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TNF-Related Weak inducer of Apoptosis (TWEAK) is a potent skeletal muscle-wasting cytokine

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Abstract

TWEAK cytokine has been implicated in several biological responses including inflammation, angiogenesis, and osteoclastogenesis. Here we have investigated the role of TWEAK in regulation of skeletal muscle mass. Addition of soluble TWEAK protein to cultured myotubes reduced the mean myotube diameter and enhanced the degradation of specific muscle proteins such as CK and MyHCf. The effect of TWEAK on degradation of MyHCf was stronger than its structural homologue TNF-α. TWEAK increased the ubiquitination of MyHCf and the transcript levels of atrogin-1 and MuRF1 ubiquitin ligases. TWEAK inhibited the phosphorylation of Akt kinase and its downstream targets GSK-3β, FOXO1, mTOR, and p70S6K. Furthermore, TWEAK increased the activation of NF-κB transcription factor in myotubes. Adenoviral-mediated overexpression of IκBαΔN (a degradation resistant mutant of NF-κB inhibitory protein IκBα) in myotubes blocked the TWEAK-induced degradation of MyHCf. Chronic administration of TWEAK in mice resulted in reduced body and skeletal muscle weight with an associated increase in the activity of ubiquitin-proteasome system and NF-κB. Finally, muscle specific transgenic over-expression of TWEAK decreased the body and skeletal muscle weight in mice. Collective, our data suggest that TWEAK induces skeletal muscle atrophy through inhibition of the PI3K/Akt signaling pathway and activation of the ubiquitin-proteasome and NF-κB systems.

Keywords

TWEAK; Skeletal Muscle atrophy; Atrogin-1; MuRF1; NF-κB; Akt

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INTRODUCTION

Skeletal muscle atrophy, or wasting, is a major component of chronic disease states such as diabetes, AIDS, sepsis, chronic obstructive pulmonary disease, chronic heart failure, cystic fibrosis, and cancer. In addition to a reduced survival rate, muscle atrophy is also related to poor functional status and health-related quality of life during starvation, immobilization, disuse, space flight, aging, as well as numerous other conditions (1). Muscle atrophy occurs as a result of an imbalance between anabolic and catabolic processes (1). Accumulating evidence suggests that a common set of biochemical changes augment the cell capacity for protein breakdown in different types of atrophying muscle (2, 3). Studies of experimental animals and patients have consistently demonstrated that the ubiquitin-proteasome system is the primary regulator of protein breakdown in various types of atrophies (4, 5). Recently it has been shown that increased expression of one or more of three muscle specific ubiquitin-conjugating enzymes (E3 ligases), atrogin-1 (also known as MAFbx), muscle RING finger protein 1 (MuRF1), and E3IIα are responsible for degrading the bulk of muscle proteins in unrelated conditions (3, 5–7). Furthermore, skeletal muscle mass is also controlled by coordinated action of key regulatory pathways including the phosphoinositide-3 kinase (PI3K)/Akt and nuclear factor-kappa B (NF-κB) (1, 8, 9).

Several lines of evidence suggest that proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon (IFN)-γ play a key role in the development of muscular abnormalities resulting in a loss of muscle mass and function (4, 10). It has been reported that: I) Inflammatory cytokines such as TNFα and IL-1β inhibit skeletal muscle differentiation *in vitro* (11–13); II) TNFα and other inflammatory cytokines are elevated under conditions which lead to muscle atrophy, such as cachexia induced by bacteria, human immunodeficiency virus (HIV), chronic heart failure, and cancer, (10, 14–16); and III) Proinflammatory cytokines induce the degradation of specific muscle proteins in differentiated myotubes (17–20). Although inhibiting the activity of some of these cytokines has been found to ameliorate muscle atrophy in some conditions, there was no effect of neutralization of one or more of these cytokines in many diseases where even their serum levels were higher (16, 21, 22). This suggests that the classical proinflammatory cytokines cannot be the sole mediators of muscle loss in all conditions and that other cytokines may play an additional role. Identification of such cytokines/molecules and understanding their mode of action leading to loss of skeletal muscle mass is important for development of new therapeutics.

TNF-related weak inducer of apoptosis (TWEAK) is a member of the TNF super family that acts on responsive cells via binding to fibroblast growth factor-inducible14 (Fn14), a small cell surface receptor (23–25). TWEAK is primarily expressed as a 249-amino acid (aa) type II transmembrane protein which is proteolytically processed into a soluble biologically active 156-aa cytokine (23). Soluble TWEAK protein has been shown to induce a variety of biological responses including cellular growth and proliferation (26, 27), angiogenesis (28), osteoclastogenesis (29), and stimulation of apoptosis under some experimental conditions (23, 30). Recently, Maecker et al. (31) have demonstrated that in contrast to TNF-α, TWEAK attenuates the transition from innate to adaptive immunity. TWEAK also activates both the classical and alternative NF-κB signaling pathways and induces the expression of

NF-κB-regulated proinflammatory cytokines and cell adhesion molecules (32–35), suggesting that TWEAK might be an important mediator of inflammatory responses.

Although TWEAK and its receptor Fn14 are highly expressed in a variety of tissues, including skeletal muscle, the physiological or pathophysiological roles of this ligand-receptor dyad remain largely unknown. We have recently shown that soluble TWEAK protein inhibits the differentiation of myoblasts into myotubes (36). However, the effects of TWEAK on differentiated skeletal muscle *in vitro* and *in vivo* have not yet been determined. Therefore, in the present study, we have investigated the role and the mechanisms by which TWEAK regulates skeletal muscle mass. Our results demonstrate that TWEAK is a strong muscle-wasting cytokine that induces degradation of muscle proteins through activation of the ubiquitin-proteasome and NF-κB pathways. TWEAK also inhibits activation of the PI3K/Akt signaling pathway in skeletal muscle. Furthermore, chronic administration of soluble TWEAK protein or skeletal muscle-specific transgenic over-expression of TWEAK diminishes overall body weight and skeletal muscle mass in mice.

MATERIALS AND METHODS

Materials

Ham's F-12 nutrient mixture and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Carlsbad, CA). Antibodies against phospho-IκBα, phospho Akt, phospho FKHR, phospho GSK3β, phospho mTOR, and total Akt protein were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antibodies against tropomyosin, β-actin, and sarcomeric α actin were obtained from Sigma Chemical Company (St. Louis, MO). Antibody against p52 protein was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid pNF-κB-Luc was from BD Biosciences (San Diego, CA). Rabbit polyclonal ubiquitin antibody was from CalBiochem (San Diego, CA). Mouse monoclonal MF-20 antibody specific to myosin heavy chain-fast twitch (MyHCf) protein was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. AlexaFluor 546 goat anti mouse antibody was purchased from Molecular Probes (Eugene, OR). Protein A sepharose beads were obtained from Pierce Biotechnology (Rockford, IL). Recombinant mouse TWEAK, TNF-α protein and TWEAK ELISA kit were from R&D Systems (Minneapolis, MN). Annexin V-EGFP apoptosis detection kit was obtained from BioVision Inc. (Mountain View, CA). NF-κB consensus oligonucleotides and luciferase assay kit were purchased from Promega (Madison, WI, USA). Poly dI·dC was obtained from Amersham Biosciences (Arlington Heights, IL). CK assay kit was obtained from Stanbio Laboratory (Boerne, TX). [γ-^{32}P] ATP (specific activity, 3000 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA).

Mice

Mice (strain: C57BL6) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed and fed in stainless steel cages on a 12 h on and 12 h off lighting schedule. Institutional Animal Care and Use Committee of the Jerry L. Pettis Memorial VA Medical Center, Loma Linda, approved experimental protocols. All procedures were conducted in strict accordance with public health service animal welfare policy.

Cell culture

C2C12 (a myoblastic cell line) was obtained from American Type Culture Collection (Rockville, MD). These cells were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. Primary myoblasts from neonatal mice were prepared by a method as described previously (36, 37). C2C12 and mouse primary myoblasts were differentiated into myotubes by incubation in differentiation medium (2% horse serum in DMEM) for 96h and 48h, respectively. Myotubes were maintained in differentiation medium and medium was changed every 48h.

Immunocytochemistry and myotube analysis

To study the effect of TWEAK on cultured myotubes, immunocytochemistry was performed as described previously (36, 38). In brief, after treatment with TWEAK, myotubes were fixed with 3.7% paraformaldehyde followed by permeabilization with 0.1% Triton-X-100. After being washed with PBS, the cells were blocked with 1% bovine serum albumin in PBS for 1 h and then incubated with MF-20 antibody (specific to MyHCf protein) at 1:100 dilutions in phosphate buffered saline (PBS) for 2h. The cells were washed with PBS, incubated with goat anti-mouse IgG-Alexa 546 (2μg/ml) for 1h, and counter-stained with DAPI for 5 min. Stained cells were analyzed under a fluorescent microscope (Olympus IX 70). Pictures were captured using Olympus MagnaFire Digital Camera and software. DAPI and MyHCf staining images were finally merged using MagnaFire software. We used Image Pro 4 software to measure myotube diameter. Myotube diameter was quantitated as follows: 10 fields were chosen randomly, and approximately 10 myotubes were measured per field. The average diameter per myotube was calculated as the mean of the 5 measurements taken along the length of the myotube.

