Fibroblast Growth Factor Inducible 14 (Fn14) Is Required for the Expression of Myogenic Regulatory Factors and Differentiation of Myoblasts into Myotubes

EVIDENCE FOR TWEAK-INDEPENDENT FUNCTIONS OF Fn14 DURING MYOGENESIS*

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Fibroblast growth factor-inducible 14 (Fn14), distantly related to tumor necrosis factor receptor superfamily and a receptor for TWEAK cytokine, has been implicated in several biological responses. In this study, we have investigated the role of Fn14 in skeletal muscle formation in vitro. Flow cytometric and Western blot analysis revealed that Fn14 is highly expressed on myoblastic cell line C2C12 and mouse primary myoblasts. The expression of Fn14 was decreased upon differentiation of myoblasts into myotubes. Suppression of Fn14 expression using RNA interference inhibited the myotube formation in both C2C12 and primary myoblast cultures. Fn14 was required for the transactivation of skeletal α-actin promoter and the expression of specific muscle proteins such as myosin heavy chain fast type and creatine kinase. RNA interference-mediated knockdown of Fn14 receptor in C2C12 myoblasts decreased the levels of myogenic regulatory factors MyoD and myogenin upon induction of differentiation. Conversely, overexpression of MyoD increased differentiation in Fn14-knockdown C2C12 cultures. Suppression of Fn14 expression in C2C12 myoblasts also inhibited the differentiation-associated increase in the activity of serum response factor and RhoA GTPase. In addition, our data suggest that the role of Fn14 during myogenic differentiation could be independent of TWEAK cytokine. Collectively, our study suggests that the Fn14 receptor is required for the expression of myogenic regulatory factors and differentiation of myoblasts into myotubes.

Myogenesis or skeletal muscle formation is a highly complex process that involves the expansion of mononucleated progenitor cells, their progression along a myogenic lineage pathway to become fusion-competent myoblasts, their migration and alignment, and finally their differentiation into multinucleated myotubes that further develop to become the myofibers of mature skeletal muscle (1–3). Myogenesis is regulated by the sequential expression of myogenic regulatory factors (MRF), a group of basic helix-loop-helix transcription factors that include Myf-5, MyoD, myogenin, and MRF4 (4, 5). Furthermore, myogenic differentiation also requires the coordination of multiple signaling pathways that regulate cell cycle withdrawal and specify myogenesis (i.e. activates MRFs). A promyogenic role has been assigned to the Akt kinase (6, 7), the p38 mitogen-activated protein kinase (MAPK) (8, 9), and the calcineurin-NFATc3-dependent signaling pathways (10). Conversely, the activation of the Ras/raf/p44-p42-MAPK cascade seems to be detrimental to the differentiation process (11). During myogenesis, fusion of myoblasts into multinucleated myotubes is the terminal step of differentiation after which no further mitotic divisions occur within the myotubes or muscle fibers. The extra nuclei required for muscle growth are provided by satellite cells, which are located under the basal lamina of the muscle fiber (12). Satellite cells also account for the majority of muscle regenerative potential in response to injury and muscular adaptation to exercise (12, 13).

Accumulating evidence suggests that in addition to growth factors, cytokines produced by muscle cells and/or invading inflammatory cells after injury may play an important role in skeletal muscle repair and regeneration (14). Muscle-derived interleukin-4 has been shown to act as a myoblast recruitment factor during mammalian muscle growth (15, 16). TNF receptor null mice showed reduced muscle regeneration in response to cryoinjury (17, 18). Conversely, systemic injection of TNF-α induced the proliferation of satellite cells in myofibers of adult mice (19). Furthermore, TNF-α and interleukin-1β have also been reported to block myoblast differentiation in vitro (20–22).

TNF-related weak inducer of apoptosis (TWEAK) is a recently identified member of the tumor necrosis factor superfamily of structurally related cytokines that generally function as both type II transmembrane proteins and as cleaved soluble...
molecules (23, 24). We have shown that soluble TWEAK protein stimulates myoblast proliferation and inhibits their differentiation into myotubes (25). However, the molecular pathways through which TWEAK modulates myoblast proliferation and differentiation remain poorly understood. Based on different binding analyses indicating a physiologically relevant affinity between TWEAK and fibroblast growth factor-inducible 14-kDa protein (Fn14), Fn14 has been suggested as the receptor for TWEAK (26, 27). Fn14 is characterized as a type Ia transmembrane receptor that is distantly related to the TNF receptor superfamily (27). It contains a single cysteine-rich domain in the extracellular region and a TNF receptor-associated factor-binding motif, but lacks the death domain in the cytoplasmic region (26, 28). The expression of Fn14 has been reported to increase on exposure of fibroblasts to growth factors, fetal calf serum, and phorbol ester (29, 30). Although TWEAK binds to Fn14, it is still controversial whether Fn14 is the only receptor for TWEAK and vice versa. It has been shown that Fn14 was not responsible for the osteoelastic effect of TWEAK on RAW264.7 cells that do not express Fn14 (31). Similarly, overexpression of Fn14 has been reported to promote neurite outgrowth independent of TWEAK cytokine (32). However, involvement of Fn14 in the process of skeletal muscle formation remains unknown. Furthermore, it remains unclear if TWEAK affects myogenic differentiation through the recruitment of the Fn14 receptor.

