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Increasing $\alpha_7\beta_1$ -integrin promotes muscle cell proliferation, adhesion, and resistance to apoptosis without changing gene expression

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Abstract

The dystrophin-glycoprotein complex maintains the integrity of skeletal muscle by associating laminin in the extracellular matrix with the actin cytoskeleton. Several human muscular dystrophies arise from defects in the components of this complex. The $\alpha_7\beta_1$ -integrin also binds laminin and links the extracellular matrix with the cytoskeleton. Enhancement of α_7 -integrin levels alleviates pathology in *mdx/utrn*^{-/-} mice, a model of Duchenne muscular dystrophy, and thus the integrin may functionally compensate for the absence of dystrophin. To test whether increasing α_7 -integrin levels affects transcription and cellular functions, we generated α_7 -integrin-inducible C2C12 cells and transgenic mice that overexpress the integrin in skeletal muscle. C2C12 myoblasts with elevated levels of integrin exhibited increased adhesion to laminin, faster proliferation when serum was limited, resistance to staurosporine-induced apoptosis, and normal differentiation. Transgenic expression of eightfold more integrin in skeletal muscle did not result in notable toxic effects *in vivo*. Moreover, high levels of α_7 -integrin in both myoblasts and in skeletal muscle did not disrupt global gene expression profiles. Thus increasing integrin levels can compensate for defects in the extracellular matrix and cytoskeleton linkage caused by compromises in the dystrophin-glycoprotein complex without triggering apparent overt negative side effects. These results support the use of integrin enhancement as a therapy for muscular dystrophy.

Keywords

myoblast proliferation; integrin transgenic mice; microarray

CELLS CAN RECOGNIZE AND INTERACT with their environment through membrane receptors specific for different extracellular matrix proteins. The heterodimeric integrins are evolutionarily conserved receptors for matrix proteins found in all metazoans (37). In skeletal muscle, the association of the extracellular matrix with the cytoskeleton is essential for maintaining the integrity of the sarcolemma, sustaining appropriate myofiber architecture, and correctly transmitting the forces produced by muscle contractions (5,42). The $\alpha_7\beta_1$ -integrin is part of a linkage system that maintains such transmembrane associations in skeletal muscle (10,66). It binds to laminin in the basal lamina that surrounds muscle fibers. Within muscle fibers, the integrin associates with the subsarcolemmal cytoskeleton network through focal adhesion complexes (5). The $\alpha_7\beta_1$ -integrin is distributed along the sarcolemma at costameres, and it is also concentrated at neuromuscular and myotendinous junctions (2,48). This

localization suggests that the integrin has a pivotal role in maintaining these specialized structures and their functions in skeletal muscle. Congenital myopathies caused by mutations in the human integrin α_7 -gene (*ITGA7*) confirm the importance of the $\alpha_7\beta_1$ -integrin in maintaining normal skeletal muscle physiology (34). Secondary deficiencies in the integrin are also common in patients with other muscular dystrophies and myopathies of unknown etiology and likely contribute to the pathologies that develop (54,55).

The dystrophin-glycoprotein complex also binds laminin and has similar roles in skeletal muscle as the integrin transmembrane linkage system (14). Mutations in the genes encoding components of the dystrophin complex result in different types of muscular dystrophies, including Duchenne muscular dystrophy (DMD) (18). The *mdx* mouse, a genotypic model of DMD, has been used extensively to study this disease and possible therapies. However, unlike DMD patients, the *mdx* mouse has a mild dystrophic phenotype and near-normal life span (8, 15). Utrophin, a mammalian homolog of dystrophin, is increased in DMD patients and *mdx* mice and has been hypothesized to compensate in part for the loss of dystrophin (44,56,71). The *mdx/utrn*^{-/-} mice lacking dystrophin and utrophin exhibit progressive muscular dystrophy, markedly reduced mobility and life span, and severe abnormalities at the neuromuscular and myotendinous junctions (20,30). Since the pathology of skeletal and cardiac muscle of *mdx/utrn*^{-/-} mice more closely resembles that in DMD patients than the pathology of *mdx* mice, the doubly deficient mouse is a useful and appropriate model for developing and testing new therapies for muscular dystrophy (4,12,13,27,31).

In addition to increased utrophin expression, DMD patients and *mdx* mice also have more $\alpha_7\beta_1$ -integrin (36,74). Other cytoskeleton-associated proteins in integrin focal adhesions, such as vinculin and talin, are also elevated in *mdx* mice (44). This increase in integrin and other components of adhesion complexes led us to suggest that the integrin and dystrophin complexes have overlapping roles in linking the extracellular matrix and cell cytoskeleton (36). Thus increasing the amount of integrin may functionally compensate for the absence of dystrophin in DMD patients. This hypothesis was tested and validated by showing that transgenic expression of α_7 alleviates the development of severe muscular dystrophy in *mdx/utrn*^{-/-} mice (12,13). Furthermore, in the absence of both the integrin and dystrophin linkage systems (*mdx/α7*^{-/-} or *gmi* mice), extreme severe muscular dystrophies develop (1,32,58). These results confirm that the $\alpha_7\beta_1$ -integrin and dystrophin complexes are functionally complementary and important in maintaining skeletal muscle integrity. The beneficial effects of increasing integrin in *mdx/utrn*^{-/-} mice include increased longevity, maintaining the structure of the myotendinous and neuromuscular junctions, reduced apoptosis and kyphosis, enhanced regeneration, and hypertrophy (12,13). Thus exploring the therapeutic potential of increasing $\alpha_7\beta_1$ -integrin in dystrophin deficient muscle remains promising.

As a next step in evaluating an integrin-mediated therapy for DMD, we have generated $\alpha_7\beta_1$ -integrin-inducible C2C12 myoblasts and myotubes using a tetracycline-inducible system and evaluated the effects of increasing α_7 on cellular functions and differentiation. We have used Affymetrix microarrays to explore whether increasing the amount of integrin affects the transcription profile of skeletal muscle in vitro and in vivo. A threefold increase in α_7 levels in C2C12 cells promoted their adhesion to laminin, stimulated proliferation, decreased apoptosis when challenged with staurosporine, and did not affect differentiation. Moreover, neither threefold more integrin in C2C12 cells, nor an eightfold increase of integrin in skeletal muscle in vivo, altered normal transcription. Likewise, no overt toxic effects were observed. Therefore enhancing α_7 -integrin expression may be useful as a therapy for muscular dystrophies characterized by compromised dystrophin complexes.

METHODS

Tetracycline-inducible integrin α_7 plasmid

The cDNA encoding the rat integrin α_7 BX2 isoform was amplified from MCK α_7 BX2 plasmid (13) with *pfu* polymerase (Stratagene, La Jolla, CA) using the following conditions: 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 65°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The forward primer (5'-ATGAATTCTCCCATGGCCAGGATTCCGAG-3') and reverse primer (5'-TATCTAGAGCGAATTGGGTACACTTACCTG-3') were used. The amplified α_7 BX2 was cloned into pcDNA4/TO vector of the T-Rex system (Invitrogen, Carlsbad, CA), and the sequence of the final plasmid was verified.