Construction and use of adenoviruses

Adenoviral vectors encoding human FLAG-IκBαΔN cDNA were constructed as described (39). Briefly, FLAG-IκBαΔN cDNA was isolated from pcDNA3-FLAG-IκBαΔN plasmid using *Hin*dIII and *Xba*I restriction enzymes and ligated at *Hin*dIII and *Xba*I sites into pAdTrack-CMV vector. The positive clones were linearized by the restriction endonuclease *Pme*I and cotransformed with the supercoiled adenoviral vector AdEasy-1 into *E. coli* strain BJ5183 (Stratagene, La Jolla, CA). Recombinant adenoviral constructs were selected, digested with restriction endonuclease *Pac*I, and transfected 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 (GFP) 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 (5mM Tris-Cl (pH8.0), 50mM NaCl, 0.05% bovine serum albumin and 25% glycerol). The titer of the virus was determined by infecting 293T cells with serial dilutions of adenoviruses and monitoring the viral plaques for expression of green fluorescence protein. C2C12 myoblasts (5×10^5 /well) were seeded in 6-well plates on day 0. On day 1, myoblasts were transduced

with Ad.β-Gal (kindly provided by Drs. Michael E. Berens and Nhan L. Tran) or Ad.IκBαΔN at a multiplicity of infection (moi) of 500 particles/cell in 1 ml of growth medium and incubated for 1.5h at 37 °C followed by addition of 1 ml of growth medium. After 24h, the medium was changed with fresh growth medium or differentiation medium without adenoviruses. The expression of proteins in transduced myoblasts/myotubes was confirmed by western blotting.

Protein extraction, immunoprecipitation, and western blotting

C2C12 myotubes or skeletal muscle from mice were lysed in lysis buffer A (50 mM Tris-Cl [pH 8.0], 200μM NaCl, 50mM NaF, 0.3% NP-40, 2 mg/ml leupeptin, 2 mg/ml aprotinin, phenylmethylsulfonylfluoride, 0.5 mg/ml benzamidine, 1 mM dithiothreitol, and 1 mM sodium orthovanadate) and clarified by centrifugation as previously described (40). Muscle extracts were normalized based on protein amount using the BioRad protein assay reagent. Protein extracts (300 μg/ml) were immunoprecipitated with ubiquitin antibody (4 μg/per sample). The immunoprecipitates were collected using protein A Sepharose beads, separated 8 % sodium dodecyl sulfate-polycrylamide gel (SDS-PAGE), transferred to nitrocellulose membrane, and blotted with MF-20 antibody. To check total or phosphorylated levels of the specific protein, 80 μg protein extracts from each sample were separated via 8–10% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibodies as described (36, 40). A 1:1000 dilution was used for the all the primary antibodies except MF-20, for which 1:100 dilution was used.

Creatine Kinase (CK) assay

CK activity was measured using a spectrophotometric based kit as described (36). Specific activity of CK was calculated after correction for total protein and defined as units per milligram of protein (U/mg).

Electrophoretic Mobility Shift Assay (EMSA)

NF-κB activation was analyzed by EMSA as described earlier (36, 40). Briefly, 10 μg of nuclear extract prepared from cultured myotubes or skeletal muscle tissue were incubated with 16 fmol ^{32}P-γATP-end-labeled NF-κB consensus double stranded oligonucleotide for 20 min at 37°C. The incubation mixture included 2–3 μg of poly dI.dC in a binding buffer (25 mM HEPES [pH 7.9], 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl). The DNA-protein complex thus formed was separated from free oligonucleotides on a 7.5% native polyacrylamide gel. The gel was dried, and the radioactive bands were visualized and quantitated by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR)

RNA isolation and QRT-PCR was performed by a method as previously described (36, 37). The sequences of primers used to measure the mRNA levels are as follows: Atrogin-1: 5'-AAG GCT GTT GGA GCT GAT AGC A - 3' (forward), and 5'-CAC CCA CAT GTT AAT GTT GCC C-3' (reverse); MuRF1: 5'-TGC CTG GAG ATG TTT ACC AAG C-3' (forward), and 5'-AAA CGA CCT CCA GAC ATG GAC A-3' (reverse);

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-ATG ACA ATG AAT ACG GCT ACA GCA A-3' (forward), and 5'-GCA GCG AAC TTT ATT GAT GGT ATT-3' (reverse). All reactions were carried out in triplicate to reduce variation. The data were analyzed using SDS software version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using GAPDH, and the normalized values were subjected to a $2^{-\Delta\Delta Ct}$ formula to calculate the -fold change between the control and experiment groups.

Generation of TWEAK-transgenic Mice

We used pCCLMCK-II vector (kindly obtained by Prof. Ulla Wewer), which drives the expression of the target cDNA using muscle creatine kinase (MCK) promoter (41). Full-length mouse TWEAK cDNA was ligated at the *Pac*I site of the pCCLMCK-II vector. The insert and orientation of TWEAK cDNA in the vector was confirmed by automated DNA sequencing. The expression cassette DNA containing the MCK promoter, TWEAK cDNA, and poly A tail was excised by *Swa*I restriction enzyme and provided to the Core Transgenic Facility of the University of Southern California for injection into the pronucleus of a fertilized ovum. Founder mice obtained were screened for the presence of transgene by PCR using DNA from the tail tip of the mice. Increased expression of TWEAK in transgenic mice was confirmed by measuring TWEAK protein content in skeletal muscle extracts using a commercially available mouse TWEAK ELISA kit (R&D Systems, Minneapolis, MN).

Analysis of myofiber cross-sectional area

Transverse paraffinized muscle sections (8 μm) were prepared and subjected to Hematoxylin and Eosin (H &E) staining as described recently (40). Stained sections were visualized under Olympus IX 70 microscope and pictures were captured using Olympus MagnaFire digital camera and software. Fiber cross-sectional area was measured for around 100 adjacent muscle fibers in each section for each mouse using Image Pro 4.0 software. In brief, the outline of each muscle fiber was manually marked using "define the object" tool in the "measurements" parameter of the software, the software then calculated the areas of the defined fiber using all the possible diameters and finally displaying the mean of the various areas.

Statistical analysis

Results are expressed as mean + standard deviation (SD). 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

TWEAK induces muscle atrophy in vitro

Muscle hypertrophy is characterized by an increase in myotube diameter whereas atrophy is characterized by a reduction in diameter. To investigate the *in vitro* effects of TWEAK on skeletal muscle metabolism, we utilized C2C12 myotubes. C2C12 myoblasts grown in a 24-well tissue culture plate were differentiated into myotubes by incubation in differentiation

medium for 96h. The medium of the cultures was changed with fresh differentiation medium and incubated for an additional 72h with or without soluble TWEAK (10 ng/ml). At the end of the incubation period, the cultures were fixed and immunostained with MyHCf. As shown in Fig.1A, myotubes in the TWEAK-treated cultures had a reduced mean diameter compared to the untreated cultures. Quantitative estimation from multiple fields of view further confirmed a significant reduction in average myotube diameter in TWEAK-treated cultures (Fig. 1B). TWEAK-treatment did not induce apoptosis in myotubes measured using the Annexin V-EGFP apoptosis detection kit (data not shown). These data provide the initial evidence that TWEAK induces skeletal muscle atrophy *in vitro*.

TWEAK stimulates degradation of muscle proteins in vitro

We then determined the *in vitro* effects of TWEAK on total protein content and the expression of specific muscle proteins. C2C12 myotubes were treated with increasing concentrations of soluble TWEAK protein for 72h. At the end of the incubation period, all cells from each well were collected, cell extracts were made, and total protein content was measured. As shown in Fig.2A, TWEAK treatment significantly decreased the total protein content in C2C12 myotubes in a dose-dependent manner. We then determined the effect of TWEAK on the level of specific muscle proteins such as creatine kinase (CK), myosin heavy chain fast type (MyHCf), tropomyosin, and sarcomeric α actin. Treatment of C2C12 myotubes with TWEAK significantly reduced the levels of CK (Fig 2B) and MyHCf without affecting the levels of tropomyosin and sarcomeric α actin (Fig. 2C). These results are consistent with other published reports suggesting that only specific muscle proteins (not all) are degraded during muscle atrophy conditions such as in response to cytokines and/or tumor growth (42). Similar to C2C12 myotubes, TWEAK-treatment also reduced the levels of MyHCf protein in myotubes prepared from mouse primary myoblasts (Fig. 2D). TWEAK did not affect the synthesis of MyHCf in cultured myotubes in our ^{35}S-methionine pulse-chase experiments (data not shown).

Since TWEAK and TNF-α are structural homologues and TNF-α is a widely known muscle wasting cytokine (4, 17, 42), we also compared the effects of TWEAK and TNF-α on degradation of MyHCf in C2C12 myotubes. Interestingly, at equimolar concentrations TWEAK was more potent in inducing the degradation of MyHCf than TNF-α (Fig. 2E).

TWEAK augments activation of the ubiquitin-proteasome pathway and the expression of atrogin-1 and MuRF1 in cultured myotubes

To determine the underlying mechanisms responsible for skeletal muscle atrophy in response to TWEAK, we studied activation of the ubiquitin-proteasome system. Cultured C2C12 myotubes were treated with soluble TWEAK protein for different time intervals ranging from 0 to 24h, protein extracts were made, an equal amount of protein was immunoprecipitated with ubiquitin antibody, and immunoblotted with MyHCf antibody. As shown in Fig. 3A, the level of ubiquitinated MyHCf was increased upon treatment of myotubes with TWEAK, indicating that TWEAK augments the activity of the ubiquitin-proteasome system.

