Differentiation through Negative Regulation of NFATc3

requires NFATc3. Collectively, our data demonstrate negative
in NFATc3-deficient myoblasts, indicating that this response
depth was in muscle growth, we investigated if inhibition of GSK-3
as this depended on regulation of the transcription factor nuclear
factor of activated T-cells (NFAT). In both myogenically con-
verted mouse embryonic fibroblasts and C2C12 myoblasts, defi-
ciency of GSK-3β protein (activity) resulted in enhanced myo-
tube formation and muscle-specific gene expression during
differentiation, which was reversed by reintroduction of wild
type but not kinase-inactive (K85R) GSK-3β. In addition,
GSK-3β inhibition restored myogenic differentiation following
calcineurin blockade, which suggested the involvement of
NFAT. GSK-3β-deficient mouse embryonic fibroblasts or myo-
blasts displayed enhanced nuclear translocation of NFATc3 and
increased NFAT-sensitive promoter transactivation, which was
reduced by reintroducing wild type, but not K85R GSK-3β.
Overexpression of NFATc3 increased muscle gene promoter
transactivation, which was abolished by co-expression of wild
type GSK-3β. Finally, stimulation of muscle gene expression
observed following GSK-3β inhibition was strongly attenuated
in NFATc3-deficient myoblasts, indicating that this response
requires NFATc3. Collectively, our data demonstrate negative
regulation of myogenic differentiation by GSK-3β through a
transcriptional mechanism that depends on NFATc3. Inhibi-
tion of GSK-3β may be a potential strategy in prevention or
maintenance of muscle mass to prepare for health, and loss of
skeletal muscle mass compromises human physical condition
and survival in chronic diseases, such as chronic obstructive
pulmonary disease (1, 2). Restoring lost muscle mass is impor-
tant for improving quality of life and ultimately disease progno-
sis (3). To restore muscle mass and improve muscle function in
various diseases conditions, a better understanding of the
molecular mechanisms of skeletal muscle (re)growth is
required. Skeletal muscle differentiation is a critical element of
certain types of postnatal growth of the skeletal musculature
and is mainly dependent on satellite cells (quiescent myoblasts),
which upon activation proliferate, differentiate, and fuse with
existing muscle fibers or with each other to form new myofibers
(4). Myoblast fusion allows additional muscle growth by myo-
nuclear accretion, beyond the limitations imposed by the myo-
nuclear domain (i.e. the maximal cytoplasm/nucleus ratio) (5).

The protein kinase GSK-3β is ubiquitously expressed, and,
although it was originally identified as a suppressor of glycogen
synthase (6), GSK-3β has been implicated in a myriad of meta-
bolic and signaling pathways (7). Recent studies have identified
GSK-3β as a negative regulator of both cardiac and skeletal
muscle hypertrophy (8, 9) as well as muscle differentiation (10).

Regulation of the transcriptional regulator nuclear factor of
activated T-cells (NFAT) depends on the balance between
inhibitory phosphorylation by GSK-3β and stimulatory
dephosphorylation by the calcium-dependent serine/threonine
phosphatase calcineurin. Activated calcineurin exposes the
nuclear localization signal of NFAT, resulting in its nuclear
translocation (11). Conversely, GSK-3β-dependent phospha-
rylation masks the nuclear localization signal, resulting in
nuclear export of NFAT and termination of calcineurin-in-
duced gene transcription (12, 13).

Although NFAT was identified in T-cells, several studies
have postulated a role for NFAT in skeletal muscle gene expres-
sion (14, 15). Five NFAT isoforms have been identified (16), and
four isoforms (NFATc1, -c2, -c3, and -5) are expressed in skel-
et muscle (17, 18). NFATc1, -c2, -c3, and -5 have overlapping
but also nonredundant functions in skeletal muscle, including
control of myogenic differentiation, myoblast migration and
fusion, and fiber type specification (18–21). Calcineurin has
been implicated in the regulation of skeletal muscle growth
(22), regrowth (23), and myogenic differentiation (24).
NFATc1, -c2, and -c3, but not NFAT5, are responsive to cal-
cineurin (25), and stimulation of myogenic differentiation via
activation of calcineurin involves NFATc3 (17, 24).

