Rhabdomyosarcoma development in mice lacking Trp53 and Fos: Tumor suppression by the *Fos* protooncogene

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Summary

The Fos protein, a major component of the AP-1 transcription factor, is essential for osteoclast differentiation, acts as an oncogene, potentiates transforming signals, and controls invasive growth and angiogenesis during tumor progression. To investigate a potential genetic interaction between the Trp53 and Fos pathways, *Trp53*/*Fos* double knockout mice were generated. These mice develop highly proliferative and invasive rhabdomyosarcomas of the facial and orbital regions, with more than 90% penetrance at 6 months of age. Rhabdomyosarcoma cell lines established from the primary tumors express characteristic muscle-specific markers, and reexpression of Fos is associated with enhanced apoptosis in vitro. Moreover, Fos is able to repress *Pax7* expression in rhabdomyosarcoma cell lines and primary myoblasts, suggesting a molecular link to genetic alterations involved in human rhabdomyosarcomas.

Introduction

Rhabdomyosarcoma is the most common soft-tissue sarcoma of childhood. It displays skeletal muscle differentiation but is considered to originate from a multipotential mesenchymal cell type. Rhabdomyosarcomas occur most often in the head and neck region, including the orbita, and are classified histologically as embryonal, alveolar, and pleomorphic. In humans, the most consistent genetic alterations correlating with rhabdomyosarcoma development of the alveolar subtype are t(2;13)(q35;q14) and t(1;13)(p36;q14) translocations, which result in overexpression of chimeric PAX3-Forkhead (PAX3-FKHR) and PAX7-FKHR transcription factors (Barr et al., 1993; Davis et al., 1994; Galili et al., 1993). PAX-FKHR fusion proteins enhance tumor cell proliferation and invasive growth (Anderson et al., 2001; Ayyanathan et al., 2000; Bernasconi et al., 1996) and protect tumor cells against apoptosis, most likely by inducing BCL-XL (Margue et al., 2000), c-MET (Epstein et al., 1996; Ginsberg et al., 1998), and other regulators of myogenic differentiation (Khan et al., 1998, 1999). In addition, many human rhabdomyosarcomas overexpress survival factors, such as IGF2, and carry mutations in the *TP53* tumor suppressor gene and in other cell cycle regulators such as *MDM2*, *CDKN2A* ($p16^{INK4}$), and *CDK4* (Merlino and Helman, 1999).

The Fos protein is a major component of the AP-1 transcription factor, which regulates various biological processes by converting extracellular signals into changes in the expression of specific target genes (Jochum et al., 2001). Mice lacking Fos develop osteopetrosis due to the lack of osteoclasts, the resorbing cell type of bone (Grigoriadis et al., 1994; Johnson et al., 1992; Wang et al., 1992). Consistent with its original identification as the transforming activity of the mouse osteosarcoma viruses FBJ-MuSV and FBR-MuSV (Finkel et al., 1966), ectopic expression of Fos in embryonal stem cells and transgenic mice results in oncogenic transformation and the development of chondrosarcoma and osteosarcoma, respectively (Grigoriadis et al., 1993; Wang et al., 1991). In addition, Fos mediates both pro- and antiapoptotic signals and regulates invasive growth and angiogenesis during tumorigenesis (Hafezi et al., 1997; Orlandini et al., 1996; Saez et al., 1995; Schreiber et al., 1995). In contrast to the prototype *Fos* protooncogene, *Trp53* prevents oncogenic transformation by blocking cell cycle progression or inducing apoptosis (Levine, 1997). *Trp53* is frequently mutated in human tumors, and *Trp53*-deficient mice develop a wide spectrum of malignancies with a high predisposition toward lymphomas (Harvey et al., 1993; Jacks et al., 1994).