Among the hundreds of known E3 ubiquitin ligases, atrogin-1 and MuRF1 have been recently identified as inducible muscle-specific ubiquitin ligases that are involved in degradation of muscle proteins in a variety of conditions (3, 5–7). We investigated if TWEAK can induce the expression of atrogin-1 and MuRF1 in cultured myotubes. C2C12 myotubes were treated with TWEAK for different time intervals and the mRNA levels of atrogin-1 and MuRF1 were determined by QRT-PCR technique. Interestingly, TWEAK caused almost three-fold increase in the mRNA level of atrogin-1 (Fig. 3B) and five-fold increase in the mRNA level of MuRF1 (Fig. 3C) within 12h. These data suggest that TWEAK-induced degradation of muscle protein occurs through the increased activation and expression of the ubiquitin-proteasome system components.

TWEAK inhibits activation of PI3K/Akt pathway in myotubes

Several recent studies have shown that inhibiting the activity of the PI3K/Akt pathway can lead to skeletal muscle atrophy and increase the expression of atrogin-1 and MuRF1 ubiquitin ligases (43–45). We examined whether TWEAK can affect the activation of PI3K/Akt signaling pathway in differentiated myotubes. C2C12 myotubes were treated with soluble TWEAK protein (10 ng/ml) for varying time intervals and phosphorylation of the specific proteins was studied by western blotting using antibodies, which recognize their phosphorylated form. Interestingly, the basal level of phosphorylation of Akt kinase was suppressed on treatment of myotubes with TWEAK (Fig. 4, top panel). The phosphorylation levels of the downstream target of Akt kinase such as GSK3β, FOXO1a, mTOR, and p70S6K were also reduced in response to TWEAK treatment (Fig. 4). There was no effect on the total protein level of Akt, GSK3β, FOXO1a, mTOR, and p70S6K upon treatment of myotubes with TWEAK (Fig. 4 and data not shown). These data suggest that TWEAK inhibits the activity of PI3K/Akt signaling system in skeletal muscle.

NF-κB transcription factor is involved in TWEAK-induced degradation of muscle protein

Accumulating evidence suggests that the activation of NF-κB can lead to muscle atrophy especially in response to tumor growth and inflammatory molecules (46–48). We investigated if TWEAK can activate NF-κB in skeletal muscles and whether increased activation of NF-κB is responsible for the degradation of muscle proteins *in vitro*. C2C12 myotubes were treated with 10 ng/ml TWEAK for different time intervals and the activation of NF-κB was measured by electrophoretic mobility shift assay (EMSA). As shown in Fig 5A (upper panel), treatment of C2C12 myotubes with TWEAK significantly increased the DNA-binding activity of NF-κB.

NF-κB can be activated through two parallel pathways. The canonical NF-κB activation pathway requires the phosphorylation and degradation of NF-κB inhibitory protein IκBα by upstream kinases (49). On the other hand, the activation of the alternative NF-κB pathway involves the proteasome-mediated processing of the NF-κB2 precursor p100 to produce p52 (50). We found that TWEAK-induced activation of NF-κB involves phosphorylation of IκBα protein. The level of phosphorylated IκBα protein, determined by western blotting, was significantly increased on treatment of myotubes with TWEAK (Fig. 5A, middle panel). However, there was no change in the relative levels of p100 and p52 on treatment with TWEAK (Fig. 5A, bottom panel). Furthermore, super-shift analysis of NF-κB/DNA

complex seen in EMSA revealed that it mainly contained p50 and p65 subunits but not p52 (data not shown). Collectively, our data suggest that in myotubes, TWEAK predominantly activates classical NF-kB signaling pathway.

We also measured NF-κB transcriptional activity in C2C12 myotubes in response to TWEAK using a luciferase reporter assay. C2C12 myoblasts were transiently transfected with pNF-κB-Luc plasmid (contains multiple kappa enhancer elements upstream of the luciferase gene) and differentiated into myotubes by incubation in DM for 96h. The myotubes were then treated with 10ng/ml TWEAK for 24h and the luciferase activity was measured using a luciferase assay kit and a luminometer. As shown in Fig. 5B, the transcriptional activity of NF-κB was significantly increased in response to TWEAK treatment.

To study the role of NF-κB in TWEAK-induced muscle protein degradation, we determined the effect of the inhibition of NF-κB on the levels of MyHCf in cultured myotubes. C2C12 myoblasts were transduced with adenoviral vectors Ad.β-gal or Ad.IκBαΔN (a dominant negative inhibitor of NF-κB) for 24h followed by treatment with TWEAK and measurement of NF-κB activity by EMSA. As shown in Fig. 5C, overexpression of IκBαΔN protein inhibited the TWEAK-induced activation of NF-κB. In another experiment, C2C12 myoblasts transduced with Ad.β-gal or Ad.IκBαΔN were differentiated into myotubes by incubation in differentiation medium for 96h followed by treatment with different concentrations of TWEAK for 72h. As shown in Fig. 5D, the inhibition of NF-κB through overexpression of IκBαΔN protein completely blocked the TWEAK-induced degradation of MyHCf. These data suggest that TWEAK activates NF-κB and the increased activation of NF-κB is responsible, at least, for the TWEAK-induced degradation of MyHCf in myotubes.

Chronic administration of TWEAK causes muscle atrophy in mice

The *in vivo* effects of TWEAK in mice were also investigated. Mice at the age of 3 weeks were given either an intraperitoneal injection of soluble TWEAK (100 μg/Kg body weight in 100 μl sterile saline solution) or saline solution alone every week for a total of 4 weeks. We did not find any significant difference in either food or water uptake between TWEAK-treated and untreated groups of mice (data not shown). At the end of the protocol the mice were sacrificed, total body weight was recorded, and soleus and tibial anterior (TA) muscles were isolated tendon-to-tendon from left and right hind limb of the mice. Muscles were fixed in 10% formalin solution, embedded in paraffin, 5μM sections were cut from mid belly, and stained with Hematoxylin and Eosin (H&E) stain. The cross-sectional area of all the myofibers of soleus muscle from individual mice was measured. Consistent with the *in vitro* data, *in vivo* administration of TWEAK significantly decreased overall body weight, soleus, and TA muscle weight (Fig. 6A). Morphological analysis of muscle sections showed significantly reduced fiber diameter in TWEAK-treated mice compared to controls (Fig. 6B and C).

In another experiment we also investigated whether TWEAK can affect the activation of ubiquitin-proteasome system and NF-κB in skeletal muscle *in vivo*. Mice were given a single intraperitoneal injection of either phosphate buffered saline (PBS) or PBS containing soluble TWEAK protein (100 μg/Kg body weight). After 6h diaphragm and soleus muscles

were isolated and processed for biochemical analysis. A significant increase in ubiquitination of MyHCf (Fig. 6D) and activation of NF-κB (Fig. 6E) was observed in skeletal muscle of mice upon *in vivo* administration of TWEAK. These data suggest that similar to the cell culture system, increased activation of ubiquitin-proteasome system and NF-κB might be responsible for TWEAK-induced degradation of muscle proteins *in vivo*.

Skeletal Muscle-specific TWEAK transgenic mice have reduced body and skeletal muscle mass

We also investigated the effect of skeletal muscle-specific transgenic overexpression of TWEAK in mice. TWEAK was expressed in mice using the muscle creatine kinase (MCK) promoter. The design of the construct is shown in Fig. 7A. In our first microinjection into the pronucleus of a fertilized ovum, we found that the founder TWEAK transgenic mice were significantly smaller in size and died within 2 weeks after birth, except one in which the copy number of transgene and expression of TWEAK was low. In the subsequent microinjections, we carefully monitored the body weight of the founder transgenic mice and littermate control mice after birth. Again the size of the founder mice was smaller even at the time of birth and a few founder transgenic mice died with a week of their birth. At the age of 8 days, we took pictures and measured body weight of the entire surviving founder transgenic (n=5) and littermate control (n=21) mice. As shown in Fig.7B TWEAK-transgenic (Tg) mice at the age of 8 days looked smaller than the corresponding littermate control mice. Furthermore, the average body weight of the transgenic mice was significantly lower than the control mice (Fig. 7C). We had to sacrifice two founder transgenic mice at the age of 8 days, which had low body weight, abnormal breathing rate, and were highly fragile. Measurement of the TWEAK concentration in skeletal muscle extracts and serum using an ELISA assay kit showed a drastic increase in the TWEAK levels in skeletal muscle of founder TWEAK-Tg mice compared to littermate control mice (Fig. 7D). The serum levels of TWEAK protein were also significantly (but not drastic) higher in TWEAK-Tg compared to littermate control mice (Fig. 7D). Gross comparison of lower hind limb showed that TWEAK-transgenic mice had reduced muscle mass compared to littermate control mice (Fig. 7E, upper panel). Additionally, the size of the gastrocnemius muscle of transgenic mice was smaller as compared to littermate control mice (Fig. 7E, lower panel).

We also investigated whether the reduced muscle mass in TWEAK-transgenic mice was associated with increased activity of the ubiquitin-proteasome pathway. Protein extracts made from gastrocnemius muscle of normal and TWEAK-transgenic mice were immunoprecipitated with anti-ubiquitin rabbit polyclonal antibody and blotted with MyHCf antibody. As shown in Fig. 7F, the ubiquitination of MyHCf was significantly higher in gastrocnemius muscle of TWEAK-transgenic mice compared to littermate control mice. As expected, activation of NF-κB was also higher in skeletal muscle of TWEAK-transgenic mice (Fig. 7G). Similar higher ubiquitination of MyHCf and activation of NF-κB was also observed in skeletal muscle isolated from the carcass of the founder transgenic mice died within a week of their birth. These data suggest that the reduced muscle mass in TWEAK-transgenic mice could be due to increased activation of ubiquitin-proteasome and NF-κB pathways.