In this study, we hypothesized that inhibition of GSK-3β
increases myogenic differentiation by promoting NFATc3
nuclear localization and transcriptional activity. Two different

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2 The abbreviations used are: GSK-3, glycogen synthase kinase 3β; NFAT,
nuclear factor of activated T-cells; GM, growth medium; DMEM, Dulbecco’s
modified Eagle’s medium; FBS, fetal bovine serum; DM, differentiation
medium; MEF, mouse embryonic fibroblast; TnI, troponin I; shRNA, short hair-
pin RNA; shGSK, short hairpin glycogen synthase kinase; WT, wild type; ca,
constitutively activated; IGF, insulin-like growth factor; pRS, pRetro-Super.
myogenic models were employed to examine the effects of genetic modulation of GSK-3β on skeletal muscle differentiation and NFAT regulation. The results revealed negative regulation of myogenic differentiation by GSK-3β through suppression of NFATc3 nuclear localization and subsequent inhibition of NFATc3-mediated muscle gene transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The murine skeletal muscle cell line C2C12 obtained from the American Type Culture Collection (ATCC number CRL1772) was cultured in growth medium (GM), composed of low glucose Dulbecco’s modified Eagle’s medium (DMEM) containing antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) and 9% (v/v) fetal bovine serum (FBS) (all from Invitrogen), or differentiation medium (DM), which contained low glucose DMEM with 0.5% heat-inactivated FBS and antibiotics. Both cell types were grown on Matrigel (BD Biosciences)-coated (1:50 in buffer and stored at −80°C). Supernatants were produced by transfection of phoenix packaging cells (Dr. G. Nolan, Stanford University), cultured in high glucose DMEM containing antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) and 9% (v/v) FBS, with the pRS GSK-3β shRNA vector or the pRS control vector by calcium phosphate precipitation. 48 h post-transfection, the tissue culture medium was filtered through a 0.45-μm filter, and supernatant containing the viral particles was used for infection of C2C12 cells after the addition of 4 μg/ml Polybrene (Sigma). Cells were infected for at least 6 h and allowed to recover for 24 h with fresh GM. Infected cells were selected with puromycin (2.5 μg/ml) for at least 96 h, and silencing efficacy was evaluated by Western blot analysis for GSK-3β or NFATc3, respectively, and tubulin as a loading control. Of the three target sequences tested, the pRS vector containing the 19-nt target sequence 5′-GTGTATATGTAGCCTG-3′ demonstrated the strongest RNA silencing for GSK-3β, whereas the targeting sequence 5′-TACTAGAGTCCGACTTGTGTA-3′ proved most successful in NFATc3 silencing.

**Western Blotting**—GSK-3β, tubulin, or NFATc3 protein abundance was evaluated by Western blotting. Adherent cells were washed in PBS, and whole cell lysates were prepared by the addition of lysis buffer (20 mM Tris, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM dithiothreitol, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1% (v/v) aprotinin). Lysates were incubated on ice for 30 min, followed by 30-min centrifugation at 16,000 × g. For nuclear and cytoplasmic fractions, adherent cells were washed in PBS, and the cytoplasm fraction was prepared by the addition of buffer C (10 mM Tris, pH 7.6, 10 mM KCl, 1.5 mM MgCl2, 1% (v/v) Triton X-100, 1 mM dithiothreitol, 0.2 mM Na3VO4, 0.4 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.2 mM NaF). After a 15-min incubation on ice, lysates were spun for 5 min (10,000 × g) at 4°C. Supernatant containing the cytoplasm fraction was saved, and the pellet, containing the nuclear fraction, was washed once with buffer C and resuspended in buffer N (20 mM Tris, pH 7.6, 160 mM KCl, 1 mM MgCl2, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM Na3VO4, 0.4 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.2 mM NaF). Nuclear lysates were incubated for 30 min on a rotating platform at 4°C and spun (16,000 × g) for 15 min at 4°C. A fraction of the supernatant was saved for protein determination, and 4× Laemmli sample buffer (0.25 mM Tris–HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4 mM dithiothreitol, and 0.04% (w/v) bromophenol blue) was added, followed by boiling of the samples for 5 min and storage at −20°C.