Results

To study a possible genetic interaction between germline mutations in the *Trp53* and *Fos* genes, $Trp53^{-/-}$ and $Fos^{-/-}$ mice

SIGNIFICANCE

Rhabdomyosarcoma is the most common soft-tissue sarcoma in children, yet little is known about the underlying genetic changes causing this disease. *Trp53*/*Fos* double knockout mice provide an important mouse model for rhabdomyosarcoma. Several aspects of human rhabdomyosarcoma development, such as a short latency period, localization of tumors to the head and neck region, and histological and immunohistochemical characteristics, are faithfully recapitulated in *Trp53*/*Fos* mutant mice. This study identifies a novel and unexpected tumor suppressive function of the *Fos* protooncogene.

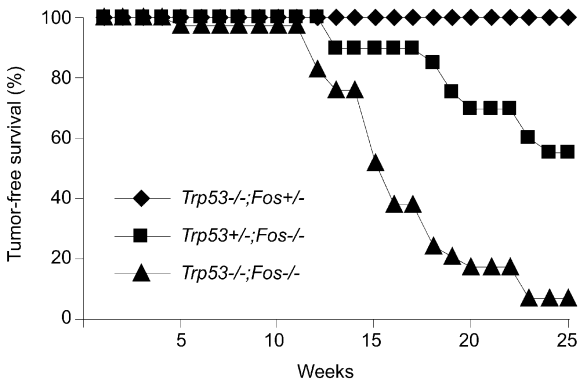

Figure 1. Rhabdomyosarcoma development in *Trp53/Fos* mutant mice

Rhabdomyosarcoma incidence was analyzed in $Trp53^{-/-};Fos^{-/-}$ (n = 29) and $Trp53^{+/-};Fos^{-/-}$ mice (n = 20) in comparison to $Trp53^{-/-};Fos^{+/-}$ controls (n = 20). $Trp53^{-/-};Fos^{-/-}$ mice develop rhabdomyosarcomas with high penetrance and short latency. Rhabdomyosarcomas were observed in $Trp53^{+/-};Fos^{-/-}$ with lower penetrance but never developed in $Trp53^{-/-};Fos^{+/-}$ mice.

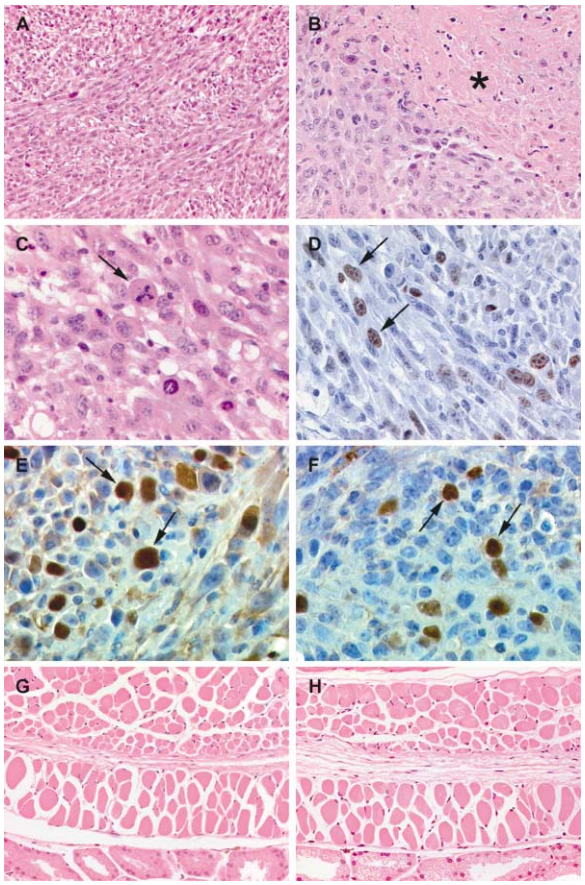

Figure 2. Histology of rhabdomyosarcomas and muscle tissue in *p53/Fos* mutant mice