In this study we have investigated the role and the mechanisms by which Fn14 receptor regulates myogenic differentiation. Our data demonstrate that Fn14 is required for the differentiation of myoblasts into multinucleated myotubes. Suppression of Fn14 expression using RNA interference inhibits the expression of myogenic transcription factors MyoD and myogenin and the activation of serum response factor (SRF) and RhoA GTPase. Furthermore, our data provide evidence that the promyogenic role of Fn14 might be independent of TWEAK cytokine.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ham’s F-12 nutrient mixture was obtained from Invitrogen. Antibodies against β-actin and Myf-5 and SRF consensus oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor mixture, horse serum, and fetal bovine serum were from Sigma. Fn14 antibody was obtained from Cell Signaling Technology (Beverly, MA). Phycoerythrin (PE)-conjugated anti-mouse and anti-human Fn14 and PE-conjugated mouse IgG isotype control antibodies were obtained from eBioscience (San Diego, CA). Monoclonal antibodies against myogenin and MyoD were obtained from BD Biosciences. MF-20 antibody was obtained from the Developmental Studies Hybridoma Bank of the University of Iowa. Alexa Fluor 546 goat anti-mouse antibody and LIVE/DEAD Viability Assay Kit were purchased from Molecular Probes (Eugene, OR). Pfu DNA polymerase and electrophoretic Escherichia coli strain B51583 were obtained from Stratagene. Transfection reagent Effectene was from Qiagen (Valencia, CA). SMARTpool Fn14 siRNA, control siRNA, and DharmaFECT3 siRNA transfection reagent were purchased from Dharmacon RNA Technologies (Lafayette, CO). Recombinant mouse TWEAK protein and Fn14-Fc chimera protein were obtained from R&D Systems, Inc. (Minneapolis, MN). NF-κB and AP-1 consensus oligonucleotides, luciferase assay kit, and a T7 based in vitro translation kit were purchased from Promega (Madison, WI). Rhoketin Rho binding domain-agarose was purchased from Upstate Biotechnology (Lake Placid, NY). CK assay kit was obtained from Stanbio Laboratory (Boerne, TX). Poly(dI-dC) was obtained from Amersham Biosciences. [γ-32P]ATP (specific activity, 3000 (111 TBq) Ci/mmol) was from PerkinElmer Life Sciences.

**Cell Culture**—C2C12 (a mouse myoblastic cell line) and 293T (human embryonic kidney cell line) were obtained from American Type Culture Collection (Rockville, MD). These cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Primary myoblasts from neonatal mice were prepared by a method as described previously (25, 33). Differentiation in C2C12 and primary myoblast cultures was induced by replacing the medium with differentiation medium (2% horse serum in Dulbecco’s modified Eagle’s medium) as described (25, 33).

**Construction of Adenoviruses**—Full-length mouse TWEAK and MyoD were cloned from cDNA made from C2C12 myoblasts. Total RNA was isolated and first strand cDNA was generated using Ambion’s oligo(dT) primer and Qiagen’s OmniScript reverse transcriptase according to the manufacturer’s instructions (Qiagen). TWEAK construct was prepared by amplifying the murine TWEAK cDNA (GenBank accession number: NM_011614) with primers: sense 5′-AAAGCTTATGGCCGCCGGTGGC CTCGG-3′ (TWEAKF); and antisense, 5′-TCTAGAGGCC CCCTGCTGAACTTTG-3′ (TWEAKR). FLAG epitope on TWEAK cDNA was added by amplifying using 5′-ATGGC TGA TCA AAG GGA CGA GAT GAC AAG GCC CGG CCG CTC GAG GCCA-3′ (FLAG-TWEAKF) and TWEAKR primers. MyoD construct was generated by PCR amplification of murine MyoD cDNA (GenBank accession number: NM_010866) with primers: 5′-GGAACTGGGATA TGGGAGTCTT-3′ (MyoDF) and 5′-CCAGTGCAAGCTC TTCAAGAGCA-3′ (MyoDR). All PCR amplifications were performed using Pfu DNA polymerase (Stratagene). PCR products were cloned into pCR®Blunt II TOPO vector (Invitrogen) and the authenticity of cDNA was confirmed by automated DNA sequencing. Finally, FLAG-TWEAK and MyoD cDNA were excised from pCR®Blunt II TOPO vector using HindIII and XbaI restriction enzymes and inserted into pcDNA3 plasmid (Invitrogen) at HindIII and XbaI sites. The expression of proteins from plasmid constructs was confirmed using T7 based in vitro translation kit (Promega) and by transfection of cultured cells followed by Western blotting.

Adenoviral vectors encoding murine TWEAK or MyoD cDNA were constructed as described (34). Briefly, the cDNA were inserted at HindIII and XbaI sites into pAdTrack-CMV vector. The positive clones were linearized by the restriction endonuclease Pmel and cotransformed with the supercoiled adenoviral vector AdEasy-1 into E. coli strain B51583 (Stratagene). Recombinant adenoviral constructs were selected, digested with restriction endonuclease Pacl, and transected into packaging cell line 293T using Effectene transfection reagent (Qiagen). Production of adenovirus in 293T cells was observed after 6–7 days and was monitored by expression of
green fluorescence protein in viral plaques. The cells were collected 7–8 days after transfection, the adenoviruses were released by three freeze-thaw cycles and amplified by infecting 293T cells in one 100-mm tissue culture plate. After 3 days the adenoviruses were harvested as described above and further amplified by infecting 293T cells. The amplified adenoviruses were harvested 3 days later, purified by centrifugation in CsCl, and stored at −80 °C in storage buffer (5 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.05% bovine serum albumin, and 25% glycerol). The titer of the virus was determined by infecting 293T cells with serial dilutions of adenovirus and monitoring the viral plaques for expression of green fluorescence protein.

**Flow Cytometric Analysis**—Flow cytometric analysis was done to study the expression of the Fn14 receptor on myogenic cells. Approximately, 1 × 10⁶ cells of each cell type were harvested in phosphate-buffered saline, and incubated for 30 min on ice with 5 μg/ml PE-conjugated anti-mouse and anti-human Fn14 or PE-conjugated mouse IgG isotype controls (eBioscience, San Diego, CA). Cells were washed three times with phosphate-buffered saline containing 0.5% bovine serum albumin and analyzed for Fn14 expression by FACSscan using CellQuest software (BD Biosciences).

