but not fat mass compared to WT mice (Fig. 1B and C), suggesting that growth retardation of these animals results from impaired skeletal muscle development, since skeletal muscle represents 40 to 50% of total body mass. To assess whether loss of Gαi2 affects skeletal muscle growth and maintenance, WT and Gαi2 KO mice were sacrificed at 8 weeks of age, and skeletal muscles were isolated and phenotyped. H&E-stained muscle sections from mutant mice were indistinguishable from those of WT mice and thus did not show evidence of gross abnormalities, such as degeneration, immune cell infiltration, or fibrosis, which are commonly observed in muscular dystrophies (Fig. 1D). However, all the skeletal muscles analyzed in the Gαi2 KO mice were significantly smaller than those of WT mice (Fig. 1E). When muscle weights were normalized to body weight, we observed that the quadriceps, gastrocnemius, and soleus muscles were still significantly smaller in the KO than in the WT mice (Fig. 1F), indicating hypotrophy, or relative lack of growth, of these muscles caused by the absence of Gαi2 in skeletal muscle. Ablation of Gαi2 did not affect fiber numbers (Fig. 2A) but resulted in a

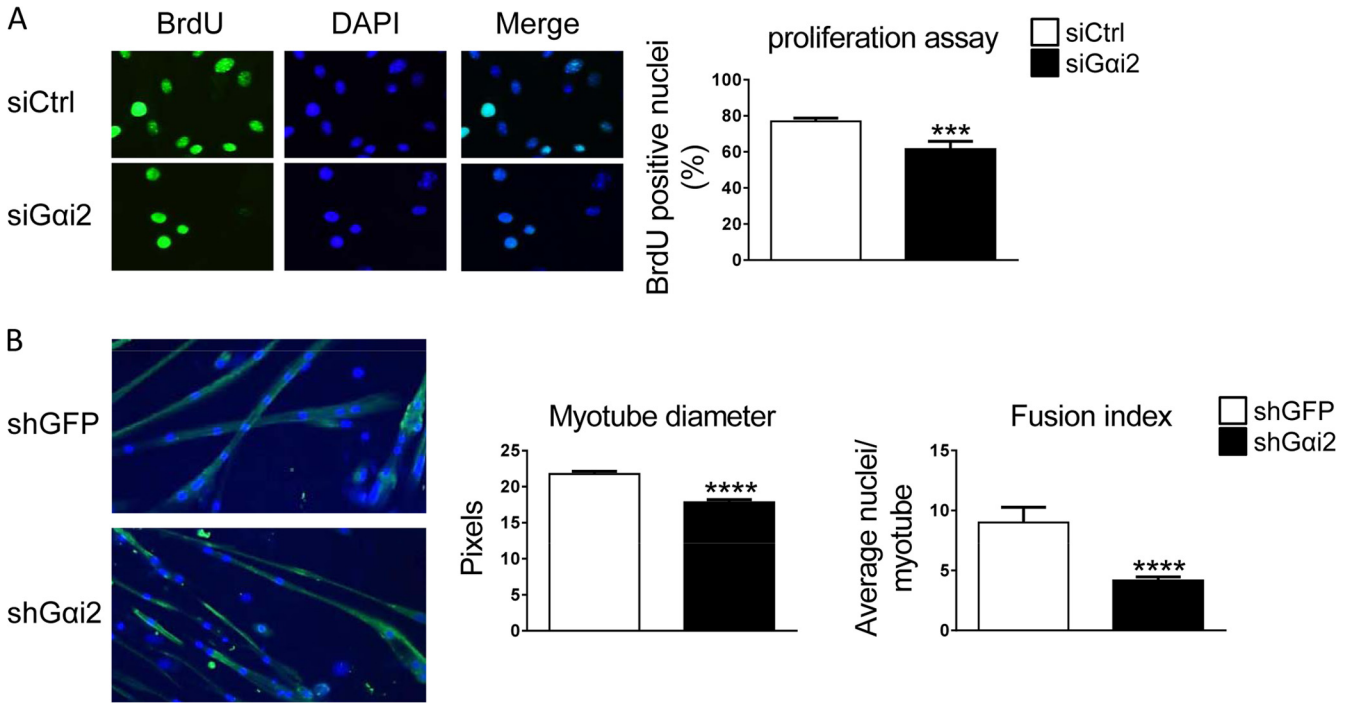

FIG 5 Knockdown of Gαi2 results in decreased proliferation, fusion, and differentiation. (A) Satellite cells were isolated and transfected with siGαi2 or siCtrl, cultured in growth medium for 3 days, and then pulsed with 10 μM BrdU for 8 h. Cell proliferation was determined by immunostaining using antibodies against BrdU (green); DAPI-counterstained nuclei are in blue. The histogram represents the percentage of BrdU incorporation, and the data are shown as the means and SEM. from three independent experiments. ***, $P < 0.001$. (B) Satellite cells were isolated, cultured in differentiation medium for 48 h, and then infected with an shRNA directed against Gαi2 (shGαi2) or shRNA control (shGFP). The cells were cultured for an additional 72 h, fixed, and stained with an antibody to MyHC (green) and DAPI (blue). The diameter and the fusion index (number of nuclei per myotube) of MyHC-stained myotubes were determined. The results are the means and SEM. ***, $P < 0.001$; ****, $P < 0.0001$.

significant decrease in mean fiber cross-sectional area (CSA) in the gastrocnemius muscle (Fig. 2B). Furthermore, the gastrocnemius muscles of Gαi2 KO mice had a greater number of small myofibers (with a CSA of ≤1,450 pixels) and fewer large myofibers (with a CSA of ≥1,700 pixels) than those of WT mice (Fig. 2C).

