Dystrophic Muscle in Mice Chimeric for Expression of α5 Integrin

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Abstract. α5-deficient mice die early in embryogenesis (Yang et al., 1993). To study the functions of α5 integrin later in mouse embryogenesis and during adult life we generated α5 −/−;+/+ chimeric mice. These animals contain α5-negative and positive cells randomly distributed. Analysis of the chimerism by glucose-6-phosphate isomerase (GPI) assay revealed that α5 −/− cells contributed to all the tissues analyzed. High contributions were observed in the skeletal muscle. The perinatal survival of the mutant chimeras was lower than for the controls, however the subsequent life span of the survivors was only slightly reduced compared with controls (Taverna et al., 1998). Histological analysis of α5 −/−;+/+ mice from late embryogenesis to adult life revealed an alteration in the skeletal muscle structure resembling a typical muscle dystrophy. Giant fibers, increased numbers of nuclei per fiber with altered position and size, vacuoli and signs of muscle degeneration–regeneration were observed in head, thorax and limb muscles. Electron microscopy showed an increase in the number of mitochondria in some muscle fibers of the mutant mice. Increased apoptosis and immunoreactivity for tenascin-C were observed in mutant muscle fibers. All the alterations were already visible at late stages of embryogenesis. The number of altered muscle fibers varied in different animals and muscles and was often increased in high percentage chimeric animals. Differentiation of α5 −/− ES cells or myoblasts showed that in vitro differentiation into myotubes was achieved normally. However proper adhesion and survival of myoblasts on fibronectin was impaired. Our data suggest that a novel form of muscle dystrophy in mice is α5-integrin-dependent.

Key words: muscular dystrophy • chimeric mice • integrin α5β1 • apoptosis

The interactions of extracellular matrix (ECM)[1] components with each other or with cell surface receptors have an important role in many biological processes such as embryonic development, wound healing, malignant transformation and many others (Hynes, 1990, 1992; Ruoslahti, 1991; Hynes and Lander, 1992; Giancotti and Mainiero, 1994). Cell–ECM interactions are mediated by cell surface receptors called integrins. Integrins are transmembrane glycoproteins which consist of noncovalently linked heterodimers each composed of an α and a β chain (Hynes, 1992).

α5β1 integrin is a specific receptor, which binds to the arginine/glycine/aspartic acid region of one of the most common ECM molecules, fibronectin (FN) (Pytela et al., 1985). α5β1 is involved in many cellular processes including cell proliferation and oncogenic transformation (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990; Schreiner et al., 1991), cell survival (Varner et al., 1995; Zhang et al., 1995), cell migration (Akiyama et al., 1989; Giancotti and Ruoslahti, 1990), assembly of FN-rich matrices (Fogerty et al., 1990), wound healing (Guo et al., 1991), T cell activation (Shimizu and Shaw, 1991), and gene expression (Werb et al., 1989). It also plays an important role during embryogenesis (Yang et al., 1993). Indeed, the α5 subunit is expressed at high levels during embryogenesis in *Xenopus* (Whittaker and De Simone, 1993), chicken (Muschler and Horwitz, 1991) and mouse (Goh et al.,

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; ES, embryonic stem; FN, fibronectin; GPI, glucose-6-phosphate isomerase; H&E, hematoxylin and eosin; LM, laminin; MTJ, myotendinous junction; PTAH, phosphotungstic acid haematoxylin; TN-C, tenascin-C; WT, wildtype.

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1997); its expression is more restricted in adult tissues (Muschler and Horwitz, 1991).

Development of skeletal muscle is a multistep process that starts when the somitic mesoderm differentiates into the dermamyotome. Soon after that, primary myoblasts proliferate and migrate to their peripheral locations where they differentiate into postmitotic multinucleated myotubes, the primary myotubes (primary fusion). The migration of secondary myoblasts that align with the primary myotubes leads to the formation of secondary myotubes (secondary fusion). Finally the myotubes specialize as fast or slow contracting fibers and become striated and innervated (Kelly and Rubinstein, 1994). Several adhesion molecules, integrins, cadherins, and immunoglobulin superfamily members are thought to be involved in myogenesis (Knudsen et al., 1990). The extracellular matrix of skeletal muscle consists of a basal lamina around every myotube and interstitial connective tissue (endomysium) between the fibers. Collagens and fibronectins are abundant in the endomysium whereas the basal lamina contains type IV collagen, laminin, heparan sulfate proteoglycan, entactin (nidogen), and fibronectins (Sanes, 1994). α and β integrin subunits are expressed on skeletal muscle cells at different times and subcellular locations. α1, α3, α5, α6, and αv are highly expressed during muscle development and downregulated after full differentiation (Bronner-Fraser et al., 1992; Duband et al., 1992; Enomoto et al., 1993; McDonald et al., 1995). α7 integrin is abundant through all stages of muscle development (Bao et al., 1993), whereas α4 integrin expression rises during secondary myogenesis then is not expressed anymore (Rosen et al., 1992). αv subunit is concentrated at the costameres and at the myotendinous junction (MTJ); α3 is localized to the MTJ whereas α5 is present in adhesion plaque-like structures along the myotube. α7 is concentrated at the MTJ but can also be detected at the neuromuscular junction and along the sarcolemmal membrane. The β1 subunit is present on myoblasts and on muscle fibers along the entire membrane with maximum concentrations at the MTJ and costameres or Z discs (Bozyczko et al., 1989).

A key question is what are the functions of these various integrins in muscle biology? One way to address this question is via genetic elimination of specific integrins. α5-deficient mice die at approximately day 10 or 11 of gestation (Yang et al., 1993). The null embryos have pronounced defects in posterior trunk and yolk sac mesodermal structures. No somites, a kinky neural tube, and vascular defects are observed in the posterior end. To study the functions of the α5 molecule after day 10 or 11 and in adult animals, we generated α5 −/− embryonic stem (ES) cells and injected them into wild-type (WT) blastocysts to obtain α5 −/−;+/+ chimeric animals. Here we present data on the characterization of these chimeras.

Materials and Methods

Growth, Selection, and Differentiation of ES Cells

ES cells, D3 line (Doetschman et al., 1985), were grown as described previously (George et al., 1993). α5 heterozygous ES cells were obtained as described in Yang et al. (1993). One heterozygous α5 ES cell line, clone 47, was expanded and selected with 4–5 mg/ml G418 (GIBCO BRL, Gaithersburg, MD). After 7–9 d of selection, drug-resistant clones were picked and expanded on feeder cells. Half of the cells from each clone were frozen in 10% DMSO in fetal bovine serum and half were lysed for extraction and analysis of DNA. From three independent selection experiments 5 clones that were null for α5 from Southern blot analysis were obtained (154, 162, 194, 201, and 305). Further Southern blot analysis of one of these clones (154) also showed that the vicinity of the mutated genomic region was not altered during the selection (data not shown). Some heterozygous clones that did not become null after G418 selection were used as control clones (152, 155, 98) as well as D3 wild-type cells and ES cells heterozygous for P and E selectins (Robinson et al., 1997). All these cells had characteristics of wild-type cells.

