Conditional Activation of MET in Differentiated Skeletal Muscle Induces Atrophy*

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Skeletal muscle atrophy is a common debilitating feature of many systemic diseases, including cancer. Here we examined the effects of inducing expression of an oncogenic version of the Met receptor (Tpr-Met) in terminally differentiated skeletal muscle. A responder mouse containing the Tpr-Met oncogene and GFP (green fluorescent protein) as a reporter was crossed with a transactivator mouse expressing tTA under the control of the muscle creatine kinase promoter. Tpr-Met induction during fetal development and in young adult mice caused severe muscle wasting, with decreased fiber size and loss of myosin heavy chain protein. Concomitantly, in the Tpr-Met-expressing muscle the mRNA of the E3 ubiquitin ligases atrogin-1/MAFbx, MuRF1, and of the lysosomal protease cathepsin L, which are markers of skeletal muscle atrophy, was significantly increased. In the same muscles phosphorylation of the Met downstream effectors Akt, p38 MAPK, and IxBα was higher than in normal controls. Induction of Tpr-Met in differentiating satellite cells derived from the double transgenics caused aberrant cell fusion, protein loss, and myotube collapse. Increased phosphorylation of Met downstream effectors was also observed in the Tpr-Met-expressing myotubes cultures. Treatment of these cultures with either a protesomeal or a p38 inhibitor prevented Tpr-Met-mediated myotube breakdown, establishing accelerated protein degradation consequent to inappropriate activation of p38 as the major route for the Tpr-Met-induced muscle phenotype.

The Met receptor and its ligand, hepatocyte growth factor/scatter factor (HGF/SF),4 play an important role in skeletal muscle biology. Mouse embryos homozygous for a null mutation of the met or the hgf/sf locus fail to form muscles in the limbs, in the diaphragm, and in some areas of the tongue (1) because of the inability of myogenic precursors to migrate from the somites and to reach these sites (2–4). In addition, hypomorphic mouse mutants with attenuated Met signaling show strong reduction of secondary fibers due to impaired proliferation of fetal myoblasts (3).

Conditional mouse models with muscle-specific Met loss or gain of function have not yet been described. Thus, our knowledge of Met-HGF/SF function in mature mammalian skeletal muscle derives by and large from in vitro studies. The Met-HGF/SF ligand/receptor pair is a positive regulator of satellite cell activation, proliferation, and migration during muscle regeneration (5–7). Satellite cells lie in close apposition to the myofiber beneath the basal lamina. Upon muscle injury, HGF/SF is released from the extracellular matrix (8). Quiescent satellite cells express the Met receptor (9), and the released HGF/SF stimulates their entry into the cell cycle. Besides increasing their proliferation, HGF/SF promotes their migration to the site of injury, as shown by its in vitro chemotactic activity (5, 10). After injury, satellite cells enter the cell cycle, proliferate, and eventually cease proliferation and fuse with each other and with adjacent fibers to form multinucleated myotubes, effectively replacing the damaged fibers. Terminal differentiation of skeletal muscle requires the irreversible withdrawal of myoblasts from the cell cycle coupled to the up-regulation of muscle-specific genes (11). Met activation counteracts the exit of satellite cells from the cell cycle and delays their myogenic differentiation (6, 12–14).

Activation of the HGF/Met axis has been implicated in development of rhabdomyosarcoma (RMS), a soft tissue tumor deriving from skeletal muscle cells. Recently we showed that Met silencing by RNA interference significantly impairs cell

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4 The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; RMS, rhabdomyosarcoma; tTA, tetracycline transactivator; GFP, green fluorescent protein; PBS, phosphate-buffered saline; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; DM, differentiation medium; GM, growth medium; Dox, doxycycline; MCK, muscle creatine kinase; MEK, MAPK kinase; C-26, colon-26; FKHR, Forkhead related transcription factors; TRE, TetO7-responsive element; MyHC, myosin heavy chain; TAMRA, 6-carboxytetramethylrhodamine; 6FAM, 6-carboxyfluorescein.
replication, survival, invasiveness, and anchorage-independent growth of RMS cells (15). We had previously shown that Met is up-regulated in vivo by the chimeric transcription factor PAX3-FKHR (16), the pathogenetic marker for Alveolar RMS, and that cells from mutant mouse embryos expressing a signaling-dead Met receptor are unable to form colonies in soft agar after PAX3-FKHR transduction (15). Furthermore, transgenic mice ubiquitously expressing HGF/SF are predisposed to develop RMS (17) and show a very high incidence of RMS when combined with Ink4a/Arf inactivation (18). All the above evidences suggest that Met plays a critical role in rhabdomyosarcoma genesis. Recently, a conditional mouse model of alveolar RMS obtained by targeting PAX3-FKHR expression to differentiating skeletal muscle has led to the hypothesis that this tumor may derive from terminally differentiating myofibers (19). In this work to further explore this concept we adopted the tetracycline transactivator (tTA) system to induce expression of an oncogenic form of Met (Tpr-Met) in differentiating muscle fibers. In the Tpr-Met oncoprotein the N-terminal region of Tpr, which includes two strong dimerization motifs, is fused to fibers. In the Tpr-Met oncoprotein the N-terminal region of Met, which is, thus, constitutively active in the absence of the ligand (20). Expression of Tpr-Met in differentiating muscle did not result in development of musculoskeletal tumors but, rather, caused dramatic muscle wasting concomitant with the induction of proteasomal and lysosomal proteolysis.