Animals

Protocols for animal use were approved by the Institutional Animal Care and Use Committee, University of Illinois at Urbana-Champaign. The α_7 -integrin transgenic mice used in this study were previously described (7). Weights of 30 animals of each sex were measured weekly for up to 30 wk. Five-week-old female transgenic mice and their wild-type controls (SJ6/C57BL6) were used for microarray studies. Animals were euthanized by CO₂ asphyxiation, and muscles were immediately dissected and snap frozen in liquid nitrogen.

Cell culture and transfection

C2C12 and L8E63 cells were cultured as previously described (9). Cells were transfected with linearized plasmids by using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stably transfected cells were selected with 7.5 μ g/ml blasticidin and 500 μ g/ml zeocin and were analyzed by immunofluorescence. Clones of the Tet α_7 -C2C12 cells with the lowest basal level and the highest expression level of rat α_7 inducible by 1 μ g/ml tetracycline were used for further studies.

Antibodies

Mouse monoclonal antibody O26 was used at a concentration of 10 μ g/ml to selectively detect rat α_7 -integrin by immunofluorescence (9). Polyclonal rabbit antibody against the α_7 B cytoplasmic domain (CDB347) was used to recognize both mouse and rat α_7 -integrin (67). Monoclonal antibody O5 and CDB347 were used in Western blots to detect rat and total integrin α_7 , respectively (67). Rabbit polyclonal antibody against the cytoplasmic domain of the β_1 D integrin chain was generously provided by Dr. W. K. Song (Department of Life Science, Kwangju Institute of Science and Technology, Kwangju, Korea). Monoclonal antibody against myosin heavy chain (MF20) was used to determine the fusion index. Rabbit anti-caspase-3 antibody (Cell Signaling Technology, Danvers, MA) was used to detect apoptotic myoblasts. Fluorophore and horseradish peroxidase-labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Skeletal muscle histology and fibers cross-section area

Ten-micrometer sections of gastrocnemius muscle from 5-wk-old animals were frozen in liquid nitrogen-cooled isopentane (Sigma, St. Louis, MO), were cut by using a Leica CM1900 series cryostat (Nussloch, Germany), and were placed on microscope slides (Surgipath, Richmond, IL). Sections were fixed in 100% acetone at -20°C for 1 min, were rinsed in tap water for 10 min, and were stained with hematoxylin and eosin using standard histological procedures. Fiber cross-sectional areas were measured by using the advanced measurements component of OpenLab software (Improvision, Lexington, MA). The cross-sectional areas of 1,000–1,200 fibers from gastrocnemius muscle of three 12-wk-old animals per genotype were measured, and the distributions were displayed using SigmaPlot (Systat Software, San Jose, CA).

Immunofluorescence

To detect rat α_7 -integrin, live cells were incubated sequentially with 10 $\mu\text{g/ml}$ O26 antibody and secondary antibody, each for 1 h at 37°C, with extensive washing in between, and were then fixed in ice-cold methanol for 5 min and rehydrated in PBS for 30 min. For myosin heavy chain staining, myotubes were fixed in ice-cold methanol for 5 min, rehydrated in PBS for 30 min, and incubated with MF20 antibody in 1% horse serum-PBS and secondary antibody, each for 1 h. For staining of active caspase-3, cells were fixed in ice-cold methanol for 5 min, washed in PBS, and incubated with a 1:100 dilution of primary antibody for 1 h, followed by secondary antibody for 1 h. Coverslips were mounted by using Vectashield (Vector Labs, Burlingame, CA). Tissue sections were fixed in -20°C acetone for 1 min, rehydrated in PBS for 10 min, and blocked in PBS containing 10% horse serum for 30 min. Primary and secondary antibodies in PBS containing 1% horse serum were applied sequentially, each for 1 h, followed by extensive washings. Immunofluorescent images were acquired using a Leica DMRXA2 microscope equipped with an AxioCam digital camera (Zeiss, Thornwood, NY) and analyzed with the use of OpenLab software. Immunofluorescence analyses of α_7 were performed on cells used in all experiments.

Immunoblot analysis

Cells were washed once and harvested in PBS containing 2 mM PMSF. Gastrocnemius and soleus muscles were powdered in liquid nitrogen before extraction. Proteins were extracted and quantified as previously described (12,13). Equal amounts of proteins were separated on 8% polyacrylamide-SDS gels and were transferred to nitrocellulose membranes. Blocked membranes were incubated with the respective primary antibodies in 2% nonfat milk for 1 h. Horseradish peroxidase-conjugated secondary antibodies were used to detect bound primary antibodies. Immunore-active protein bands were detected using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Bands were quantified by using ImageQuant software. Data were obtained from three independent experiments.

Adhesion assay

Cell adhesion was measured as previously described (19). Briefly, Costar 24-well polystyrene plates (Sigma) were coated with substrates in 0.1% BSA in PBS for 2 h at room temperature. The wells were then washed three times with PBS and were blocked with 1% BSA in PBS for 2 h. DMEM (250 μl) with 0.1% BSA was added to the wells and was preincubated for 1 h at 37°C. Cells were washed once with PBS, detached by 0.2 g/l EDTA in PBS, pelleted, and resuspended in DMEM containing 1% BSA. 5×10^4 cells were added to each well and incubated at 37°C for 30 min. Nonadherent cells were removed by gentle rinsing with PBS, and the attached cells were fixed with 4% paraformaldehyde-1% glutaraldehyde in PBS and stained with 1% toluidine blue. After extensive rinses in distilled water, the stained cells were counted using a Nikon inverted microscope equipped with an ocular micrometer. For each well, at least 10 randomly selected fields were measured. Data from three experiments in triplicates are presented.

Proliferation

Tet α_7 -C2C12 cells were grown for 36 h with or without tetracycline, then detached and seeded in growth medium containing different concentrations of fetal bovine serum (FBS). Cell numbers in triplicate samples were measured daily. Doubling times were calculated from linear regression analysis of cell numbers versus time. Data were derived from three independent experiments.

Flow cytometry

Tet α ₇-C2C12 cells were grown for 36 h with or without tetracycline and were starved for 48 h in serum free growth medium to synchronize them at G1/G0. Growth medium containing 20% FBS with or without tetracycline was then added. Cells were collected and fixed in suspension with 70% ethanol 0, 8, 24, and 32 h later. Cells were treated with RNase and stained with propidium iodide. Flow cytometry was carried out using a Coulter XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA). The percentages of cells in each phase of the cell cycle were determined by using ModFit software (Verity Software, Topsham, ME). The data from three experiments in triplicates are presented.

Apoptosis

Tet α ₇-C2C12 cells were cultured on laminin-coated glass coverslips for 36 h with or without tetracycline. Staurosporine (0, 1, or 2 μ M; Cell Signaling Technology) was added for 3 h to induce apoptosis. After immunostaining, the numbers of active caspase-3-positive cells and the total numbers of cells were counted in 100 randomly selected $\times$ 40 fields. Apoptosis assays were done in triplicate in three experiments.