DISCUSSION

Skeletal muscle wasting associated with several chronic states (e.g. cancer, chronic heart failure, diabetes, and sepsis) can cause greater risk than the primary causative disease. Prevention of muscle loss in various diseases requires insight into the etiology of muscle wasting. In the present study we provide convincing evidence that TWEAK is a potent skeletal muscle wasting cytokine. Addition of TWEAK to C2C12 and primary myotube cultures led to both a reduction in myotube diameter and degradation of specific muscle protein. Similarly, chronic administration of TWEAK *in vivo* or muscle-specific overexpression of TWEAK in mice resulted in a loss of skeletal muscle mass. Our data suggest that TWEAK causes muscle atrophy through increased activation of the ubiquitin-proteasome system and NF-κB transcription factor. Additionally, TWEAK-induced inhibition of the Akt signaling pathway might also contribute to the loss of skeletal muscle mass.

TWEAK was originally identified in 1997 by Chicheportiche et al during a search for erythropoietin-related mRNAs in macrophages (23). Although TWEAK transcripts were detected in several tissues including brain, heart, skeletal muscle, pancreas, spleen, and in peripheral blood monocytes (23), TWEAK-knockout mice were viable, healthy, and fertile suggesting that TWEAK is not crucial for normal development (31). However, recent studies have clearly shown that the expression of TWEAK and its receptor Fn14 is upregulated in response to diverse stimuli and TWEAK has pleiotropic effects on both immune and non-immune cell types (26, 29, 31, 51–54). Increased expression of TWEAK has also been observed in space-flown WI38 human fibroblasts (53) and in several human tumor cell types (55, 56) which are known to cause muscle wasting. This further indicates that TWEAK might be an important mediator of skeletal muscle atrophy in different physiological and/or pathophysiological conditions.

Accumulating evidence suggests that ubiquitin-proteasome system, which provides a mechanism for selective degradation of regulatory and structural proteins, is responsible for degradation of the bulk of muscle proteins in various atrophying conditions (2, 57, 58). It distinguishes damaged or misfolded proteins and labels the target proteins by conjugation with the polypeptide cofactor ubiquitin (59). Ubiquitin-conjugated proteins are then subsequently recognized by the 26S proteasome, a large multisubunit multicatalytic protease complex, which degrades ubiquitinated proteins to small peptides (60). Although the upstream signaling events remain unknown, our data show that TWEAK rapidly increases the conjugation of ubiquitin to MyHCf (Fig. 3A) and ubiquitination preceded the degradation of MyHCf (Fig. 2C and Fig. 3A). Similar higher ubiquitination of MyHCf was also seen in skeletal muscle of TWEAK-treated mice (Fig. 6D) and transgenic mice expressing TWEAK in skeletal muscles (Fig. 7F). These evidences suggest that activation of the ubiquitin-proteasome system might be a major pathway that leads to increased proteolysis in skeletal muscle in response to TWEAK.

Even though the ubiquitin-proteasome system involves the concerted action of many proteins, the key enzyme in this system is E3 (ubiquitin ligase), which couples the activated ubiquitin to lysine residues on the protein substrate and confers specificity to the system.

Recently, using transcriptional screening, two E3 ubiquitin ligases, atrogin-1 and MuRF1, have been identified to be highly induced in the process of muscular atrophy (6, 61). Increased expression of atrogin-1 and/or MuRF1 has been reported in animal models of muscular atrophy induced by immobilization, denervation, hindlimb suspension, starvation, tumor load, lipopolysaccharide and glucocorticoids (3, 4). The catabolic role of atrogin-1 and MuRF1 has been supported by the fact that targeted deletion of atrogin-1 or MuRF1 showed reduced muscle atrophy in response to denervation and hindlimb suspension (61). Interestingly, TWEAK treatment significantly increased the mRNA levels of both atrogin-1 (Fig.3B) and MuRF1 (Fig. 3C) in cultured myotubes and on *in vivo* administration of TWEAK in mice (data not shown), further indicating that inducible expression of these E3 ligases might be responsible for TWEAK-induced muscle atrophy. Collectively, our data indicate that accelerated muscle loss in response to TWEAK involves the activation of the components of the ubiquitin proteolytic system.

An interesting observation of the present study was that in contrast to other muscle wasting cytokines, TWEAK inhibited the phosphorylation of Akt kinase and its downstream targets in skeletal muscles (Fig 4). Several recent studies have shown that inhibition of the PI3K/Akt pathway can lead to skeletal muscle atrophy (9). Pharmacological or molecular inhibition of Akt reduces the average diameter of myotubes in cultures (44). Akt1 and Akt2 knockout mice have reduced muscle mass compared to their normal counterparts (45). Conversely, activation of Akt kinase can prevent muscle loss in response to denervation (43, 62). It has been suggested that activation of Akt increases skeletal muscle mass by activating anabolic and suppressing catabolic pathways. The phosphorylation of GSK-3β by Akt releases its inhibitory effect on the translation initiation factor eIF2B (63, 64). Akt phosphorylates and activates mammalian target of rapamycin (mTOR) (62), which in its turn, causes phosphorylation of p70S6K and PHAS-1/4E–BP1 (63, 65). Phosphorylation of p70S6K and PHAS-1/4E–BP1 leads to the activation of pathways promoting protein synthesis and translation initiation, respectively. In addition, Akt is also known to prevent muscle loss by phosphorylating the forkhead family of transcription factors such as FOXO1 and FOXO3a, which prevents their translocation from the cytosol to the nucleus (66). Recent studies have shown that atrogin-1 and MuRF1 are the transcriptional targets of FOXO transcription factors. Thus the activation of PI3K/Akt signaling pathway in skeletal muscle inhibits the expression atrogin-1 and MuRF1 by inhibiting the activity FOXO1 and FOXO3a transcription factors (7, 66, 67). Our results demonstrating that along with Akt kinase, the basal level of phosphorylation of GSK-3β, mTOR, p70S6K, and FOXO1 was also diminished in TWEAK-treated myotubes (Fig. 4) suggests that an inhibition of the PI3K/Akt pathway might contribute to skeletal muscle atrophy in response to TWEAK.

Prevailing evidence suggests that activation of NF-κB can lead to muscle atrophy in conditions such as tumor growth, glucocorticoids, and cytokines (1, 8). Recently, Cai et al showed that muscle-specific transgenic expression of IκB kinase β (an upstream activator of NF-κB pathway) causes profound muscle loss in mice and skeletal muscle specific inhibition of NF-κB attenuated the denervation- and tumor-induced muscle loss in mice (46). Furthermore, atrophy resulting from hindlimb unloading was blocked in *nfkb1* and *bcl3* gene knockout mice (47), suggesting that activation of NF-κB subunits causes muscle

atrophy in diverse conditions. As our data in Fig.5 show treatment of myotubes with TWEAK caused a rapid and sustained increase in both DNA-binding and transcriptional activities of NF-κB. These data are consistent with previously published reports from us (36) and another group (35), which suggest that TWEAK causes long lasting activation of NF-κB in several cell types including myogenic cells. In addition, we found that TWEAK also activates NF-κB *in vivo* in skeletal muscle of mice (Fig. 6 and Fig. 7). Since the inhibition of NF-κB activity completely blocked the TWEAK-induced degradation of MyHCf in myotubes (Fig. 5D), NF-κB constitutes the pathways, which are responsible for TWEAK-induced skeletal muscle atrophy. Although the exact mechanisms by which NF-κB causes muscle atrophy remains enigmatic, there is a possibility that the activation of NF-κB augments the expression of certain genes, which are involved in degrading muscle proteins. In fact, several genes including MyoD (68), ubiquitin conjugating E2 enzyme UbcH2/E220k (69), and proteasome C3 subunit are the targets of NF-κB (70). In addition, NF-κB seems to regulate the expression of MuRF1 that is also unregulated in response to TWEAK (Fig. 3B). The promoter region analysis of mouse MuRF1 gene using online PromoterInspector software showed that it contains multiple NF-κB binding sites (our unpublished data). The role of NF-κB in regulation of MuRF1 expression is further supported by the study demonstrating that transgenic expression of IKKβ causes almost three fold increase in the expression of MuRF1 gene; muscle wasting in these mice was partially rescued by crossing them with MuRF1-knockout mice (46).

Our *in vitro* observations are well supported by *in vivo* studies, which demonstrate that chronic administration of soluble TWEAK protein in mice or transgenic overexpression of TWEAK in skeletal muscle reduces the body and muscle weight in mice (Fig.6 and Fig. 7). Although more investigations are required to determine the mechanisms and type of muscle fibers TWEAK affects *in vivo*, it is possible that reduced muscle mass in TWEAK-treated mice or transgenic mice is a result of both TWEAK-induced muscle protein degradation as supported by data in this study and an inhibition of muscle regeneration because TWEAK also inhibits differentiation of myoblasts into myotubes (36). We did not find any effect of TWEAK on the synthesis of MyHCf in cultured myotubes (data not shown). However, we cannot completely rule out the possibility that TWEAK might inhibit the synthesis of certain muscle proteins. Furthermore, since TWEAK is a secreted protein, reduced body weight of muscle specific TWEAK-transgenic mice could also be attributed to a general retardation in overall body growth. Indeed, the serum level of the TWEAK-transgenic mice was higher than the control mice (Fig. 7D). TWEAK may also interfere with the actions of growth factors during development and/or directly inhibits the activation of certain signal transduction pathways (e.g. PI3K/Akt) which are required for normal development (45).