To generate chimeric mice, ES cells were prepared and injected into C57BL6 blastocysts as described by George et al. (1993) and Bradley et al. (1987). Chimeric progeny were identified by coat color 1 wk after birth. Before and around birth the animals were screened by PCR for the presence of the neo gene and by glucose-6-phosphate isomerase assay (see below). Differentiation of ES cells followed the protocol of Yang et al. (1996). The embryoid bodies were analyzed for muscle differentiation after 15–30 d of culture in a leukemia inhibitory factor–free medium. Differentiated cultures were stained with an antibody against skeletal muscle myosin heavy chain (MY32; Sigma Chemical Co., St. Louis, MO) as described (Yang et al., 1996).

DNA Extraction, Southern Blot, and PCR Analysis

DNA was extracted from ES cells or myoblasts as described in Yang et al. (1996). Southern blot analyses were performed as described in Yang et al. (1993). PCR analysis for the neo gene was performed on tail DNA as described in Taverna et al. (1998).

Glucose-6-Phosphate Isomerase Analysis of Tissue

Glucose-6-phosphate isomerase (GPI) analysis was performed on extracts of different tissues, e.g., limb or pectoral muscle, as described in Yang et al. (1996). Densitometric analysis of the GPI assays was performed using either 1 D-multilane scan or Spot Denso scan of the IS-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA). The percentage of ES cell-derived (129Sv) isoform of GPI was calculated as described in Yang et al. (1996).

Histological Analysis

Pregnant mothers were killed towards the end of pregnancy and embryonic day E16–E18 embryos were analyzed. Embryos, newborns, 1–4 wk-old mice, or several month-old animals were killed in a CO_2 chamber, opened ventrally and dorsally, and then the opened carcasses were immersed in 10% formalin (3.7% formaldehyde in phosphate-buffered saline) and kept in fixative until processed. In some cases embryos or newborns were cut transversely into four portions, each portion embedded in mounting medium (OCT compound; Miles Laboratories, Elkhart, Milwaukee, WI) and immediately frozen in liquid nitrogen-cooled isopentane. 6-μm sections were cut. The same freezing procedure was used to generate sections from dissected limb or pectoral muscle of adult mice. Transverse or longitudinal paraffin-embedded or frozen sections were processed for hematoxylin and eosin (H&E) or phosphotungstic acid hematoxylin (PTAH) staining according to the supplier's protocol (Sigma Chemical Co.).

Apoptosis

Apoptotic cells were analyzed in the animals using terminal transferase biotinylated-dUTP nick-end labeling (TUNEL) on paraffin sections from E17 or E18 formalin-fixed embryos as described by Morganbesser et al. (1995). To analyze apoptosis in vitro, myoblasts (see below) were plated on mouse laminin-1 (GIBCO BRL) or FN for 2 h before being trypsinized and resuspended in buffer containing 1 μg/ml annexin-FITC as described by the manufacturer's protocol (Zymed, South San Francisco, CA). After a 10-min incubation, the cells were washed, stained with 1 μg/ml Hoechst 33342, and then mounted on a slide. In five separate fields, both the total number of cells and the number of annexin-positive cells were counted.

Immunohistochemistry

To analyze ECM molecules, 6-μm paraffin-embedded or frozen sections from E18 embryos were used. Frozen sections were also used to analyze the distribution of α5 integrin. Paraffin-embedded sections from overnight formalin-fixed embryos were treated with trypsin (0.1%) for 30 min at

room temperature and then stained as described in George et al. (1993). Frozen sections from unfixed embryos were stained with the same protocol except that they were not pretreated with trypsin and the incubations were shortened to 15 min for primary and secondary antibodies. Primary antibodies were used at 1:100 dilution: rabbit polyclonal anti-LM (Sigma Chemical Co.); rabbit polyclonal anti-collagen IV (Becton Dickinson Labware, Franklin Lakes, NJ); rabbit polyclonal anti-entactin (nidogen), a gift of A. Chung (University of Pittsburgh, Pittsburgh, PA); rabbit 24 polyclonal anti-FN (Mautner and Hynes, 1977); rat monoclonal anti–tenascin-C (Sigma Mtn-12; Sigma Chemical Co.); rat monoclonal anti α5 integrin (PharMingen, San Diego, CA). Secondary antibodies were used at 1:200 dilution of FITC-conjugated goat anti–rabbit or anti–rat (Biosource International, Camarillo, CA).

Isolation of Myoblasts

Limb muscles from neonatal chimeric mice (−/−;+/+ or +/−;+/+) were dissociated to isolate pure populations of myoblasts as described in Rando and Blau (1994). Primary cultures were plated on laminin-1–coated dishes and grown in growth medium consisting of Ham's F-10 nutrient mixture (BioWhittaker, Walkersville, MD), 20% fetal bovine serum (FBS) (BioWhittaker), 2.5 ng/ml basic fibroblast growth factor (bFGF) (Promega Corp., Madison, WI), and penicillin (200 U/ml)/streptomycin (200 μg/ml) (GIBCO BRL). ES cell-derived myoblasts were purified by maintaining the cells in G418 (50 μg/ml) for at least 2 wk. To induce differentiation, myoblast cultures were maintained in medium consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse serum and penicillin/streptomycin. For analysis of differentiation of each cell population, the differentiated cultures were fixed with MeOH (−20°C) and stained with Hoechst (1 μg/ml). The fusion index was determined microscopically as the ratio of the number of nuclei in cells with three or more nuclei to the total number of nuclei. 10 random fields in each of three separate cultures were counted at 40× magnification, with each field having between 50 and 100 nuclei.

Retroviral Infection

α5 cDNA was introduced into α5-deficient myoblasts by retroviral-mediated gene transfer (Guan et al., 1990) using a human α5 cDNA insert (Argraves et al., 1987). Retroviral infection of myoblast populations was performed as described previously (Rando and Blau, 1994). To ensure a high level of infection, cells were infected on three successive days, which gives >90% infection (Rando and Blau, 1997). To control for the infection procedure, α5-deficient myoblasts were infected with a retrovirus lacking a cDNA insert. Expression of α5 in retrovirally transduced cells was examined by Western blot analysis (see below).

Cell Adhesion

Cells were plated on 5 μg/ml LM-1, 5 μg/ml FN (both from GIBCO BRL), or collagen type I (100 μg/ml; Sigma Chemical Co.) and allowed to attach for 1 h at 37°C. Unattached cells were removed by washing. Adherence was then assessed by hemacytometer counts of attached cells after trypsinization.