**RNA Interference and Retroviral Infection**—Vectors expressing hairpin small interference RNAs were constructed by inserting pairs of annealed, HindIII/BglII-digested oligonucleotides containing the 19-nt target sequence into the pRetroSuper (pRS) vector (31, 32), kindly provided by Dr. R. Agami (The Netherlands Cancer Institute, Amsterdam). Ectropic retroviral supernatants were produced by transfection of phoenix packaging cells (Dr. G. Nolan, Stanford University), cultured in high glucose DMEM containing antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) and 9% (v/v) FBS, with the pRS GSK-3β shRNA vector or the pRS control vector by calcium phosphate precipitation. 48 h post-transfection, the tissue culture medium was filtered through a 0.45-μm filter, and supernatant containing the viral particles was used for infection of C2C12 cells after the addition of 4 μg/ml Polybrene (Sigma). Cells were infected for at least 6 h and allowed to recover for 24 h with fresh GM. Infected cells were selected with puromycin (2.5 μg/ml) for at least 96 h, and silencing efficacy was evaluated by Western blot analysis for GSK-3β or NFATc3, respectively, and tubulin as a loading control. Of the three target sequences tested, the pRS vector containing the 19-nt target sequence 5′-GTGTATATGTAGCCTG-3′ demonstrated the strongest RNA silencing for GSK-3β, whereas the targeting sequence 5′-TACTAGAGTCCGACTTGTGTA-3′ proved most successful in NFATc3 silencing.

**Transfections and Plasmids**—Transient transfections were performed using Lipofectamine 2000™ (Invitrogen) or Nanofectin (PAA, Pasching, Austria) according to manufacturers’ instructions. Troponin I (Tnl)-luciferase plasmid, kindly provided by Dr. Albert Baldwin (University of North Carolina, Chapel Hill, NC), was used as a reporter for the activity of muscle-specific transcription factors (0.25 μg/transfection). An NFAT-sensitive luciferase plasmid (28) used as a reporter for NFAT transcriptional activity (0.25 μg/transfection) and plasmids encoding NFATc3, a constitutively activated form of NFATc3 (29), were kindly provided by Dr. Leon de Windt (Hubrecht Laboratory, Utrecht, The Netherlands). pEMSV-MyoD, used for myogenic conversion of fibroblasts, was a kind gift from Dr. Barbara Winter (University of Braunschweig, Germany). Plasmids encoding GSK-3β, GSK-3β S9A, and GSK-3β K85R were kindly provided by Dr. S Sokol (Harvard Medical School, Boston, MA) (30). pSV-β-gal (0.25 μg/transfection) was employed to correct for differences in transfection efficiency (Promega, Madison, WI). To determine luciferase and β-galactosidase activity, cells were lysed in luciferase lysis buffer and stored at −80°C. Luciferase (Promega, Madison, WI) and β-galactosidase (Tropix, Bedford, MA) were measured according to the manufacturers’ instructions.

**RNA Interference and Retroviral Infection**—Vectors expressing hairpin small interference RNAs were constructed by inserting pairs of annealed, HindIII/BglII-digested oligonucleotides containing the 19-nt target sequence into the pRetroSuper (pRS) vector (31, 32), kindly provided by Dr. R. Agami (The Netherlands Cancer Institute, Amsterdam). Ectropic retroviral supernatants were produced by transfection of phoenix packaging cells (Dr. G. Nolan, Stanford University), cultured in high glucose DMEM containing antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) and 9% (v/v) FBS, with the pRS GSK-3β shRNA vector or the pRS control vector by calcium phosphate precipitation. 48 h post-transfection, the tissue culture medium was filtered through a 0.45-μm filter, and supernatant containing the viral particles was used for infection of C2C12 cells after the addition of 4 μg/ml Polybrene (Sigma). Cells were infected for at least 6 h and allowed to recover for 24 h with fresh GM. Infected cells were selected with puromycin (2.5 μg/ml) for at least 96 h, and silencing efficacy was evaluated by Western blot analysis for GSK-3β or NFATc3, respectively, and tubulin as a loading control. Of the three target sequences tested, the pRS vector containing the 19-nt target sequence 5′-GTGTATATGTAGCCTG-3′ demonstrated the strongest RNA silencing for GSK-3β, whereas the targeting sequence 5′-TACTAGAGTCCGACTTGTGTA-3′ proved most successful in NFATc3 silencing.
**GSK-3β Inactivates NFATc3 and Suppresses Myogenesis**

Santa Cruz Biotechnology). After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody, 1:5000 (Vector Laboratories, Burlingame, CA), and visualized by Supersignal WestPico chemiluminescent substrate (Pierce) according to the manufacturer’s instructions.

