Proinflammatory Cytokine Tumor Necrosis Factor (TNF)-like Weak Inducer of Apoptosis (TWEAK) Suppresses Satellite Cell Self-renewal through Inversely Modulating Notch and NF-κB Signaling Pathways*

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Background: TNF-like weak inducer of apoptosis (TWEAK) cytokine is an important regulator of inflammation and skeletal muscle mass. Results: TWEAK represses Pax7 expression and Notch signaling and activates NF-κB in satellite cells. Conclusion: TWEAK suppresses satellite cell self-renewal through reciprocally regulating Notch and NF-κB signaling. Significance: Inhibition of TWEAK can be used as a therapeutic approach to maintain the satellite cell pool in skeletal muscle.

Satellite cell self-renewal is an essential process to maintaining the robustness of skeletal muscle regenerative capacity. However, extrinsic factors that regulate self-renewal of satellite cells are not well understood. Here, we demonstrate that TWEAK cytokine reduces the proportion of Pax7+ satellite cells (an index of self-renewal) on myofiber explants and represses multiple components of Notch signaling in satellite cell cultures. The number of Pax7+ cells is significantly increased in skeletal muscle of TWEAK knock-out (KO) mice compared with wild-type in response to injury. Furthermore, Notch signaling is significantly elevated in cultured satellite cells and in regenerating myofibers of TWEAK KO mice. Forced activation of Notch signaling through overexpression of the Notch 1 intracellular domain (N1ICD) rescued the TWEAK-mediated inhibition of satellite cell self-renewal. TWEAK also activates the NF-κB transcription factor in satellite cells and inhibition of NF-κB significantly improved the number of Pax7+ cells in TWEAK-treated cultures. Furthermore, our results demonstrate that a reciprocal interaction between NF-κB and Notch signaling governs the inhibitory effect of TWEAK on satellite cell self-renewal. Collectively, our study demonstrates that TWEAK suppresses satellite cell self-renewal through activating NF-κB and repressing Notch signaling.

Satellite cells are skeletal muscle resident stem cells that are located between the basal lamina and sarcolemma in a mitotically quiescent state (1). These cells are responsible for growth, maintenance, and repair of adult myofibers (2, 3). Upon injury, satellite cells are rapidly activated to reenter the cell cycle, undergo several rounds of proliferation, and differentiate into myocytes that eventually fuse with each other or pre-existing myofibers to complete the repair (4, 5). Although the majority of the activated satellite cells differentiate into myogenic lineage, a portion of them self-renew and return to the quiescent state to respond to the next round of muscle injury and repair (4, 5). The paired-box transcription factor Pax7 is specifically expressed in satellite cells (4–7). Pax7 determines satellite cell fate in cooperation with other myogenic regulatory factors such as MyoD (7, 8). Pax7 maintains satellite cells in a quiescent state (7). By contrast, MyoD stimulates myogenesis through promoting the expression of muscle-specific genes (9, 10) and impeding p21/WAF1 (also known as cyclin-dependent kinase inhibitor 1) (11, 12). Therefore, the expression pattern of Pax7 and MyoD specifies the myogenic status of satellite cells as quiescent (Pax7+/MyoD−), activated (Pax7+/MyoD+), or differentiated (Pax7−/MyoD+) (6, 13, 14). Moreover, activated satellite cells (Pax7+/MyoD+) must repress MyoD and maintain Pax7 to promote self-renewal (Pax7+/MyoD−) (4, 6, 14).

Notch signaling is a key pathway involved in embryonic myogenesis, adult myofiber regeneration, and regulation of satellite cell fate (15, 16). Notch signaling is initiated when a Notch ligand such as Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3, or DLL4 binds to the extracellular domain of a transmembrane cell surface Notch receptor (Notch1–4) on neighboring cell (17). These ligand-receptor interactions lead to proteolytic cleavage of the Notch receptors via the γ-secretase complex, releasing the Notch intracellular domain, which translocates to the nucleus and binds the transcriptional repressor, RBP-Jκ, converting it into an activator and inducing the expression of downstream target genes (17, 18). Some of the most well-defined RBP-Jκ-dependent Notch target genes include specific members of the Hes/Hey family such as Hes1, Hes5, Hes6, Hey1, Hey2, and Heyl (19). Although the molecular machinery involved in the regulation of satellite cell self-renewal remains less understood, several lines of evidence suggest that Notch signaling plays a critical role in this process (3, 20–22). Over-

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expression of Notch 1 intracellular domain (N1ICD)$^2$ inhibits satellite cell proliferation and differentiation while increasing their self-renewal on cultured myofibers (13). Activation of the Notch pathway also inhibits differentiation through repressing the levels of MyoD (23). Moreover, a γ-secretase inhibitor reduced the proportion of Pax7$^+/\text{MyoD}^-$ cells and increased Pax7$^-$/MyoD$^+$ cells on cultured myofibers (14, 22). Furthermore, inactivation of RBP-Jk through a genetic approach dramatically reduced the proportion of Pax7$^+$ cells by spontaneously increasing terminally differentiated cells in both normal and injured skeletal muscle of mice (24, 25). Altogether, these observations suggest that the activation of Notch signaling promotes satellite cell self-renewal through augmenting Pax7 expression and repressing MyoD levels. Although intracellular pathways involved in regulation of satellite cell fate have been somewhat elucidated, extrinsic signals that govern satellite cell fate determination remain poorly understood.