Western Blot Analysis

Myoblasts were lysed in RIPA buffer. Proteins (50 μg) were electrophoresed on 7.5% SDS-PAGE gels under nonreducing conditions then transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The membranes were probed with an antibody to α5 integrin (1:5,000; Chemicon, Temecula, CA) followed by a peroxidase-linked donkey anti–rabbit secondary antibody (1:10,000; Amersham Corp., Arlington Heights, IL). The enhanced chemiluminescence (ECL) system was used to visualize the bound secondary antibody.

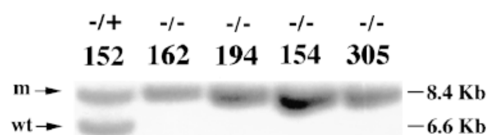


Figure 1. Southern Blot analysis of ES cells for α5 integrin. Clone 152, an α5-heterozygous clone of ES cells; both the wt (6.6-kb) and the mutated (8.4-kb) bands are present. *162*, *194*, *154*, *305*, four independent homozygous ES cell clones. Only the mutated band is present.

Table I. Generation and Survival of α5 −/−;+/+ Chimeric Mice

Clone	Blastocysts injected	Chimeras at E17-E18	Chimeras at weaning
α5 −/− (5 clones)	135	76 (56%)	—
α5 ± or D3 (wt)	139	31 (22%)	—
α5 −/− (5 clones)	205	—	37 (18%)
α5 ± or D3 (wt)	258	—	39 (15%)

Results

Generation of Chimeric Mice

ES cells heterozygous for α5 integrin were selected in the presence of high concentrations of G418 (4–5 mg/ml) to obtain homozygous clones. Southern blot analysis indicated that five different α5 −/− ES cell clones were generated (Fig. 1). The five clones were injected into WT C57BL6 blastocysts. Control chimeras were produced by injecting clones heterozygous for α5 integrin or for P and E selectins (Robinson et al., 1997) or WT ES cells (D3) into WT C57BL6 blastocysts. Chimeric animals had both black (from C57BL6) and agouti (from 129Sv-derived ES cells) coat color.

For embryos and newborns the chimerism was determined by PCR analysis for the neo gene (Taverna et al., 1998) in combination with a GPI assay for quantitation (Yang et al., 1996). The chimerism in adults was evaluated by the coat color. A representative population of chimeric animals obtained using α5 −/− or control ES cells was the following (Table I): at day 17 or 18 of pregnancy, 56% (76 out of 135) of the C57BL6 blastocysts injected with five independent α5 −/− clones developed as chimeric embryos, whereas only 22% (31 out of 139) of the C57BL blastocysts injected with control ES cells (D3 or α5 +/− clones) developed as chimeric embryos. The number of embryos

Table II. Percentage Contributions of α5 −/− Cells to Tissues of Chimeric Mice

Chimeras	Percentage ES cell contribution to chimeras												
	Control ES cells				α5 −/− ES cells								
Tissue	1	2	3	4	5	6	7	8	9	10	11	12	13
Lung	30	10	10	50	30	10	—	—	—	—	—	—	20
Liver	10	10	30	50	10	40	—	—	—	—	10	20	15
Spleen	60	50	30	50	80	50	30	—	—	—	10	50	60
Kidney	60	50	50	80	30	30	—	—	—	—	20	40	50
Brain	10	10	—	—	40	30	—	—	—	—	—	—	—
Gut	50	50	—	—	40	30	40	—	—	—	—	—	—
Heart	60	10	50	65	50	—	30	—	—	80	10	50	20
Diaphragm	10	10	5	60	50	50	—	—	—	5	—	40	70
Limb muscle	90	90	30	80	80	90	90	80	90	80	80	80	80
Pectoral muscle	90	90	—	80	90	90	—	80	90	80	—	80	80
Coat color*	95	90	40	98	90	90	95	95	95	90	90	98	95

*Judged by eye; —, not done.