The ability of skeletal muscles of adult mammals to sustain correct postnatal growth and regeneration is attributed to a population of cells situated within the basal lamina of the myofibers called SCs. Upon injury or muscle growth, SCs become activated and differentiate into proliferating myoblasts, which eventually fuse to preexisting myotubes or to each other to form new myotubes (14). The transcription factor Pax7 is a marker of SCs and is required for their development and maintenance in adult life (8). To investigate whether the pool of satellite cells was affected in the Gαi2 KO mice, we evaluated the number of $Pax7^+$ cells in uninjured tibialis anterior (TA) muscles from WT and KO mice by immunofluorescence and found that there was no difference in the number of $Pax7^+$ cells in the Gαi2 KO and WT mice (Fig. 2D). These results were also confirmed at the gene expression level by real-time PCR for *Pax7*, which demonstrated no difference in *Pax7* levels in KO and WT mice (Fig. 2E).

To investigate whether the phenotype of the muscles in Gαi2 KO mice was attributable to a defect in SC activation and proliferation, we subjected the mice to an injury requiring regeneration, since it is in this setting that the requirement for satellite cell activation is most evident. The TA muscles from WT and Gαi2 KO mice were subjected to CTX injection and assayed for regeneration by H&E staining at day 7 postinjury. Regenerating myofibers are easily identifiable by the presence of centralized nuclei compared to preexisting myofibers, in which the nuclei are positioned at the periphery. The TA muscles of WT mice were composed mainly of regenerating myofibers at day 7 post-CTX injection (Fig. 3A, left). TA muscles from Gαi2 KO mice had no centralized nuclei, indicating that there was a lack of regeneration activity, and instead were composed of degenerating necrotic fibers, fibrotic tissue, and inflammatory cells (Fig. 3A, right). Quantitative analysis showed that the extent of necrosis and muscle weight loss was significantly higher in the TA muscles of Gαi2 KO mice than in those of WT mice (Fig. 3B). By day 14 postinjury, the TA muscles from WT mice demonstrated a higher number of myofibers with 3 or more central nuclei, indicating a higher rate of fusion and regeneration (Fig. 3C). In contrast, fewer Gαi2 KO myofibers had 3 or more central nuclei at day 14 postinjury (Fig. 3C). In addition, the fiber CSA of the muscles isolated from Gαi2 KO mice was smaller than that of muscles from WT mice (Fig. 3D). Frequency histograms of the single-fiber area delineate the different size distributions in muscles from Gαi2 KO versus WT mice, with a shift toward smaller areas, especially for the CSA of the fibers with 3 or more nuclei, indicating defective regeneration and a lower rate of fusion (Fig. 3D). We also surveyed the expression of Gαi2 during the differentiation process *in vitro* and during the regeneration process *in vivo* and observed that Gαi2 mRNA levels were upregulated during C2C12 differentiation (Fig. 4A) and during skeletal muscle regeneration (Fig. 4B), further indicating that Gαi2 is required for both differentiation and regeneration.