Affymetrix microarray analysis

RNAs were extracted from cells and gastrocnemius and soleus complex muscles. Triplicate cultures of Tet α ₇-C2C12 myoblasts were grown with or without tetracycline for 36 h and were extracted using TRIzol (Invitrogen). RNA pellets were resuspended in diethyl pyrocarbonate-treated water and subjected to a cleanup protocol using an RNeasy minikit (Qiagen, Valencia, CA). The RNA was prepared from 18 mice of each genotype. Triplicate samples for each genotype were made by pooling the RNA from 6 mice. RNA quality was examined using a bioanalyzer (Agilent Technologies, Santa Clara, CA), denaturing gel analysis, and OD₂₆₀/OD₂₈₀ ratios. Total RNA was further processed for use on Affymetrix mouse 430 2.0 microarray slides (Affymetrix, Santa Clara, CA) at the University of Illinois Keck Center for Comparative and Functional Genomics. Affymetrix CEL files were analyzed by using GeneSifter (VizX Labs, Seattle, WA), and the probe level analysis algorithm GC-RMA (robust multichip average with adjustment for GC content of probes) was applied. These data have been deposited in the National Center for Biotechnology Information Gene Expression omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession numbers GSE 8312 and GSE 8313. Microarray statistical analysis was performed using the *t*-test with Benjamini and Hochberg [false discovery rate (FDR)] multiple-testing correction for two group comparisons. Except where indicated, probe sets were defined as differentially expressed if the Benjamini and Hochberg (FDR) corrected *P* was <0.05 and the absolute value of the average fold difference was $\geq$ 2. The correlation coefficient of arrays between groups was calculated using bioconductor and R software packages (28,69).

Reverse transcription-polymerase chain reaction

RNA extracted from cells or skeletal muscle was treated with RNase-free DNase I (Fisher Scientific, Pittsburgh, PA) for 10 min at room temperature to remove genomic DNA. cDNA was synthesized by using RETROscript kits (Ambion, Austin, TX) with random decamers using 100 ng of cDNA as templates. GAPDH was used as an internal control. Primer sequences for each gene are listed in Table 1. PCR reactions were performed by using the following conditions: start at 94°C for 2 min, cycle at 94°C for 30 s, 55°C to 61°C (depending on the primer used) for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. Cycle numbers were determined to be within the exponential amplification range for each gene. PCR products were electrophoresed in 1.5% agarose gels. Intensities of amplicon bands were quantified using ImageQuant software and were normalized to GAPDH control bands from the same samples.

To provide independent confirmation, RNAs for RT-PCR and microarray analyses were purified from separate animals.

Statistical analyses

All averaged data are presented as means $\pm$ SE. Comparisons between two groups were performed by unpaired *t*-test. One-way ANOVA followed by Tukey's posthoc analysis was used if more than two groups were compared. Statistics were computed by using SYSTAT software (Systat Software, San Jose, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Inducible expression and localization of α_7 -integrin in C2C12 myogenic cells

Alleviation of severe muscular dystrophy in *mdx/utrn*^{-/-} mice by $\alpha_7\beta_1$ -integrin demonstrates the overlapping functions between the integrin and the dystrophin glycoprotein complexes (12,13). To further explore developing a therapy for muscular dystrophy on the basis of enhancing α_7 -integrin levels, we have examined the functional and transcriptional consequences of increasing $\alpha_7\beta_1$ -integrin in muscle cells. Mouse C2C12 cells were used to derive α_7 -integrin tetracycline-inducible clones of myogenic cells that express the rat α_7 -integrin. RT-PCR confirmed expression of the rat α_7 -integrin transgene after tetracycline induction, whereas noninduced cells did not express rat α_7 RNA (Fig. 1A). Western blot analysis of myoblasts and myotubes induced with tetracycline detected the rat integrin, whereas no transgene-derived protein was observed in noninduced cells (Fig. 1, B and C). A threefold increase in total integrin was detected in myoblasts and a 1.5-fold increase was present in myotubes (Fig. 1B). To be functionally active, the integrin must be targeted to the cell membrane. Immunofluorescence staining of live cells with anti-rat α_7 monoclonal antibody (O26) demonstrated that induced rat α_7 -integrin was localized to the cell membrane in both myoblasts and myotubes (Fig. 1C). Thus the induced integrin localized to the cell membrane to form $\alpha_7\beta_1$ -integrin focal adhesions.

Induced α_7 -integrin selectively promotes myoblast adhesion to laminin

To test whether rat α_7 -integrin expressed in C2C12 cells is functional, we determined whether the induced α_7 -integrin promotes adhesion to different extracellular matrix proteins. Induced and noninduced Tet α_7 -C2C12 cells were detached and plated in complete medium on BSA-, laminin-, or fibronectin-coated 24-well plates. The numbers of cells that adhered after 30 min were determined (Fig. 2A). Increased integrin promoted cell adhesion on laminin and decreased adhesion on fibronectin (Fig. 2B). Virtually no cells adhered to BSA-coated wells, which confirms that adhesion was substrate specific.

$\alpha_7\beta_1$ -Integrin stimulates cell proliferation and promotes cell cycle progression

Integrins can function with receptors for growth factors such as EGF and hepatocyte growth factor to regulate cell proliferation (16,59). We therefore tested whether induced Tet α_7 -C2C12 cells, with approximately threefold more integrin, have a different proliferation rate. Induced or noninduced Tet α_7 -C2C12 cells were seeded at the same density in growth medium containing different concentrations of FBS, with or without tetracycline. Wild-type C2C12 cells were used to exclude the possibility that tetracycline itself affects cell proliferation. No differences in doubling times were observed in induced or noninduced cells grown in medium containing 20% or 10% FBS. However, lower concentrations of FBS (0.5%, 1%, 2%, and 5%) slowed cell proliferation of noninduced cells but did so to a lesser extent in cells with elevated integrin levels (Fig. 3A). These results suggest that $\alpha_7\beta_1$ -integrin may sensitize myoblasts to growth factors to activate cell proliferation.

To further test whether additional $\alpha_7\beta_1$ -integrin renders myoblasts more sensitive to growth factors, sparsely seeded myoblasts were grown for 48 h in serum-free medium, with or without tetracycline, to synchronize the cells at G1/G0 without inducing differentiation. The medium was then changed to contain 20% FBS, with or without tetracycline, and cell cycle progression was determined by flow cytometry. Tet α_7 -C2C12 cells induced with tetracycline progressed into G2/M faster than noninduced cells (Fig. 3B). This is the first report to show that $\alpha_7\beta_1$ -integrin regulates myoblast proliferation and cell cycle progression, and it supports previous findings that myoblasts proliferate more rapidly when grown on laminin (26).

Enhanced integrin expression does not affect myoblast differentiation

The $\alpha_7\beta_1$ -integrin is expressed on replicating myoblasts, quiescent satellite cells, and mature skeletal muscle fibers, and it is involved in myoblast differentiation and maintaining muscle integrity (10). Myoblast fusion and expression of myosin heavy chain were used to monitor whether increased $\alpha_7\beta_1$ affected cell differentiation. C2C12 and Tet α_7 -C2C12 cells were grown to confluence, at which point differentiation medium with or without tetracycline was added. The percentage of myosin heavy chain-positive cells and the cell fusion index indicate no significant differences due to increased integrin (Fig. 4, A and B).