**Short Interfering RNA (siRNA)**—Silencing or knockdown of the Fn14 gene was achieved using the short interfering RNA (siRNA) technique. SMARTpool siRNA (containing RNA duplex with sequences: 5′-GGAUUCGCG UUG GUG UUG AUU3′ (sequence 1); 5′-CGU CGUCCAUCAUCAUCAAUU-3′ (sequence 2); 5′-GGACUGGGCUUAGAGUUCAU-3′ (sequence 3) and 5′-CUAAGGAACUGCAGCAUUUUU-3′ (sequence 4) or the above individual siRNA duplexes for mouse Fn14 mRNA (GenBank accession number: NM_013749) and non-targeting control siRNA were obtained from Dharmacon RNA Technologies (Lafayette, CO). C2C12 myoblasts plated in either a 6-well (100,000 cells/well) or 24-well (20,000 cells/well) plate were transfected with siRNA duplexes using DharmaFect-3 transfection reagent following the manufacturer’s instructions. After 36 h of transfection, the protein levels of Fn14 were measured by Western blotting and flow cytometry analysis.

**Generation and Use of Short Hairpin (shRNA)-expressing Lentivirus**—We used pLL3.7 plasmid (provided by Dr. Luk Van Parijs, Massachusetts Institute of Technology) to generate lentivirus encoding Fn14 shRNA (35). We used a sequence from the SMARTpool Fn14 siRNA mixture (i.e. sequence 5′-GCGCTGGGGCTTAATGC-3′ from 545 to 563 of Fn14 mRNA) and a control sequence that does not target any known mouse mRNA (i.e. 5′-GCTCGGGCATCCCTCTAAGA-3′). Oligonucleotides including control or Fn14 siRNA sequence and hairpin structure were chemically synthesized (Integrated DNA Technologies, Inc.). The sequences of the oligonucleotides were as follows: Fn14, 5′-TGACTGGGGCTTAATGC-3′ (forward) and 5′-TGAGAAAAAGCCTGGGGCTTAATGC-3′ (reverse); control, 5′-TGCGCATCCCTCTAAGATCGAGAT-3′ (reverse), and 5′-TCGAGAAAAAGCCTGGGGCTTAATGC-3′ (forward) and 5′-TCGAGAAAAAGCCTGGGGCTTAATGC-3′ (reverse). The oligonucleotides were annealed, phosphorylated, and inserted into pLL3.7 vector using HpaI and XhoI sites. To produce lentivirus, plasmid DNA and helper plasmids (pMDLG/pRRE, pMD2.G, pRSVRev) were transfected into 293T cells. Forty-eight hours later, culture supernatants were collected, spun at 2,000 × g for 10 min, and filtered through a 0.45-μm filter. Titers were determined by infecting 293T cells with serial dilutions of lentivirus. For transduction of C2C12 myoblasts with lentivirus, C2C12 myoblasts were plated at 2 × 10⁵ cells per well in 6-well plates. The cells were transduced by adding culture supernatants containing lentivirus to the wells in the presence of 8 μg/ml Polybrene. After 48 h, the lentivirus containing culture medium was replaced by differentiation medium and myogenic differentiation was studied.

**Transient Transfection and Reporter Assay**—Transfection of skeletal α-actin promoter was studied by transiently transfecting the cells with the pSK-Luc construct that contains luciferase cDNA under the control of skeletal α-actin promoter (36). C2C12 myoblasts grown in a 6-well plate were transfected with pSK-Luc (0.5 μg/well) using Effectene transfection reagent (Qiagen). Transfection efficiency was controlled by cotransfection of myoblasts with pSV-galactosidase (0.1 μg/well). When cells were around 90% confluent, differentiation was induced by replacing growth medium by differentiation medium. Specimens were processed for luciferase and β-galactosidase expression using the luciferase and β-galactosidase assay systems with reporter lysis buffer as per the manufacturer’s instructions (Promega). Luciferase measurements were made using a luminometer (Analytic Scientific Instrumentation, model 3010).

**Immunocytochemistry**—Immunofluorescence was performed to study the myotube formation in C2C12 and primary myoblast cultures as described recently (25, 37).

**Immunoprecipitation and Western Blotting**—C2C12 myoblasts were incubated in differentiation medium for different time intervals and lysed in lysis buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.3% Nonidet P-40, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and protease inhibitor mixture). Protein concentration was determined using the Bio-Rad protein assay reagent. Two milligrams of protein extract was immunoprecipitated with Fn14 antibody (2 μg/per sample). The immunoprecipitates were collected using protein A-Sepharose beads, separated by 12% SDS-PAGE, and blotted with either RhoA or Rac-1 antibody. To check the cellular level of specific proteins, 80 μg of protein extracts from each treatment were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibody as described previously (38–40). The dilution of primary antibody was as follows: MF-20 (1:100), anti-myogenin (1:500), anti-MyoD (1:500), anti-Myf5 (1:100), anti-RhoA (1:1000), anti-Rac-1 (1:1000), anti-Fn14 (1:2000), and anti-β-actin (1:3000). Immunoblots were quantified using ImageQuant software (GE Healthcare).

**Creatine Kinase (CK) Assay**—CK activity was measured using a spectrophotometry-based kit as described previously (25, 37).
Rho GTPase Activity Assay—The activation of RhoA was measured using coprecipitation assays as described previously (41).

Electrophoretic Mobility Shift Assay (EMSA)—The activation of SRF nuclear factor-κB (NF-κB), and activator protein-1 (AP-1) transcription factors was measured by EMSA as described previously (25).

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR)—RNA isolation and QRT-PCR was performed to measure the mRNA level of MyoD, myogenin, and Myf-5 in C2C12 myoblasts following a method as described recently (25, 33).