After damage, injured skeletal muscle tissue is infiltrated by

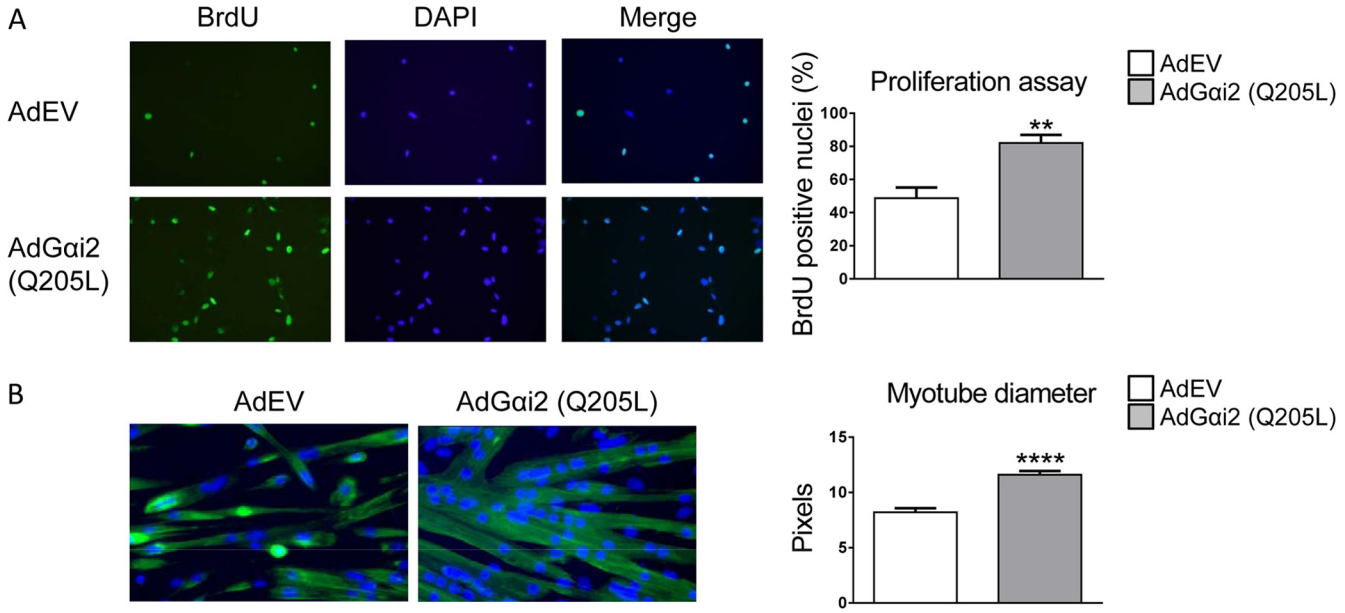

FIG 6 Expression of constitutively active Gαi2 increases satellite cell proliferation and differentiation. (A) Satellite cells were isolated, infected with AdEV or AdGαi2(Q205L), cultured in growth medium for 48 h, and then pulsed with 10 μM BrdU for 8 h. Cell proliferation was determined by immunostaining using antibodies against BrdU (green); DAPI-counterstained nuclei are in blue. The histogram represents the percentage of BrdU incorporation, and the data are shown as the means and SEM from three independent experiments. **, $P < 0.01$. (B) Satellite cells infected as described for panel A were cultured in differentiation medium for 48 h, fixed, and stained with an antibody to MyHC (green) and DAPI (blue), and the myotube diameter was measured. The results are the means and SEM of three independent experiments. ****, $P < 0.001$.

inflammatory cells, and SCs are activated, proliferate, differentiate, and contribute to repair of injured tissue. In the Gαi2 KO mice at days 7 and 14 after CTX injection, the expression of Pax3 was significantly decreased compared to WT mice (Fig. 4C). In contrast, Pax7 expression was not affected by ablation of Gαi2 (Fig. 4C). During muscle repair, newly formed and regenerating myofibers express differentiation and regeneration markers, such as Myf5, myogenin, and embryonic and neonatal MyHC (Myh3 and Myh8, respectively). As maturation and regeneration progress, adult MyHC isoforms (fast and slow) are expressed (15–17). In Gαi2 KO mice, expression of Myf5, myogenin, Myh3, and Myh8 was significantly higher than in WT mice, suggesting that at day 14 post-CTX injection, the fibers of the Gαi2 KO mice were still in the regeneration process while the fibers of WT mice had fully regenerated (Fig. 4C). Collectively the data show that Gαi2 is required for skeletal muscle development and normal regeneration in the CTX model of muscle injury. However, the difference in regenerative capacity between Gαi2 KO and WT mice cannot be attributed to a smaller pool of quiescent SCs, since the numbers of $Pax7^+$ cells did not differ.

Gαi2 regulates proliferation and promotes differentiation of SCs. To explore the regeneration defect in the Gαi2 KO mice, we next investigated the requirement for Gαi2 during SC activation and differentiation, analyzing the effects of silencing Gαi2 in SCs under growth and differentiation culture conditions. To this end, SCs were transfected at day 3 of culture in growth medium, with siGαi2 or siControl (siCtrl), and the proliferation rate was analyzed by BrdU incorporation. SCs transfected with siGαi2 demonstrated a significant decrease in the percentage of cells incorporating BrdU into their DNA compared to SCs transfected with siCtrl (Fig. 5A).

We used shRNA to downregulate Gαi2 expression and to study its role under differentiation conditions. SCs were cultured in differentiation medium for 48 h and then infected with shGαi2 or control shRNA (shGFP) lentivirus. Fusion was scored as the average number of nuclei per MyHC-positive myotube. The fusion index was significantly lower in the SCs infected with shGαi2 lentivirus than in those infected with shGFP lentivirus (Fig. 5B). Additionally, myotube size was also negatively affected, since the average diameter of myotubes from SCs infected with shGαi2 was significantly smaller than those from SCs infected with control shGFP lentivirus (Fig. 5B). These data indicate that Gαi2 is required for SC proliferation and differentiation.