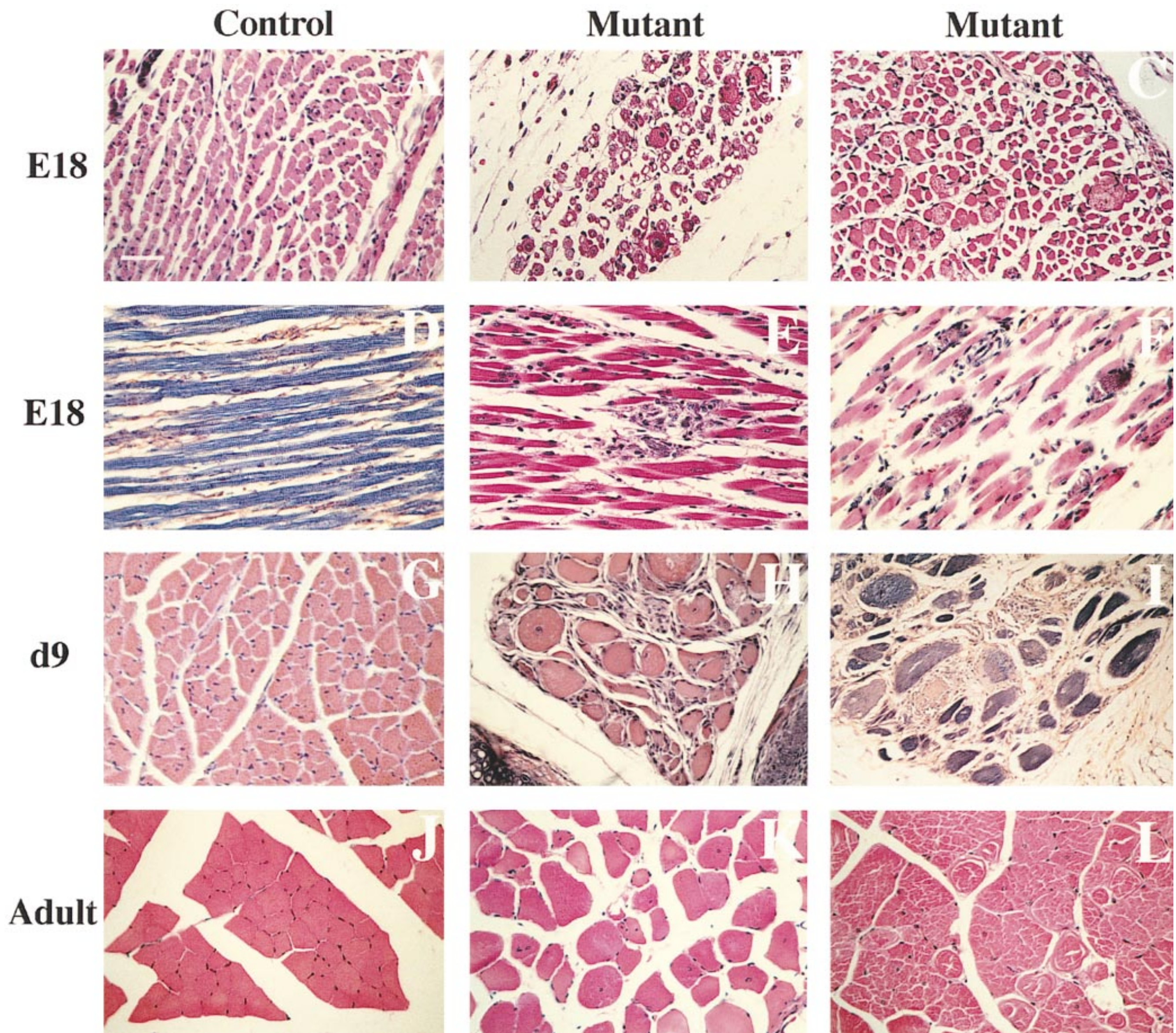


Figure 2. Morphology of skeletal muscle in α5 −/−;+/+ chimeric mice. (*A–C*) Transverse sections of muscles from E18 chimeric embryos: (*A*) control thoracic, (*B*) mutant thoracic, and (*C*) mutant limb muscles. Giant fibers, central nuclei, vacuoles, and size variability can be observed in the mutant muscles. (*D–F*) Longitudinal sections of muscles from E18 chimeric embryos: (*D*) control thoracic muscle, (*E*) mutant head, and (*F*) mutant thorax muscles. Increase of collagen among fibers (*E*) and degeneration of fibers (*F*) appear in mutant muscles. (*G–I*) Transverse sections of head muscle of 9-d-old animals: (*G*) control and (*H* and *I*) mutants. Mutant muscles show a high variability in fiber size, increased connective tissue, central nuclei, and fiber degradation. (*J–L*) Transverse sections of limb muscle of adults: (*J*) control and (*K* and *L*) mutants. Note the presence of central nuclei (*K*) and ring fibers (*L*) in mutant chimeras. *D* and *I* are PTAH stained, all others are H&E stained. Bar, 50 μm.

in uterus (chimeric and nonchimeric) was similar in both kind of injections; therefore the higher number of α5 −/−;+/+ chimeric embryos indicates a better contribution of α5 −/− ES clones in the blastocysts; i.e., the null ES clones are highly competent, more so than the parent and control ES cells, presumably as a result of the subcloning involved in their selection. In contrast, at weaning (4-wk-old animals) 18% (37 out of 205) of α5 −/−;+/+ and 15% (39 out 258) of control chimeras were observed. The marked decrease of α5 −/−;+/+ chimeras from 56% at E17 or E18 to 18% at weaning suggests that not all the chimeras present in uterus or born can survive. Indeed we found that many α5 −/−;+/+ newborn chimeras died within the 24 h after delivery. The lower survival rate for α5-null chimeras suggested requirements for α5 integrin in the perinatal development of certain tissues or organs.

Developmental Capacities of α5-null ES Cells

The chimerism of various tissues was analyzed by GPI assay in adult animals (see Table II). The contribution of 129Sv cells in some organs was lower than expected from

coat color in both mutant and control chimeras. This was probably due to a disadvantage of 129Sv cells in specific organs. However the α5-null cells contributed equally as well as did the control ES cells (Table II). We analyzed the populations of mature 129Sv-derived T and B lymphocytes by FACS® analysis and observed equal contributions in mutant and in control chimeras (data not shown). Clone 154 was tested for germ-line transmission of the α5-null allele: germline transmission was obtained from both a male and a female chimera indicating the presence of both α5 −/− sperm and oocytes. We conclude that α5 −/− cells contribute to all tissues analyzed, albeit in somewhat different percentages.

Muscle Defects in Animals Chimeric for α5

Histological analysis of E16–E18 embryos, newborns, young, and adult animals revealed no major defects. The chimeras were normal in size, weight, and appearance and showed no obvious defects in behavior (walking, climbing, swimming, or mating). The only defects we detected consistently were structural alterations in skeletal muscles. Approximately 40% of the 150 α5 −/−;+/+ chimeric embryos and newborn animals analyzed showed abnormal skeletal muscles in the head, thorax, or limb (i.e., muscles derived from several different embryonic origins—neural crest, lateral mesoderm, somites). Chimerism varying from 10 to 90% was observed for both α5 −/−;+/+ chimeras and control animals. Embryos with the most noticeable alterations had high chimerism as evaluated by GPI analysis of either the upper portion of the right posterior leg (including cartilage, muscle, and skin) or the dissected limb muscle of that leg. Examples of these alterations are illustrated in Fig. 2. Fig. 2, *B* and *C* show transverse sections through the thorax (*B*) or limb (*C*) of E18 α5 −/−;+/+ chimeric embryos and they illustrate irregularity in the sizes of the fibers with presence of giant fibers, central nuclei, and vacuoles. Increased collagen deposition between fibers and fiber degeneration were also observed in muscle from E18 α5 −/−;+/+ chimeric embryos (Fig. 2, *E* and *F*). Sections of the thoracic muscles of control E18 embryos (Fig. 2, *A* and *D*) showed good regularity in the muscle structure and no fiber degeneration or collagen accumulation could be observed. Analysis of α5 −/−;+/+ chimeras in the first month after birth confirmed the presence of muscle degeneration and regeneration in the skeletal muscles. Fig. 2, *H* and *I* show examples of degeneration in the head muscles of a 9-d-old chimera. Variability in fiber size, central nuclei, empty spaces in the fibers, and increase of connective tissue (especially evident in Fig. 2 *I* with PTAH staining) were observed. A control muscle is shown in Fig. 2 *G*. Signs of muscle degeneration–regeneration were still visible in some adult animals (several months old). Many fibers with central nuclei (Fig. 2 *K*, limb) ring fibers indicative of fiber degeneration and regeneration (Fig. 2 *L*, limb) and infiltration of adipose tissue (data not shown) were found in head, thorax, and limb muscles. Control limb muscle is shown in Fig. 2 *J*. The diaphragms and the heart of α5 −/−;+/+ chimeric mice appeared normal at all stages (data not shown), perhaps because the heart and diaphragm generally showed a lower 129Sv (ES cell) contribution than other muscles for both −/− and control chimeras (Table II). Alternatively, it is possible that mice with a high percentage of α5 −/− cells in the diaphragm are among those that die early. Ultrastructural analysis of limb muscle of E17 embryos revealed an increase in the number of mitochondria in some fibers of mutant chimeric mice (Fig. 3, *B* and *C*) compared with control chimeras (Fig. 3 *A*). It is possible that the fibers with more mitochondria correspond to the giant fibers we observed by histology.