Proliferation Assay—Cellular proliferation was measured using alamarBlue® dye (BioSource International, Camarillo, CA) as described previously (25, 33). Cells transfected with control siRNA or Fn14 siRNA were seeded into a 24-well plate and incubated in either growth medium or differentiation medium. After 48 h the medium was removed and 200 μl of 10% alamarBlue diluted in phenol red-free Dulbecco’s modified Eagle’s medium was added. The fluorescence was determined 2 h later using a fluorescence plate reader (Fluorolite 1000, Dynex Technologies, Chantilly, VA). AlamarBlue was evaluated using the optimal excitation and emission wavelengths of 546 and 590 nm, respectively. Cellular proliferation was also confirmed by measuring the total protein concentration in each well and by counting the total number of viable cells at the end of the incubation period.

Statistical Analysis—Results are expressed as mean ± S.D. The Student’s t test or analysis of variance was used to compare quantitative data populations with normal distributions and equal variance. A value of p < 0.05 was considered statistically significant unless otherwise specified.

RESULTS

In this study we have investigated the role of the Fn14 receptor in myogenesis. Because the major muscle differentiation steps can be reproduced in vitro with C2C12, a mouse myoblastic cell line (42), we have employed C2C12 myoblasts to study the involvement of Fn14 in myogenesis.

Expression of Fn14 Receptor on Myoblasts and Its Suppression by RNA Interference—Fn14 expression has been reported in certain but not all cell types (31, 43). Using flow cytometric analysis we first examined if the cells of the myogenic lineage express Fn14 receptor on their cell surface. As shown in Fig. 1A, the expression of Fn14 was strong on C2C12 and mouse primary myoblasts. Consistent with the published report (31), the expression of Fn14 was not detected on RAW264.7 cells (Fig. 1A). To examine whether the expression of Fn14 protein changes during myogenic differentiation, C2C12 myoblasts were incubated in differentiation medium for different time intervals ranging from 0 to 120 h and the expression of Fn14 was measured by Western blotting. The expression of myosin heavy chain fast type (MyHCf), a muscle-specific protein, was used as a marker for myoblast differentiation (33). As shown in Fig. 1B and C, the expression of Fn14 in C2C12 myoblasts was significantly reduced upon differentiation of myoblasts into myotubes indicating that Fn14 is present in primitive myoblastic cells but its expression is reduced after myogenic differenti-

![FIGURE 1. Expression of Fn14 receptor on myogenic cells.](image-url)

A, C2C12, mouse primary myoblasts, and RAW264.7 cells were stained with PE-conjugated anti-human and mouse Fn14 (closed histogram) and analyzed by flow cytometry. Nonspecific binding was determined with cells incubated with PE control mouse IgG (open histogram). Data presented here show that Fn14 is expressed on C2C12 and mouse primary myoblasts but not on RAW264.7 cells. B, C2C12 myoblasts were incubated in differentiation medium for different time intervals and the expression of Fn14 and MyHCf (a marker of myoblast differentiation) was studied by Western blotting. A representative immunoblot and quantification from three independent experiments presented here show that expression of Fn14 is decreased on differentiation of myoblasts into myotubes. *, p < 0.05, values significantly different from control cultures at the 0-h time point. C, C2C12 myoblasts were differentiated into myotubes by incubation in differentiation medium for 72 h. The expression of Fn14 on the myotube surface was measured by flow cytometry. Data presented here show that the expression of Fn14 was lower in C2C12 myotubes compared with C2C12 myoblasts (in A, left panel). D, C2C12 myoblasts transfected with control siRNA or Fn14 siRNA duplexes and the expression of Fn14 was studied using flow cytometric analysis. The data presented here show that transfection with Fn14 siRNA drastically reduced the expression of Fn14 on the cell surface of C2C12 myoblasts. E, Western blotting analysis confirmed the reduced expression of the Fn14 protein in Fn14 siRNA-transfected C2C12 myoblasts. F, C2C12 myoblasts plated in a 6-well tissue plate were transfected with control or Fn14 siRNA and after 36 h the number of viable cells was measured using the trypan blue dye exclusion method. Data presented here show that knockdown of Fn14 did not affect the viability of C2C12 myoblasts. DM, differentiation medium.
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-to C2C12 myoblasts, transfection with Fn14 siRNA also resulted in decreased myoblast size and reduced myotube formation compared with control siRNA-transfected C2C12 cultures (Fig. 2). To confirm the inhibition of C2C12 differentiation after transfection with Fn14 siRNA, we performed additional experiments that addressed this issue. We first examined the expression of specific muscle proteins during myogenic differentiation. In another experiment, the expression of muscle-specific proteins CK and MyHCf (25) in control and Fn14-knockdown C2C12 cultures after 72 h of differentiation was also examined. The expression of both CK and MyHCf was significantly reduced in Fn14-knockdown cultures compared with controls (Fig. 3, B and C) indicating that Fn14 receptor is required for transactivation of the skeletal α-actin promoter and the expression of specific muscle proteins during myogenic differentiation.

We also investigated the effect of suppression of Fn14 expression on differentiation of C2C12 myoblasts using a vector-based RNA interference technique. C2C12 myoblasts were transfected with lentivirus expressing either control or Fn14 shRNA for 48 h (resulting in >80% cell transduction) followed by incubation in differentiation medium for 72 h. Transduction of C2C12 myoblasts with Fn14 shRNA-expressing lentivirus reduced the levels of Fn14 protein (Fig. 3, middle panel). As shown in Fig. 3D (lower panel), the level of MyHCf protein was significantly decreased in Fn14 shRNA lentivirus-transduced cultures compared with control shRNA-transduced cultures (0.4 versus 1-fold, respectively).