$\alpha_7\beta_1$ -Integrin protects myoblasts from apoptosis induced by staurosporine

Cell adhesion regulates proliferation, migration, and differentiation, and it also supports cell survival (63,75). Integrin-mediated adhesion regulates apoptosis in a variety of cells, including skeletal muscle (43,68). In laminin-2-deficient congenital muscular dystrophy, the amount of $\alpha_7\beta_1$ -integrin is secondarily reduced, leading to a loss of muscle fiber adhesion to the extracellular matrix, apoptosis, and muscle wasting (74). Likewise, α_7 -transgenic *mdx/utrn*^{-/-} mice with elevated levels of integrin also have fewer apoptotic nuclei than *mdx/utrn*^{-/-} mice as measured by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling staining (12). We therefore used the Tet α_7 -C2C12 cells to examine whether integrin can prevent apoptosis in myoblasts in vitro. Tet α_7 -C2C12 myoblasts were grown in tetracycline to express excess integrin and were then treated with staurosporine. Staurosporine-induced apoptosis in C2C12 cells can be identified by staining for active caspase-3 (35,50). Apoptotic cells also shrink and can be identified morphologically (Fig. 5A, a, c, and e). Immunofluorescence staining revealed less caspase-3 activity in tetracycline-induced cells compared with noninduced and control

C2C12 cells (Fig. 5A, b, d, and f). The enhanced expression of integrin in myoblasts reduced apoptosis by approximately twofold in cells grown on laminin (Fig. 5B). Thus it appears that enhanced expression of $\alpha_7\beta_1$ -integrin can protect myoblasts from apoptosis.

The histology of α_7 -integrin transgenic skeletal muscle appears normal

Transgenic mice expressing the integrin α_7 BX2 subunit, under control of the muscle creatine kinase promoter, were generated as described previously (7). Immunoblot analysis of protein extracts of the gastrocnemius and soleus muscles from α_7 -transgenic and wild-type control mice revealed an eightfold increase in α_7 BX2 protein in the transgenic muscle (Fig. 6A). Immunofluorescence staining with the rat-specific O26 monoclonal antibody and a polyclonal antibody against the α_7 B cytoplasmic domain demonstrated a high level of expression of the rat integrin α_7 -transgene (Fig. 6B). The overwhelming majority of the integrin was localized to the sarcolemma; however, some cytoplasmic localization was also detected (Fig. 6B) and is likely due to an excess of α_7 -chain. Hematoxylin and eosin staining revealed no discernable differences between the skeletal muscles of the transgenic mice and their wild-type controls (Fig. 6B). Similarly, no significant differences in body weight (Fig. 6C) or average fiber cross-sectional areas were observed (Fig. 6D), although there was a 12% increase in the percentage of smaller fibers in the transgenic mice. The eightfold more α_7 -chain expressed in the transgenic

skeletal muscle did not produce any overt negative changes under normal physiological conditions.

Increasing the amount of $\alpha_7\beta_1$ -integrin in myoblasts and skeletal muscle does not markedly alter normal transcription

Integrin $\alpha_7\beta_1$ can interact with signaling molecules and transcription factors such as FHL2 and FHL3 in skeletal muscles (60,76). Therefore, for integrin overexpression to be an effective therapy, it would be best if it did not disrupt normal cell functions and gene expression. As a global index of that, transcription profiles of noninduced and induced Tet α_7 -C2C12 cells and of wild-type mice and integrin α_7 -transgenic mice were determined by using Affymetrix microarray analysis.

Comparison of the data from the noninduced and induced Tet α_7 -C2C12 cells revealed relatively few differences in their overall transcriptional profiles (Fig. 7A). The correlation coefficient of the induced and noninduced myoblast arrays was 0.996, which demonstrated the high similarity in their transcription profiles. Seven probes representing six genes indicated greater than twofold changes with Benjamini and Hochberg adjusted *P* values < 0.05 (Table 2). These six genes are involved in cell metabolism (*Cth*, *Ugt1a2*, *Ank2*, and *pigt*), regulation of apoptosis (*Trib3* and *Angptl4*), and endoplasmic reticulum (ER) overloading responses (*Trib3*). Among the six genes, only *Trib3* had two separate probe sets, and both revealed similar changes in expression levels. This was confirmed by semiquantitative RT-PCR (Fig. 7B). A full list of probe sets that revealed changes in expression of ~1.5-fold, with adjusted *P* values < 0.05, is in Supplemental Table 1. (The online version of this article contains supplemental data.)

Trib3, the mammalian homolog 3 of *Drosophila* tribbles, was first identified as an inhibitor of the Akt pathway, and it is highly expressed in liver (22). More recent reports demonstrated that *Trib3* expression can be induced by stress-related signals downstream of *CHOP* (53,77). Interestingly, *CHOP* was also increased 1.6-fold in our array analysis. These increases in expression of *Trib3*, *CHOP*, and several other genes involved in cellular stress responses may represent a generalized response to the overexpression of membrane receptors and adapt the myoblast to increased α_7 -chain. However, *Trib3* is not normally expressed in adult skeletal muscle, and its expression is dramatically decreased upon differentiation of C2C12 cells (38). Thus this increase of *Trib3* transcripts in myoblasts is not likely significant to the response of muscle fibers to increases in $\alpha_7\beta_1$ -integrin.

Array analysis of gastrocnemius and soleus muscles from α_7 -transgenic mice was done to determine the effect of increasing integrin *in vivo*. Twenty-nine probe sets representing 20 genes reported more than twofold increases in transcription, and three probe sets identified more than twofold decreases in expression of three genes (Table 3). A 36-fold increase in α_7 -transcripts was found in the transgenic mice. Genes whose expression significantly changed ~1.5-fold are listed in Supplemental Table 2. Similar to the results from the myoblast array analysis, relatively few genes were found to be differentially expressed in the integrin transgenic mice (Fig. 7A). The 0.999 correlation coefficient of the wild-type and α_7 -integrin transgenic mice array analysis indicates the high similarity of their transcription profiles. Significant changes of several genes noted in the array data (*Teme58*, *pttg1*, *hspa5*, and *arinet*) were confirmed by semiquantitative RT-PCR (Fig. 7B).

Gene ontology analysis revealed that many of the changes in gene expression are related to cell metabolism, including protein synthesis, posttranslational processing, and protein transport. These genes include ribosomal protein S4 (1110033J19Rik), branched-chain amino acid aminotransferase (*Bcat2*), stromal cell-derived factor 2-like protein 1 (*SDF2L1*), thyroid hormone receptor interactor 11 (*Trip11* or *TRIP230*), transmembrane protein 58 (*Tmem58*,

similar to rat *Scotin*), progressive myoclonic epilepsy, type 2 gene- α (*Epm2a*), disulfide isomerase-associated 6 (*Pdia6*), coatamer protein complex subunit- α (*Copa*), and heat shock 70-kDa protein 5 (*Hspa5*, *Grip*, or *Bip*).

Other groups of genes that were differentially transcribed are related to cell division, G protein signaling, and the interferon-inducible p200 family of proteins. *Tacc2*, *Prc1*, and *pttg1* (securin) are regulators of cell division and showed increased expression. Their relation to the integrin is unclear, but an increase in satellite cell proliferation was noted in $\alpha_7mdx/utrn^{-/-}$ mice (12). *RGS5* and *xpr1* are involved in G protein signaling (3,17), and their expression was increased in the integrin transgenic mice. Five probes representing three proteins in the interferon-inducible p200 family were significantly increased in the integrin transgenic mice (Table 3). Members of this family have specific functions in skeletal and cardiac muscles (46). *Ifi204* promotes myoblast differentiation and is increased following MyoD-dependent differentiation of muscle precursor cells (46). Therefore, the increased expression of these genes may enhance skeletal muscle differentiation in the α_7 -integrin transgenic mice.