**EXPERIMENTAL PROCEDURES**

*Generation and Identification of Transgenic Mice—*Mice harboring the MCK-tTA (skeletal muscle-specific) promoter construct were kindly donated by Dr. P. Plotz (21) and kept in a C57BL/6 background. MCK-tTA heterozygotes were interbred, and homozygous mice were selected by Southern and dot blot. The Tpr-Met cDNA (22) was digested with EcoRI, and overhangs were blunted using T4 DNA polymerase and ligated into the PvuII site of pBl-GFP vector (Clontech) to generate the bidirectional Tpr-Met-TRE-GFP responder construct. The Tpr-Met-TRE-GFP construct was cut with Asel, and a 5.9-kilobase gel-purified fragment was microinjected into the fertilized eggs of FVB mice in the San Raffaele-Telethon Core Facility for Conditional Mutagenesis (Milan, Italy). Founder mice were identified by PCR analysis of genomic DNA prepared from tail biopsies. A total of seven transgenic founders were obtained upon two series of microinjections. Ear fibroblasts from all responders but one (#20) expressed GFP upon infection with LV-TA1 lentiviral vector carrying the tTA under the phosphoglycerate kinase promoter. The various founders showed strong GFP expression variability (#12 > #8 > #9 > #4 > #2 > #15), with the highest levels found in mouse #12. Lines were established from these founders, analyzed by Southern blots, and maintained in a FVB background. Two independent target lines segregated in the F1 progeny from founder #12 (12.2 and 12.9) and were chosen for breeding to MCK-tTA transactivator mice. Mice were genotyped by PCR analysis of tail genomic DNA. The tTA transgene was identified with primers annealing to tTA cDNA, 5’-AGA GGA GCC CCT GCT TAT CC-3’ and the other to the β-globin poly(A) sequence downstream of the Tpr-Met transgene, 5’-GCG CAT TAG AGC-3’ and 5’-TTC AAC GCC ACG TAA GGA GCC TGG-3’, resulting in a 547-bp fragment. The Tpr-Met-TRE-GFP responder transgene was identified with a primer annealing to exon 20 of Met, 5’-AGA GGA GCC CCT CCT TAT CC-3’, and the other to the β-globin poly(A) sequence downstream of the Tpr-Met transgene, 5’-GCG CAT TAG AGC-3’ and 5’-TTC AAC GCC ACG TAA GGA GCC TGG-3’, resulting in a 665-bp fragment. Expression of GFP in vivo was detected 2–3 weeks after induction. Animals developing massive atrophy (50% weight loss with respect to uninduced controls) within two months of age were analyzed. Samples of muscle derived from an experimental mouse model of cancer cachexia were kindly provided together with the controls by Dr. Paola Costelli. Briefly, BALB/c mice were injected subcutaneously with the colon carcinoma cell line C-26 (5 x 10^5 cells per mouse) according to Matsumoto et al. (23), and the hindlimb muscles were recovered 12 days later for Western blot analysis. All animal procedures were approved by the Ethical Commission of the University of Torino, Italy, and by the Italian Ministry of Health.

*Ear Fibroblasts and Satellite Cells Cultures—*Ear biopsies from transgenic founders were minced and digested with 1 μg/μl collagenase/dispase (Roche Applied Science) in Dulbecco’s PBS supplemented with CaCl2 and MgCl2 (D-PBS; Sigma) for 120 min at 37 °C with shaking. The digestion was stopped by adding 1:3 fetal bovine serum (FBS) (Euroclone), and crushed tissue was plated in Dulbecco’s modified Eagle’s medium (Euroclone) plus 10% FBS in 24-well plates. Fibroblasts adhered to the bottom of the plate after 1 week. Cells were passaged every

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**The TPR-MET Oncogene Induces Muscle Atrophy**

*In vivo* imaging of GFP expression was detected 2–3 weeks after induction. Animals developing massive atrophy (50% weight loss with respect to uninduced controls) within two months of age were analyzed. Samples of muscle derived from an experimental mouse model of cancer cachexia were kindly provided together with the controls by Dr. Paola Costelli. Briefly, BALB/c mice were injected subcutaneously with the colon carcinoma cell line C-26 (5 x 10^5 cells per mouse) according to Matsumoto et al. (23), and the hindlimb muscles were recovered 12 days later for Western blot analysis. All animal procedures were approved by the Ethical Commission of the University of Torino, Italy, and by the Italian Ministry of Health.