To further explore the role of Fn14 in myoblast differentiation, we used the siRNA technique to suppress expression of Fn14 protein in myoblasts. C2C12 myoblasts were transfected with either control siRNA or Fn14 siRNA and expression of the Fn14 receptor was studied using FACS. As shown in Fig. 1A, transfection of C2C12 myoblasts with Fn14 siRNA significantly reduced the expression of the Fn14 receptor compared with controls (6.3 versus 93.4%, respectively). The FACS data were also confirmed by Western blotting. A drastic decrease (94 ± 4.3%) in Fn14 protein level was observed in Fn14 siRNA-transfected C2C12 myoblasts, whereas control siRNA-transfected C2C12 myoblasts remained low up to 5 days after transfection with Fn14 siRNA (data not shown). Furthermore, there was no significant difference in the viability between Fn14 siRNA and control siRNA-transfected C2C12 myoblasts measured by trypan blue dye exclusion uptake assay (33) after 36 h of siRNA transfection (Fig. 1F).

Silencing of Fn14 Receptor Inhibits Myotube Formation in C2C12 and Primary Myoblast Culture—C2C12 myoblasts transfected with either control or Fn14 siRNA were incubated in differentiation medium and myotube formation was monitored using fluorescence microscopy after immunostaining with MF-20 antibody (specific to MyHCf). Representative photomicrographs presented here show a significant reduction in myotube formation in Fn14 siRNA-transfected C2C12 cultures and mouse primary myoblast cultures compared with control cultures. In C2C12 cultures, the myotube formation (red colored cells) in C2C12 cultures was drastically increased after 72 h of induction of differentiation. However, compared with control siRNA-transfected cultures, the myotube formation was significantly lower in Fn14 siRNA-transfected C2C12 cultures. Furthermore, a few myotubes that were formed in Fn14 siRNA-transfected C2C12 cultures were relatively smaller in size compared with those formed in control siRNA-transfected C2C12 cultures (Fig. 2A). Similar to C2C12 myoblasts, transfection with Fn14 siRNA also inhibited myotube formation in mouse primary myoblast cultures after 48 h of induction of differentiation (Fig. 2B). These results provide the first evidence that the Fn14 receptor is required for the differentiation of myoblasts into multinucleated myotubes.

Fn14 Is Essential for Transactivation of Skeletal α-Actin Promoter and Expression of MyHCf and CK—Although suppression of the Fn14 receptor using siRNA inhibited myotube formation, it remained unclear if Fn14 was required only for the fusion of myogenic cells or if it was also required for expression of specific muscle proteins during differentiation. To address this issue, we first examined transactivation of the skeletal α-actin promoter, which controls the expression of several structural genes in skeletal muscle. C2C12 myoblasts were first transfected with control or Fn14 siRNA duplexes for 24 h followed by transfection with the pSK-Luc plasmid that contains luciferase cDNA under control of the skeletal α-actin promoter (33). The medium of the cells was replaced by differentiation medium and the cells were incubated for an additional 72 h. As shown in Fig. 3A, suppression of Fn14 significantly blocked activation of the skeletal α-actin promoter in C2C12 myoblasts.

To confirm the inhibition of C2C12 differentiation after transfection with Fn14 siRNA, we performed additional experiments that addressed this issue. We first examined the expression of specific muscle proteins during myogenic differentiation. In another experiment, the expression of muscle-specific proteins CK and MyHCf (25) in control and Fn14-knockdown C2C12 cultures after 72 h of differentiation was also examined. The expression of both CK and MyHCf was significantly reduced in Fn14-knockdown cultures compared with controls (Fig. 3, B and C) indicating that Fn14 receptor is required for transactivation of the skeletal α-actin promoter and the expression of specific muscle proteins during myogenic differentiation.

We also investigated the effect of suppression of Fn14 expression on differentiation of C2C12 myoblasts using a vector-based RNA interference technique. C2C12 myoblasts were transduced with lentivirus expressing either control or Fn14 shRNA for 48 h (resulting in >80% cell transduction) and then incubated in differentiation medium for 72 h. Transduction of C2C12 myoblasts with Fn14 shRNA-expressing lentivirus reduced the levels of Fn14 protein (Fig. 3, middle panel). As shown in Fig. 3D (lower panel), the level of MyHCf protein was significantly decreased in Fn14 shRNA lentivirus-transduced cultures compared with control shRNA-transduced cultures (0.4 versus 1-fold, respectively).

To confirm the inhibition of C2C12 differentiation after transfection with Fn14 siRNA, we performed additional experiments that addressed this issue. We first examined the expression of the Fn14 gene and not due to any off-target effects of siRNA, we obtained individual siRNA duplexes in the Fn14 siRNA SMARTpool mixture and transfected C2C12 myoblasts with each of them. All four siRNA duplexes (described under “Experimental Procedures”) were able to suppress the expression of Fn14, although to different levels, and inhibited myogenic differentiation accordingly when transfected individually into C2C12 myoblasts (data not shown). In Fn14 SMARTpool siRNA, three of the four sequences (i.e. sequences 2, 3, and 4) were in the
3'-untranslated region of mouse Fn14 mRNA. C2C12 myoblasts were first stably transfected with pcDNA3-FLAG-Fn14 (containing only the coding region mouse Fn14 gene) or pcDNA3 vector alone. These cells were then transfected with control siRNA or a pool of the three siRNA (from 3'-untranslated region of mouse Fn14 mRNA, i.e. sequences 2, 3, and 4). As shown in Fig. 3E, transfection of C2C12 myoblasts with mouse Fn14 cDNA significantly reversed the inhibitory effect of silencing the endogenous Fn14 gene on myogenic differentiation by suppressing the expression of the Fn14 gene in C2C12 myoblasts and not due to possible off-target effects.

**Fn14 Is Required for the Expression of MRFs**—Because the expression of MRFs is a prerequisite for specification and formation of skeletal muscle (4, 5), we examined the effect of silencing of the Fn14 receptor on levels of MyoD, Myf-5, and myogenin. Control or Fn14 siRNA-transfected C2C12 myoblasts were incubated in differentiation medium for 72 h and the mRNA levels of MyoD, Myf-5, and myogenin were measured by QRT-PCR. Transfection of C2C12 myoblasts significantly reduced the mRNA levels of MyoD, Myf-5, and myogenin (Fig. 4A). Furthermore, Western blot analysis showed that protein levels of MyoD and myogenin were reduced by 56.8 ± 7.3 and 65.4 ± 5.2%, respectively, in Fn14-knockdown cultures compared with control C2C12 cultures. There was no significant difference in the levels of Myf5 protein between Fn14 siRNA and control siRNA-transfected cultures (Fig. 4B).