Histological analyses of a tumor derived from the orbita of a $Trp53^{-/-};Fos^{-/-}$ mouse reveals a spindle cell sarcoma (**A**) with areas of necrosis ([**B**]; note asterisk), nuclear pleomorphism ([**C**]; arrow), high Ki-67 labeling index ([**D**]; arrows), and nuclear immunoreactivity for rhabdomyoblastic markers, MyoD ([**E**]; arrows), and myogenin ([**F**]; arrows). Muscle tissue from the orbita of a $Trp53^{-/-};Fos^{-/-}$ mouse (**H**) shows no abnormalities compared to an age-matched $Trp53^{+/-};Fos^{+/-}$ control (**G**). Magnification is 90× in **A**, 180× in **B**, 220× in **C**–**F**, and 110× in **G** and **H**.

were intercrossed to generate *Trp53/Fos* double mutant mice. These compound mice were obtained at Mendelian numbers and initially were phenotypically indistinguishable from the osteopetrotic $Fos^{-/-}$ mice (data not shown). However, $Trp53^{-/-};Fos^{-/-}$ mice started to develop tumors of the facial and orbital regions at 10 weeks of age, and tumor penetrance was 93% at 25 weeks (Figure 1). Tumors at these sites also grew in $Trp53^{+/-};Fos^{-/-}$ mice, although with lower penetrance, but were not observed in $Trp53^{-/-};Fos^{+/-}$ and $Trp53^{+/-};Fos^{+/-}$ mice (Figure 1, data not shown). Histological analyses of tumors revealed broad anastomosing fascicles of tumor cells with small areas of necrosis (Figures 2A and 2B). Tumor cells were polygonal or elongated and had pleomorphic nuclei and abundant eosinophilic cytoplasm with cross-striations (Figure 2C). Mitotic figures were numerous, and staining for the cell cycle-associated Ki-67 antigen revealed high proliferative activity (Figure 2D). Tumors displayed invasive growth into facial and external orbital muscles, suggesting an origin in the muscle compartments of these sites. Immunoreactivity for well-established markers of myogenic differentiation, including desmin (data not shown), myogenin, and MyoD, was observed in most tumor cells (Figures 2E and 2F). Tumor cell morphology and marker profile were remarkably similar to the embryonal type of human rhabdomyosarcoma. To analyze whether inactivation of the *Trp53* and *Fos* genes interferes with normal muscle development, histological analysis of orbital and femoral muscles was performed in 6- to 10-week-old, tumor-free $Trp53^{-/-};Fos^{-/-}$ mice. No morphological abnormalities were found, and comparable numbers of satellite cells and Ki-67-reactive cells were present in muscles derived from $Trp53^{-/-};Fos^{-/-}$ and $Trp53^{+/-};Fos^{+/-}$ mice (Figures 2G and 2H, data not shown). Therefore, muscle tumor development in *Trp53/Fos* mutant mice is not associated with a general defect in muscle cell differentiation and increased numbers of progenitor cells.

To study the cellular mechanisms underlying muscle tumor development, tumor cell lines were established. They all showed characteristic myoblast morphology and expressed the muscle-specific markers MyoD and desmin (Figure 3A). When grown under appropriate conditions, cells fused to form differentiated myotubes with cytoplasmic cross-striations, providing additional evidence for the rhabdomyoblastic phenotype of the tumor cells (Figure 3B). We next analyzed the effects of reexpressing *Fos* and *Trp53* in double mutant cell lines using retroviral gene transfer. Expression of *Trp53* resulted in massive cell death, and no cell lines could be established (data not shown). This is in agreement with Trp53 function to induce cell cycle arrest and apoptosis when overexpressed in cultured cells (Diller et al., 1990). In contrast, we were able to generate $Trp53^{-/-};Fos^{-/-}$ rhabdomyosarcoma cell lines reexpressing Fos as judged by RNase protection and Western blot analysis (Figure 4A and data not shown). Fos reexpression in rhabdomyosarcoma cell lines did not alter cellular morphology. However, cell lines expressing Fos grew significantly slower than noninfected or empty-vector controls, resulting in 30%–60% fewer cells after 6 days in culture (Figure 3C). Therefore, we next analyzed potential effects of Fos reexpression on proliferation and cell death. Whereas cell cycle analysis by flow cytometry did not