*Ear Fibroblasts and Satellite Cells Cultures—*Ear biopsies from transgenic founders were minced and digested with 1 μg/μl collagenase/dispase (Roche Applied Science) in Dulbecco’s PBS supplemented with CaCl2 and MgCl2 (D-PBS; Sigma) for 120 min at 37 °C with shaking. The digestion was stopped by adding 1:3 fetal bovine serum (FBS) (Euroclone), and crushed tissue was plated in Dulbecco’s modified Eagle’s medium (Euroclone) plus 10% FBS in 24-well plates. Fibroblasts adhered to the bottom of the plate after 1 week. Cells were passaged every
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3–4 days. Satellite cells were isolated from muscles of 17-day-old double transgenic mice (MCKtTA/Tpr-Met-TRE-GFP) kept in Dox from conception. After removal of skin, fat tissue, and bones, hindlimb muscles were digested with 1 μg/μl collagenase/dispase in D-PBS for 40–50 min at 37 °C with shaking. During the proteolytic digestion, tissues were occasionally fragmented by repeated pipetting. The digestion was stopped by adding 1:3 fetal bovine serum in D-PBS. The debris was removed by filtration through a 70-μm sterile filter, and cells were collected by centrifugation. Cells were then resuspended in complete GM growth medium (F-10 HAM; Sigma) containing 20% fetal bovine serum, 3% chicken embryo extract and 2.5 ng/ml bovine fibroblast growth factor (Peprotech). Cells were preplated overnight to discard contaminating fibroblasts and then non-adherent cells were plated in GM on collagen (0.1 mg/ml, Sigma)-coated plates. Single satellite cells started to be visible after 2–3 days of culture. Cells were passaged every 3 days, when they were ∼70% confluent using EDTA 0.5 mm in PBS for detachment. The cellular population underwent a proliferation crisis after 2–3 weeks, from which immortalized satellite cells arose. Under such conditions purity of satellite cells exceeded 99%. Proliferating cells were cultured for 20–25 passages at maximum. To obtain differentiation into myotubes, cells were plated at subconfluence on gelatin (0.5%, Sigma)-coated plates, maintained in GM for 24 h, and then shifted to DM differentiation medium (Dulbecco’s modified Eagle’s medium containing 5% horse serum (Euroclone)). Incubation was performed at 37 °C in a 5% CO₂, water-saturated atmosphere, and all media were supplemented with 2 mM l-glutamine, 100 units of penicillin and 0.1 mg/ml streptomycin.

Lentiviral Vector Production and in Vitro Transduction—High titer lentiviral vector stock was produced in 293T cells by calcium phosphate-mediated transfection of the modified transfer and packaging vectors pMDlg/pRRE, pRSV-Rev, and pMD2.VSVG (24). The viral p24 antigen concentration was determined by human immunodeficiency virus-1 p24 core profile enzyme-linked immunosorbent assay (PerkinElmer Life Sciences). Ear fibroblasts (1 × 10⁵ cells in 35-mm diameter culture dishes) from Tpr-Met responder mice were transduced with 70 ng of p24 of lentiviral vector in presence or absence of doxycycline (1 μg/ml) and Polybrene (8 μg/ml).

Inhibitors, Reagents, and Antibodies—PD98059 (20 μM), SB203580 (5–20 μM), wortmannin (100 nM), and MG132 (5 μM) were added after 24 h from DM shift. All inhibitors were purchased from Calbiochem. All reagents used were from FlukaChemie and Sigma. The following antibodies were used: anti-human Met (Santa Cruz, #sc-10); anti-mouse Met (Santa Cruz, #Sc-8057; Zymed Laboratories Inc., #3D4); anti-phospho-Erk-1,2 MAPK (Sigma, #M8159); anti-phospho-Akt (Ser-473; Cell Signaling, #9271S); anti-phospho-p38 MAPK (Cell Signaling, #4631); anti-α-tubulin (Sigma, #T5168); anti-desmin (Dako, #M0724); anti-GFP (Molecular Probes, #A-11122); anti-phospho-IκBα (Ser-32; Cell Signaling, #9241S); anti-Ki67 (Immunotech, #M1920). Anti-myoins heavy chain (MyHC) MoAb (MF20) was kindly provided by Dr. M. Prat. Horseradish peroxidase-conjugated goat anti-rabbit, and rabbit anti-mouse antibodies were from Pierce.

Western Blot Analysis—Myotube cultures and muscle tissues were lysed at room temperature in lysis buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, and 2% SDS) and sonicated. For phospho-protein analysis, myotubes were lysed at 4 °C in EB buffer (20 mM Tris HCl, pH 7.4, 160 mM NaCl, 10% glycerol, 5 mM EDTA, pH 8, 1% Triton-X-100, protease inhibitor mixture, and 1 mM sodium orthovanadate). Protein concentration was determined by BCA assay (Pierce), and proteins were resolved in 10% SDS-PAGE gels and transferred to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences). Immunoblots were developed with Super Signal West Pico chemiluminescent substrate (Pierce) and visualized on Amersham Biosciences Hyperfilm.