**Assessment of Myogenic Differentiation**—Myogenic differentiation of C2C12 cells was assessed biochemically via determination of muscle creatine kinase (MCK) activity and morphologically by determination of the myogenic index. For MCK activity, cells grown and differentiated in 35-mm dishes for 72 h were washed twice in cold PBS, lysed in 0.5% Triton X-100, and scraped off of the dish with a rubber policeman. Lysates were centrifuged for 2 min at 16,000 x g, and the supernatant was stored in separate aliquots at −80 °C for determination of protein content or MCK activity in the presence of 1% bovine serum albumin. MCK activity was measured using a spectrophotometry-based (33) kit from Stanbio (Stanbio, Boerne, TX).

serum albumin. MCK activity was assessed by the Bradford method (34).

**RNA Isolation and Assessment of mRNA Abundance**—Total RNA from C2C12 cells was isolated using the Totally RNA kit (Ambion, Austin, TX) according to the manufacturer’s instructions. After isolation, RNA was dissolved in 1 mM sodium citrate (pH 6.4) and stored at −80 °C. One μg of RNA was reverse transcribed to cDNA using the Reverse iT First Strand Synthesis kit (ABgene, Epsom, UK) with anchored oligo(dT) primers. MCK and β-actin mRNA were determined by quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and obtained from Sigma Genosys (Haverhill, UK). MCK was amplified using the following primers: MCK FP, 5′-AGGTTTTCTGGCCGCTTCTT-3′; RP, 5′-CGGTGCCCAGGTTGGA-3′. PCRs (25 μl total volume) contained 1× MasterMix Plus for SYBR green I (Eurogentec, Seraing, Belgium) and primers (300 nm). Standard curves were made in duplicate by performing serial dilutions of pooled cDNA aliquots. Ct values were obtained for each sample, and the relative DNA concentrations were derived from the standard curve. The expression of the genes of interest was normalized to β-actin (primers obtained from Ambion). Real time PCRs were performed in an ABI PRISM™ 7700 Sequence Detector (Applied Biosystems).

**Immunofluorescence**—GSK-3β shRNA or control pRS-C2C12 myoblasts were grown on glass coverslips and were fixed and stained after 24 or 48 h of culture in DM for NFATc3 using a polyclonal antibody 1:100 (catalog number sc-8321; Santa Cruz Biotechnology) and a fluorescein isothiocyanate-fluorophore-conjugated anti-rabbit secondary antibody, 1:1000 (Molecular Probes, Leiden, The Netherlands). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (20 μg/ml). Images were taken at a ×400 magnification, using a fluorescent microscope connected to a digital DXM 1200F camera, both from Nikon (Kanagawa, Japan).

**RESULTS**

Enhanced Myogenic Conversion of Mouse Embryonic Fibroblasts in the Absence of GSK-3β—Myogenic conversion of fibroblasts by overexpression of the muscle regulatory factor...
MyoD is a useful tool to study activation of muscle-specific gene transcription (35). To address the suppressive effects of endogenous GSK-3β on myogenesis by genetic rather than pharmacological modulation, we compared myogenic conversion of GSK-3β−/− and WT MEFs by measuring transactivation of the troponin-I (TnI) promoter. MEFs lacking endogenous GSK-3β displayed increased TnI promoter transactivation compared with WT MEFs (Fig. 1A). Moreover, restoring GSK-3β expression by co-transfecting GSK-3β−/− MEFs with a plasmid encoding WT GSK-3β decreased TnI promoter transactivation, whereas expression of a kinase-dead mutant (K85R) did not show any effects (Fig. 1B). This finding demonstrates that GSK-3β kinase activity suppresses muscle-specific gene transcription, suggesting a negative regulatory role for GSK-3β in myogenic differentiation.

dephosphorylation by calcineurin (12). Previous reports have revealed that calcineurin activity is required for muscle differentiation (22). To establish whether pharmacological inhibition of calcineurin resulted in repression of myogenic differentiation in our model, C2C12 myoblasts were cultured in DM for 72 h with or without the calcineurin inhibitor FK506, and MCK activity was assessed. MCK was significantly decreased in a dose-dependent fashion in response to FK506 (Fig. 3A). In contrast, pharmacological inhibition of GSK-3β by LiCl resulted in increased MCK activity (Fig. 3B) and MCK mRNA abundance (Fig. 3C) (10). Simultaneous inhibition of GSK-3β and calcineurin completely prevented the repressive effect of calcineurin blockade on myogenic differentiation based on assessment of MCK enzyme activity (Fig. 3B) and MCK mRNA abundance (Fig. 3C). These data demonstrate that calcineurin