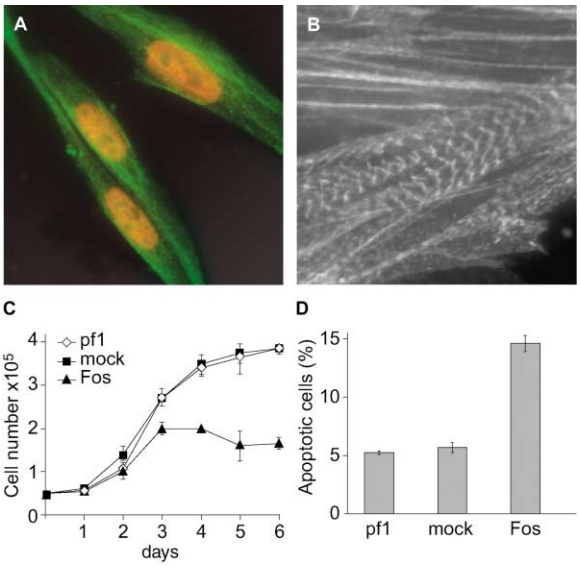

Figure 3. Fos-induced apoptosis in rhabdomyosarcoma cell lines

A: Indirect immunofluorescence analysis of rhabdomyosarcoma cell lines established from *Trp53*/*Fos*-deficient muscle tumors. Under nondifferentiating conditions, cells express the muscle-specific markers MyoD (red, nuclear) and desmin (green, cytoplasmic).

B: Formation of myotubes with cross-striations visualized by α-actinin staining in the cultures when grown at high density and in low serum medium.

C and **D**: Reexpression of Fos in *Trp53*/*Fos*-deficient rhabdomyosarcoma cell lines leads to reduced cell numbers (**C**) and increased spontaneous apoptosis (**D**) of tumor cells.

reveal significant changes in cell cycle progression (data not shown), we found a 2- to 3-fold increase in the number of apoptotic cells upon ectopic Fos expression (Figure 3D). These data demonstrate that reexpression of Fos in *Trp53*/*Fos*-deficient rhabdomyosarcoma cells leads to increased cell death without affecting cell proliferation.

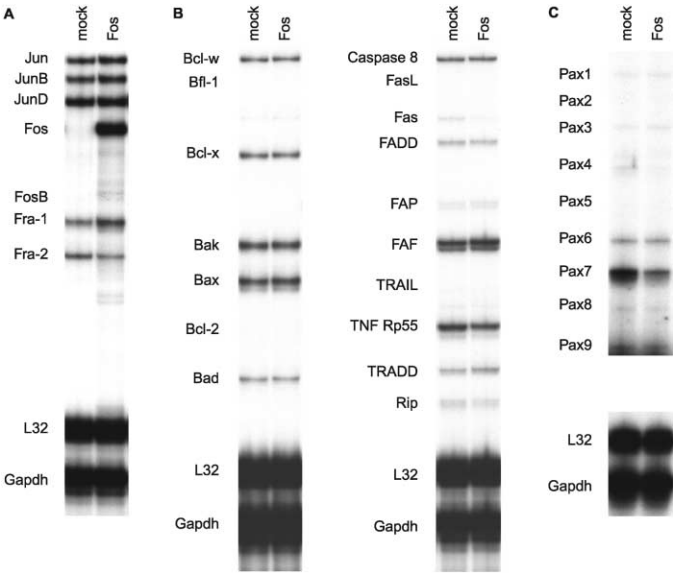

Figure 4. Fos is able to downregulate Pax7 in rhabomyosarcoma cell lines

mRNA expression levels of AP-1 family members (**A**), of genes that modulate apoptosis (**B**), and of Pax genes (**C**) were analyzed by RNase protection assay after mock transfection or reexpression of Fos.