Proinflammatory cytokines are some of the important extraacellular cues that affect the proliferation and differentiation of myoblasts (26, 27). However, their role in satellite cell self-renewal has not been yet investigated. Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a multifunctional proinflammatory cytokine belonging to a TNF superfamily (28, 29). TWEAK acts on the target cells through binding to fibroblast growth factor induced 14 (Fn14) receptor, a member of the TNF receptor superfamily (28). Accumulating evidence suggests that TWEAK-Fn14 signaling plays an important role in the acquisition and maintenance of skeletal muscle mass (29). TWEAK and Fn14 are expressed by a variety of cell types including satellite cells and myoblasts (30, 31). Although addition of TWEAK protein induces proliferation, it inhibits differentiation of cultured myoblast into multinucleated myotubes (31, 32). Levels of both TWEAK and Fn14 are increased in response to myoblast growth factor induced 14 (Fn14) receptor, a member of the TNF receptor superfamily (28). Accumulating evidence suggests that TWEAK-Fn14 signaling plays an important role in the acquisition and maintenance of skeletal muscle mass (29). TWEAK and Fn14 are expressed by a variety of cell types including satellite cells and myoblasts (30, 31). Although addition of TWEAK protein induces proliferation, it inhibits differentiation of cultured myoblast into multinucleated myotubes (31, 32). Levels of both TWEAK and Fn14 are increased in response to injury (31, 33). Moreover, it has been found that muscle-specific transgenic overexpression of physiological levels of TWEAK inhibits skeletal muscle regeneration after cardiotoxin (CTX)-mediated injury (33). Conversely, skeletal muscle regeneration is improved in TWEAK-KO mice upon injury (33).

One of the important mechanisms by which TWEAK regulates myogenesis is through activation of transcription factor nuclear factor-κB (NF-κB). The NF-κB family contains five members: RelA (also known as p65), RelB, c-Rel, p105/p50, and p100/p52, which make homo- and heterodimers (26, 34). Depending on the type of stimuli, the activation of NF-κB occurs through canonical or non-canonical signaling pathways. Canonical NF-κB signaling involves the upstream activation of inhibitors of κB (IκB) kinase-β (IKKβ) and subsequent phosphorylation and degradation of the IκB protein. By contrast, activation of the non-canonical NF-κB pathway requires the activation of NF-κB-inducing kinase and IκKα leading to phosphorylation and proteolytic processing of the p100 subunit into p52 (34). Although the role of the non-canonical pathway has not yet been investigated using genetic mouse models, several studies have suggested that activation of canonical NF-κB signaling inhibits regenerative myogenesis (35, 36). We have previously reported that high amounts of TWEAK inhibit myogenic differentiation through the activation of canonical NF-κB signaling and reducing stability of the MyoD protein (32). Furthermore, the activation of NF-κB is increased in regenerating myoblasts of TWEAK-Tg mice and reduced in TWEAK-KO mice suggesting that TWEAK mediates NF-κB activation in injured myofibers (33). A recent study has also demonstrated that TWEAK represses the expression of Notch1 in differentiated myotubes (37). Importantly, cross-talk between NF-κB and Notch signaling has been implicated in regulation of various cellular responses such as proliferation, differentiation, and apoptosis (38). Despite these observations, the role of TWEAK in regulation of satellite cell fate and potential interplay between Notch and NF-κB has not yet been investigated.

In the present study, we have investigated the role and the mechanisms by which TWEAK regulates satellite cell self-renewal. Our results demonstrate that TWEAK inhibits satellite cell self-renewal through repressing the expression of the components of Notch signaling pathways. Furthermore, our study demonstrates that reciprocal interaction between Notch and NF-κB signaling regulates satellite cell self-renewal in response to TWEAK.

**EXPERIMENTAL PROCEDURES**

**Animals**—TWEAK-KO mice were provided by Dr. Avi Ashkenazi (Genentech, South San Francisco, CA) and have been previously described (39). C57BL/6 were purchased from Jackson Laboratory (Bar Harbor, ME). To induce the necrosis-regeneration response in skeletal muscle, at the age of 8 weeks, 100 μL of 10 μM cardiotoxin (Sigma) dissolved in phosphate-buffered saline (PBS) was injected into the TA muscle of wild-type and TWEAK-KO mice. After 5 days, the tibial anterior (TA) muscle was collected from euthanized mice for histological and biochemical studies. All experimental protocols with mice were approved in advance by the Institutional Animal Care and Use Committee of the University of Louisville.

**Isolation, Culturing, and Immunostaining of Single Myofibers**—Single myofibers were isolated from the extensor digitorum longus muscles after digestion with collagenase type I (catalog number LS004196; Worthington, Lakewood, NJ) and trituration as previously described (22). Suspended fibers were cultured in horse serum-coated 6-well plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2% chicken embryo extract (Accurate Chemical, Westbury, NY), and 1% penicillin-streptomycin for 72 h. Cultured fibers were then fixed in 4% paraformaldehyde and stained for Pax7 (1:20, Developmental Studies Hybridoma Bank) and MyoD (1:200, Santa Cruz Biotechnology, Inc.).