FIGURE 2. GSK-3β knockdown stimulates differentiation of skeletal muscle cells. RNA interference against GSK-3β was achieved in C2C12 myoblasts as described under “Experimental Procedures.” pRS-control or pRS-GSK-3β shRNA myoblasts were drug-selected for 96 h and passaged when appropriate. Cells were cultured in GM for 24 h, and soluble protein (5 μg) from lysates was separated by SDS-PAGE to assess GSK-3β abundance by Western blot analysis (A). Alternatively, control or GSK-3β shRNA myoblasts were differentiated in DM for 72 h to assess myotube formation qualitatively (B) and quantitatively by calculating the myogenic index as the fraction of nuclei residing in myotubes divided by the total number of nuclei (C). Control or GSK-3β shRNA myoblasts were also differentiated in DM for 72 h to assess MCK enzyme activity (D) or MCK mRNA abundance (E). Shown are representative graphs of three independent experiments (n = 3 ± S.E.).
and GSK-3β have opposing effects on myoblast differentiation, which may be conveyed through their mutual phosphosubstrate NFAT, which has been implicated in myogenic differentiation (24).

**GSK-3β Suppresses NFATc3 Nuclear Localization in Skeletal Muscle Cells**—Next, NFATc3 nuclear localization was assessed in myoblasts by immunostaining during differentiation. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue), and NFATc3 (green) localization was determined by immunocytochemistry (A). To evaluate whether NFAT expression is also suppressed by GSK-3β in skeletal muscle cells, C2C12 cells were transfected with the NFAT reporter construct and NFATc3 overexpression (Fig. 5A). Co-transfection of NFATc3 with plasmids encoding WT GSK-3β further enhanced NFAT transcriptional activity in WT MEFs but not in GSK-3β−/− MEFs (Fig. 5A). To evaluate whether NFAT transcriptional activity is also suppressed by GSK-3β in skeletal muscle cells, C2C12 cells were transiently transfected with the NFAT reporter construct and NFATc3. Overexpression of NFATc3 caused a strong increase of NFAT transcriptional activation, which was further increased by inhibition of endogenous GSK-3β using LiCl (Fig. 5B). Co-transfection of NFATc3 with plasmids encoding WT GSK-3β but not kinase-inactive (K85R) GSK-3β decreased NFAT transcriptional activation, whereas transcriptional activity of a non-phosphorylatable mutant (ca) of NFATc3 was not affected by GSK-3β over-expression (Fig. 5C). Combined, these data position NFAT downstream of GSK-3β signaling and show a direct regulatory role of GSK-3β in NFATc3-mediated transcriptional activity in C2C12 cells.
NFAT-dependent Muscle-specific Gene Expression Is Inhibited by GSK-3β—To examine whether NFATc3 positively affects myogenic differentiation, C2C12 cells were transiently transfected with the TnI promoter reporter construct to assess muscle-specific gene expression during differentiation. Co-expression of NFATc3 increased TnI promoter transactivation after 24 h (not shown) and 48 h following induction of differentiation (Fig. 6A). TnI promoter transactivation was further enhanced by GSK-3β inhibition (Fig. 6A). NFATc3 overexpression also increased TnI promoter transactivation following myogenic conversion of GSK-3β−/− MEFs (Fig. 6B). Reintroducing WT, but not K85R GSK-3β, repressed NFATc3-stimulated TnI promoter transactivation. In contrast, increased TnI promoter activity in response to ca NFAT overexpression was refractory to GSK-3β (Fig. 6B). Importantly, TnI promoter transactivation was also increased in differentiating C2C12 myoblasts when NFATc3 was overexpressed, which was in turn suppressed by WT but not K85R GSK-3β (Fig. 6C). Overexpression of ca NFATc3 also stimulated muscle-specific gene expression but was insensitive to GSK-3β (Fig. 6C). These results show that muscle gene expression during myogenic differentiation is promoted by NFATc3, which in turn is negatively regulated by GSK-3β.