To begin to understand the molecular pathways responsible for the apoptosis in *Trp53*/*Fos* mutant rhabdomyosarcoma cell lines after ectopic Fos expression, we analyzed mRNA levels of other AP-1 genes and genes known to modulate apoptosis in rhabdomyosarcoma cells, including Pax genes. RNase protection assays revealed that reexpression of Fos did not change mRNA levels of other AP-1 family members in all three rhabdomyosarcoma cell lines tested, except for *Fosl1* (*Fra-1*), which has previously been shown to be a transcriptional target of Fos (Bergers et al., 1995) (Figure 4A). Expression of *Bcl-2* family members and death signaling molecules of the *TNF*-α and *Fas* pathways were also not significantly affected by ectopic Fos expression (Figure 4B). However, expression of *Pax7* was substantially repressed by Fos in two of three cell lines analyzed (Figure 4C and data not shown). Expression of the other *Pax* genes was not significantly changed.

Since Pax7 expression is downregulated during myogenic terminal differentiation (Seale et al., 2000), we tested whether ectopic Fos expression would induce differentiation in rhabdomyosarcoma cell lines. Semiquantitative and quantitative RT-PCR analysis of myogenic marker gene expression revealed that Fos reexpression resulted in decreased levels of *MyoD*, *Myogenin*, and *myosin heavy chain* (*MHC*) *IIb* (Figures 5A and 5B). Moreover, Fos reexpression inhibited differentiation into mature myotubes (data not shown). These observations are consistent with published data identifying Fos/AP-1 as a negative regulator of *MyoD* expression and myoblast differentiation (Pedraza-Alva et al., 1994).

Finally, we tested whether ectopic Fos expression in primary myoblasts would cause changes in gene expression similar to those observed in rhabdomyosarcoma cell lines. Primary myoblast cultures were prepared from *Trp*53-deficient newborn mice, transfected with a Fos expression vector, and mRNA was analyzed by quantitative RT-PCR. Ectopic Fos expression resulted in upregulation of *Mmp3* expression, a known Fos target gene (data not shown). Fos expression led to a decrease in *MyoD* and *Myogenin* expression and, importantly, a decrease in *Pax7* expression below the detection limit (Figure 5C). These data confirm that Fos negatively modulates the expression of myogenic markers and show that Fos can repress *Pax7* expression both in primary myoblasts and rhabdomyosarcoma cell lines.

Discussion

This study shows that inactivation of the bona fide oncogene *Fos* in *Trp53* mutant mice specifically leads to the formation of sarcomas that morphologically resemble the embryonal variant of human rhabdomyosarcoma. In humans, most rhabdomyosarcomas of this type localize to the head and neck region, where rhabdomyosarcoma development consistently occurs in *Trp53*/*Fos* mutant mice. The mechanism for this selective localization to the face and orbita, both in human and mouse, remains unexplained. Reexpression of Fos in *Trp53*/*Fos* mutant rhabdomyosarcoma cell lines did not alter cell cycle progression but led to increased cell death of tumor cells, suggesting that impaired apoptosis contributes to rhabdomyosarcoma formation in *Trp53*/*Fos* mutant mice. The increase in apoptosis upon Fos reexpression is consistent with previous observations that Fos