The ongoing research in our laboratory further suggests that the actions of TWEAK in skeletal muscle are regulated by the expression of its receptor Fn14. The activation of catabolic pathways such as NF-κB in response to TWEAK was completely inhibited by knockdown of Fn14 receptor in myogenic cells using RNA interference technique (Dogra et al., submitted for publication). The expression of Fn14 in skeletal muscle was higher only at the initial stages (up to 6 weeks) of mouse development, which decreased to almost undetectable levels in older (3 months) mice (data not shown). More importantly, we found

that skeletal muscle atrophy was more prominent when TWEAK was administered in 3-week old mice (Fig. 6) but not in 12-week old mice (data not shown). Similarly, the TWEAK-transgenic mice that survived (due to low copy number of transgene) initially had lower body weights, but after 10–14 weeks, the body weight of normal and TWEAK-transgenic mice was similar (data not shown). These observations clearly suggest that TWEAK regulates muscle mass by recruiting Fn14 receptor and the biological actions of TWEAK depend on the expression of Fn14 in skeletal muscle.

In summary, we provide the first evidence that TWEAK is a strong muscle wasting cytokine. While more investigations are required to determine the mechanisms, which regulate TWEAK and Fn14 expression in skeletal muscle under different atrophying conditions, the findings in this study could be of high clinical significance because TWEAK might be the major mediator of skeletal muscle loss in many disease states, by itself or in combination with other catabolic molecules.

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Abbreviations

CK	creatine kinase
EMSA	electrophoretic mobility shift assay
Fn14	fibroblast growth factor inducible 14
GSK-3β	glycogen synthase kinase-3 beta
IκB	I kappa B
IKKβ	IκB kinase β
mTOR	mammalian target of rapamycin
MyHCf	myosin heavy chain fast type
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide-3 kinase
p70S6K	p70 S6 kinase
NF-κB	nuclear factor-kappa B
QRT-PCR	quantitative real time-PCR
TNF	tumor necrosis factor
TWEAK	TNF-related weak inducer of apoptosis

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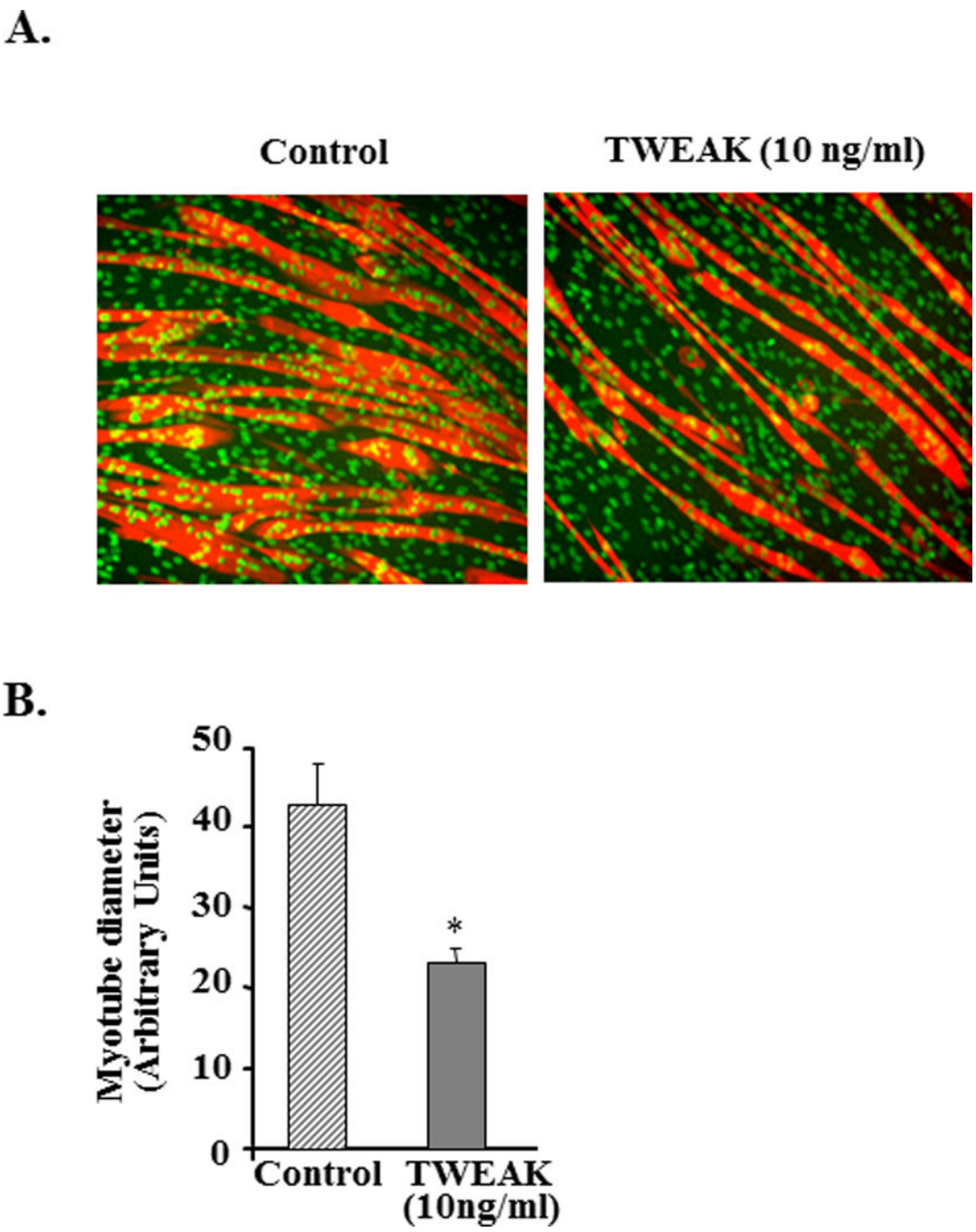

Fig. 1. Effect of soluble TWEAK protein on cultured myotubes

C2C12 myoblasts were cultured on 24-well tissue culture plates and allowed to differentiate into myotubes by incubation in differentiation medium for 96h. **A**). Differentiated C2C12 myotubes were incubated with soluble TWEAK (10 ng/ml) for 72h, the cells were fixed in 10% paraformaldehyde, and immunostained with MyHCf antibody. Representative photomicrographs presented here demonstrate that TWEAK-treated myotubes had reduced mass than the corresponding untreated myotubes. **B**). Quantitative estimation of diameter of at least 100 random myotubes in control and TWEAK-treated C2C12 cultures using Image-

Pro4.5 software revealed that TWEAK-treatment significantly decreased the diameter of the myotubes. *p<0.05, values significantly different from corresponding TWEAK-untreated cultures.