The distribution of α5 integrin in mutant and control embryos was investigated by immunohistochemistry. α5 integrin immunoreactivity was found around each muscle fiber and at some MTJ in control animals (Fig. 4, *A* and *B*). In mutant embryos a conspicuous decrease of α5 integrin staining was found around many muscle fibers; however, fibers completely negative for α5 were rare (Fig. 4 *C*) due to the fact that null and wild-type myoblasts fuse together.

The composition of the ECM in the muscles of chimeric mice was analyzed by immunohistochemistry. Fibronectin, the ligand for α5β1 integrin, was present around and between all the fibers of both mutant and control chimeras. No major, consistent differences in intensity were observed between giant- and regularly sized fibers of mutant

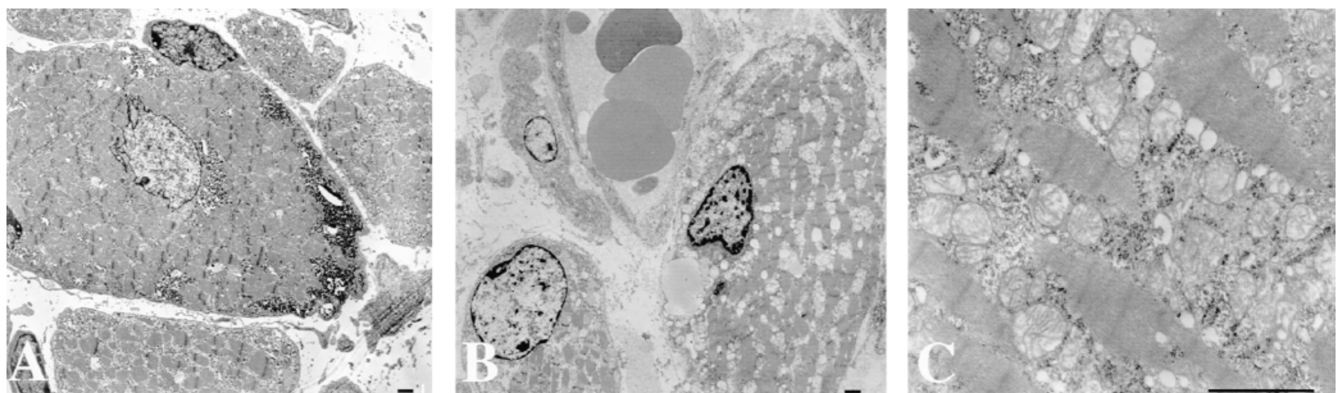


Figure 3. Electron micrographs of limb muscle. Limb muscles from E17 control (*A*) and α5 −/−;+/+ chimeras (*B* and *C*) are shown. An increase in the number of mitochondria is observed in some fibers of the mutant chimeras (*B* and *C*). Bar, 500 nm.

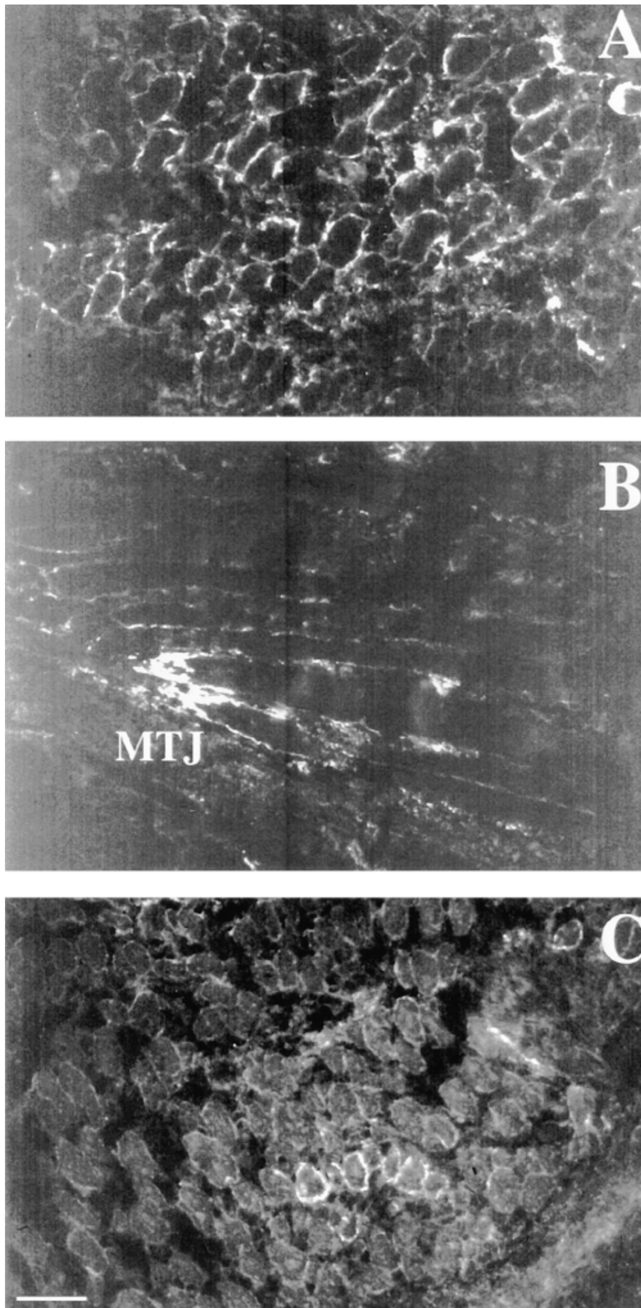


Figure 4. α5 integrin expression in muscle. Transverse (*A* and *C*) and longitudinal (*B*) sections of thoracic muscle from E18 control (*A* and *B*) and mutant (*C*) chimeric embryos were stained for α5 integrin. α5 integrin is expressed around the muscle fibers (*A*) and at the myotendinous junctions (*MTJ*) in control animals, while it is weakly expressed or missing around some fibers in mutant animals (*C*). Bar, 50 μm.

chimeras (data not shown). The basement membrane components, nidogen (entactin), collagen IV, and laminin-1 were also analyzed and no differences in staining were observed between mutant and control chimeras (data not shown). Expression of tenascin-C (TN-C) has been correlated with muscle regeneration in several muscular dystrophies (Settles et al., 1995). In normal muscle it is present only at the MTJ and on the nerves crossing the muscle (Chiquet and Fambrough, 1984). The analysis of TN-C in E17 or E18 embryos showed an enhanced deposition of TN-C around and between some muscle fibers of α5 −/−;+/+ chimeric embryos compared with controls (Fig. 5 *A*), suggesting a similar process of degeneration–regeneration as in other myopathies.