To analyze whether Gαi2 was sufficient to induce SC proliferation and hypertrophy, we infected SCs with an adenovirus encoding the constitutively active mutant of Gαi2 [AdGαi2(Q205L)] or adenovirus control (AdEV) and analyzed proliferation and differentiation (Fig. 6). SCs infected with AdGαi2(Q205L) had a higher percentage of BrdU-positive cells than those infected with AdEV, indicating that constitutively active Gαi2 is sufficient to induce proliferation of SCs (Fig. 6A). Furthermore, upon differentiation, SCs infected with AdGαi2 formed significantly larger myotubes (Fig. 6B). These results suggest that Gαi2 expression plays a critical role in SC proliferation and differentiation and that the phenotype of Gαi2 KO mice is due to a defect in the proliferation and differentiation of SCs.

Gαi2 controls the mRNA levels of markers of SC activation and differentiation. To determine the molecular basis of the requirement for Gαi2 in SCs, we analyzed the mRNA of genes encoding activation and differentiation markers under growth conditions or differentiation conditions, respectively. SCs infected with shGαi2 lentivirus in growth medium had decreased mRNA levels of markers of quiescence and activation, such as Pax3/Pax7 and Myf5, as well as markers of differentiation, such as MyoD,

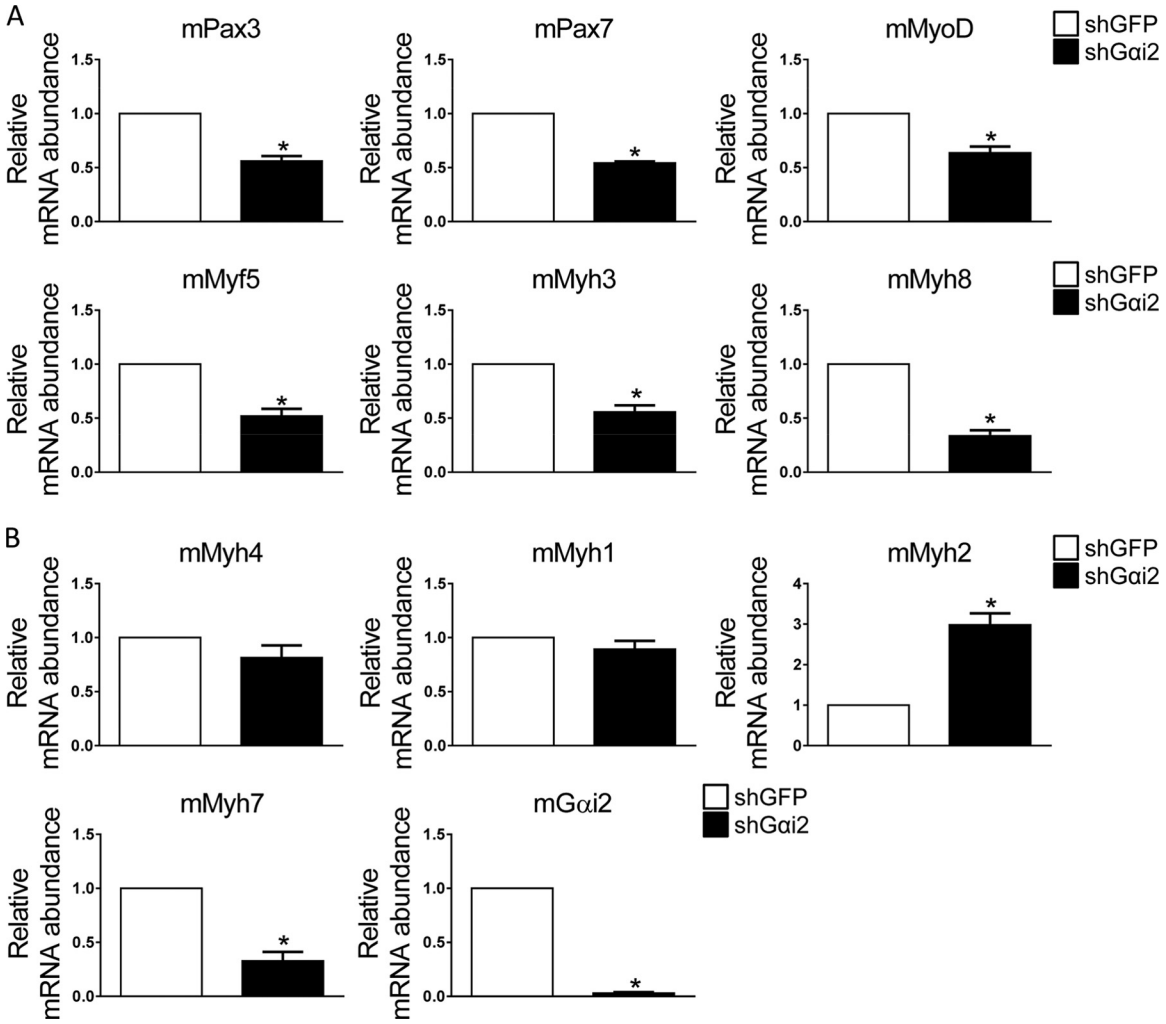

FIG 7 Knockdown of Gαi2 perturbs the expression of markers of satellite cell activation and differentiation. Satellite cells were infected with shGαi2 or shRNA control (shGFP) and cultured in growth medium (A) or differentiation medium (B) for 48 h. Markers of satellite cell activation (A) and differentiation (B) were analyzed by qRT-PCR. *, $P < 0.05$. The error bars indicate SEM.