Immunohistochemistry, Fiber Size Measurements, and Immunofluorescence—Either E20 embryos, P0 newborn mice, or dissected tibialis anterior muscle were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Sections for histological analysis (6–8 μm thick) were rehydrated and stained with hematoxylin-eosin. For immunohistochemistry, rehydrated sections were treated with 3% H₂O₂ and microwaved for 30 min in 10 mM Antigen Retrieval Citra (Biogenex). Sections were then incubated with primary antibody 1 h at room temperature. Incubation with biotinylated secondary antibody and peroxidase-conjugated streptavidin (both 15 min room temperature) were performed using the Dako LSAB 2 system peroxidase kit. Staining was developed by liquid diaminobenzidine chromogen (Biogenex) followed by hematoxylin counterstain. For double staining experiments, after Ki-67 peroxidase staining sections were decorated with MF-20 primary antibody followed by biotinylated secondary antibody and phosphatase-conjugated streptavidin using the same kit as above. Blue staining was developed by Fast Blu chromogen (Sigma). Fiber cross-sectional areas were measured using IMAGE J software (rsb.info.nih.gov/ij) at 40× magnification. GFP fluorescence was monitored in living myotubes. For indirect immunofluorescence studies, myotubes differentiated on 24-well plates were fixed with methanol/acetone and permeabilized with 0.5% Triton X-100, incubated with MF20 primary antibody and then with Cy3-conjugated anti-mouse antibody (Sigma). Nuclei were stained with 4′,6-diamidino-2-phenylinodole. Samples were viewed under a fluorescence-equipped inverted microscope (Leica). Pictures were taken with Evoluo VF color cool camera and Image-Pro software.

Quantitative Reverse Transcription-PCR—Total RNA was extracted using the Qiagen RNeasy midi kit. RNA integrity was checked using RNA 6000 Nano Assay kit and Agilent 2100 Bioanalyzer. Total RNA quantification was measured by PCR and fluorogenic 5′ nuclease assay (Taqman assay). cDNAs were synthesized using Taqman reverse transcription reagents kit (Applied Biosystems). For each RNA sample a control reaction without reverse transcriptase was also run to exclude genomic contamination. 25 ng of each sample was loaded in triplicate in the optical reaction plate. Taqman PCR Mastermix (Applied Biosystems), Taqman probe (200 nM), and primers targeting the gene of interest (900 nM) were added to each sample. A standard curve generated from known amounts of mouse genomic DNA was used to determine the absolute quantities of each RNA. The reactions were read using the ABI PRISM
7900HT sequence detection instrument. The results were normalized to endogenous control (cyclophilin). The following primers and probes sequences were used: atrogin-1 forward, 5′-CAGTCGCA-CACAGCTTCA-3′; atrogin-1 probe, 6FAM-AGCAAGAGAAGCCT-TAMRA; cyclophilin forward, 5′-CTGTGGCTTCCTGTCGTC-3′; cyclophilin reverse, 5′-CCCTCTTCTATGTGGCC-3′; cyclophilin probe, 6FAM-TTGCCTGCAGCCTCG-TAMRA. All probes were synthesized by Applied Biosystems.

**Statistics**—Data are expressed as the mean ± S.E. or ± S.D. as indicated. Differences between the induced mice and the control groups were tested by using the χ² test. The level of significance was set at $p < 0.05$. A Pearson correlation coefficient was used to test the strength and relationship between variables.

**RESULTS**

**Generation of Bitransgenic Mice with Inducible Expression of Tpr-Met in Skeletal Muscle**—To obtain transgenic mice in which expression of Tpr-Met can be specifically induced in muscle in a regulated manner, we adopted the Tet-on/off technology described by Gossen and Bujard (25). The Tpr-Met-TRE-GFP responder construct was assembled by inserting the cDNAs of Tpr-Met and of the GFP reporter into the bidirectional minimal promoters ($P_{\text{minCMV1}}, P_{\text{minCMV2}}$). The -globin and late SV40 polyadenylation sites were downstream to Tpr-Met and GFP, respectively. kb, kilobases. The responder mouse was crossed with the transactivator line containing the TetR/VP16 tTA under control of the MCK promoter. The Tpr-Met-TRE-GFP expression is induced in skeletal muscle by Dox withdrawal conditions.

**FIGURE 1. Generation of bitransgenic mice with inducible expression of Tpr-Met in skeletal muscle.** A, the Tpr-Met-TRE-GFP responder mouse was produced by microinjecting the construct containing the Tpr-Met and GFP (reporter gene) cDNA sequences under control of the TetO7 responsive element fused to the bidirectional minimal promoters ($P_{\text{minCMV1}}, P_{\text{minCMV2}}$). The -globin and late SV40 polyadenylation sites were downstream to Tpr-Met and GFP, respectively. kb, kilobases. B, the responder mouse was crossed with the transactivator line containing the TetR/VP16 tTA under control of the MCK promoter. The Tpr-Met-TRE-GFP expression is induced in skeletal muscle by Dox withdrawal conditions. C, the Tpr-Met-TRE-GFP double transgensics embryos (E20) were raised in the absence (Tpr-Met Ind.) or presence (uninduced) of Dox during pregnancy. The Tpr-Met Ind. embryos showed a strong green fluorescence localized in skeletal muscle (left panel, upper row) and lacked the S-shaped curvature of the vertebral column seen in uninduced embryos (middle panel). Analysis of muscle extracts with anti-Met and anti-GFP antibodies showed that the Tpr-Met and GFP proteins were expressed only in the Tpr-Met Ind. mice (right panel). D, hematoxylin and eosin-stained sagittal sections of dorsal muscles, diaphragm (arrows), and tongue. Scale bars, 100 μm (left and middle panels) and 500 μm (right panels).