In mdx/mdx dystrophic mice, degeneration of fibers is sometimes preceded by apoptosis (Matsuda et al., 1995; Sandri et al., 1995; Tidball et al., 1995). We investigated whether the same is true in α5 −/−;+/+ chimeric mice. Fig. 5 *B* shows that, although almost no apoptosis was observed in E18 control chimeras, some E18 mutant animals (seven out of 10 chosen at random) showed an increase of apoptotic fibers in various muscles (Fig. 5 *B*, *dark fibers*).

Myogenesis In Vitro in the Absence of α5 Integrin (ES Cells and Myoblasts)

Our in vivo data suggest that muscle differentiation can occur even in almost complete absence of α5-positive myoblasts (GPI of the limb ~95%). However, long-term integrity of the muscle is compromised. We examined the ability of α5-null ES cells to differentiate into myoblasts and myotubes. Three null and two heterozygous (control) ES cell clones were grown in suspension to produce embryoid bodies. The embryoid bodies were then plated on gelatin-coated coverslips to induce differentiation. The differentiated cultures were stained with an antibody against skeletal muscle myosin heavy chain. 42% of the wells (19 out of 45) with α5-null embryoid bodies (all three clones) and 28% of the wells (14 out of 50) with control embryoid bodies stained positively. The degree of differentiation (fusion, myosin expression) appeared similar in control and mutant cultures (Fig. 6 *A*).

To test further the role of α5 integrin in muscle cell adhesion, growth, differentiation, and survival we isolated primary myoblast cultures from control and mutant chimeric mice. Myoblasts derived from α5-null or heterozygous ES cells were selected by culture in G418. The purity of the cell populations was analyzed by Southern blot analysis (data not shown) (Yang et al., 1993, 1995) and the expression of α5 integrin was analyzed by Western blot analysis (Fig. 7 *A*). Control (+/−) myoblasts expressed α5 integrin whereas α5-null myoblasts were negative for α5 expression (Fig. 7 *A*). When maintained in low-serum medium, both control (+/−) and mutant (−/−) myoblasts differentiated into multinucleated myotubes (Fig. 6 *B*), and the rate and extent of myotube formation was similar in the two populations (Fig. 6 *C*). Control and α5-deficient myoblasts grown on laminin-1 had similar doubling times (data not shown) and displayed similar morphologies (Fig. 7 *B*). However, the phenotypes of the two populations were very different when plated on fibronectin, the ligand for the α5β1 integrin receptor. Whereas α5-expressing cells attached and spread readily on fibronectin, α5-deficient cells adhered poorly, showed very little spreading, and displayed a rounded morphology, typical of poor cell–substrate adherence (Fig. 7, *B* and *C*). As further evidence that the different substrate requirements were indeed due to α5 deficiency (and not to some other property of the α5-deficient cell population), we restored α5 expression to

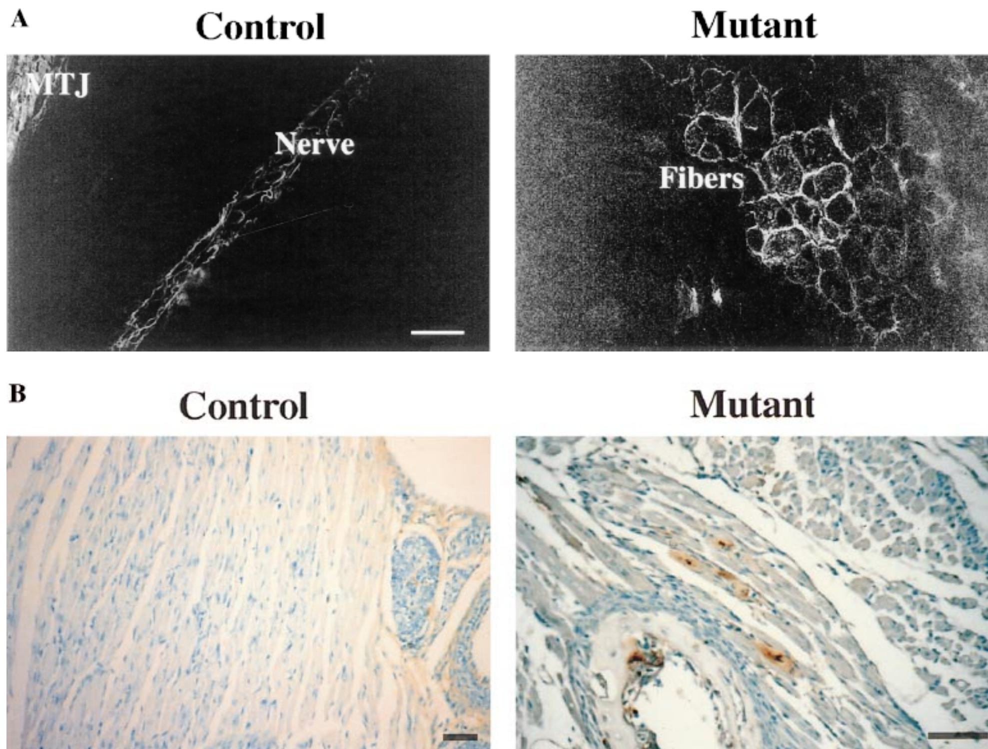


Figure 5. Muscle lesion–associated phenotypes: tenascin-C (TN-C) expression and apoptosis. (*A*) Transverse sections of thoracic muscle from E18 mutant or control chimeric embryos were stained for tenascin-C. Sections from control animals show TN-C staining in the nerve (*center*) crossing the muscle and at the MTJ (*upper left*), but the muscle itself is negative. In contrast, tenascin is found distributed around muscle fibers in the mutant embryos and is not restricted to the MTJ. (*B*) Terminal transferase biotinylated-dUTP nick-end labeling (TUNEL) analysis in transverse sections of thoracic muscles from mutant or control E18 embryos is shown. More apoptotic cells are observed in mutant chimeras. Bars, 50 μm.

the α5-deficient cells by retrovirus-mediated gene transfer. A control population of α5-deficient cells was infected with a retrovirus expressing only the neo gene. Expression of α5 in rescued cells (but not in control infected cells) was confirmed by Western blot analysis (Fig. 7 *A*). The rescued cells appeared to adhere as well to fibronectin as did wild-type cells (Fig. 8, *A* and *B*).

Because of the apoptosis we observed in vivo (Fig. 5 *B*) and since interference with integrin-mediated adhesion has been reported to cause apoptosis (Frisch and Francis, 1994; Zhang et al., 1995; Goh et al., 1997), we tested for apoptosis in the myoblasts cultured on various substrates. Whereas α5-expressing cells showed similar percentages of annexin-positive cells on fibronectin or laminin-1, α5-deficient cells showed a fourfold increase in annexin-positive cells on fibronectin compared with laminin-1 (Fig. 9). Clearly, the absence of α5 expression increases the propensity of the cells to undergo apoptotic cell death when FN is the major component of the ECM.