**Primary Satellite Cell Culture**—Satellite cells were isolated from the hind limbs of 8-week-old mice following a protocol as described (22). Briefly, mice were sacrificed and TA and gastro-
cnemius muscles were isolated. Excess connective tissues and fat were cleaned in sterile PBS followed by mincing of skeletal muscle in DMEM and enzymatic dissociation with 0.1% Pronase. The digested slurry was spun, pelleted, and triturated several times and then passed through a 70-µm cell strainer (BD Falcon). The filtrate was spun at 1000 × g and resuspended in myoblast growth medium (Ham’s F-10 medium with 20% FBS supplemented with 5 ng/ml of basic fibroblast growth factor). Cells were first re-fed after 3 days of initial plating. During the first few passages, cells were also enriched by pre-plating for selection of pure myoblast population. Upon selection, the cells were cultured in a 1:1 ratio of myoblast growth medium and growth medium (DMEM with 20% FBS) until 80% confluence was reached.

**Cell Proliferation Assay**—Satellite cell proliferation was assayed using Click-it® EdU Cell Proliferation Assay kit (Invitrogen). In brief, satellite cells were seeded on 24-well cell culture plates coated with 10% Matrigel. After 24 h, the cells were treated with 10 ng/ml of TWEAK protein for 24 h. 5-Ethynyl-2′-deoxyuridine (10 μM, Invitrogen) was added in culture medium for the final 0, 0.5, 1, and 2 h. Cells were then fixed with 3.7% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100. The 5-ethyl-2′-deoxyuridine+ cells were identified using Click-IT reaction mixture. Nuclei were counterstained with 1× Hoechst 33342 for 30 min at room temperature. Images were visualized on Nikon Eclipse TE 2000-U microscope (Nikon), a digital camera (Nikon Digital Sight DS-Fi1), and Nikon NIS Elements BR 3.00 software (Nikon).

**Immunocytochemistry**—Cultured primary satellite cells were fixed by 4% paraformaldehyde, blocked in 1% bovine serum albumin (BSA) in PBS for 1 h, and incubated with anti-Pax7 (1:20, Developmental Studies Hybridoma Bank) and anti-MyoD (1:150, Santa Cruz Biotechnology) in blocking solution at overnight at 4 °C under humidified conditions. The cells were washed briefly with PBS before incubation with Alexa Fluor 488- or 546-conjugated secondary antibody (1:1000, Invitrogen) for 1 h at room temperature and then washed 3 times for 5 min with PBS. The cells were then stained with DAPI for nuclei detection. The cells were cultured in a 1:1 ratio of myoblast growth medium and growth medium (DMEM with 20% FBS) until 80% confluence was reached.

**Gene Transfer by Electroporation**—To overexpress specific proteins in primary satellite cells, plasmid DNA was introduced into cells by electroporation (1500 V, 10 ms for duration, 3 pulses) using the Neon transfection system following a protocol suggested by the manufacturer (Invitrogen). After the electroporation, cells were allowed to recover in F-10 medium with 20% FBS for 48 h followed by treatment with soluble mouse TWEAK protein (R&D Systems).

**Electrophoretic Mobility Shift Assay (EMSA)**—DNA binding of NF-κB was measured by performing EMSA as described (40). Briefly, 20 μg of nuclear extracts prepared from satellite cells were incubated with 16 fmol of 32P-end-labeled NF-κB consens-sus oligonucleotide (Promega) at 37 °C for 30 min, and the DNA-protein complex was resolved on a 7.5% native polyacryl-amide gel. The radioactive bands from the dried gel were visual-ized and quantified by PhosphorImager (GE Healthcare) using ImageQuant TL software.

**Quantitative Real-Time PCR (QRT-PCR)**—RNA isolation and QRT-PCR were performed using a method as described in Ref. 40. Briefly, total RNA was isolated using the TRIzol reagent (Invitrogen) and an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturers’ protocols. First strand cDNA for PCR analyses were made using a commercially available kit (Applied Biosystems, Grand Island, NY). The quantification of mRNA expression was performed using the SYBR Green dye (Applied Biosystems) method on a sequence detection system (Applied Biosystems, model 7300). Primers were designed using Vector NTI software (Invitrogen). Primer sequence has been previously described (22). Data normalization was accomplished using the endogenous control (β-actin), and the nor-malized values were subjected to a 2−ΔΔCt formula to calculate the fold-change between control and experimental groups.

**Western Blot**—Skeletal muscle tissues or cultured cells were washed with PBS and homogenized in lysis buffer (50 mM Tris-Cl (pH 8.0), 200 mM NaCl, 50 mM NaF, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 0.3% IGEPA, and protease inhibitors). Approximately 100 μg of protein was resolved on each lane on 10 or 12% SDS-PAGE, electrotransferred onto nitrocellulose or polyvinylidene difluoride membrane, and probed using anti-Pax7 (1:400, Developmental Studies Hybridoma Bank), anti-cleaved Notch1 (1:500, Cell Signaling Technology), anti-Notch1 (1:500, Santa Cruz Biotechnology), anti-Hes6 (1:1000, Santa Cruz Biotechnology), anti-MyoD1 (1:5000, Santa Cruz Biotechnology) and detected by enhanced chemiluminescence. For loading controls, the membranes were stripped and reprobed with anti-α-tubulin (1:3000, Cell Signaling Technology).

**Statistical Analysis**—All results are expressed as mean ± S.D. Data were analyzed by t test with Bonferroni correction. p < 0.05 was considered statistically significant.