The TPR-MET Oncogene Induces Muscle Atrophy from E14 onward (28) when secondary myogenesis and terminal differentiation occur. Pregnant mothers were either given doxycycline (Dox) starting at the time of mating to block the responder transgene expression in the offspring (uninduced) or were left without treatment to induce its expression (Tpr-Met-induced). Fig. 1C shows E20 bitransgenic embryos, which developed in mothers kept with and without Dox (uninduced/Tpr-Met-induced). Induced bitransgenic embryos were imme-
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Immediately identifiable for their fluorescence and expressed Tpr-Met in their muscles (Fig. 1, upper row and right panel). Dox administration prevented tTA induction keeping GFP and Tpr-Met at undetectable levels (Fig. 1, lower row and right panel). The Tpr-Met-induced embryos had a characteristic “hunchback” appearance and lacked the S-shaped curvature of the vertebral column seen in control mice (Fig. 1, middle panel). This is likely to be an indirect consequence of the strong reduction and disorganization of back muscles (Fig. 1D, left panel) that are required to support proper alignment of the vertebrae during development. As shown in Fig. 1D, middle and right panels, in Tpr-Met-induced mice the diaphragm was much thinner, and the tongue muscles were reduced with respect to controls. Induced newborn mice died perinatally, presumably due to respiratory and feeding defects.

Induction of Tpr-Met in Skeletal Muscle Causes Muscle Atrophy—To better visualize the morphology of the muscle fibers, transverse sections of hindlimbs from newborn (P0) mice were paraffin-embedded and stained with anti-desmin antibody followed by peroxidase reaction. Scale bars, 250 μm (left panels) and 10 μm (right panels). B, distribution of muscle fiber cross-sectional areas. Individual tibialis anterior muscle fiber sizes from Tpr-Met Ind. mice (green bars) are shifted to the left side of the curve in comparison with those from uninduced mice (blue bars). The mean cross-sectional area of muscle fibers is significantly smaller in the Tpr-Met Ind. compared with uninduced mice (p < 0.01). C, longitudinal sections of tibialis anterior were stained with Masson trichrome to detect transverse banding pattern. Scale bars, 10 μm. D, tongue sections were stained with monoclonal antibody anti-MyHC followed by alkaline phosphatase (blue) and rabbit polyclonal anti-Ki-67 followed by peroxidase (brown). Scale bars, 10 μm.

FIGURE 2. Skeletal muscle atrophy in newborn Tpr-Met-induced mice. A, transverse sections taken at the midpoints of hindlimbs (knee-foot) from uninduced and Tpr-Met-induced (Tpr-Met Ind.) newborn (P0) mice were paraffin-embedded and stained with anti-desmin antibody followed by peroxidase reaction. Scale bars, 250 μm (left panels) and 10 μm (right panels). B, distribution of muscle fiber cross-sectional areas. Individual tibialis anterior muscle fiber sizes from Tpr-Met Ind. mice (green bars) are shifted to the left side of the curve in comparison with those from uninduced mice (blue bars). The mean cross-sectional area of muscle fibers is significantly smaller in the Tpr-Met Ind. compared with uninduced mice (p < 0.01). C, longitudinal sections of tibialis anterior were stained with Masson trichrome to detect transverse banding pattern. Scale bars, 10 μm. D, tongue sections were stained with monoclonal antibody anti-MyHC followed by alkaline phosphatase (blue) and rabbit polyclonal anti-Ki-67 followed by peroxidase (brown). Scale bars, 10 μm.
FIGURE 3. Postnatal induction of Tpr-Met induces skeletal muscle atrophy. To prevent expression of Tpr-Met during development, bitransgenic mice were conceived and raised in the presence of doxycycline (200 μg/ml) continuously administered in the drinking water to pregnant mothers. When Tpr-Met was induced upon Dox withdrawal at weaning, it caused severe muscle wasting within the 2 months of life. A, body weights are expressed as percentages of weight increase relative to 100% measured at the time of induction. Overall body weight of Tpr-Met Ind. mice was significantly reduced after 4 weeks of induction (n = 3 in each group). B, skin was stripped from the 8-week-old mice for gross comparisons of muscle mass. C, transversal sections taken at the midpoints of legs from 6-week-old control and Tpr-Met Ind. mice were paraffin-embedded and stained with hematoxylin and eosin. Scale bars, 100 μm. D, cross-sectional areas for individual tibialis anterior muscle fibers were measured in transverse fixed sections, and the distribution from Tpr-Met Ind. mice (green bars) was shifted to the left side of the curve in comparison with that from control mice (blue bars). The mean cross-sectional area of muscle fibers is significantly different between the induced mice and the control group (p < 0.01).
The TPR-MET Oncogene Induces Muscle Atrophy

![Graph](image)