Discussion

The results presented here show that α5-null cells can participate in a wide variety of differentiative processes; animals with a high degree of chimerism in many organs survive and reproduce. The only defect which we observed consistently in chimeric animals containing a significant proportion of α5-null cells was a novel form of muscular dystrophy. This allowed us to focus on the differentiation and survival of muscle cells and tissues and the dependence of these processes on α5β1 integrin.

Earlier results have shown blockade of migration of myoblasts and of their differentiation into myotubes by inhibitory antibodies against various integrin subunits, including β1 (Jaffredo et al., 1988; Menko et al., 1987), α4 (Rosen et al., 1992) or α7 (Echtermeyer et al., 1996; Yao et al., 1996). However, in vitro differentiation of ES cells and myoblasts lacking β1 or α4 proceeds normally (Yang et al., 1996; Brakebush et al., 1997) and β1-null and α4-null cells can participate in myogenesis in chimeric mice (Faessler and Meyer, 1995; Yang et al., 1996). In the experiments reported here, we obtained similar results for α5-null cells. In vitro, neither α5-null ES cells nor α5-null myoblasts showed any deficit in myogenesis and, in chimeric mice, muscles with a high proportion of α5-null cells could form. These results demonstrate that α5β1 integrin is not essential for the proliferation, migration, or differentiation of myoblasts, myotubes, and skeletal muscles. However, in contrast with the results for α4-null chimeras (Yang et al., 1996), we observed a significant level of abnormalities in the skeletal muscles of the α5-null chimeras (Figs. 2–4). These muscles showed many characteristics of muscular dystrophy, including giant fibers, central nuclei, vacuoles, fibrosis, and fiber degeneration. Later in life we observed signs of regeneration such as ring fibers. We also observed increased apoptosis and ectopic expression of TN-C (Fig. 5) as has been reported for other forms of muscular dystrophy (Settles et al., 1996).

These results are reminiscent of the observations of Mayer et al. (1997) on α7 integrin-deficient mice, which also exhibit muscular dystrophy, and of recent reports of deficiencies in α7β1 in human and murine muscular dystrophies (Hodges et al., 1997; Mayer et al., 1997; Vachon et al., 1997). It appears that these two integrins, α5β1 and α7β1, one a receptor for fibronectin and the other a recep-

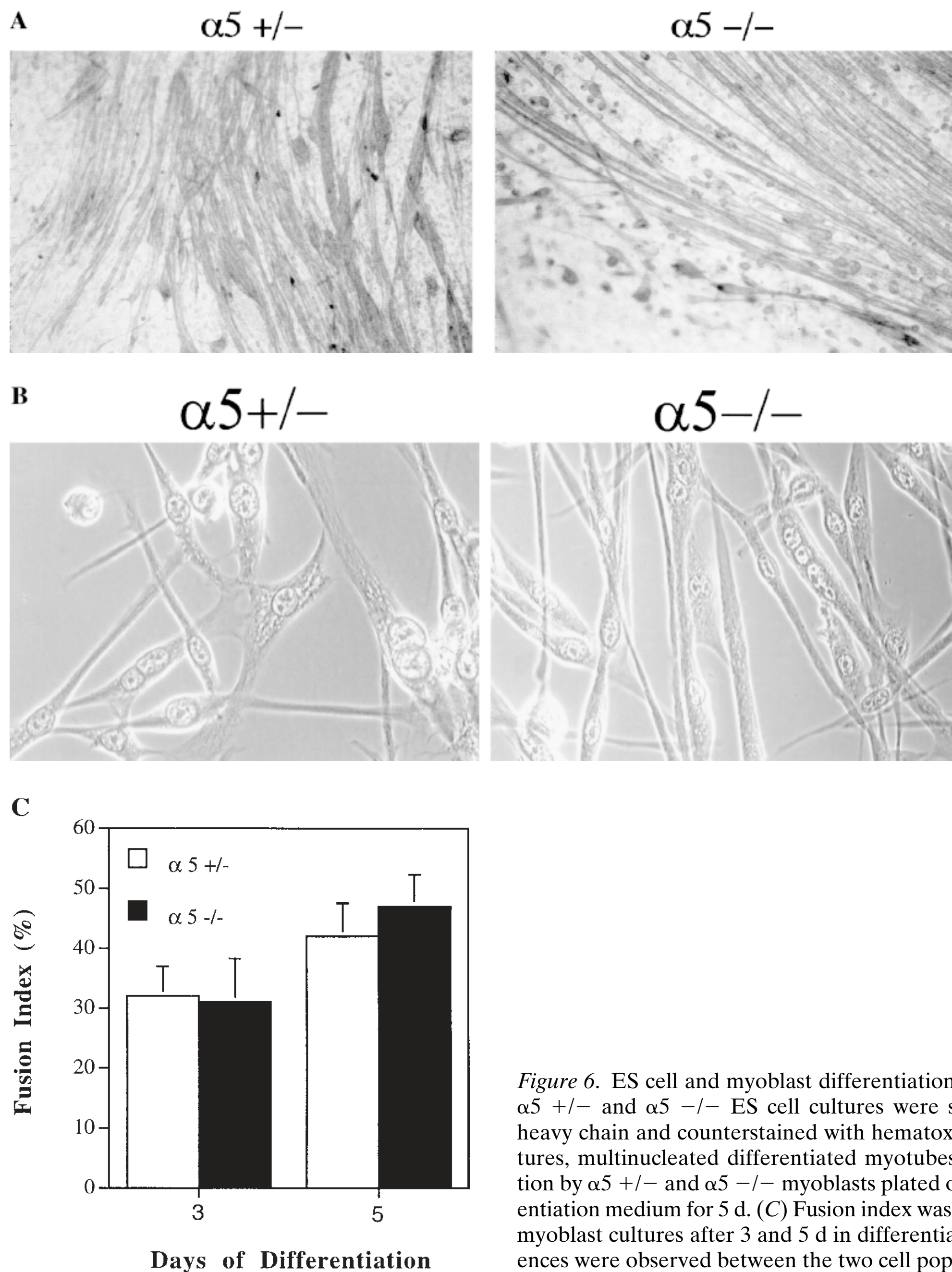


Figure 6. ES cell and myoblast differentiation into myotubes. (*A*) Differentiated α5 +/− and α5 −/− ES cell cultures were stained for skeletal muscle myosin heavy chain and counterstained with hematoxylin (nuclear staining). In both cultures, multinucleated differentiated myotubes are present. (*B*) Myotube formation by α5 +/− and α5 −/− myoblasts plated on laminin and maintained in differentiation medium for 5 d. (*C*) Fusion index was determined for α5 +/− and α5 −/− myoblast cultures after 3 and 5 d in differentiation medium. No significant differences were observed between the two cell populations.

tor for laminin, are both necessary for long-term integrity of myotubes, although not for their initial development.