Myh3, and Myh8, compared to cells infected with control shGFP lentivirus (Fig. 7A). Under differentiation conditions, knockdown of Gαi2 decreased gene expression of Myh7 but did not affect Myh1 and Myh4; in contrast, Myh2 levels were increased (Fig. 7B). In a previous study, expression of AdGαi2(Q205L) *in vitro* and *in vivo* also resulted in upregulation of Myh2 expression (11). These results could be attributed to the different cells used in the previous study (e.g., human primary fetal myotubes) and/or to a shift of myosin expression following the obligatory pathway—Myh7→Myh2→Myh1→Myh4—in the absence of Gαi2 and a change in the opposite direction—Myh4→Myh1→Myh2→Myh7—in the presence of AdGαi2(Q205L). Similar effects have been described during muscle inactivity and increased activity or functional overload, respectively (18). More studies are needed to clarify the molecular mechanisms governing the regulation of myosin expression by Gαi2.

It is known that the muscle microRNAs (miRNAs) (miR-1, miR-27, miR-133a/b, and miR-206) play a critical role in the regulation of muscle differentiation (19, 20). The levels of all four miRNAs were found to be proportional to the stage of muscle development and elevated during *in vitro* differentiation (21). To determine whether there is a defect in differentiation upon knockdown or overexpression of Gαi2, we analyzed the expression of the four muscle-specific miRNAs under differentiation conditions in SCs. Infection of SCs with AdGαi2(Q205L) resulted in significant upregulation of miR-1 and miR-206 compared to SCs infected with AdEV (Fig. 8A). In contrast, SCs in which Gai2 was knocked down showed significantly lower levels of miR-1 and miR-27b (Fig. 8A). The data shown in Fig. 8 indicate that Gai2 is required and sufficient to regulate miR-1 levels. We have previously shown that, in human skeletal muscle cells, the expression of a constitutively active Gαi2 induces skeletal myotube hypertrophy via a PKC-dependent pathway (11). To further investigate how Gai2 controls miR-1 expression, C2C12 myoblasts were pretreated for 1 h with PKCi and then infected with AdEV or AdGαi2(Q205L) for 24 h in the absence or presence of PKCi, and expression of miR-1 was analyzed by real-time PCR. As observed in SCs, expression of AdGαi2(Q205L) significantly increased the levels of miR-1, and the increase was partially reduced by pharmacological inhibition of PKC (Fig. 8B), indi-