**FIGURE 4.** Met activation in vivo leads to up-regulation of atrogin-1/MAFbx, MuRF1, ubiquitin ligases, and cathepsin L. Tpr-Met expression was induced (black columns) in mice \( n = 8 \) at p21, and body weight was monitored. Upon reaching a 50% weight loss with respect to controls from the same litter (white columns), mice were sacrificed, and RNA was extracted from the tibialis anterior muscle. A. expression analysis by real time PCR shows an increase in atrogin-1/MAFbx, MuRF1, and cathepsin L mRNA in Tpr-Met-expressing muscle compared with controls. Values are shown ± S.D. Cyclophilin was used as internal standard. ***,** \( p < 0.01 \). B. representative Western blot analysis of Tpr-Met expression in samples of atrophic and control muscle shows that the expression level of the transgene is comparable among mice that became atrophic at early and late times after induction. The upper band (aspecific) can be used as a loading control. C. left, representative Western blot analysis of the level of myosin heavy chain and of phosphorylated (p-) forms of known Met effectors in the muscle of mice, where 50% weight loss occurred within 15–20 days. Right, densitometric quantification of the proteins indicated.

endoreplication. Moreover, we did not observe apoptotic nuclei (data not shown).

We then asked whether Tpr-Met induction would also cause muscle wasting in mature mice. Tpr-Met was switched off throughout development by continuous administration of Dox (200 \( \mu \)g/ml) in the drinking water given to pregnant mothers. To avoid a possible immune reaction to the GFP or the Tpr-Met proteins, the transgenes were switched on by Dox withdrawal at weaning, when the immune system has not yet reached complete maturation. Starting at 2–3 weeks of induction, the mice showed a reduction in body weight due to decreased skeletal muscle mass (Fig. 3, A and B). GFP expression could be detected in differentiated skeletal muscle as soon as the mice started to lose weight. Muscles of both the limbs and trunk were affected, and the reduction in muscle mass significantly shortened their life span. The morphology of skeletal muscles was examined using hematoxylin and eosin staining on transverse sections.

There were no signs of degeneration, regeneration, or mononuclear infiltration in control or Tpr-Met-induced mice (Fig. 3C). However, the mean cross-sectional area of the Tpr-Met-induced mice showed a 38% decrease (Fig. 3D) compared with that of control mice. The corresponding histogram showed a significant leftward shift in distribution, with an evident increase in the percentage of small fibers in the Tpr-Met-induced mice.

During muscle atrophy there is an increased transcription of genes coding for proteins belonging to the ubiquitin-mediated degradation and the lysosomal pathways. Atrogin-1/MAFbx and MuRF1 are two muscle-specific E3 ubiquitin ligases playing a critical role in the atrophy process (29, 30). Cathepsin L mRNA is also consistently overexpressed in muscle wasting (31). Quantitative reverse transcription-PCR analysis revealed a significant increase in mRNA levels for MuRF1, atrogin-1/MAFbx, and cathepsin L in the skeletal muscle of Tpr-Met-induced mice when compared with healthy control subjects (2.05, 2.84, and 2.45-fold, respectively; Fig. 4A). Furthermore, there was a significant negative correlation between the ubiquitin ligases and cathepsin L mRNA content and total body weight (data not shown).

To identify the signaling pathway(s) that mediates the Tpr-Met-induced atrophy program, we analyzed the phosphorylation level of the major Met effectors, i.e. Akt, p38 MAPK, Erk1/2, and IxB\( \alpha \) (32, 33) in Tpr-Met expressing adult muscle. Tpr-Met mice induced postnataally reached a 50% weight loss with respect to controls in a variable time ranging from 15 to 36 days. The level of Tpr-Met protein was the same in the atrophic muscle samples regardless of the time necessary for reaching the desired weight loss (Fig. 4B), and in all samples myosin heavy chain was decreased with respect to controls (Fig. 4C).

In the muscle of mice where 50% weight loss occurred within 15–20 days, phosphorylation of Akt, p38, and IxB\( \alpha \) was increased with respect to controls, whereas the level of Erk1/2 phosphorylation was unchanged (Fig. 4C). Conversely, in the muscle of mice where atrophy reached the desired level at 32–36 days of induction (not shown), phosphorylation of phospho-Akt was higher, but that of p38 and IxB\( \alpha \) did not differ from controls.
Induction of Tpr-Met Causes Collapse of Differentiated Myotubes in Vitro and Proteasome-dependent Protein Degradation—Satellite cells isolated from 17-day-old bitransgenic mice (MCKtTA/Tpr-Met-TRE-GFP) kept in Dox from conception were immortalized by serial passages. Two cell lines, TPR30 and TPR31, were established. Myoblasts displayed a round shape (Fig. 5A and B) and expressed satellite cell markers such as endogenous c-Met and desmin (not shown). They did not express GFP when cultured in GM (Fig. 5C) or absence of Dox and then assessed for protein content, expression of MyHC, and response to proteasome inhibitors. A, protein concentration was determined after solubilization in 2% SDS and expressed as total protein/3.5-cm well (5 wells/condition) ± S.E. The asterisk indicates significant difference versus control (p < 0.05). B, down-regulation of MyHC was detected by Western blot, using tubulin as endogenous standard. C, the myotubes were grown for 48 h after the DM shift and then treated for further 48 h without (−) and with (+) MG132 proteasome inhibitor. The myotube breakdown of Tpr-Met-expressing myotubes was prevented by MG132 treatment. Arrows indicate collapsed myotubes. Scale bars, 200 μm.