Stimulation of Muscle Gene Expression during Myogenic Differentiation by GSK-3β Inhibition Requires NFATc3—Finally, we investigated whether stimulation of myogenic differentiation by inhibition of GSK-3β is dependent on NFATc3 signaling. Using the same RNA interference approach as for GSK-3β knockdown, C2C12 cell lines harboring a stably integrated NFATc3 targeting sequence (pRS-NFATc3 shRNA) were generated. Compared with control cells, NFATc3 protein abundance was efficiently reduced (~75%) in pRS-NFATc3 shRNA myoblasts (Fig. 7A). Next, these and control myoblasts were transiently transfected with the TnI promoter reporter construct to assess muscle-specific gene expression during differentiation. Differentiation-induced TnI promoter transactivation was strongly attenuated in myoblasts with reduced NFATc3 levels compared with control myoblasts (4-fold at 48 h in DM) compared with pRS-NFATc3 shRNA myoblasts (2-fold at 48 h in DM). These data demonstrate that stimulation of muscle gene expression during differentiation by inhibition of GSK-3β is dependent on NFATc3.

DISCUSSION

Skeletal muscle growth involves different processes, including a net increase in protein synthesis as well as proliferation, differentiation, and fusion of satellite cells and simultaneous expression of muscle-specific genes (5, 36). These cellular and intracellular processes are coordinated at multiple levels by insulin-like growth factor-1 (IGF-1)/Akt/mammalian target of rapamycin and IGF-1/Akt/GSK-3β signaling pathways (9, 37). Various studies have shown that GSK-3β is a negative regulator of both cardiac and skeletal muscle growth (9, 38, 39). Recently, GSK-3β inactivation was associated with myonuclear accretion and myogenic differentiation in skeletal muscle recovering from atrophy (10). In addition, in vitro studies have suggested that GSK-3β suppresses myogenesis, since pharmacological inhibition of GSK-3β was reported to stimulate myogenic differentiation (10,41). The results presented in the current study extend these observations, since loss of endogenous GSK-3β was demonstrated to stimulate muscle-specific gene expression and myotube formation during differentiation and implicate
regulation of NFATc3 nuclear translocation and transcriptional activity as the mechanism of suppression of muscle gene expression by GSK-3β during differentiation.

In the present study, ablation of GSK-3β signaling increased muscle-specific promoter transactivation, muscle gene expression, and myotube formation in two independent models of myogenic differentiation. This is in line with the stimulatory effect of IGF-I-mediated or pharmacological inhibition of GSK-3β activity on myogenic differentiation reflected by increased myotube formation and enhanced expression of muscle-specific genes such as TnI, slow and fast, MCK, and MyoD (10). Conversely, reintroduction of enzymatically active GSK-3β, but not kinase-inactive GSK-3β, reduced muscle-specific gene expression, indicating that GSK-3β kinase activity is required for its suppressive effects on myogenesis.

Previous reports have demonstrated that stimulation of myogenic differentiation can be accomplished via activation of calcineurin, which antagonizes the phosphorylation of certain GSK-3β substrates (15, 17, 24). Conversely, inhibition of calcineurin by cyclosporin A treatment suppressed biochemical and morphological differentiation of skeletal muscle cells (17). In line with these findings, calcineurin inhibition using cyclosporin A (data not shown) or FK506 blocked myotube formation (data not shown) and accumulation of MCK mRNA and enzyme activity.

Interestingly, pharmacological inhibition by LiCl or genetic ablation (data not shown) of GSK-3β kinase activity not only stimulated MCK expression and myotube formation (10) but also completely restored the adverse effect of calcineurin inhibition on myogenic differentiation, suggesting a dominant role of GSK-3β over calcineurin in muscle growth. In line with these observations, calcineurin-induced cardiac hypertrophy is completely prevented by
The current study does not exclude regulation of other NFAT isoforms by GSK-3β during differentiation, since four NFAT isoforms, NFATc1 to -c3 and NFAT5, are expressed in skeletal muscle (17, 18). Although NFAT5, -c2, and -c3 have all been attributed a role in the regulation of myoblast differentiation, we focused on NFATc3 in this work for a number of reasons. First, NFAT5 is not subject to calcineurin regulation, yet our data and other studies (19, 24) imply that calcineurin signaling is required for basal differentiation. Conversely, stimulation of myogenesis was also observed in these studies by overexpression of ca calcineurin or calcineurin activation by increasing intracellular calcium levels. Moreover, previous studies have postulated a specific role for NFATc3 during stimulation of myoblast differentiation (17, 24). In line with these reports, myoblasts with reduced NFATc3 protein levels displayed decreased TnI promoter transactivation during differentiation. Together with our data, this suggests a model of NFATc3 regulation in which the balance between inhibitory phosphorylation by GSK-3β and stimulatory dephosphorylation by calcineurin is in favor of GSK-3β-mediated nuclear exclusion of NFATc3 during basal, nonstimulated differentiation. In contrast, signals increasing calcineurin activity (e.g. calcium) or decreasing GSK-3β activity (e.g. IGF-1) result in NFATc3 nuclear translocation and stimulation of myogenic differentiation.