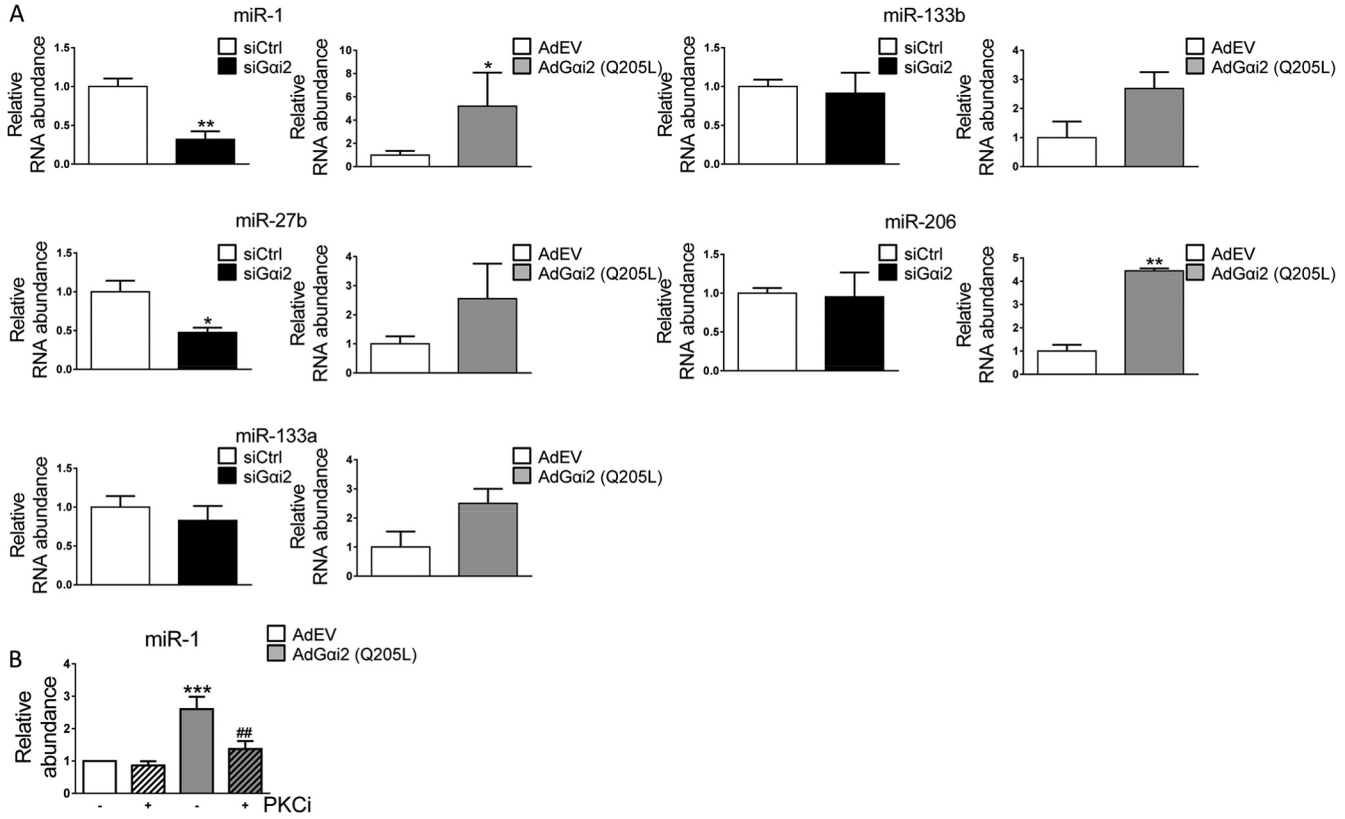

FIG 8 Gαi2 regulates the expression of muscle-specific miRNAs in satellite cells and in C2C12 myoblasts via PKC. (A) Satellite cells transfected with siGαi2 or siCtrl or infected with AdEV or AdGαi2(Q205L) were cultured in differentiation medium for 72 h. Expression of muscle-specific miRNAs was analyzed by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$. (B) C2C12 myoblasts were pretreated or not for 1 h with 1 μM PKCi, infected with AdEV or AdGαi2(Q205L), and cultured in differentiation medium for an additional 48 h. Expression of miR-1 was analyzed by qRT-PCR. $n = 3$; ***, $P < 0.001$ versus AdEV; ##, $P < 0.01$ versus vehicle control-treated cells. The error bars indicate SEM.

cating that the upregulation of miR-1 induced by Gαi2 is partially PKC dependent.

Altogether, these results indicate that Gai2 has a role in the regulation of SC activation and differentiation and that Gαi2 partially controls miR-1 expression via PKC.

Gαi2 induces SC activation via a PKC/GSK3β pathway independently of AKT and via HDAC inhibition. We have previously shown that, in human skeletal muscle cells, the expression of a constitutively active Gαi2 induces skeletal myotube hypertrophy via PKC/GSK3β and mTOR/p70S6K pathways independently of AKT (11). It was important to determine which pathway downstream of Gαi2 was mediating the effects on SCs. To assess the contribution of the PKC/GSK3β pathway to the increase in differentiation induced by Gαi2(Q205L) in SCs, we employed a pharmacological approach. A specific PKC inhibitor (PKCi) (11) significantly reduced the increase in myotube width and length induced by AdGαi2(Q205L) expression (Fig. 9A). Furthermore, AdGαi2(Q205L)-mediated activation of Gαi2 in SCs significantly increased the phosphorylation of GSK3β, p70S6K, and rpS6, and treatment with PKCi blocked this increase without affecting AKT phosphorylation (Fig. 9B).

Deacetylase inhibitors are known to increase muscle cell size by promoting myoblast recruitment and fusion (22). It has also been shown that treatment *in vivo* with deacetylase inhibitors leads to functional and morphological recovery of dystrophic muscles, improving fusion and regeneration (23). Interestingly, the activity of HDACs in SCs was suppressed significantly (~30%) by Gαi2(Q205L), indicating that the effects of Gαi2 in SC proliferation and muscle regeneration might occur via HDAC inhibition (Fig. 9C).

Altogether, the data in Fig. 8 and 9 demonstrate that the AKT pathway is dispensable for the effect induced by Gαi2 and instead point to Gαi2/PKC as the signaling intermediate linking the Gαi2, p70S6K, and GSK3β pathways. Both Gαi2/PKC and the Gαi2/HDAC pathways contribute to miR-1 induction and SC activation and differentiation.

DISCUSSION

The Gα protein Gαi2 is a required component for a variety of G-protein-coupled receptor (GPCR) actions, which play important roles in many tissue and organ systems. In a prior study (11), it was demonstrated that activation of Gαi2 is sufficient to induce the hypertrophy and differentiation of myotubes in culture. Activation of Gαi2 resulted in hypertrophy, improved regeneration, and a switch to oxidative fibers *in vivo* (11). The signaling pathways downstream of Gαi2 mediating these effects were mapped, resulting in several previously undiscovered pathway connections. However, whether Gαi2 signaling was required for normal muscle growth and maintenance was not previously studied, nor were the downstream pathways mediating the effects of endoge-