**RESULTS**

**TWEAK Inhibits Satellite Cells Self-renewal**—Suspension culture of myofiber explants represents an *ex vivo* model that
TWEAK Regulates Satellite Cell Self-renewal

FIGURE 1. TWEAK inhibits self-renewal of satellite cells on cultured myofibers. Single myofiber cultures were established from extensor digitorum longus muscle of WT mice and treated with TWEAK (10 ng/ml). After 72 h, satellite cell-myoblast clusters (containing >4 cells) on single myofibers were labeled with antibodies against Pax7 and MyoD. Nuclei were counterstained with DAPI. Representative merged images of Pax7-, MyoD-, and DAPI-stained cells on myofibers in control (A) and TWEAK-treated cultures (B) are presented. The percentage of Pax7+/MyoD- cells (C) per cluster and MyoD+ cells (D) per cluster in untreated and TWEAK-treated myofiber cultures are indicated. Quantitative analysis of the percentage of self-renewing (Pax7+/MyoD-)(E), proliferating (Pax7+/MyoD+)(F), and differentiating (Pax7-/MyoD+)(G) satellite cells/myoblasts on myofibers in untreated and TWEAK-treated myofiber cultures, n = 4 in each group. Error bars represent S.D. *p < 0.05 values significantly different from untreated cultures.

TWEAK mimics muscle injury in vivo with respect to satellite cell activation, proliferation, and differentiation (14). Upon isolation, each myofiber is associated with a fixed number of satellite cells (Pax7+/MyoD-) resting in quiescence. At around 24 h in culture, satellite cells undergo their first round of cell division, through up-regulating MyoD (Pax7+/MyoD+) and proliferating to form cell aggregates. Cells then either terminally differentiate (Pax7-/MyoD+) or self-renew (Pax7+/MyoD-) (14, 22). We first employed myofiber cultures to study the effects of TWEAK on satellite cell self-renewal. Single myofiber were isolated from extensor digitorum longus muscle of WT mice and cultured. Myofibers were treated with recombinant TWEAK protein (10 ng/ml) for 72 h followed by performing immunostaining to detect Pax7+ and MyoD+ cells (Fig. 1, A and B). Quantitative analysis of cells on myofibers showed that TWEAK significantly reduced the percentage of total Pax7+, and the fractions of Pax7+/MyoD- and Pax7+/MyoD+ cells per cluster (Fig. 1, C, E, and F). Although TWEAK did not affect the total number of MyoD+ cells (Fig. 1D), the proportion of Pax7−/MyoD+ cells per cluster was significantly higher in TWEAK-treated cultures compared with untreated controls (Fig. 1G).

In a separate experiment, we also studied the effects of TWEAK on self-renewal of satellite cells in myofiber-free cultures. Freshly isolated satellite cells from hind limb muscle of WT mice were treated with 10 ng/ml of TWEAK for 24 h followed by performing double immunostaining for Pax7 and MyoD. Results showed that TWEAK significantly reduced the proportion of Pax7+ cells without affecting the count of MyoD+ cells (Fig. 2, A and B). We also studied the effects of TWEAK on mRNA and protein levels of Pax7 by performing a QRT-PCR assay and Western blot, respectively. Consistent with immunocytochemistry results, treatment with TWEAK considerably reduced mRNA and protein levels of Pax7 without affecting the levels of MyoD in satellite cell cultures (Fig. 2, C–E). By performing pulse labeling with 5-ethyl-2′-deoxyuridine, we also investigated whether 10 ng/ml of TWEAK affects the proliferation of cultured satellite cells. However, there was no significant difference in satellite cell proliferation in control and TWEAK-treated cultures (data not shown). Taken together, these results demonstrate that TWEAK inhibits satellite cell self-renewal without affecting their proliferation.

TWEAK Inhibits Notch Signaling in Satellite Cells—Recent reports suggest that the expression of Pax7, at least in part, is regulated through the activation of Notch signaling (13, 24, 25). However, it remains unknown whether TWEAK affects the activation of Notch signaling in satellite cells. To understand the mechanisms by which TWEAK inhibits satellite cell self-renewal, we next evaluated whether TWEAK regulates Notch signaling in satellite cells. Freshly isolated satellite cells from hind limb muscle of wild-type mice were treated with 10 ng/ml of TWEAK for 24 h followed by measuring the transcript levels of the Notch-related genes by performing QRT-PCR assay. Interestingly, TWEAK significantly reduced the mRNA levels of Notch receptors Notch1 and Notch3, ligands Jagged2 and Dll1, and target genes Hey1, Heyl, and Hes6 in satellite cells (Fig. 3, A–C). We also performed Western blot analysis to study the effects of TWEAK on Notch pathways. Consistent with QRT-PCR results, we found that TWEAK reduced the pro-
TWEAK Regulates Satellite Cell Self-renewal

To further understand the relationship between TWEAK and Notch signaling, we compared the transcript levels of several Notch-related genes in satellite cells prepared from wild-type and TWEAK-KO mice under naive conditions. As shown in Fig. 3F, the mRNA levels of Pax7, Notch3, Jagged2, Dll1, and Hes6 were significantly higher in satellite cells of TWEAK-KO mice compared with WT mice. Furthermore, mRNA levels of Pax7 were significantly higher in TWEAK-KO cultures compared with WT cultures (Fig. 3F). Taken together, these results support the premise that TWEAK inhibits Notch signaling in satellite cells.