Several expressed sequence tags are also differentially expressed. Among these, increased expression of Armet, an extracellular protein, may represent a modification of the extracellular environment by muscle fibers with enhanced integrin.

Neither β_1 -integrin gene expression nor the mRNAs encoding other focal adhesion components increased commensurate with the increases in α_7 -chain in both myogenic cells and skeletal muscle. Immunofluorescence staining also did not show an increase in β_1 D in the α_7 -transgenic mice (Fig. 6B). The localization of α_7 -chain inside the transgenic muscle fibers (Fig. 6B, arrows) and the decreased expression of integrin α_4 (*ITGA4*) transcripts seen in the microarray data indicates the β_1 -chain may be limiting in the α_7 -transgenic mice. Normal expression of β_1 -integrin in α_7 -knockout mice also suggests that the regulation of transcription of α_7 and β_1 is independent in skeletal muscle (25,58), and it explains the lack of changes in β_1 mRNA evidenced in our arrays. Since no alterations in transcription of the genes encoding the components of the dystrophin glycoprotein complex were observed in both array analyses, it is likely that the enhanced structural linkage and signaling provided by increased integrin accounts for the alleviation of dystrophic pathology in α_7 -transgenic *mdx/utrn*^{-/-} mice (12, 13).

Array analysis of ADAM12 transgenic mice, in which an increase in ADAM12 partially rescued *mdx* mice, reported an increase of α_7 -protein but not mRNA in skeletal muscle (41, 51). In both our cell and tissue arrays, no significant change in transcription of any of the ADAM genes was detected, which suggests that $\alpha_7\beta_1$ -integrin may function downstream or in concert with ADAM12 in promoting muscle regeneration and preventing muscular dystrophy. This conclusion is also supported by the inability of overexpressing ADAM12 to alleviate the pathology of laminin-2-deficient mice in which integrin $\alpha_7\beta_1$ is secondarily reduced (33).

The array analyses reported in the present study show that few perturbations of transcription result from increasing $\alpha_7\beta_1$ -integrin in myoblasts or skeletal muscle and encourage the development of an integrin-mediated therapy for muscular dystrophy.

DISCUSSION

The $\alpha_7\beta_1$ -integrin is a laminin receptor on muscle progenitor cells and mature skeletal muscle fibers. Developmentally regulated expression and alternative splicing of the α_7 -integrin chain are important for appropriate myogenic cell proliferation, adhesion, migration, and differentiation (19,78). Alleviation of muscular dystrophy by transgenic overexpression of the integrin in *mdx/utrn*^{-/-} mice revealed the potential for using it as a therapy for muscular dystrophy (13). This led us to examine the biological effects of enhancing integrin levels in

myoblasts and skeletal muscle fibers. Using tetracycline-inducible integrin-expressing myoblasts, we have demonstrated that increasing the amount of the $\alpha_7\beta_1$ -integrin improves myoblast adhesion and proliferation and imparts resistance to staurosporine-induced apoptosis but does not interfere with myogenic differentiation. These effects were achieved without markedly altering gene expression.

The alleviation of severe muscular dystrophy in mice by enhanced expression of the $\alpha_7\beta_1$ -integrin results from the functional enhancement of muscle fiber adhesion, strengthening of myotendinous and neuromuscular junctions expansion of regenerative capacity, and enhanced signaling, leading to decreased apoptosis and fiber survival (12,13). As shown here, transgenic overexpression of the α_7 -chain in wild-type mice did not alter body weight or skeletal muscle histology, nor did it dramatically change the skeletal muscle global gene expression. Thus, increasing α_7 -integrin is beneficial to muscle and has only minimal adverse effects.

In myoblasts, tetracycline induction resulted in threefold overexpression of α_7 -integrin, whereas only a 1.5-fold increase was observed in myotubes. The smaller fold increase observed in myotubes is likely due to increased synthesis of endogenous mouse α_7 -integrin during C2C12 differentiation (67,79). Furthermore, consistent with previous reports that α_7 -integrin increases non-muscle-cell adhesion to laminin (19,23,72), we confirmed that enhanced expression of integrin $\alpha_7\beta_1$ in C2C12 myoblasts also increases their adhesion to laminin; however, it correspondingly decreases adhesion to fibronectin. Similar effects were reported when α_7 -integrin was overexpressed in Chinese hamster ovary cells, suggesting that there may be a competition between α_7 and other integrin α -subunits for the common β_1 -chain (72). In support of this hypothesis, a decrease of α_4 integrin transcripts was also detected in the α_7 -integrin transgenic mice. These data suggest a potential competition between different integrin α -chains for their common β_1 -subunit as a mechanism of regulating myoblast affinity for different matrix proteins.

The increased capacity of cells with enhanced levels of $\alpha_7\beta_1$ -integrin to proliferate in low serum concentrations suggests that the integrin may render satellite cells more responsive to limiting concentrations of growth factors in vivo. These quiescent muscle stem cells that are activated by low levels of growth factors may contribute to an increase in the number of precursor cells and enhanced regeneration and repair such as seen in α_7 -integrin transgenic *mdx/utrn*^{-/-} mice (12). Similarly, the $\alpha_7\beta_1$ -integrin renders myofibers highly sensitive to respond to low levels of agrin, and this promotes clustering of acetylcholine receptors, an early stage in the development of neuromuscular junctions (9,11).

Increased apoptosis of both myofibers and myoblasts in dystrophic muscle has been reported (49,65,73), and caspase-3 activation has been shown to participate in this process. We observed an approximate 50% reduction in the number of caspase-3 activated cells following staurosporine treatment of cells with enhanced expression of $\alpha_7\beta_1$, which suggests that the integrin can function as an antiapoptotic signal in myogenic cells. α_7 -Integrin transgenic *mdx/utrn*^{-/-} mice likewise have fewer apoptotic cells than their nontransgenic counterparts (12). In accordance with this hypothesis, increase in integrin does protect against exercise-induced muscle damage (7). Thus, enhancing α_7 -integrin expression can spare skeletal muscle from injury or apoptosis without compromising normal physiological functions.

Several “complementary” genes have been shown to rescue or alleviate the pathology of dystrophic muscle when their expression levels are increased (24). However, transcriptional changes induced by overexpression of these genes in myoblasts and skeletal muscle have yet to be fully examined. Evaluation of the global changes in transcription resulting from complementary genes in transgenic mice is essential for determining potential side effects of these gene therapies. Here we utilized array profiling to evaluate the overexpression of α_7 -

integrin as a therapy for muscular dystrophy. Neither the threefold increase in α_7 -protein in myoblasts nor the eightfold increase in mature skeletal muscle significantly altered their respective transcriptional profiles. In both cases, relatively few genes had greater than twofold changes in expression, and the majority of these changes in transcription are likely related to mild ER stress posed by overexpression of the α_7 -chain. Others are related to cell division, G protein signaling, and the interferon-inducible p200 family of proteins. Among them is RGS5, a marker for pericytes (6), and these cells are myogenic in humans and may be used in stem cell therapy for muscular dystrophy (21). The increased expression of RGS5 in α_7 -integrin transgenic mice may result in an increase in the number of pericytes and the higher regenerative capacity and reduced pathology seen in $\alpha_7^{\text{mdx/utrn}^{-/-}}$ mice (12,13). Similarly, the interferon-inducible p200 family of proteins such as p204 may also promote muscle differentiation in α_7 -transgenic mice.