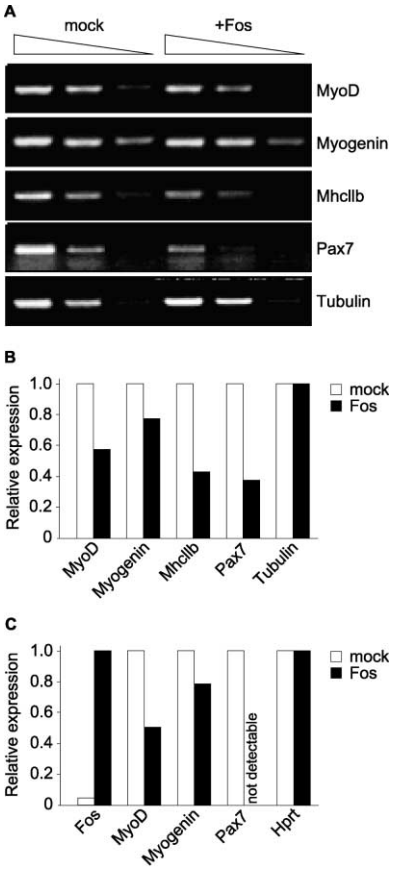

Figure 5. Effects of ectopic Fos expression on myogenic marker gene and Pax7 expression in rhabomyosarcoma cell lines and primary myoblasts

Expression analysis of myogenic marker genes in *Trp53*/*Fos*-deficient rhabdomyosarcoma cell lines upon reexpression of Fos. RNA expression levels were analyzed by semiquantitative (**A**) and quantitative (**B**) RT-PCR. For **A** and **B**, a representative example of data obtained from three independent cell lines is shown. **C**: Expression analysis of myogenic marker genes in *Trp53*-deficient primary myoblasts upon ectopic expression of Fos. Cells were transfected with pBabe control or Fos expressing vector, and RNA expression levels were analyzed by quantitative RT-PCR.

can have proapoptotic functions in various cell types, including neurons, retinal photoreceptors, prostatic epithelial cells, and B lymphocytes (Jochum et al., 2001). Ectopic expression of Fos was associated with a substantial reduction of *Pax7* expression in primary myoblasts and in two of three *Trp53*/*Fos* mutant rhabdomyosarcoma cell lines. Pax7 has previously been shown to be essential for the differentiation of myogenic satellite cells (Seale et al., 2000), and elevated Pax7 levels have been found in a human embryonal rhabdomyosarcoma cell line (Bernasconi et al., 1996). Moreover, treatment of this rhabdomyosarcoma cell line with *Pax7* antisense oligonucleotides induced cell death (Bernasconi et al., 1996). These data suggest that reduced Pax7 levels could account for the increased apoptosis after reexpression of Fos. However, altered *Pax7* expression was observed in only two of three *Trp53*/*Fos* mutant rhabdomyosarcoma cell lines. Future experiments using cDNA microarray analysis will aim to unravel additional mechanisms involved in Fos-dependent rhabdomyosarcoma development.

Genetically engineered models for rhabdomyosarcoma are rare, and the application of most models is limited by low tumor penetrance or long latency (Hahn et al., 1998; Harvey et al., 1993; Takayama et al., 1997; Teitz et al., 1993; Vogel et al., 1999). Since rhabdomyosarcoma development in *Trp53*/*Fos* mutant mice is frequent and similar to tumorigenesis in mice carrying a hepatocyte growth factor/scatter factor (HGF/SF) transgene in an *Ink4a*/*Arf*-deficient background (Sharp et al., 2002) and in mice carrying a HER-2/neu transgene in a *Trp53* mutant background (Nanni et al., 2003), these mutant mice may provide unique models for therapeutic studies. Moreover, the rhabdomyosarcomas in *Trp53*/*Fos* mutant mice closely resemble human rhabdomyosarcoma with respect to location and histology; therefore, we speculate that *Fos* inactivation might also play a role in human rhabdomyosarcoma development.