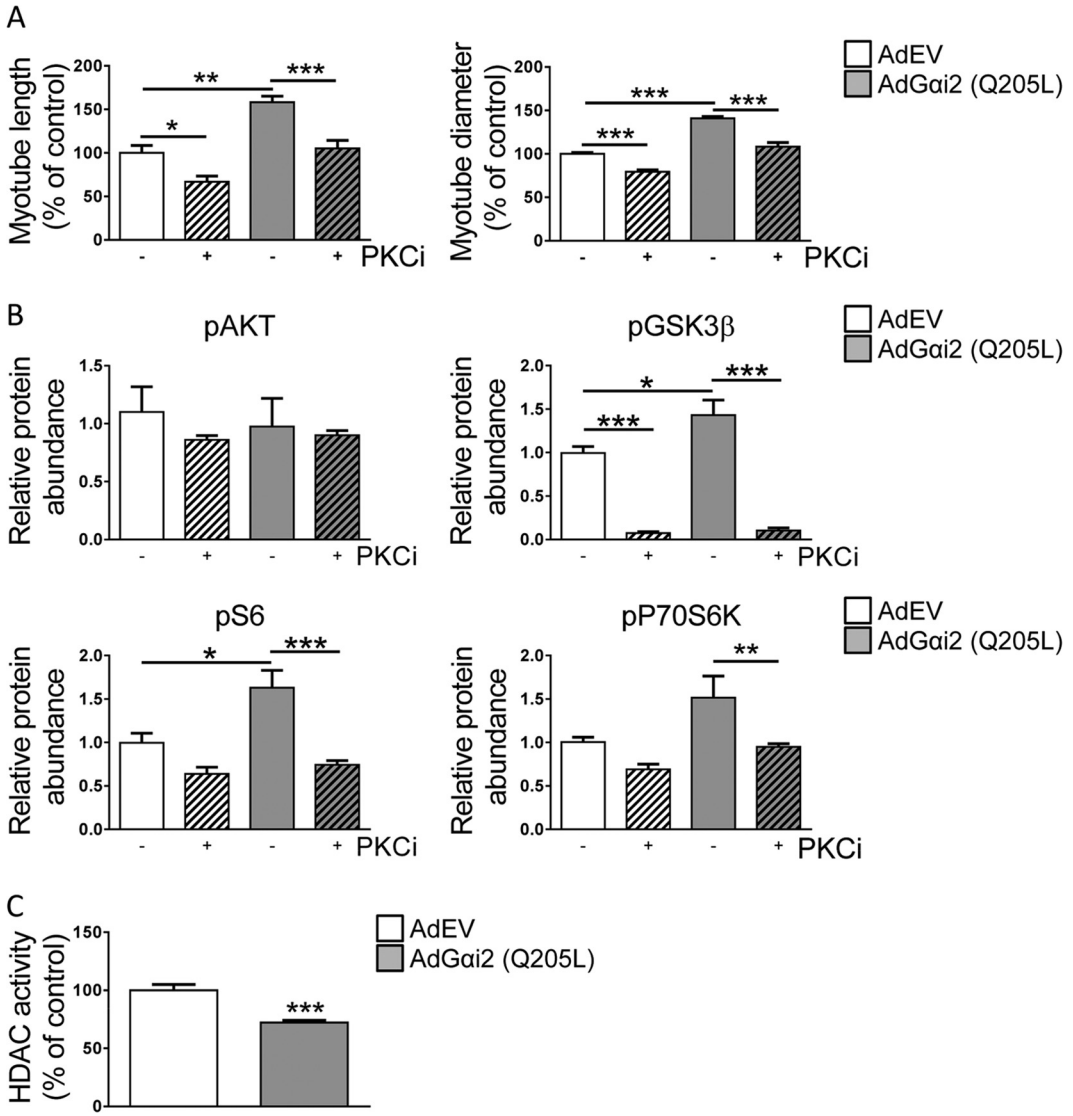

FIG 9 Constitutively active Gαi2 induces satellite cell differentiation into myotubes through activation of a PKC pathway and inhibition of GSK3β and HDAC activity. Satellite cells were pretreated or not for 1 h with 1 μM PKCi, infected with AdEV or AdGαi2(Q205L), and cultured in differentiation medium for 72 h. The cells were stained with MyHC for analysis of myotube length and diameter (A) or lysed for analysis of phosphorylated proteins by ELISA (B) and HDAC activity (C). The data are expressed as means and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

nous Gαi2. These were the aims of the current study, and for this purpose, Gαi2-null (KO) animals were employed, as were SCs in which the Gαi2 pathway was activated by transfection of a constitutively active mutant or in which the pathway was perturbed via siRNA or shRNA to downregulate Gαi2.

Gαi2 KO mice have smaller muscles than WT controls. Formally, this decrease in muscle size is not thought of as "atrophy," since there was never a normal muscle to atrophy; rather, given the developmental nature of this effect, the finding is labeled muscle hypotrophy, and it was quite significant, especially in the soleus muscle, where almost a 50% relative decrease in muscle size was observed, indicating a critical role for Gαi2 signaling in physiological muscle growth. The relative decrement in growth suggested a potential requirement for Gαi2 in SC function, since these cells are recruited in normal muscle development (8).