Increased Number of Pax7⁺ Cells and Notch Signaling in Regenerating Myofibers of TWEAK-KO Mice Upon Injury—We have previously reported that TWEAK regulates muscle regeneration in response to CTX-mediated injury (33). However, it remains unknown whether TWEAK also affects the number of resident satellite cells in regenerating skeletal muscle. We next evaluated the in vivo role of TWEAK on the number of satellite cells and the expression of the components of Notch signaling in regenerating myofibers. TA muscle of 8-week-old wild-type and TWEAK-KO mice were given intramuscular injection of CTX. After 5 days, the TA muscle was isolated and processed for immunostaining for Pax7 and laminin. Interestingly, the number of Pax7⁺ cells was found to be significantly increased in CTX-injected TA muscle of TWEAK-KO mice compared with WT mice (Fig. 4A and B). To further understand the role of TWEAK in satellite cell self-renewal, we also established suspension cultures of myofibers from TWEAK-KO mice and their corresponding wild-type mice. After 72 h of culturing, myofiber-associated satellite cells/myoblasts were immunostained for Pax7 and examined under a fluorescence microscope. As shown in Fig. 4C, the proportion of Pax7⁺ cells per cluster was significantly increased in myofiber cultures of TWEAK-KO mice compared with wild-type mice. Furthermore, QRT-PCR analysis showed that mRNA levels of Pax7, Notch3, Jagged1, and Heyl were significantly higher in CTX-injected TA muscle of TWEAK-KO mice compared with WT mice (Fig. 4D). These results are suggestive that TWEAK also inhibits the expression of Pax7 and Notch signaling in satellite cells in vivo.

Overexpression of N1ICD Rescues TWEAK-mediated Repression of Satellite Cell Self-renewal—After establishing that TWEAK inhibits Pax7 expression and Notch signaling, we next sought to investigate whether TWEAK functions through Notch to regulate the levels of Pax7. Primary satellite cells prepared from WT mice were transfected (through electroporation) with vector alone or a N1ICD-expressing plasmid. After 48 h of transfection, the cells were treated with TWEAK (10 ng/ml) for 24 h followed by immunostaining for Pax7 and DAPI. Results showed that TWEAK significantly reduced the proportion of Pax7⁺ cells in cultures transfected with vector alone (Fig. 5A and B). By contrast, TWEAK failed to reduce the proportion of Pax7⁺ cells in N1ICD-transfected cultures (Fig. 5A and B). Moreover, there was no significant difference in the percentage of MyoD⁺ cells in control and N1ICD-expressing cultures in response to TWEAK treatment (data not shown). We also performed Western blot to measure the levels of Pax7 protein in N1ICD-transfected cells. As shown in Fig. 5C, TWEAK reduced the levels of Pax7 protein in satellite cell cul-

FIGURE 2. TWEAK represses Pax7 levels in cultured satellite cells. Primary satellite cells/myoblasts were isolated from WT mice. After purification, the cells were treated with 10 ng/ml of TWEAK for 24 h. A, representative photomicrographs of satellite cells after staining with antibodies against Pax7 and MyoD. Nuclei were counterstained with DAPI. Scale bar: 50 μm. B, percentage of Pax7⁺ and MyoD⁺ cells in control and TWEAK-treated cultures. n = 4 in each group. C, relative mRNA levels of Pax7 and MyoD in satellite cell cultures after 24 h of TWEAK (10 ng/ml) treatment. n = 4 in each group. D, satellite cells were treated with 10 ng/ml of TWEAK for the indicated times followed by immunoblotting to detect protein levels of Pax7, MyoD, and tubulin. Representative immunoblots are presented here. E, densitometry quantification of the percentage decrease in Pax7 protein levels in response to TWEAK treatment in three independent experiments. Error bars represent S.D., *p < 0.05, values significantly different from untreated cells.
TWEAK Regulates Satellite Cell Self-renewal

**FIGURE 3.** TWEAK inhibits the expression of Notch-related molecules in satellite cell/myoblast cultures. Primary satellite cells prepared from WT mice were treated with 10 ng/ml of TWEAK for 24 h. Total RNA from untreated and TWEAK-treated cells was extracted and processed to measure mRNA levels of: A, Notch receptors: Notch1, Notch2, Notch3; B, Notch ligands: Jagged1, Jagged2, Dll1; and C, Notch target genes: Hey1, Heyl, and Hes6 by performing QRT-PCR. n = 3–4 in each group. Error bars represent S.D. *, p < 0.05 values significantly different from TWEAK-untreated cells. D, Western blot analysis of Notch1, N1ICD, and Hes6 proteins in control and 24-h TWEAK-treated satellite cells. E, densitometry quantification of immunoblots for Notch1 protein levels from four independent experiments. F, relative mRNA levels of Pax7, Notch1, Notch3, Jagged2, Dll1, and Hes6 in primary satellite cell/myoblast cultures measured by QRT-PCR assay. n = 3–4 in each group. Error bars represent S.D. *, p < 0.05, values significantly different from WT cells.

**FIGURE 4.** Genetic ablation of TWEAK improves the number of Pax7+ cells and Notch signaling in cardiotoxin-injected TA muscle of mice. TA muscle of 3-month-old WT and TWEAK-KO mice were injected with 100 μl of cardiotoxin (CTX, 10 μM) and 5 days later the muscle was isolated and processed for immunostaining for Pax7 and laminin. Nuclei were identified by counterstaining with DAPI. Representative photomicrographs presented here demonstrate increased immunostaining for Pax7 in CTX-injected TA muscles of TWEAK-KO mice compared with WT mice. B, quantification of the number of Pax7+ cells per myofiber in TA muscle of WT (n = 3) and TWEAK-KO mice (n = 5) 5 days post-CTX injection. C, single myofiber cultures were prepared from WT and TWEAK-KO mice and immunostained for Pax7 and MyoD and the percentage of Pax7+ cells per cluster was calculated (n = 8 in each). D, relative mRNA levels of Pax7, Notch1, Notch2, Notch3, Jagged1, Dll1, Hey1, Heyl, and Hes6 in 5-day CTX-injected TA muscle of WT and TWEAK-KO mice measured by QRT-PCR assay. n = 3–4 in each group. Error bars represent S.D. *, p < 0.05, values significantly different from WT mice.