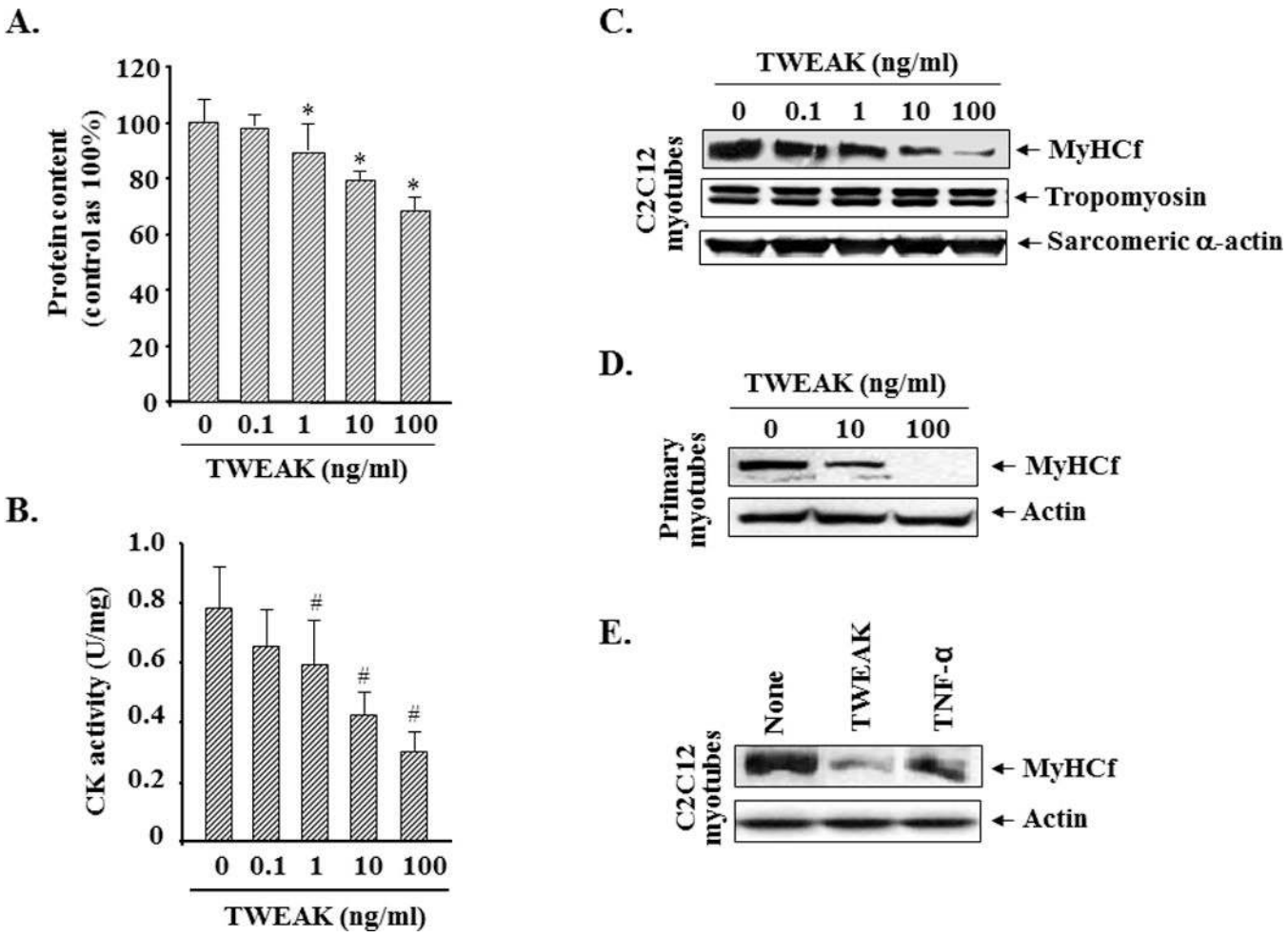

Fig. 2. Effect of soluble TWEAK protein on the total protein content and level of specific muscle proteins

A). C2C12 myotubes were treated (in triplicate) with indicated concentration of TWEAK for 72h, all the cells in each well were collected, protein extracts were made, and total protein content in each well was measured. Data presented here show that **A**). TWEAK significantly decreased the total protein content in myotubes. *$p<0.05$, values significantly different from TWEAK-untreated cultures. **B**). CK activity, measured by a commercially available kit, showed that TWEAK-treatment significantly decreased the levels of CK in myotubes. #$p<0.05$, values significantly different from TWEAK-untreated cultures. **C**) Equal amount of protein were analyzed for levels of MyHCf, tropomyosin, and sarcomeric α actin by western blotting. Representative immunoblots from three independent experiments presented here show that TWEAK reduced the level of MyHCf in a dose-dependent manner. TWEAK-treatment did not affect the level of tropomyosin and sarcomeric α actin in myotubes. **D**). Cultured mouse primary myotubes were treated with indicated concentrations of soluble TWEAK protein for 72h and the levels of MyHCf were measured by western blot. Data presented here show that TWEAK reduced the levels of MyHCf in primary myotubes. **E**). C2C12 myotubes were incubated with either 1nM TWEAK or 1nM TNF-α protein for 72h and the levels of MyHCf were measured by western blotting. Representative

immunoblot from four independent experiments presented here show that at equimolar concentrations TWEAK was more potent in inducing MyHCf degradation than TNF-α.

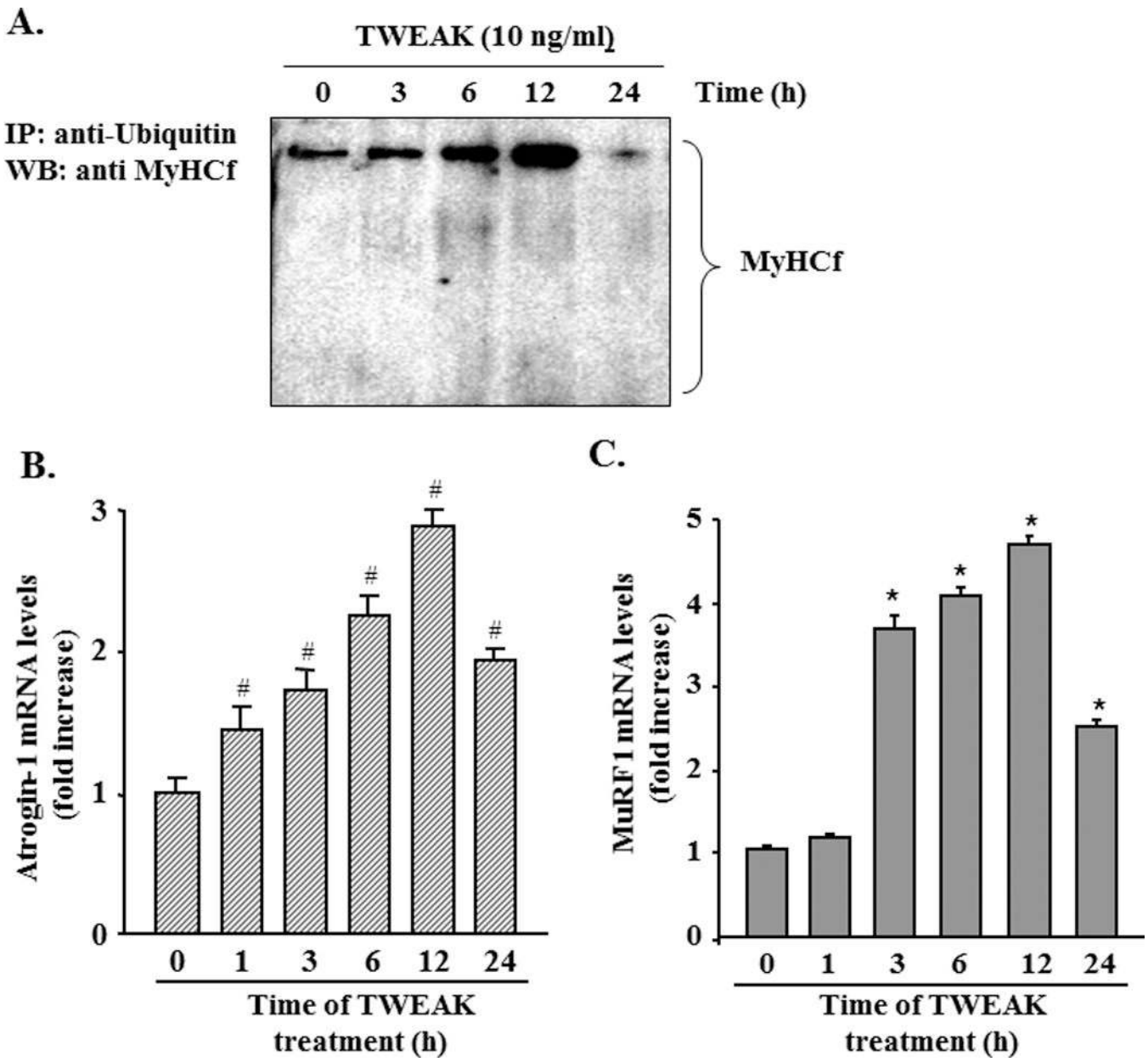

Fig. 3. TWEAK induces ubiquitination of MyHCf and expression of atrogin-1 and MuRF1 in myotubes

A). C2C12 myotubes were incubated with soluble TWEAK (10 ng/ml) for indicated time intervals. At the end of the incubation periods, myotubes were collected, protein extracts were made, equal amount of protein (300 μg/sample) were immunoprecipitated with rabbit polyclonal ubiquitin antibody (4 μg/sample), and subjected to western blotting for MyHCf. Data presented here show that treatment of myotubes with TWEAK increases the ubiquitination of MyHCf. C2C12 myotubes were treated with soluble TWEAK (10 ng/ml) for different time intervals and the mRNA levels of atrogin-1 and MuRF1 were measured by QRT-PCR. Data presented here show that TWEAK augments the mRNA level of **B**). Atrogin-1 and **C**). MuRF1 in cultured myotubes. *p<0.05, values significantly different

from that of untreated myotubes (at 0h). $^{\#}p<0.05$, values significantly different from that of untreated myotubes.