Several possibilities may explain the lack of more dramatic changes in the transcription profiles of α_7 -integrin-overexpressing myoblasts and skeletal muscle. First, $\alpha_7\beta_1$ -integrin primarily functions as a receptor, mediating the transsarcolemma linkage of muscle fibers to the extracellular matrix. Its relatively short cytoplasmic tail suggests it does not have enzymatic activity, and thus it has less potential for initiating signals compared with other receptors (67). Therefore, although enhanced expression of the integrin improves structural linkages through the sarcolemma, it does not necessarily promote major changes in transcription. Accordingly, alleviation of pathology in dystrophic mice by enhancing integrin levels is likely to be largely due to the restoration of the transsarcolemma linkages and specialized skeletal muscle structures such as the myotendinous and neuromuscular junctions (12,13).

Second, recent reports of changes in signaling induced by exercise in α_7 -integrin transgenic wild-type mice provide evidence that the integrin is a mechanosensor in skeletal muscle (7). In α_7 -transgenic mice, our array analysis also reveals that the genes involved in G protein signaling (*RGS5* and *xpr*) are changed in accordance with the enhanced α_7 -integrin. Since integrin and G protein signaling regulate one another (64) and both are involved in initiating mechanical signaling (45), the coordinated expression of these genes in skeletal muscle may regulate mechanosignal transduction and adaptation of fibers to contraction and stretch. Hence, much larger changes in transcriptional profiles may result from activation of $\alpha_7\beta_1$ -integrin by mechanical loading that is known to promote signaling. Alternatively, excess integrin may promote structural integrity or amplifications of transient signaling that do not result in altered gene expression. Further array analysis of integrin α_7 -transgenic mice following exercise is needed to determine whether activation of the integrin by mechanical stimuli regulates gene expression.

Third, although the α_7 -transgenic mice have a 36-fold increase in α_7 -RNA and an eightfold increase in α_7 -protein, no major changes were detected in either mRNA or protein levels of its interacting β_1 -subunit. Thus the amount of $\alpha_7\beta_1$ -integrin does not regulate β_1 -transcription. Likewise, there is no decrease in β_1 -chain in α_7 -null mice (36). Similarly, we did not observe changes in mRNA levels of other components of focal adhesion complexes in our array analysis. Limited amounts of β_1 -chain and/or other components of focal adhesion complexes (focal adhesion kinase, integrin-linked kinase, vinculin, and talin) may restrict signaling by the integrin and result in a lack of transcriptional regulation. Immunofluorescence localization of the α_7 -chain in transgenic mice revealed some staining within fibers, and this likely reflects limiting amounts of the β_1 -subunit. Unable to dimerize with the β_1 -subunit, excess α_7 -chain remained inside the fibers and likely triggered a mild ER-stress response as revealed in the array analyses. It will be interesting to determine whether enhancing the expression of both the α_7 - and β_1 -subunits will result in further regulation of signaling, altered transcription, or a greater alleviation of dystrophic pathology.

It should be noted that transcription is not uniform in all nuclei in muscle fibers; nuclei in proximity to junctional sites transcribe genes encoding proteins specific to those sites (47,52, 61,62). Therefore, it is possible that enhanced integrin expression triggers locally restricted transcriptional changes in nuclei near myotendinous and/or neuromuscular junctions, sites where the integrin is highly enriched. In our analysis, such changes may have been obscured by the relatively large amount of total RNA from other portions of the muscle fibers.

Although enhancing integrin levels in wild-type skeletal muscle did not dramatically alter transcription, in the environment of dystrophic muscle, that may not be the case. Dystrophic and healthy skeletal muscle differ with regard to the composition of their extracellular matrix, oxidative condition, sarcoplasmic Ca^{2+} concentration, protease activity, and other metabolic processes (14,29,70), and all of these differences may regulate α_7 -integrin activation and/or downstream transcription. In fact, an increase in α_7 -RNA and protein, as well as talin and vinculin, are seen in Duchenne patients and in *mdx* mice (36,44). Therefore, expression profiling of α_7 -transgenic *mdx/utrn*^{-/-} is needed to test whether increased $\alpha_7\beta_1$ -integrin affects transcription in dystrophic muscle and how those altered genes may contribute to alleviating pathology.

Lastly, the $\alpha_7\beta_1$ -integrin was recently reported to have tumor suppressor activity in several tissues and mutations in the α_7 -gene related to tumor formation (57). This interesting finding is consistent with earlier results in which developmentally defective myogenic cells were shown to be transformed and tumorigenic (40) and did not express the H36 antigen (39) [later shown to be α_7 -integrin (66)], and with the recent finding of a high rate of tumor formation in α_7 -null mice (DJ Burkin, unpublished observations). Thus, enhancing the levels of α_7 -integrin may also be useful as a therapeutic approach to cancer with minimal transcription effects.

In summary, the effects of increasing integrin levels in both muscle precursor cells and in skeletal muscle fibers are encouraging with respect to developing integrin enhancement as a therapy for muscular dystrophies. Whereas the integrin can promote myoblast adhesion, proliferation, and resistance to apoptosis, increasing integrin in precursor cells would yield increased numbers and more effective cells to repair dystrophic fibers. In addition, muscle fibers derived from such precursors would have more integrin to strengthen the linkage between the extracellular matrix and cytoskeleton and to diminish apoptosis. The increased proliferative capacity of such myogenic cells may also contribute to a more effective regenerative capacity and counter the depletion of stem cell populations that underlies progressive muscle wasting such as that seen in Duchenne patients. Moreover, increasing integrin levels in myoblasts and skeletal muscle does not promote major changes in gene expression, and therefore an integrin-based therapy is not likely to impart serious negative side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Tetracycline-inducible expression of rat $\alpha 7$ -integrin in C2C12 mouse myoblasts and myotubes. **A:** RT-PCR of RNA from noninduced (lane 1) and induced Tet $\alpha 7$ -C2C12 myoblasts (lane 2) was used to detect rat $\alpha 7$ RNA. Rat $\alpha 7$ -transcripts were only detected under induced conditions. C2C12 myoblast RNA (lane 3) was negative for rat $\alpha 7$ -transcripts. Reactions with RNA from induced cells without reverse transcriptase show that amplification is not due to DNA contaminants (lane 4). Detection of GAPDH RNA was used as an internal control for equivalent loading. M, markers. **B:** immunoblots of protein extracts from noninduced and induced Tet $\alpha 7$ -C2C12 myoblasts probed with monoclonal antibody O5 against the rat $\alpha 7$ -integrin extracellular domain and polyclonal antibody against the common $\alpha 7$ B cytoplasmic domain detect rat $\alpha 7$ integrin and total $\alpha 7$ B integrin, respectively. Blots were reprobed with monoclonal antibody against γ -actin as a control for loading. Induced myoblasts have threefold more total $\alpha 7$ -integrin and induced myotubes have ~1.5-fold more total $\alpha 7$ -integrin. Differences between induced and noninduced $\alpha 7$ -chain levels were statistically significant (* $P < 0.01$). **C:** immunostaining of tetracycline-induced myoblasts and myotubes with anti-rat $\alpha 7$

monoclonal antibody O26 reveals bright staining on induced cells (*e* and *f*); noninduced myoblasts and myotubes are negative (*c* and *d*). L8E63 rat myoblasts also exhibit bright staining (*b*). Staining is not detected in induced Tet α ₇-C2C12 cells incubated solely with secondary anti-mouse antibody (*a*). Bar, 100 μ m.