In support of this model, increased NFATc3 nuclear localization coincided with up-regulation of various muscle specific mRNA transcripts after either pharmacological inhibition (10) or knockdown of GSK-3β. This positions the effects of GSK-3β at the pretranslational level and suggests stimulation of muscle-specific gene transcription by NFATc3. Indeed, overexpression of NFATc3 was sufficient to stimulate transactivation of the TnI promoter during muscle differentiation. Moreover, NFATc3 was subject to negative regulation by GSK-3β kinase activity, since simultaneous overexpression of GSK-3β prevented the stimulatory effects of NFATc3 on muscle gene expression. Finally, stimulation of muscle gene expression during myogenic differentiation by GSK-3β inhibition (Fig. 5B) result in increased TnI promoter transactivation of NFATc3 in response to LiCl was strongly attenuated in myoblasts in which NFATc3 expression was silenced.

Participation of NFAT in muscle-specific gene expression has been documented previously and occurs in association with other transcription factors, such as GATA2 (15), Myf5 (45), and MEF2 (46). In addition, NFATc3 was shown to enhance the myogenic activity of MyoD (24). Since NFATc1 and -c4 did not have this effect (24), this further supports a specific role for NFATc3 in stimulation of muscle differentiation.

NFATc3 silencing did not completely block enhanced differentiation following GSK-3β inhibition (Fig. 7B). This may have resulted from the presence of residual NFATc3 protein (Fig. 7A). Alternatively, additional signaling modules that are negatively regulated by GSK-3β may contribute to increased differentiation in the absence of GSK-3β kinase activity, besides NFAT-dependent transcription. For example, eukaryotic initiation factor 2B is negatively regulated by GSK-3β (47), and inactivation of GSK-3β during IGF-1-induced muscle hypertrophy is associated with increased protein synthesis (9), which

**FIGURE 7. Stimulation of muscle gene expression during myogenic differentiation by GSK-3β inhibition requires NFATc3.** RNA interference against NFATc3 was achieved in C2C12 myoblasts as described under “Experimental Procedures.” pRS-control or pRS-NFATc3 shRNA myoblasts were drug-selected for 96 h and passaged when appropriate. Cells were cultured in GM for 24 h, and soluble protein (15 μg) from lysates was separated by SDS-PAGE to assess NFATc3 or tubulin (loading control) abundance by Western blot analysis (A). pRS-control or pRS-NFATc3 shRNA myoblasts were transiently transfected with a TnI promoter luciferase reporter construct and a plasmid encoding β-galactosidase (0.25 μg each). After incubation in GM or DM for the indicated times with or without LiCl (5 mM), cells were lysed to measure luciferase and β-galactosidase activity. TnI promoter transactivation levels were normalized to values obtained in GM for the individual cell lines (B).
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also occurs during myogenic differentiation (10). In addition, β-catenin, a crucial major downstream effector molecule in Wnt signaling is rapidly degraded upon phosphorylation by GSK-3β (48). Interestingly, β-catenin has been shown to regulate several myogenic proteins, such as MyoD and myogenin (49), suggesting a role for β-catenin in myogenesis (40), which may be stimulated in the absence of GSK-3β. Therefore, we propose that GSK-3β may act as a central mediator of myogenic differentiation, since differentiation-promoting stimuli, such as calcium/calcineurin, IGF-I, and Wnt, converge on GSK-3β, which may subsequently control multiple regulatory steps of myogenic differentiation. In conclusion, this study identifies GSK-3β as a potential target for stimulation of myogenic differentiation via NFATc3 to enhance skeletal muscle growth or promote recovery from muscle atrophy.

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