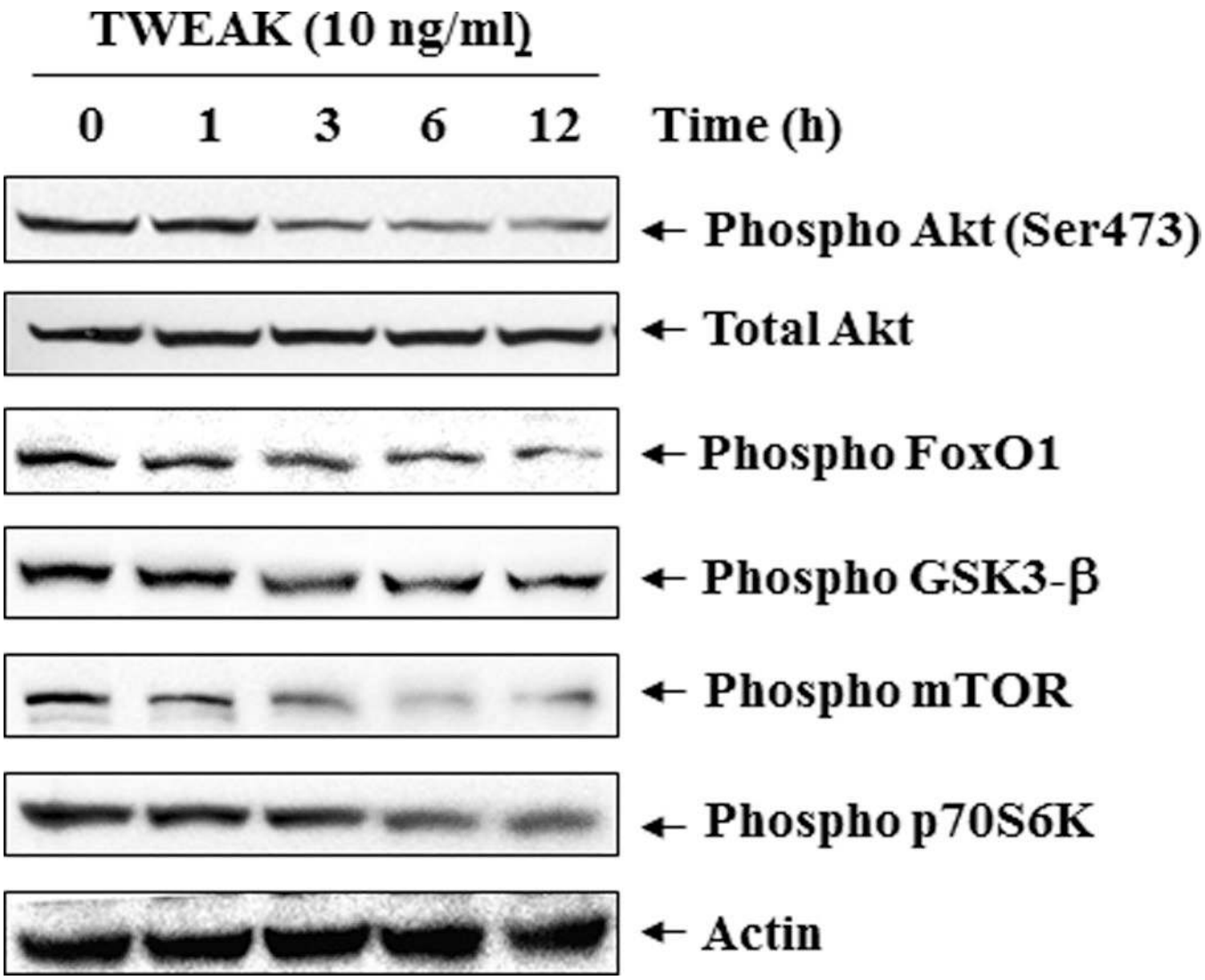

Fig. 4. TWEAK inhibits the activity of PI3K/Akt signaling pathway in myotubes

C2C12 myotubes were incubated with soluble TWEAK (10 ng/ml) for indicated time intervals. At the end of the incubation period, the cell extracts were made, and the levels of phosphorylated protein was determined by western blotting. Representative immunoblots from two independent experiments presented here show that TWEAK inhibited the basal level of phosphorylation of Akt, FOXO1, GSK-3β, mTOR, and p70S6K protein in myotubes.

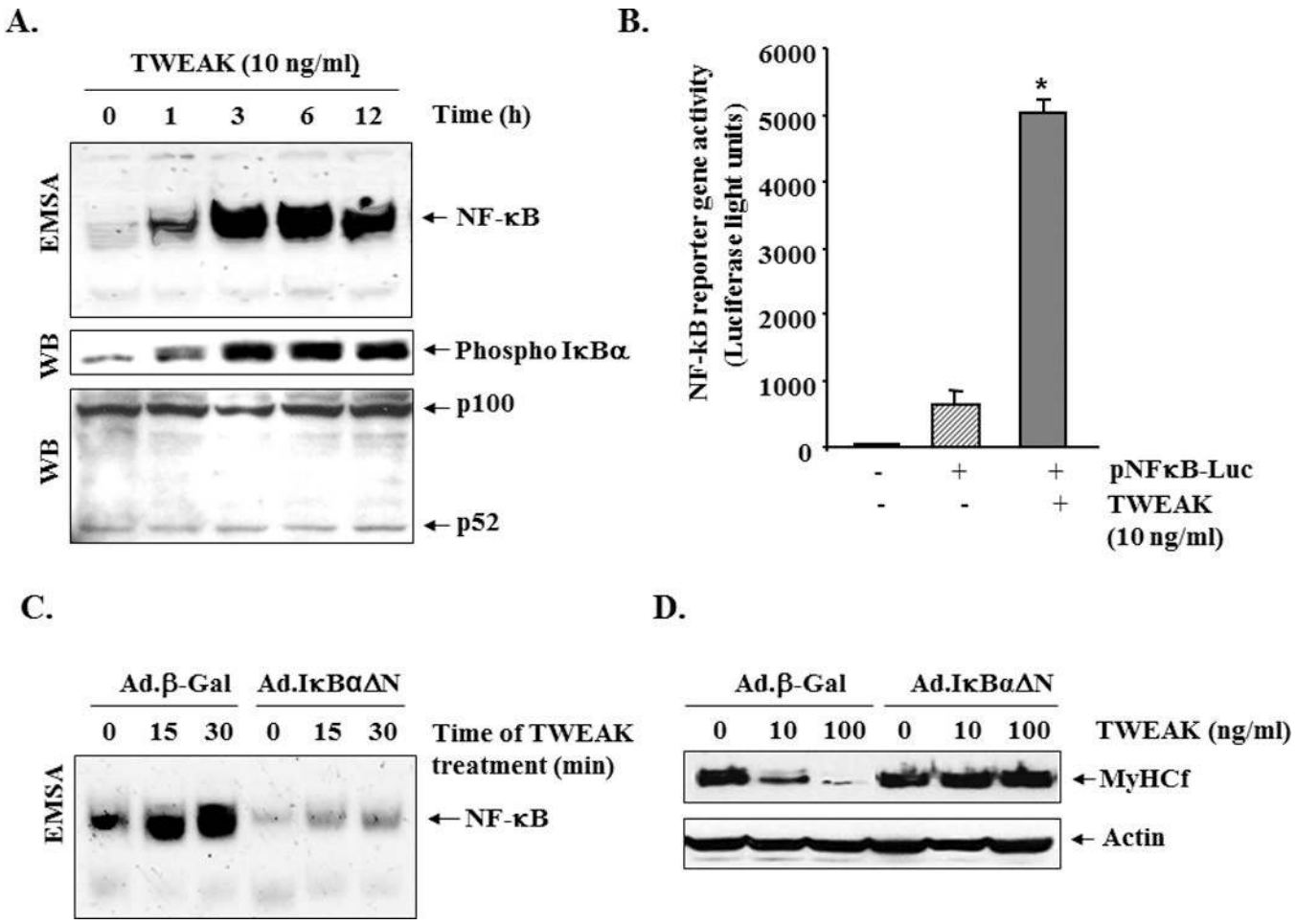

Fig. 5. Role of NF-κB in TWEAK-induced protein loss in myotubes

A) C2C12 myotubes were incubated TWEAK (10 ng/ml) for different time intervals and the activation of NF-κB was measured by EMSA. Data presented here show that TWEAK activated NF-κB in a time-dependent manner (upper panel). The level of phosphorylated IκBα protein, determined by western blotting, was also increased in response to TWEAK treatment (middle panel). Western blot analysis also showed that the treatment of myotubes with TWEAK did not affect the processing of p100 into p52 (lower panel). **B**). C2C12 myotubes transiently transfected with pNF-κB-Luc plasmid were incubated with or without soluble TWEAK protein for 24h, and the luciferase activity in cell extracts was measured as described in the "Materials and Methods". Data presented here show that TWEAK significantly increased the transcriptional activity of NF-κB transcription factor in myotubes. *$p<0.05$, values significantly different from pNF-κB-Luc plasmid transfected and TWEAK-untreated myotubes. **C**). C2C12 myoblasts were transduced with Ad.β-Gal or Ad.IκBαΔN adenoviruses (moi: 1:500) for 24h as described in the "Materials and Methods" section. These myoblasts were treated with TWEAK (500 ng/ml) for indicated time period and the activation of NF-κB was studied by EMSA. Representative EMSA gel presented here shows that overexpression of IκBαΔN protein inhibits TWEAK-induced activation of NF-κB. **D**). C2C12 myoblasts transduced with Ad.β-Gal or Ad.IκBαΔN adenoviruses were differentiated into myotubes by incubation in differentiation medium for 96h. The myotubes

were then treated with indicated concentrations of soluble TWEAK protein for 72h followed by measurement of cellular levels of MyHCf by western blotting. Data presented here show that inhibition of NF-κB through overexpression of IκBαΔN protein blocked the TWEAK-induced degradation of MyHCf in myotubes. EMSA, electrophoretic mobility shift assay; WB, western blot.