FIGURE 5. Induction of Tpr-Met in terminally differentiated myotubes causes breakdown of myotubes. Satellite cells isolated from 17-day-old bitransgenic mice kept in Dox were immortalized by serial passages in vitro. Proliferating myoblasts from TPR30 cell line were cultured in GM in the presence (A) or absence of Dox (B and C), then shifted into DM to induce muscle differentiation. Myotubes were cultured in DM in the presence or absence of Dox for 2 days (D–F), 3 days (G–I), and 6 days (J–L). Pictures of living cells were taken in contrast phase (A and B, D and E, G and H, and J and K) and fluorescence (C, F, I, and L) microscopy. The MCK-driven tTA activated GFP (and hence, Tpr-Met) expression from 2 days in DM forward (F, I, and L). Prolonged expression of Tpr-Met caused myotubes to fuse aberrantly (H and I) and collapse (K and L). Scale bars, 200 μm (A and B, D and E, G and H, and J and K) and 100 μm (C, F, I, and L).

Interfering with p38 MAPK Cascade Prevents Myotube Collapse—In myotubes kept for 4 days in DM, phosphorylation of Akt, Erk1/2, p38 MAPK, and IkBα increased in conditions of Tpr-Met induction (Fig. 7A). To assess the role of these effectors in myotubes collapse, we used selective inhibitors PD 098059 (Erk inhibitor), which inhibits the MAPK kinase MEK1, SB 203580, a specific inhibitor of p38 MAPK, and wortmannin, which inhibits the phosphatidylinositol 3-kinase/Akt pathway. Inhibitor treatment was initiated 24 h after the shift to DM and was prolonged for 48 h. The myotubes differentiated in significant variations in the Tpr-Met-induced versus uninduced myotube cultures (data not shown). These results indicate that the expression of Tpr-Met oncogene in differentiated satellite cells does not suppress cell-substrate adhesion and does not induce apoptosis. Instead, the Tpr-Met-induced myotubes showed reduced levels of total protein content (Fig. 6A) and of MyHC (Fig. 6B) when compared with uninduced myotubes. To verify whether protein degradation occurred through the ubiquitin-dependent proteasome pathway, we used a specific proteasome inhibitor, MG132 (Fig. 6C). The cultures were grown for 48 h after the DM shift and then treated for further 48 h with the inhibitor. As shown in Fig. 6C, the collapse of Tpr-Met expressing myotubes was prevented by treatment with the proteasome inhibitor.
**The TPR-MET Oncogene Induces Muscle Atrophy**

**A**

| TPR30 | TPR31 |
|-------|-------|
| Dox: + - | + - |
| Tpr-Met | | |
| pAKT | | |
| pErk | | |
| pp38 | | |
| plkBα | | |
| tubulin | | |

**B**

**Uninduced**

**Tpr-Met Ind.**

Uninduced conditions were unaffected by inhibitor treatment (Fig. 7B, left panels). On the other hand, in induced conditions, SB 203580 clearly rescued myotube integrity, PD 098059 displayed a small effect especially when compared with p38 inhibitor (Fig. 7B, right panels), whereas wortmannin was without effect (not shown). This indicates that inappropriate activation of p38 MAPK is the major mediator of the effects of Tpr-Met induction in myotube cultures.

**Met Receptor Expression in Normal Adult Skeletal Muscle and in Tumor-induced Muscle Wasting**—To assess the significance of Met activation in skeletal muscle under physiological or pathological conditions, we evaluated if Met is expressed in adult skeletal muscle in normal and tumor-bearing mice. Mice inoculated subcutaneously with colon-26 (C-26) tumor cells exhibited severe muscle wasting after 13 days from tumor inoculation (23). Extracts of muscles from controls and C-26 tumor bearing mice were analyzed by Western blot with anti-mouse Met antibody (Fig. 8). The mature β-chain of 145 kDa (p145 MET) was labeled in normal skeletal muscle (Fig. 8, controls). Significantly higher levels of p145 MET were found in cachectic muscles (158% increase, p < 0.01).

**DISCUSSION**

By forcing expression of Met oncogene in differentiated myotubes of transgenic mice, we originally intended to explore the possibility to push myonuclei back into the cell cycle and to revert differentiation as suggested by the development of skeletal tumors obtained by Capecchi and co-workers (19) by targeting PAX3-FKHR expression to differentiating skeletal muscle (19). However, constitutively active Met did not induce nuclear reentry into S-phase nor promote any de-differentiation in muscle. We instead found that in the setting of terminally differentiating myofibers, it caused muscle atrophy. Muscle wasting caused by expression of the Tpr-Met oncogene was reminiscent of the situation seen in different clinical conditions of skeletal muscle atrophy, such as cancer cachexia, AIDS, diabetes, kidney disease, aging, denervation, and immobilization (34–36). The cross-sectional area of individual fibers was reduced, and there was a marked decrease of MyHC concomitant to an increased expression of atrophy markers, such as atrogin-1/MAFbx and MuRF1, two muscle-specific E3 ubiquitin ligases (29, 30, 37, 38), and cathepsin L, a lysosomal protease (31).