Experimental procedures

Mice and histological analysis

Generation, genotyping, and phenotypic characterization of *Trp53* and *Fos* mutant mouse strains have been decribed previously (Jacks et al., 1994; Wang et al., 1992). Mutant mice were maintained on a mixed (129Sv x C57BL/6) genetic background. Tissues were immersion fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin/eosin using standard histological techniques. For immunohistochemistry, antigen retrieval with citrate buffer was performed, and tissue sections were incubated with primary antibody: desmin (clone Y-20; Santa Cruz), MyoD (clone 5.8A; DAKO), myogenin (F5D; DAKO), Ki-67 (clone TEC3; DAKO). Immunoreactivity was detected with biotinylated secondary antibodies and the biotin/streptavidin-horseradish peroxidase system (DAKO; Vector) using diaminobenzidine (DAB) as a chromogen.

Generation of rhabdomyosarcoma cell lines and primary myoblasts, immunofluorescence staining

Tumor samples were mechanically disaggregated and explanted into petri dishes. Pools of cells from three independently isolated tumors were established as cell lines and further characterized. Experiments were performed at passage 5–8 in culture. For all experiments, cells were cultured in DMEM containing 10% fetal calf serum, except for differentiation into myotubes, which was performed in DMEM containing 5% horse serum. Indirect immunofluorescence staining of cells was performed according to standard techniques using the following primary antibodies: desmin (clone Y-20; Santa Cruz), MyoD (clone 5.8A; DAKO), and α-actinin (Sigma). Primary myoblasts were prepared from newborn *Trp53*$^{-/-}$ mice (Springer et al., 1997).

Retroviral gene transfer, proliferation, and apoptosis assays

Fos and *Trp53* cDNAs were cloned into pBabe retroviral vectors containing an IRES-EGFP cassette to monitor expression. Tumor cell lines at passage 6 were infected with viruses and selected with 2.5 ug/ml Puromycin according to standard procedures. Expression of *Fos* and *Trp53* was verified by Northern blot and Western blot analysis. For proliferation assays, 0.3×10^5 cells were plated into 6-well dishes and counted every 24 hr for 6 consecutive days. Duplicates were counted for each cell line and time point. For apoptosis assays, cells were plated in duplicates at 0.5×10^5 cells per 6-well dish, and apoptosis was measured after 48 hr in culture using the Annexin-V cell death detection kit (Roche) according to the manufacturer's instructions. Primary myoblasts were transfected with either pBabe control or Fos expressing vector with Lipofectamine according to the manufacturer's recommended protocol.

RNase protection assay, quantitative RT-PCR

Total RNA was isolated with the TRIZOL protocol (Sigma). RNase protection assays were performed using the RiboQuant multiprobe RNase protection assay systems mJun/Fos, mApo-2, mApo-3, and mPax (Pharmingen) according to the manufacturer's protocol. Expression of myogenic markers and controls were analyzed by real-time PCR using DNA Engine Opticon 2 Lightcycler™ with the following primers. Hprt Forward GCTTGCTGGTGA AAAGGACCTC; Hprt Reverse CAAATCAAAAGTCTGGGGACGC; Tubulin Forward CAACGTCAAGACGGCCGTGTG; Tubulin Reverse GACAGAGG

CAAACTGAGCACC; Fos Forward ATGGGCTCTCCTGTCAACACAC; Fos Reverse TTCTCCGTTTCTCTTCCTCTTCAG; Mmp3 Forward TGTGTGGT TGTGTGCTCATCCTAC; Mmp3 Reverse GATTTCCTCCATTTTGGCGAAC; MyoD Forward GCCCGCGCTCCAACTGCTCTGAT; MyoD Reverse CCT ACGGTGGTGCGCCCTCTGC; Myogenin Forward ACCAGGAGCCCCACT TCTATGATG; Myogenin Reverse GCCCGCCCCCGCCTCTGTA; MHC IIb Forward CACCCAGAACACCAGCCTCATCAA; MHC IIb Reverse TGC GGTCCTCCTCGGTCTGGTA; Pax7 Forward CATCCGGCCCTGCGTCATC; Pax7 Reverse GTCCGGGTAGTGGGTCCTCTCAAA.

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