MyoD transcription factor regulates the expression of several muscle-specific genes including MyoD, p21, Musin, and Desmin during muscle differentiation (4, 5). Because the differentiation-associated increase in the levels of MyoD was decreased on knockdown of Fn14 receptor we next investigated if forced expression of MyoD using adenoviral vector could augment differentiation in Fn14-knockdown C2C12 cultures. Control or Fn14-knockdown C2C12 myoblasts were transduced (multiplicity of infection 1:500) with adenoviruses expressing either β-gal (Ad.β-gal) or mouse MyoD (Ad.MyoD) protein for 24 h followed by incubation in differentiation medium for additional 72 h. The differentiation of myoblasts into myotubes was monitored by measuring CK activity. The data presented in Fig. 4C show that overexpression of MyoD significantly increased CK activity in Fn14-knockdown C2C12 cultures. These results indicate that Fn14 receptor is required for the expression of MyoD and an inhibition in the expression of MyoD may be responsible, at
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**FIGURE 4.** Effect of Fn14 knockdown on mRNA and protein levels of Myf-5, MyoD, and myogenin. C2C12 myoblasts transfected with control siRNA or Fn14 siRNA were incubated in differentiation medium for 72 h and the mRNA and protein levels were measured by QRT-PCR and Western blot, respectively. A, a significant decrease in the mRNA levels of MyoD and myogenin was observed in Fn14 knockdown cultures compared with control cultures. *, p < 0.05, values significantly different from control siRNA transfected C2C12 cultures. B, representative immunoblots of three independent experiments presented here show that protein levels of MyoD and myogenin were decreased in Fn14 siRNA-transfected C2C12 cultures compared with control siRNA-transfected cultures. C, control or Fn14 siRNA-transfected myoblasts were transduced with Ad.β-gal or Ad.MyoD adenovirus (multiplicity of infection 1:500) for 24 h and incubated in differentiation medium for an additional 72 h. The differentiation of myoblasts into myotubes was assessed by measuring CK activity. Data presented here show that overexpression of MyoD significantly increased the CK activity in control and Fn14 knockdown C2C12 cultures. @, p < 0.05, values significantly different from control siRNA-transfected. #, p < 0.05, values significantly different from control siRNA-transfected and Ad.β-gal-transduced C2C12 cultures. Θ, p < 0.05, values significantly different from Fn14 siRNA-transfected and Ad.β-gal-transduced cultures.

least in part, for reduced differentiation of Fn14-knockdown C2C12 myoblasts.

**Fn14 Is Required for Activation of SRF and RhoA GTPase during Myogenic Differentiation**—SRF transcription factor is critical for the induction of muscle-specific genes including skeletal α-actin and MyoD (44–46). To understand the mechanisms by which Fn14 promotes myogenesis, we investigated if knockdown of Fn14 in C2C12 myoblasts affects the activation of SRF during differentiation. As shown in Fig. 5A, the DNA binding activity of SRF was increased in control siRNA-transfected C2C12 myoblasts upon incubation in differentiation medium. However, the differentiation-associated increase in SRF activation was blunted in C2C12 myoblasts transfected with Fn14 siRNA.

It has been reported that during myogenesis, the activation of SRF requires upstream activation of Rho family GTPases, especially RhoA (47). Recently Tanabe et al. (32) reported that Rho GTPase Rac-1 interacts with Fn14 receptor in neurons. We first investigated if Fn14 interacts with Rho GTPases in C2C12 myoblasts. C2C12 myoblasts were incubated in differentiation medium for different time intervals and protein extracts made were immunoprecipitated with Fn14 antibody followed by immunoblotting with either RhoA or Rac-1 antibody. Interestingly, both RhoA and Rac-1 were found to interact with Fn14 and their interaction was enhanced upon induction of differentiation (Fig. 5B). Because the activation of RhoA (but not Rac-1) is required for activation of SRF and expression of MyoD, we investigated the levels of activated RhoA in control and Fn14-knockdown C2C12 cultures at different time points after incubation in differentiation medium. As shown in Fig. 5C, the levels of activated RhoA were decreased in Fn14-knockdown C2C12 cultures compared with control cultures at all time points studied. There was no affect in the levels of total RhoA protein between control and Fn14-knockdown C2C12 cultures (Fig. 5C). Taken together, these data suggest that Fn14 might promote myogenic differentiation through the activation of RhoA GTPase and SRF transcription factor.

**Promyogenic Role of Fn14 during Myogenic Differentiation Is Independent of TWEAK**—Because Fn14 is considered to be a receptor for TWEAK cytokine (26), we next evaluated the effect of overexpression of Fn14 or TWEAK protein on myogenic differentiation. C2C12 myoblasts were transduced with adenoviral vectors expressing β-gal, mouse Fn14, or mouse TWEAK protein for 24 h. The cells were then incubated in differentiation medium for 72 h and the differentiation of myoblasts into myotubes was studied by measuring the levels of MyHCf and CK. Adenoviral-mediated increase in the expression of Fn14 and TWEAK protein was confirmed by Western blot and mouse TWEAK ELISA assay kit, respectively. There was a 7.4 ±