However, the levels of Pax7 protein were significantly higher in N1ICD-transfected cells and TWEAK only modestly reduced levels of Pax7 protein in these cells (Fig. 5, C and D). Our Western blot analysis also confirmed increased expression of N1ICD protein in transfected cells (Fig. 5C). These results suggest that TWEAK inhibits the expression of Pax7 in satellite cells through repressing Notch signaling.

**TWEAK Inhibits Pax7 Expression in Satellite Cells through Activation of NF-κB Signaling**—TWEAK has been reported to activate NF-κB in cultured myoblasts (30, 32, 41). However, it remains unknown whether TWEAK-mediated repression of...
satellite cell self-renewal also involves NF-κB. We first sought to determine whether a low concentration of TWEAK (i.e., 10 ng/ml), which inhibits Pax7 levels, is sufficient to activate NF-κB in satellite cells. Cultured satellite cells were treated with TWEAK for different time periods ranging from 1 to 24 h and the nuclear extracts made were subjected to EMSA to measure the DNA-binding activity of NF-κB. Time course analysis revealed that the NF-κB/DNA-binding activity was increased as early as 1 h after TWEAK treatment and remained elevated even at 24 h (Fig. 6A). These results are consistent with previous reports that TWEAK causes long lasting activation of NF-κB in cultured cells (32, 42). To understand the role of NF-κB in TWEAK-mediated suppression of Pax7, we transfected satellite cells with vector alone or a super-repressor mutant of IκBα (IκBoSR, a dominant-negative inhibitor of canonical NF-κB signaling). After 48 h, the cells were treated with TWEAK (10 ng/ml) for 24 h and immunostained for Pax7. TWEAK significantly reduced the number of Pax7+ cells in the cultures transfected with vector alone. Interestingly, the percentage of Pax7+ cells was significantly higher in IκBoSR-expressing cultures compared with those transfected with vector alone upon treatment with TWEAK (Fig. 6, B and C). Furthermore, Western blot analysis showed that TWEAK represses Pax7 levels in cells transfected with vector alone at both 12 and 24 h (Fig. 6, D and E). TWEAK also reduced the levels of Pax7 in IκBoSR-expressing cells at 12 h. However, the protein levels of Pax7 were significantly higher in IκBoSR-expressing cells compared with corresponding vector-alone transfected cells 24 h after TWEAK-treatment (Fig. 6, D and E). Overexpression of the IκBoSR protein did not affect the number of MyoD+ cells in control or TWEAK-treated cultures (data not shown). These results suggest that TWEAK reduces Pax7 levels in satellite cells through the activation of NF-κB.

Reciprocal Interaction between NF-κB and Notch Signaling in TWEAK-treated Satellite Cells—Our results showed that TWEAK inhibits the expression of Pax7 through repressing Notch signaling and activating NF-κB. However, it remains unknown whether there is any regulatory interaction between NF-κB and Notch signaling in TWEAK-treated satellite cells. To address this issue, we first studied whether blocking the activation of NF-κB can modulate the inhibitory effects of TWEAK on the expression of Notch-related molecules. Results showed that overexpression of IκBoSR significantly improved mRNA levels of Notch1, Notch3, DLL1, Heyl, and Hey1 in TWEAK-treated satellite cells (Fig. 7A). We next examined whether overexpression of N1ICD can affect the activation of NF-κB in response to TWEAK. As shown in Fig. 7B, overexpression of N1ICD inhibited the TWEAK-induced activation of NF-κB in satellite cells. Taken together, these results support the existence of a reciprocal interaction between NF-κB and Notch in TWEAK-treated satellite cells.

DISCUSSION

Satellite cells are primarily a quiescent cell population, dividing infrequently under normal conditions (2). However, they are rapidly activated in response to exercise, muscle injury, or exercise, muscle injury, or conditions that induce satellite cell activation. TWEAK is a member of the TNF family and is rapidly induced in muscle in response to exercise, muscle injury, or inflammation. TWEAK has been shown to play a role in muscle regeneration by promoting satellite cell activation and proliferation. In this study, we demonstrate that TWEAK represses satellite cell self-renewal through the activation of NF-κB and Notch signaling. The results suggest that TWEAK-induced activation of NF-κB and Notch signaling is a critical mechanism for the repression of satellite cell self-renewal.
degenerative muscle disorder, when the generation of fusion-competent myoblasts is required to support growth, repair, and regeneration (4, 5, 14). Replenishment of satellite cell population by self-renewal is pivotal for skeletal muscle homeostasis (2, 21). Defects in the process of self-renewal can compromise the muscle regenerative capacity after repeated injuries and may eventually lead to muscle wasting similar to that observed during aging (43) and in various genetic muscle disorders (44). Although endogenous properties of satellite cells provides its heterogeneity (4, 5, 14), accumulating evidence suggests that