Fig. 2.

$\alpha_7\beta_1$ -Integrin promotes substrate-specific adhesion. **A**: myoblasts after 30 min of incubation on various concentrations of fibronectin and laminin. Adhesion to laminin is increased and adhesion to fibronectin is decreased in tetracycline-induced Tet α_7 -C2C12 myoblasts. **B**: quantification of adherent cells on fibronectin and laminin. At all concentrations of fibronectin and laminin tested, differences in adhesion of induced ($\blacktriangle$) and noninduced myoblasts ($\blacksquare$) were statistically significant ($P < 0.05$).

Fig. 3.

$\alpha_7\beta_1$ -Integrin promotes myoblast proliferation and facilitates cell cycle progression. **A:** C2C12 cells and Teta7-C2C12 cells were grown for 36 h with (+) or without tetracycline (-), detached by using EDTA, and seeded onto 60-mm dishes in medium containing the indicated serum concentrations. Cell numbers were determined 24, 48, and 72 h thereafter. Increased integrin in Teta7-C2C12 cells shortened the doubling times at low concentrations of FBS (0.5%, 1%, 2%, and 5%). Tetracycline itself did not affect the doubling time of C2C12 myoblasts. Data are from three independent experiments. *Statistical significance ($P < 0.01$). **B:** Teta7-C2C12 myoblasts were grown for 36 h with or without tetracycline. Cells were then starved in serum-free growth medium $\pm$ tetracycline. After 48 h, serum-free medium was replaced with normal growth medium (20% FBS) $\pm$ tetracycline. Cell DNA content was determined by flow cytometry 0, 8, 24, and 32 h thereafter, and the percentages of cells in each phase of the cell cycle were determined using ModFit software. At 24 and 32 h, more integrin-induced cells had progressed into G2/M than the noninduced myoblasts. Data are from three independent experiments. The average percentage of cells in G1 (2N) and G2/M (4N) are indicated. *Statistical significance ($P < 0.05$).

Fig. 4. Differentiation is not affected by increased integrin. Tetα₇-C2C12 cells were grown to confluence, with or without tetracycline; differentiation medium was then added. Immunofluorescence localization of muscle-specific myosin heavy chain (MHC; A) and myoblast fusion (B) was equivalent in noninduced and tetracycline-induced cells. Bar, 100 μm. Data are from three independent experiments.

Fig. 5. $\alpha_7\beta_1$ -Integrin protects C2C12 myoblasts from staurosporine-induced apoptosis. **A:** Tet α_7 -C2C12 myoblasts were grown $\pm$ tetracycline for 36 h, and 2 μM staurosporine was then added. C2C12 cells were also treated with staurosporine. After 3 h, staurosporine-induced apoptosis was apparent as determined by rounded cellular morphology (*a*, *c*, and *e*). Immunostaining of active caspase-3 revealed a similar extent of active caspase-3 in C2C12 staurosporine-treated myoblasts (*b*) and noninduced Tet α_7 -C2C12 cells (*d*), but less activated caspase-3 was detected in myoblasts induced to express more α_7 -integrin (*f*). Bar, 100 μm . **B:** a twofold decrease in the number of cells with active caspase-3 was detected in induced Tet α_7 -C2C12 myoblasts treated with 1 μM or 2 μM staurosporine compared with noninduced cells. *Statistical significance ($P < 0.001$).

Fig. 6.

Transgenic mice expressing high levels of α_7 -integrin exhibit no negative phenotype. **A:** immunoblots of protein extracts from wild-type gastrocnemius and soleus muscle (lanes 1, 3, and 5) and α_7 -transgenic mice (lanes 2, 4, and 6) probed with anticytoplasmic domain polyclonal antibody to detect rat and mouse integrin α_7 B. Arrows indicate the full-length α_7 -integrin chain (~120 kDa) and a ~70 kDa cleavage product generated from rat, but not mouse, α_7 -integrin. The transgenic mice express eightfold more integrin α_7 B than wild-type mice. Three separate gels of samples from 18 mice of each genotype were analyzed. Differences between the two genotypes are statistically significant (* $P < 0.0001$). **B:** hematoxylin and eosin staining of sections from the gastrocnemius of 5-wk-old wild-type (a) and α_7 -transgenic (b)

female mice exhibit identical skeletal muscle histology. No signs of muscle pathology (central nuclei, mononuclear cell infiltration, or necrotic fibers) were observed in the presence or absence of the integrin transgene. Immunostaining with anti-rat α_7 antibody reveals a high level of transgenic integrin at the sarcolemma (*d*), and wild-type control sections are negative (*c*). Localization of rat and mouse integrin is seen using antibody against the common $\alpha_7\beta_1$ cytoplasmic domain. At the same exposure, transgenic mice (*f*) show significantly higher staining intensity at the sarcolemma compared with wild type (*e*). Some α_7 -integrin is detected inside muscle fibers (*j*, arrows) of transgenic mice indicating the presence of excess α_7 -chain. Similar levels of β_1 D integrin were detected in wild-type (*g*) and transgenic (*h*) mice. Sections incubated without primary antibody reveal no staining (*i*). Bar, 100 μm . *C*: the weights of α_7 -transgenic mice do not significantly differ from wild-type mice up to 30 wk. *D*: the distribution of fiber cross-sectional areas of wild-type and α_7 -transgenic gastrocnemius muscles are similar. The average fiber area was not statistically different, although there were 12% more fibers with smaller cross-sectional areas in the transgenic animals.

Fig. 7. Microarray analysis reveals highly similar gene expression profiles in wild-type and $\alpha 7$ -transgenic cells and skeletal muscle. **A:** scatterplot analyses of Affymetrix mouse genome 430 2.0 array data show a high concordance of gene expression in induced and noninduced Teta7-C2C12 myoblasts [left; correlation coefficient (cc) = 0.996] as well as in transgenic and wild-type $\alpha 7$ mice (right; cc = 0.999). Probes in which more than twofold increases were observed in induced myoblasts or transgenic mice are represented by red dots; green dots indicate decreases. **B:** confirmation of differentially expressed genes by semiquantitative RT-PCR. Six independently prepared RNA samples from each source were tested for expression of the genes indicated, and the means values $\pm$ SE are presented. Primers were designed to cover at least one intron-exon boundary to eliminate potential results from genomic DNA contamination. RT-PCR of GAPDH was used to control for equivalent loading. Representative gel images for each gene are shown. Quantification of the RT-PCR products shows that all genes tested have similar fold changes in expression to those indicated in the microarray analyses. *Statistical significance ($P < 0.05$).