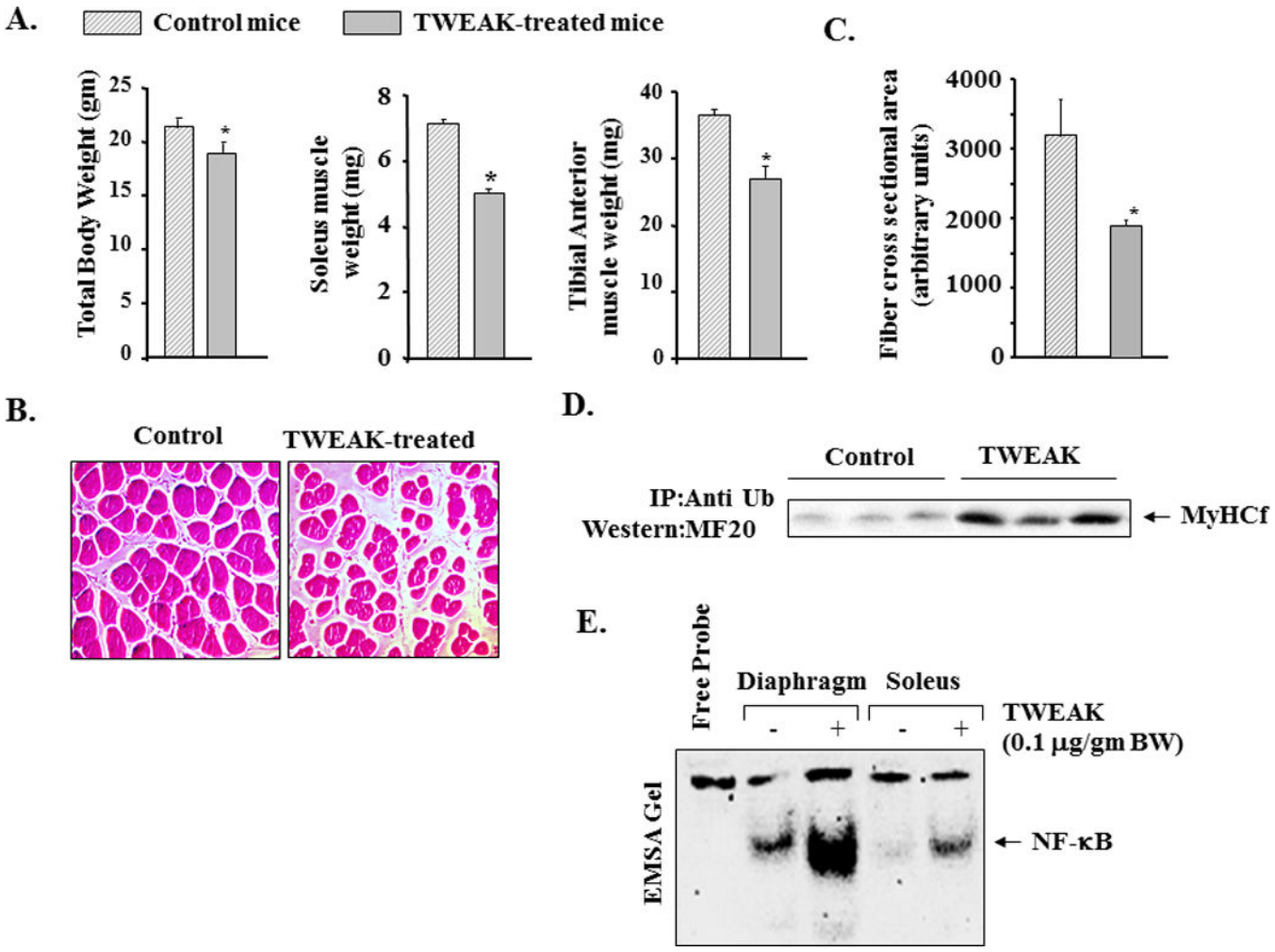

Fig. 6. Effect of *in vivo* administration of TWEAK on body weight, muscle mass, fiber cross-sectional area, and expression of MyHCf

C57BL6/J mice (3- week old) were given an intraperitoneal injections of either saline or TWEAK (100 µg/Kg body weight) at day 0, 7, 14, and 21. On day 22 the mice were sacrificed. Total body weights, as well as individual weights of soleus and tibial anterior (TA) muscle from each hind limb of the mice were measured. The data presented here show a significant decrease in: **A**) total body weight, soleus and TA muscle weight in TWEAK-treated (n=5) mice compared to control mice (n=5). **B**). Representative photomicrograph of H&E staining of soleus muscle section presented here show reduced fiber cross-sectional area in TWEAK-treated mice, **C**). Quantitative estimation of myofibers diameter (mean + s.d.) show significantly decreased fiber cross-sectional area of soleus muscle in TWEAK-treated mice compared to control mice (*$p<0.05$ values compared to corresponding TWEAK-untreated control mice). **D**) 3-week old mice were given a single intraperitoneal injection of TWEAK (100 µg/Kg BW) and after 6h the level of ubiquitinated MyHCf was determined by a method as described in the “Materials and Methods” section. The data presented here show higher ubiquitination of MyHCf in skeletal muscle of TWEAK-treated mice. **E**). A representative EMSA gel shows activation of NF-κB in diaphragm and soleus

muscle of mice after 6h of a single intraperitoneal injection of soluble TWEAK protein (100 μg/Kg BW). BW, body weight; EMSA, Electrophoretic mobility shift assay; Ub, ubiquitin.

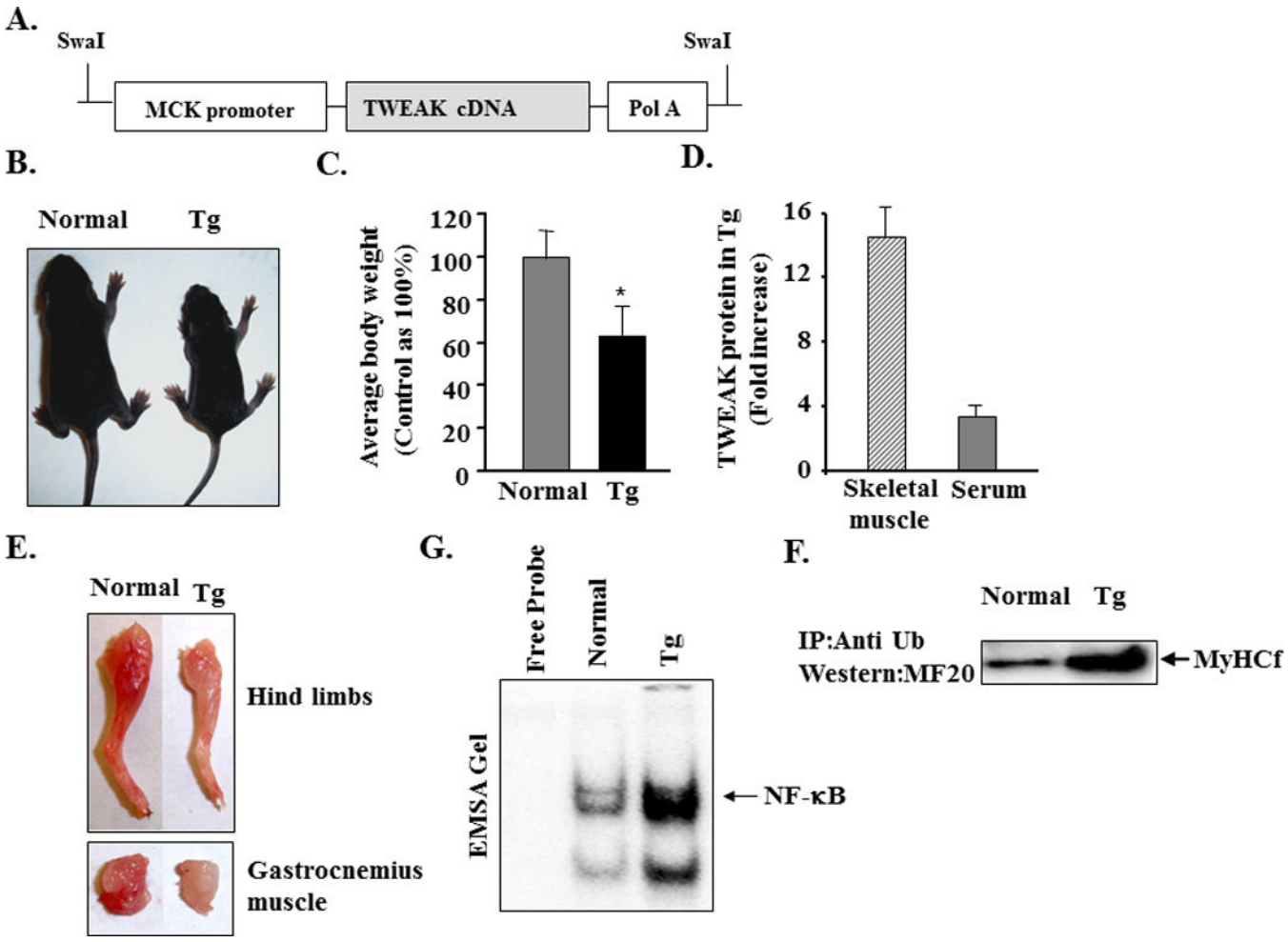

Fig. 7. Initial characterization of founder TWEAK-transgenic mice

A). Design of the transgenic construct used to generate muscle specific TWEAK transgenic mice. **B**). A representative photomicrograph shows that TWEAK-transgenic mice are smaller in size compared to littermate control mice after 8 days of birth. **C**). Average body weight of 8 days old TWEAK-transgenic mice (n=5) is significantly (*p<0.05) lower than littermate normal mice (n=21). **D**) Fold increase in the level of TWEAK protein in skeletal muscle and serum of TWEAK-transgenic mice (n=3) measured using a mouse TWEAK ELISA kit. **E**) Gross comparison of hind limbs and gastrocnemius muscle of TWEAK-transgenic mice and littermate control mice at the age of 8 days. **F**). Increased ubiquitination of MyHCf in gastrocnemius muscle of 4–8 days old TWEAK-transgenic mice (n=4) compared to littermate control mice (n=4). **G**). Higher activation of NF-κB in gastrocnemius muscle of 4–8 days old TWEAK-transgenic mice (n=4) compared to littermate control mice (n=4). EMSA, electrophoretic mobility shift assay; MCK, muscle creatine kinase; Tg, transgenic mice; Ub, ubiquitin.