FIGURE 6. TWEAK inhibits Pax7 expression in satellite cells through activation of NF-κB transcription factor. A, satellite cells/myoblasts prepared from WT mice were treated with 10 ng/ml of TWEAK for the indicated time periods and nuclear extracts made were subjected to EMSA to measure DNA-binding activity of NF-κB. A representative EMSA gel is presented. B, satellite cells/myoblasts were transfected with vector alone (pcDNA3) or a super-repressor mutant of IκBoα (IκBoSR)-expressing plasmid (pCMV-FLAG-IκBoSR). After 48 h, the cells were treated with TWEAK (10 ng/ml) for 24 h and immunostained for Pax7. Nuclei were stained with DAPI. Representative photomicrographs are presented. Scale bar: 50 μm. C, percentage of Pax7+ cells in vector- and IκBoSR-transfected cells after treatment with 10 ng/ml of TWEAK for 24 h. n = 5–6 in each group. D, primary satellite cells/myoblasts were transfected with vector alone or IκBoSR-expressing plasmid for 48 h. The cells were then treated with 10 ng/ml of TWEAK for 12 and 24 h followed by performing Western blot to detect Pax7, FLAG-IκBoAA (IκBoSR), and unrelated protein tubulin. E, densitometry quantification of changes in Pax7 protein levels upon treatment with TWEAK. Error bars represent S.D. *, p < 0.05 values significantly different from TWEAK-untreated cells transfected with vector alone. #, p < 0.05 values significantly different from TWEAK-treated cells transfected with vector alone.

FIGURE 7. Reciprocal interaction between NF-κB and Notch signaling in TWEAK-treated satellite cells. A, primary satellite cells prepared from WT mice were transfected with vector alone or IκBoSR-expressing plasmid for 48 h. The cells were treated with 10 ng/ml of TWEAK protein for 24 h and relative mRNA levels of Notch1, Notch3, DLL1, Hey1, and Hey2 were measured by performing QRT-PCR assay. n = 3–4 in each group. Error bars represent S.D. *, p < 0.05 values significantly different from control cells. #, p < 0.05 values significantly different from TWEAK-treated cells transfected with vector alone. B, primary satellite cells prepared from WT mice were transfected with vector alone or a N1ICD-expressing plasmid. After 48 h, the cells were treated with 10 ng/ml of TWEAK for 12 and 24 h and the DNA-binding activity of NF-κB was measured by performing EMSA. A representative EMSA gel is presented here.
the satellite cell niche plays an important role in satellite cell fate determination (5, 21, 45, 46). Among other cell types, TWEAK is also expressed by myogenic lineage cells (30, 31). Because the expression of TWEAK is increased in injured myofibers (33), TWEAK appears to be a bona fide candidate that can regulate satellite cell fate. Results of the present study provide experimental evidence that TWEAK inhibits satellite cell self-renewal. These results are consistent with our previously published reports that transient overexpression of TWEAK in skeletal muscle causes progressive muscle wasting (47) and attenuates myofiber regeneration in response to cardiotoxin-mediated injury (33).

The transcription factor Pax7 is a critical regulator for satellite cell biogenesis, survival, specification, and self-renewal. Skeletal muscle of Pax7-null mice lack satellite cells and show reduced muscle growth, and defects in regenerative response after injury (48, 49). Furthermore, quiescent satellite cells express high levels of Pax7 where the expression of other myogenic regulatory factors such as Myf5 and MyoD is undetectable. In proliferating satellite cells, Pax7 persist at lower levels, whereas expression of Pax7 is completely repressed in myogenic lineage cells that commit to terminal differentiation (4, 6, 14). The critical role of Pax7 in maintaining satellite cell quiescence or self-renewal is also evident by the findings that forced expression of Pax7 inhibits myogenesis and cell cycle progression in satellite cells resulting in the maintenance of their inactivated state (7). Our results suggest that one of the mechanisms by which TWEAK reduces satellite cell self-renewal is through repressing the expression of Pax7 (Fig. 5). The physiological significance of TWEAK in satellite cell self-renewal is evident by the findings that the proportion of Pax7+ cells is significantly higher in myofiber cultures of TWEAK-KO mice (Fig. 4C). Furthermore, the number of Pax7+ cells is increased in TWEAK-KO mice 5 days post-cardiotoxin injection (Fig. 4, A and B). The concentration of TWEAK used in this study did not affect the number of MyoD+ cells (Fig. 1D). However, we cannot rule out the possibility that higher amounts of TWEAK can also reduce the levels of MyoD in satellite cells/myoblast cultures resulting in their reduced differentiation into myogenic lineage. Indeed, we and others have previously reported that higher dosages of TWEAK (≥100 ng/ml) destabilize the MyoD protein in C2C12 myoblasts and inhibit their differentiation (31, 32, 41). Furthermore, there is also a possibility that the chronic presence of even low levels of TWEAK for longer durations can inhibit both satellite cell self-renewal and differentiation. Indeed, a recent study has shown that low levels of TWEAK stimulate primary myoblast fusion without affecting their proliferation or differentiation (41). Therefore, it is possible that TWEAK inhibits satellite cell self-renewal by repressing Pax7 levels and causing premature fusion of MyoD-expressing cells resulting in loss of skeletal muscle mass and regenerative capacity.