It was of interest to investigate whether activation of the same Met effectors that mediate proliferation of undifferentiated myogenic precursors (32, 33) could play a role in Tpr-Met-induced muscle atrophy. Phosphorylation of Erk1/2, which in adult muscle has been shown to be involved in the control of fiber type rather than fiber size (39), was unaffected. Phosphorylation of Akt, p38 MAPK, and IkBα was increased relative to control muscle. Previous work has shown that the transforming growth factor-β/MAPK pathways, including p38 phosphorylation, are up-regulated and constitutively activated in muscle atrophy due to acute quadriplegic myopathy (37) and, more recently, that tumor necrosis factor-α-induced p38 MAPK activation stimulates expression of atrogin-1/MAFbx in skeletal muscle (40). Whether MuRF1 and cathepsin L are downstream targets of p38 remains to be determined. Recently it has been shown that forced activation of NF-κB through muscle-specific transgenic expression of activated IkB kinase β (MIKK mice) causes severe muscle wasting (41). Skeletal muscle atrophy in MIKK mice was associated with increased MuRF1 expression, whereas the MAFbx gene was not up-regulated. NF-κB is activated by Met signaling via the canonical IkB phosphorylation/degradation cycle and via the Erk1/2 and p38 MAPK cascades (32).

By activating the Akt pathway, growth factors such as insulin-like growth factor 1 (IGF-1) not only induced skeletal muscle hypertrophy (42–45) but also interfered with atrophy, as shown by the fact that stimulation of Akt blocks glucocorticoid-induced atrophy and atrogin-1/MAFbx and MuRF1 gene
expression (46, 47). However, phosphorylation of Akt in response to IGF-1 is higher and lasts longer relative to HGF/SF (48). In our Tpr-Met transgenic model, Akt stimulation was obviously insufficient to counteract the catabolic effects induced by p38 and NF-κB in adult skeletal muscle.

Satellite-derived myotube cultures mimic embryonic and newborn muscles. At this stage of development fetal myoblasts undergo active proliferation and fusion. In myotube cultures derived from the bistransgenic mice, Tpr-Met expression led to aberrant fusion, MyHC decrease, and ultimately, myotube collapse. The addition of MG132, a proteasome inhibitor, rescued myotube integrity. The same spectrum of Met effectors that had been found activated downstream of Tpr-Met in vivo were also phosphorylated in these cultures, with the exception of Erk1/2, unaffected in vivo and strongly activated in vitro. The Ras-Raf-MEK-ERK pathway is known to be involved in cell-cell fusion (49). Before myotube collapse, we observed numerous abnormal X-Y-shaped myotubes with aggregated nuclei, indicating that cell elongation, polarity, and fusion were perturbed. However, treatment of the cultures with an Erk1/2 inhibitor resulted only in a partial rescue, whereas the most dramatic effect was obtained by inhibiting p38. The abnormal myotubes with aggregated nuclei did not form in the presence of SB 203580. Our results indicate that aberrant activation of p38 pathway is chiefly responsible for the phenotype and is in line with the concept that p38 MAPK activity, necessary at early stages of myogenic differentiation to induce withdrawal from cell cycle (50), must be suppressed at later stages of myogenesis to allow the differentiated cells to elongate, polarize, aggregate, and fuse (51).

In adult muscle Met triggers regeneration by mediating satellite cells activation, proliferation, and migration to the site of injury. In mature myotubes Met is down-regulated, but some residual expression has been documented in the literature (52). We verified by Western blot that Met is expressed in adult skeletal muscle and that its level increases in the cachetic muscle of tumor bearing C-26 mice. This finding together with our in vivo results obtained with Tpr-Met in mature muscle suggests that elevated levels of HGF, such as those that may occur in cancer (53), could be involved in inducing muscle wasting via Met activation and ensuing protein degradation. However, we cannot unambiguously establish the contribution to the Met signal detected by Western blot of the mature myotubes versus the satellite cell population. Furthermore, we were unable to reproduce the effects seen upon Tpr-Met induction by adding exogenous HGF to wild type differentiated myotubes in vitro. As an additional word of caution, it should be noted that there are important differences between Tpr-Met and the full size Met receptor. The Tpr-Met oncoprotein contains a constitutively active wild type kinase domain and C-terminal tail of Met, responsible for signaling (22). But several regulatory residues located in the juxtamembrane region of the receptor, essential for the negative feedback regulation of the kinase, are lost in Tpr-Met (54, 55). Thus, Tpr-Met leads to uncontrolled overstimulation of Met downstream effectors. Furthermore, although a fraction of the Tpr-Met oncoprotein is associated to the membrane, the majority of it is cytoplasmic, and this ectopic localization may lead to different effects with respect to the wild type receptor (56). Thus, although a link between cancer-associated cachexia and overstimulation of the HGF-Met axis is an intriguing hypothesis, further studies are needed to determine if high levels of HGF alone or in combination with other factors associated with atrophy can contribute to muscle wasting in vivo. Regardless of its relevance to the pathogenesis of cancer-associated cachexia, our model represents the first evidence in the mouse that a constitutively active growth factor receptor activates protein degradation in differentiated muscle.

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