**FIGURE 5.** Effect of silencing of Fn14 on the activation of SRF and RhoA in C2C12 myoblasts. A, C2C12 myoblasts transfected with control or Fn14 siRNA were incubated in differentiation medium for the indicated time intervals and the activation of SRF was studied using EMSA. The data presented here show that silencing of Fn14 inhibited the differentiation-associated increase in SRF activity in C2C12 myoblasts. B, C2C12 myoblasts were incubated in differentiation medium for the indicated time intervals, protein extracts were made, and equal amounts of protein (2 mg for each time point) were immunoprecipitated with Fn14 antibody followed by immunoblotting with RhoA or Rac-1 antibody as described under “Experimental Procedures.” The data presented here show that RhoA and Rac-1 interacts with Fn14 receptor during myogenesis. C, control or Fn14 siRNA-transfected C2C12 myoblasts were incubated in differentiation medium for the indicated time intervals and the levels of GTP-bound (activated) RhoA were studied by coimmunoprecipitation assay. Representative data from two independent experiments presented here show that levels of activated RhoA are decreased in C2C12 myoblasts upon silencing of Fn14 receptor.
myoblasts were incubated in differentiation medium and TWEAK protein and neutralizes its biological actions (48). Studies have shown that the Fn14-Fc chimera protein binds to the Fn14 receptor and decreases the TWEAK-induced inhibition of myoblast proliferation (25). To examine whether the TWEAK-induced proliferation of myoblasts is mediated through the Fn14 receptor, C2C12 myoblasts transfected with either control or Fn14 siRNA for 36 h were incubated with or without soluble TWEAK protein. After 48 h, the proliferation of myoblasts was measured using alamarBlue dye. Consistent with our recent report (25), TWEAK treatment significantly increased the proliferation of control siRNA-transfected C2C12 myoblasts. The proliferation of C2C12 myoblasts was reduced with knockdown of Fn14 receptor. Furthermore, TWEAK-induced proliferation was abolished on knockdown of Fn14 receptor in C2C12 myoblasts (Fig. 7A). In addition, knockdown of the Fn14 receptor inhibited the TWEAK-induced activation of NF-κB and AP-1 transcription factors in C2C12 myoblasts (Fig. 7B), suggesting that TWEAK induces myoblast proliferation via binding to Fn14 receptor.

TWEAK Activates p44-p42 MAPK but Inhibits Akt Kinase in C2C12 Myoblasts—We also investigated the effects of soluble TWEAK protein on the activation of p44-p42 MAPK and Akt kinase in C2C12 myoblasts. The activation of p44-p42 MAPK promotes proliferation, whereas the activation of Akt pathway augments myoblast differentiation (11). Treatment of myoblasts with TWEAK (100 ng/ml) led to ~3-fold (at 60-min time point) increase in the phosphorylation of p44-p42 MAPK (Fig. 7C, upper panel). On the other hand, treatment of myoblasts with TWEAK did not induce the phosphorylation of Akt kinase. Indeed, there was a 55% decrease (at 60-min time point) in the basal level of phosphorylation of Akt kinase on treatment of C2C12 myoblast with TWEAK (Fig. 7C, middle and lower panels).

DISCUSSION

In this study we demonstrate that the Fn14 receptor is an important determinant of skeletal muscle formation. Suppression of Fn14 expression using RNA interference inhibits several important steps in the myogenic program including the activation of RhoA and SRF, expression of myogenic transcription factors, and formation of multinucleated myotubes indicating that Fn14 receptor is involved in providing the initial signals that commit myoblasts to differentiate into myotubes. Furthermore, our data suggest that although mitogenic effects of TWEAK are mediated through Fn14 receptor, Fn14 may function independent of TWEAK cytokine during myoblast differentiation.

F14 was originally identified by a differential display approach to search for growth factor-inducible molecules in murine NIH3T3 fibroblasts (29). Northern blot studies of multiple tissues revealed that Fn14 mRNA is highly expressed in most of the tissues of newborn mice (29). In adult mice, high expression of Fn14 mRNA was detected in heart and ovary, with only minimal levels in other tissues including skeletal muscle (29). On the other hand, the expression of Fn14 has been reported to increase in response to injury to the rat vessel wall (26), mouse liver (30), and sciatic nerve (32). Additional evi-
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FIGURE 7. Suppression of Fn14 expression inhibits C2C12 myoblast proliferation and the activation of NF-κB and AP-1 in response to TWEAK. A, control or Fn14 siRNA-transfected C2C12 myoblasts were incubated in differentiation medium for 48 h with or without soluble TWEAK (100 ng/ml). At the end of the incubation period, cellular proliferation was measured using alamarBlue dye as described under “Experimental Procedures.” Data presented here show that TWEAK-induced proliferation of C2C12 myoblasts is blunted on Fn14 knockdown. *, p < 0.05, values significantly different from corresponding TWEAK-ununtreated and control siRNA-transfected C2C12 myoblasts. #, p < 0.05, values significantly different from corresponding TWEAK-treated and control siRNA-transfected C2C12 myoblasts. B, control or Fn14 siRNA-transfected C2C12 myoblasts were treated with recombinant TWEAK protein for different time intervals and the activation of NF-κB and AP-1 were measured by EMSA. Data presented here show that knockdown of Fn14 inhibits TWEAK-induced activation of NF-κB and AP-1 in C2C12 myoblasts. C, C2C12 myoblasts were treated with recombinant TWEAK (100 ng/ml) for different lengths of time and the activation of p44-p42 MAPK and Akt kinase was studied by Western blotting using phosphospecific antibody. Representative immunoblots from two independent experiments presented here show that TWEAK increases the phosphorylation of p44-p42 MAPK but inhibits Akt kinase phosphorylation.