Recent literature suggests that Notch signaling plays a critical role in satellite cell self-renewal and proliferation. Activation of Notch signaling is a prerequisite for the expansion of satellite cells and to prevent premature differentiation of myogenic precursors in injured myofibers (24, 25). Furthermore, Notch3 is highly expressed in a subpopulation of quiescent satellite cells indicating that Notch signaling may underlie the heterogeneity of satellite cells (50). One of the mechanisms by which Notch signaling maintains the satellite cell pool is through directly augmenting the expression of Pax7. Overexpression of N1ICD stimulates the expression of Pax7 and promotes self-renewal both in vivo and in vitro (13). Furthermore, it has been found that N1ICD stimulates the expression of Pax7 through interaction with RBP-Jκ, which binds to two consensus sites in the promoter region of Pax7 gene (13). Consistent with these findings, our results suggest that TWEAK inhibits Notch signaling in cultured satellite cells. Moreover, expression of the components of Notch-related molecules is increased in satellite cell cultures prepared from TWEAK-KO mice (Fig. 3). The role of Notch signaling in TWEAK-mediated repression of Pax7 is supported by our results that the forced activation of Notch signaling through overexpression of N1ICD blocks the inhibitory effect of TWEAK on the expression of Pax7 (Fig. 5). Intriguingly, TWEAK inhibits the expression of both Notch receptors and ligands in satellite cells (Fig. 3). Although the exact mechanisms by which TWEAK inhibits expression of multiple Notch-related genes remain unknown, it is possible that the repression of one or more Notch receptors and/or ligands results in inhibition of other components as an autoregulatory mechanism.

Another mechanism by which TWEAK inhibits satellite self-renewal is through activation of NF-κB. A number of studies have demonstrated that TWEAK activates the NF-κB signaling pathway in a variety of cell types including myogenic cells (29, 30, 32, 41). Activation of canonical NF-κB signaling in response to inflammatory cytokines inhibits the differentiation of muscle progenitor cells into myotubes (26). Blocking NF-κB signaling also attenuates myofiber regeneration in mdx (a mouse model of Duchenne muscular dystrophy) mice (35). Our results demonstrate that TWEAK also activates NF-κB in satellite cells (Fig. 6A). Inhibition of NF-κB through overexpression of the IkBoSR protein significantly improved the number of Pax7+ satellite cells in TWEAK-treated cultures suggesting that NF-κB is involved in inhibition of satellite cell self-renewal in response to TWEAK (Fig. 6, B and C). Although the role of NF-κB in myogenic differentiation has been studied using multiple approaches (26, 51, 52), whether NF-κB also regulates satellite cell self-renewal is not clearly understood. Consistent with our results, a recent study has demonstrated that inhibition of NF-κB through overexpression of the IkBoSR protein does not affect the levels of Pax7 in C2C12 myoblasts (53). However, serum from cachectic tumor-bearing mice failed to induce Pax7 levels in IkBoSR-expressing C2C12 myoblasts suggesting that NF-κB may be required for induction of Pax7 expression in some specific conditions (53). By contrast, our results suggest that in TWEAK-treated satellite cells, NF-κB functions to repress the levels of Pax7.

Cross-talk between NF-κB and Notch signaling has been observed in different cellular contexts (38, 54). Because overexpression of IkBoSR significantly increased mRNA levels of Notch-related molecules in TWEAK-treated satellite cells (Fig. 7A), we are tempted to speculate that NF-κB inhibits satellite cell self-renewal through repression of Notch signaling. Similar to our results, a recent study has also documented the role of
NF-κB in inhibition of Notch1 expression in response to TNFα (55). Such inhibition in Notch1 expression appears to involve epigenetic mechanisms such as recruitment of Ezh2 and Dnm3b to cause histone and DNA methylation, respectively (55). Whether TWEAK affects the expression of various Notch-related genes through similar mechanisms remains to be investigated. Cytoplasmic sequestration of p65 by IkBα has been found to both translocate nuclear corepressors SMRT (silence mediator for retinoic acid and thyroid receptors)/N-CoR (nuclear receptor corepressor) to the cytoplasm and up-regulate transcription of Notch-dependent genes (56). Moreover, p65 and IkBα are able to directly bind SMRT, and this interaction can be inhibited in a dose-dependent manner by the cAMP-response element-binding protein coactivator suggesting that stimuli that promote IkBα degradation, p65 acetylation, and NF-κB activation, such as TNFα, inhibit Notch-dependent transcriptional activity (56). Although our results suggest that NF-κB represses the expression of multiple Notch genes, it is also possible that NF-κB blocks the transcriptional activity of Notch in TWEAK-treated cells.

Our results also demonstrate that overexpression of N1ICD inhibits the TWEAK-induced activation of NF-κB in satellite cells (Fig. 7B). These results are in agreement with a previously published report suggesting that N1ICD can function as an IκB-like molecule and regulate NF-κB-mediated gene expression through a direct interaction with the p50 subunit of NF-κB (54). Thus a regulatory circuitry exists between NF-κB and Notch signaling in TWEAK-treated satellite cells (Fig. 8). Under naive conditions, Notch signaling is constitutively activated, which maintains the expression of Pax7 and hence satellite cell self-renewal. Addition of TWEAK activates NF-κB, which represses the expression of Notch-related molecules resulting in reduced Pax7 levels.

In summary, our study provides initial evidence that TWEAK suppresses satellite cell self-renewal through inversely regulating NF-κB and Notch signaling pathways. Inhibition of TWEAK using pharmacological approaches can be an important approach to replenishing the satellite cell pool in skeletal muscle in various disease states.
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