A muscle regeneration model was employed to determine if SCs can function normally in the absence of Gαi2, since SCs are activated and must differentiate in order for regeneration to occur (8). The muscle was subjected to cardiotoxin-induced injury to induce the requirement for subsequent regeneration; in normal animals, *Gαi2* mRNA is upregulated in regenerating muscle, suggesting a potential requirement for the protein. In the cardiotoxin-injured Gαi2 KO muscles, there was a significant decrease in the ability of the muscle to regenerate. In fact, the Gαi2 KO muscle demonstrated rather dramatic necrosis and degeneration, whereas the normal muscle regenerated appropriately. These findings demonstrate that Gαi2 signaling is required for normal regeneration to proceed and thus suggest an important role for its downstream pathways in mediating muscle repair. The combination of the negative effects on growth and regeneration in the Gαi2 KO animals caused us to focus further on the SCs and their function in these animals.

Fusion of satellite cells to form multinucleated muscle fibers is

an essential step in skeletal muscle growth, differentiation, and regeneration (8, 24, 25). Pax3 expression in particular, a marker of SCs, was decreased in Gαi2 KO animals; however, Pax7 mRNA abundance was not affected. Myf5 and myogenin, two markers of satellite cell activation and differentiation, as well as markers of regeneration (Myh3 and Myh8), were still upregulated in Gαi2 KO animals compared to WT animals at day 14 post-CTX-induced muscle damage, thus further indicating a delay in the regeneration process in Gαi2-null mice.

The Gαi2 KO animals demonstrate the necessity for Gαi2-mediated function for normal muscle growth and regeneration. We also employed acute knockdown of Gαi2, via siRNA or shRNA treatment, on SCs in culture and found significant declines in proliferation and differentiation of SCs, confirming the impression seen with the KO muscle *in vivo*. These findings were further strengthened by expressing a constitutively active mutant of Gαi2 in SCs via viral infection. This construct induced a significant increase in SC proliferation and dramatic hypertrophy of the resultant myotubes, demonstrating not only that Gαi2 is required for satellite cell function but also that its activation is sufficient for satellite cell proliferation to increase. A particularly interesting group of markers of SC differentiation that was published recently (19, 20) is a set of microRNAs that are muscle specific: miR-1, miR-27b, miR-133a and -b, and miR-206. Activation of Gαi2 in SCs caused a significant increase in miR-1 and miR-206 levels; conversely, knockdown of Gαi2 resulted in decreased abundance of miR-1 but not miR-206, suggesting that Gαi2 is required for miR-1 expression but is dispensable for miR-206 expression. miR-27b expression was also negatively regulated by Gαi2 knockdown; however, activation of Gαi2 was not sufficient to perturb its levels. The expression of miR-133b was not significantly affected by either activation or downregulation of Gαi2. Because Gαi2 is necessary and sufficient for miR-1 expression, we investigated further the molecular mechanisms underlying miR-1 regulation. Previously, we have shown that pharmacological inhibition of PKC prevented the hypertrophic effect of Gαi2 (11). In this study, inhibition of PKC prevented the upregulation of miR-1 induced by constitutively active Gαi2, demonstrating for the first time a role for PKC in the regulation of a muscle-specific microRNA. The molecular mechanisms that regulate the expression of miR-1 in the regenerating environment are still poorly characterized. It has been previously shown that HDAC4 contains two naturally occurring putative miR-1-binding sites at its 3′ untranslated region (UTR) and that miR-1 specifically represses HDAC4, which in turn contributes to the regulatory effects of this miRNA on myoblast proliferation and differentiation (19). One important aspect to consider is that myo-miRs are regulated by most of the same epigenetic regulators that typically control the expression of muscle genes. For instance, miR-1 is regulated by MyoD and MEF2, which are also regulated by HDACs (26, 27). Thus, by extension, HDAC inhibitors are predicted to promote the expression of myomiRs in myoblasts (28). In this report, we show that Gαi2 decreases the activity of HDACs in SCs; therefore, it is conceivable that the regulation of miR-1 by Gαi2 is also partly dependent on HDAC inhibition.

As for the mechanism by which Gαi2 mediates its effects on SCs, we have previously delineated several pathways that can be activated by Gαi2 (11) and thus sought to determine which of these were required for SC function. A PKC inhibitor ablated the effects of activated Gαi2 on SCs, indicating that the Gαi2/PKC pathway is required. Downstream of PKC, there is perturbation of GSK and p70S6 activation, which have previously been shown to be important mediators of myoblast proliferation and hypertrophy in the setting of IGF-1 signaling (29). Interestingly, while IGF-1 requires AKT to activate these downstream pathways, in the setting of Gαi2, PKC does this job, and indeed, this seems to be a required pathway for competent SC-mediated regeneration and to fully maximize growth and development of the muscle.

These findings delineate for the first time a requirement for Gαi2 signaling in normal muscle growth and regeneration and suggest that activation of this pathway may be quite helpful in settings of disease, where regeneration is required for repair of skeletal muscle.

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