Study provides the first experimental evidence that Fn14 receptor is required not only for proliferation but also for the differentiation of myoblasts into myotubes, as demonstrated by an inhibition in the transactivation of skeletal α-actin promoter, expression of muscle-specific proteins MyHCf and CK, and impairment of myotube formation on silencing of Fn14 receptor in myoblasts. Although early events that trigger myogenic differentiation remain poorly understood, the transcription factors of MyoD family (MyoD, Myf-5, myogenin, and MRF4) and the myocyte enhancer binding factor-2 family members are the intrinsic regulators of myogenesis both in vivo and in cell cultures (50–52). Binding of MyoD factors along with other widely expressed basic helix-loop-helix transcription factors to DNA control regions termed E-boxes activates the transcription of many muscle-specific genes (51, 53). Gene knock-out studies in mice have revealed that MyoD and Myf-5 act redundantly at an early step in myoblast specification. Muscle formation was completely inhibited in mice lacking both MyoD and Myf-5 (54). MyoD is also required for the regeneration of adult myofibers in response to injury (55). Acute over-expression of MyoD can readily convert a range of cell types to myoblasts (51, 56) further confirming that MyoD is the central regulator of the myogenic program. Our data demonstrating a significant reduction in the levels of MyoD and myogenin with knockdown of Fn14 receptor in C2C12 myoblasts suggest that one of the mechanisms by which the Fn14 receptor promotes myogenic differentiation is through induction and/or stabilization of MyoD protein during differentiation (Fig. 4, A and B). This argument is supported by our observation that forced expression of the MyoD protein using adenoviral vectors drastically increased the differentiation in Fn14-knockdown C2C12 cultures (Fig. 4C).

SRF is a transcription factor, which binds to a serum response element associated with a variety of genes including immediate early genes such as c-Fos, FosB, and JunB, and muscle genes such as Actin and Myosin (57). SRF activity is essential for the differentiation of myoblasts into myotubes (44). Recent studies have further shown that inactivation of SRF causes inhibition of MyoD expression and skeletal α-actin promoter activity in myoblasts (45, 47, 58). We observed that there was more than a 2-fold increase in the activity of SRF in control myoblasts 48 h after incubation in differentiation medium (Fig. 5A). These data...
are consistent with published reports demonstrating that the SRF expression/activity is increased during myogenic differentiation (47). Interestingly, there was no such increase in the activity of SRF transcription factor in Fn14-knockdown C2C12 myoblasts after induction of differentiation (Fig. 5A). Because both MyoD and skeletal α-actin genes contain consensus serum response elements in their promoter/enhancer region, an inhibition in the SRF activity might be responsible, at least in part, for the reduced expression of MyoD, skeletal α-actin, and the differentiation of myoblasts into myotubes upon silencing of Fn14 receptor.

Another interesting observation of the present study was that Rho family GTPases RhoA and Rac-1 physically interact with Fn14 receptor in myoblasts (Fig. 5B). Accumulating evidence suggests that Rho GTPases especially RhoA play an essential role during myogenic differentiation. Inhibition of RhoA activity through overexpression of a dominant negative mutant of RhoA (N19-RhoA) in C2C12 myoblasts inhibited the expression of muscle-specific genes and their differentiation into myotubes (47, 59). Furthermore, RhoA is also required for the activation of SRF during myogenic differentiation (60). Thus, our results demonstrating that RhoA interacts with the Fn14 receptor in myoblasts (Fig. 5B) and knockdown of Fn14 inhibits the levels of activated RhoA in C2C12 myoblasts (Fig. 5C) suggest that RhoA might be an important component of the signaling pathway that links the Fn14 receptor to downstream activation of SRF and expression of muscle-specific genes during myogenic differentiation.

Recently, TWEAK was identified to be a ligand for Fn14 (26). TWEAK mRNA has been detected in a number of tissues including skeletal muscle (61). Polek et al. (31) have examined the effect of TWEAK on differentiation of a murine monocyte cell line that does not display Fn14. Under the influence of TWEAK, the cells acquired the features of osteoclasts suggesting that Rho GTPases especially RhoA play an essential role during myogenic differentiation. Inhibition of RhoA activity through overexpression of a dominant negative mutant of RhoA (N19-RhoA) in C2C12 myoblasts inhibited the expression of muscle-specific genes and their differentiation into myotubes (47, 59). Altotropic RhoA is required for the activation of SRF during myogenic differentiation (60). Thus, our results demonstrating that RhoA interacts with the Fn14 receptor in myoblasts (Fig. 5B) and knockdown of Fn14 inhibits the levels of activated RhoA in C2C12 myoblasts (Fig. 5C) suggest that RhoA might be an important component of the signaling pathway that links the Fn14 receptor to downstream activation of SRF and expression of muscle-specific genes during myogenic differentiation.

How Fn14 regulates both proliferation and differentiation in myoblasts remains enigmatic. Based on the results of this study, it appears that the major role of Fn14 is to facilitate myogenic differentiation by activating promyogenic-signaling pathways such as phosphatidylinositol 3-kinase/Akt. However, when the level of TWEAK is increased, binding of Fn14 to TWEAK induces myoblast proliferation, possibly by activating downstream mitogenic signaling pathways. This is supported by our data that demonstrate that treatment of C2C12 myoblasts with TWEAK activates transcription factors AP-1 and NF-κB (Fig. 7B), and p44-p42 MAPK (Fig. 7C, upper panel), which are involved in proliferation, but not Akt kinase (Fig. 7C, lower panel), which promotes myogenic differentiation through the activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. Indeed, TWEAK independent effects of Fn14 have been observed in other biological systems (32, 62).

Certainly, more investigations are required to further delineate the mechanisms through which Fn14 regulates skeletal muscle formation. Because Fn14 and its ligand TWEAK belong to the TNF receptor and TNF cytokine superfamly, respectively (27), Fn14/TWEAK may represent important members of a larger group of receptor-cytokine networks involved in formation and/or regeneration of skeletal muscle in response to injury. While our manuscript was in initial review, another group published an article demonstrating defects in differentiation of myoblasts from Fn14 knock-out mice and the regeneration of skeletal muscle in response to cardiototoxin injury was delayed in Fn14 knock-out mice (63). Taken together our data in this study and recently published report (63) provide strong evidence that this receptor-ligand dyad is crucial for the genesis of skeletal muscle.

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