Table 1

Primers used in RT-PCR for microarray validation

Gene Name	Forward Primer	Reverse Primer
<i>Rat Itga7</i>	5'-TGATGGTATACTTGGACCCGG-3'	5'-TTTCCACCTTCCTTGGGTCT-3'
<i>GAPDH</i>	5'-CCATGGAGAAGGCCGGGG-3'	5'-CAAAGTTGTCATGGATGACC-3'
<i>Hspa5</i>	5'-TACTCGGGCCAAATTGAAG-3'	5'-GGGGACAAACATCAAGCACT-3'
<i>Armet</i>	5'-CTGGGACGATTTTACCAGGA-3'	5'-TCAGGTCAATCTGCTTGTCTG-3'
<i>Teme58</i>	5'-AGAGTCCCCGTCCTGTACCT-3'	5'-TGTGGCCTAGGCTCTCATCT-3'
<i>Pttg1</i>	5'-TGGGTGCCAACATAAACTGA-3'	5'-CCAGCTTCAACCCATCCTTA-3'
<i>Trib3</i>	5'-TGCCTCGCTTTGTCTTCA-3'	5'-CTGGTCCATCTCCCTTCG-3'

Table 2
Differentially expressed genes in noninduced vs. induced Tet α 7-C2C12 myoblasts (>2-fold)

Affymetrix Probe Identifier	Gene Identifier	Gene (Symbol)	Direction	Adjusted P Value	Fold Change
1426065_a_at	BC012955	Tribbles homolog 3 (<i>Trib3</i>)	Up	0.0001	2.9
1456225_x_at	BB508622	Tribbles homolog 3 (<i>Trib3</i>)	Up	0.0071	2.9
1426260_a_at	D87867	UDP glucuronosyltransferase 1 family, polypeptide A2, A6A, A10, A7C, A5, A9, A6B, A1 (<i>Ugt1a2</i> , <i>Ugt1a6a</i> , <i>Ugt1a10</i> , <i>Ugt1a7c</i> , <i>Ugt1a5</i> , <i>Ugt1a9</i> , <i>Ugt1a6b</i> , <i>Ugt1a1</i>)	Up	0.04297286	2.47
1434264_at	BQ174638	Ankyrin 2, brain (<i>Ank2</i>)	Up	0.04451635	2.23
1426243_at	BC019483	Cystathionase (cystathionine γ -lyase) (<i>Cth</i>)	Up	0.0047	2.1
1417130_s_at	NM_020581	Angiotensin-like 4 (<i>Angptl4</i>)	Down	0.0010	2.1

Table 3
Differentially expressed genes in wild-type and α_7 -integrin transgenic muscles (>2-fold)

Affymetrix Probe Identifier	Gene Identifier	Gene (Symbol)	Direction	Adjusted P Value	Fold Change
1418393_a_at	NM_008398	Integrin α_7 (<i>Itga7</i>)	Up	0.000	36.58
1447891_at	BI466416	Transforming, acidic coiled-coil containing protein 2 (<i>Tacc2</i>)	Up	0.013	7.82
1426906_at	M74124	Interferon-activated gene 204 (<i>Ifi204</i>)	Up	0.006	4.56
1438390_s_at	AV105428	Pituitary tumor-transforming 1 (<i>Pttg1</i>)	Up	0.003	4.31
1452730_at	AK004068	RIKEN cDNA 1110033J19 gene (<i>1110033J19Rik</i>)	Up	0.005	4.17
1435330_at	BG092516	Expressed sequence A1447904 similar to γ -interferon-inducible protein Ifi-16 (<i>Ifi16</i>)	Up	0.013	4.12
1443153_at	BB306866	Thyroid hormone receptor interactor 11 (<i>Tripl1</i>)	Up	0.005	3.12
1426053_a_at	AF131102	Xenotropic and polytropic retrovirus receptor 1 (<i>Xpr1</i>)	Up	0.003	2.97
1425764_a_at	AF031467	Branched chain aminotransferase 2 (<i>Bcat2</i>)	Up	0.010	2.75
1428112_at	AK014338	Arginine-rich, mutated in early-stage tumors (<i>Armer</i>)	Up	0.003	2.74
1421553_at	NM_010146	Epilepsy, progressive myoclonic epilepsy, type 2 gene- α (<i>Epm2a</i>)	Up	0.036	2.66
1424105_a_at	AF069051	Pituitary tumor-transforming 1 (<i>Pttg1</i>)	Up	0.039	2.5
1450130_at	AA215024	Xenotropic and polytropic retrovirus receptor 1 (<i>Xpr1</i>)	Up	0.007	2.47
1420940_x_at	NM_009063	Regulator of G protein signaling 5 (<i>Rgs5</i>)	Up	0.007	2.47
1416064_a_at	NM_022310	Heat shock 70-kDa protein 5 (<i>Hspa5</i>)	Up	0.003	2.41
1453749_at	BB749723	RIKEN cDNA 2610507I01 gene (<i>2610507I01Rik</i>)	Up	0.013	2.39
1452231_x_at	M74124	Interferon-activated gene 204 (<i>Ifi204</i>)	Up	0.006	2.35
1438426_at	BF468228	Transmembrane protein 58 (<i>Tmem58</i>)	Up	0.010	2.31
1418206_at	NM_022324	Stromal cell-derived factor 2-like 1 (<i>Sdf2l1</i>)	Up	0.004	2.29
1427464_s_at	AJ002387	Heat shock 70-kDa protein 5 (<i>Hspa5</i>)	Up	0.003	2.28
1458099_at	BB291417	Unknown gene	Up	0.022	2.26
1452406_x_at	AJ007909	Erythroid differentiation regulator 1 (<i>Erdrl1</i>)	Up	0.007	2.23
1423775_s_at	BC005475	Protein regulator of cytokinesis 1 (<i>Prc1</i>)	Up	0.010	2.2
1445385_at	BB551855	Adult inner ear cDNA RIKEN full-length enriched library	Up	0.038	2.12
1437274_at	BM204808	Coatomer protein complex subunit- α (Copa)	Up	0.003	2.07
1435331_at	BM241008	Expressed sequence A1447904 similar to γ -interferon-inducible protein Ifi-16 (<i>Ifi16</i>)	Up	0.010	2.06
1448775_at	NM_008328	Interferon-activated gene 203 (<i>Ifi203</i>)	Up	0.041	2.05
1423648_at	BC006865	Protein disulfide isomerase associated 6 (<i>Pdia6</i>)	Up	0.005	2.01
1439200_x_at	BE686792	Erythroid differentiation regulator 1 (<i>Erdrl1</i>)	Up	0.007	2
1456498_at	BB284583	Integrin α -4 (<i>Iga4</i>)	Down	0.014	2.68
1441092_at	BB214614	RIKEN cDNA 9330159M07 gene (<i>9330159M07Rik</i>)	Down	0.023	2.15
1425763_x_at	BC019425	Immunoglobulin heavy chain J558 family (<i>Igh-VJ558</i>)	Down	0.006	2.04