α5 is found localized at adhesion plaques (McDonald et al., 1995) and at the MTJ (Fig. 4); α7 is concentrated at the MTJ (McDonald et al., 1995) where tendons attach. Because these two integrins are present at the points in the fibers where mechanical stress occurs, it suggests an anchoring function of these molecules. Two *Drosophila* mutants show a similar situation; in *myospheroid* and *inflated* (mutations affecting integrin subunits) muscle differentiation occurs in the absence, respectively, of βPS or αPS2 integrins; however, on contraction, the muscles detach from their attachments (Volk et al., 1990; Brabant et al., 1993). We can imagine that, in the absence of α7β1 or α5β1, important points of adhesion are lost or weakened and therefore contraction leads to damage to the myotubes. The increase in the number of mitochondria in some fibers of the α5 −/−;+/+ chimeric mice could suggest that, when the fibers cannot function normally, they tend to hypercontract and they need more ATP that requires the formation of a higher number of mitochondria. Other possibilities for the increase in mitochondria include altered differentiation and compensation for the reduced level of α5 integrin.

It is noteworthy that many forms of muscular dystrophy arise from defects in connections to the extracellular matrix. That includes the classical muscular dystrophies arising from defects in dystrophin and its transmembrane linkage via dystroglycans to laminins and in the laminins

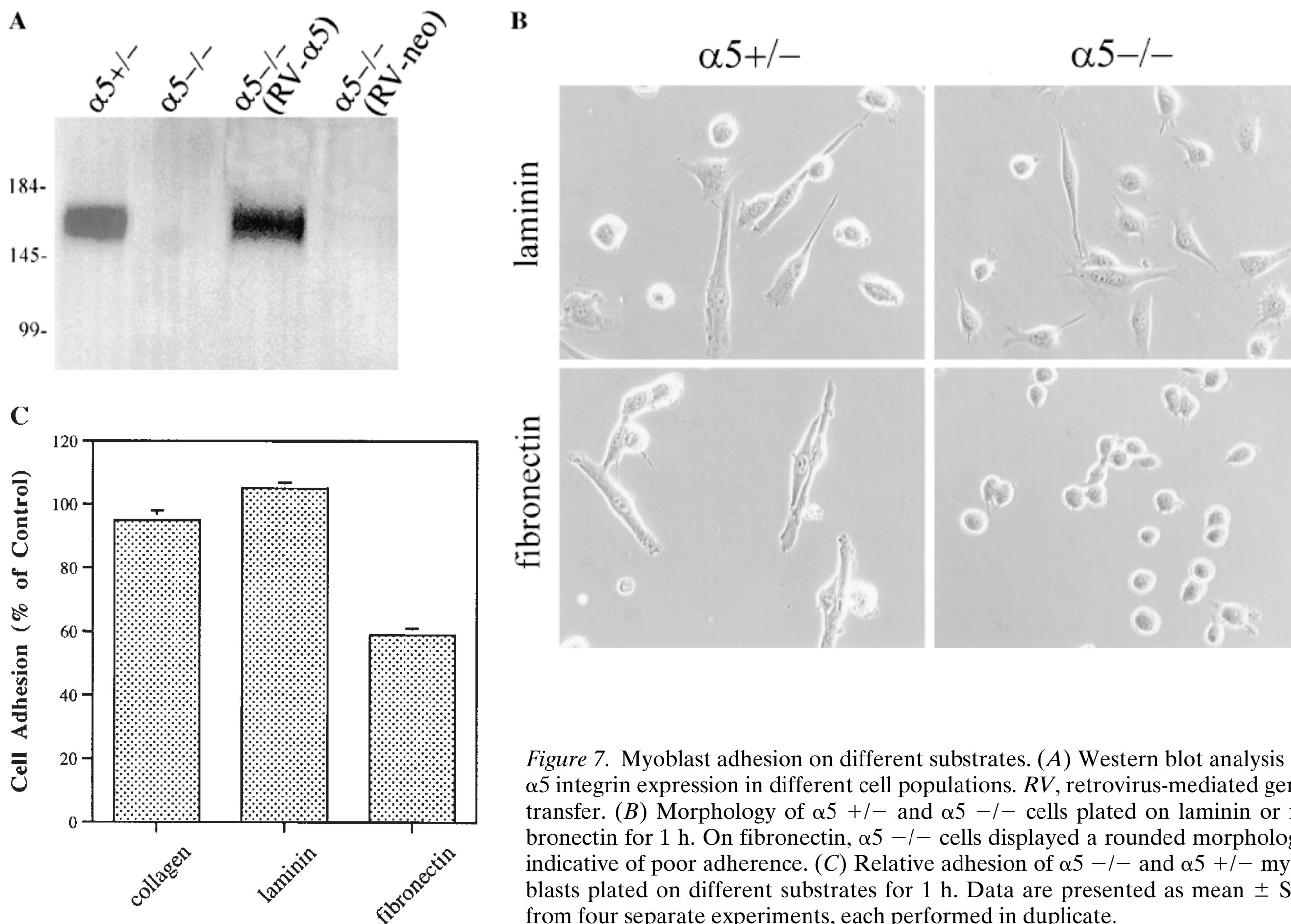


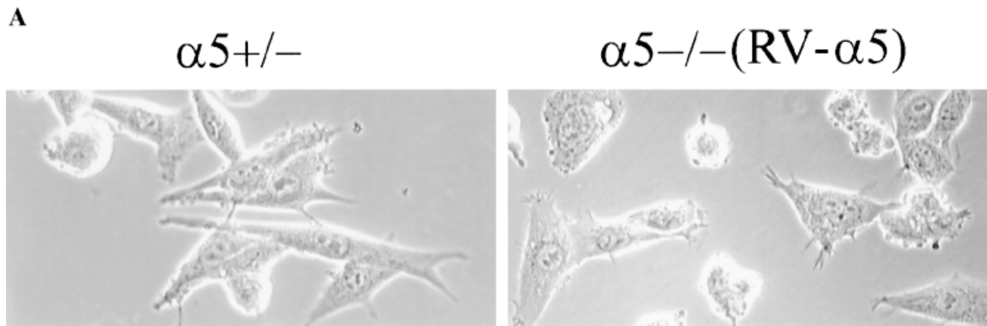
Figure 7. Myoblast adhesion on different substrates. (*A*) Western blot analysis of α5 integrin expression in different cell populations. *RV*, retrovirus-mediated gene transfer. (*B*) Morphology of α5 +/– and α5 –/– cells plated on laminin or fibronectin for 1 h. On fibronectin, α5 –/– cells displayed a rounded morphology indicative of poor adherence. (*C*) Relative adhesion of α5 –/– and α5 +/– myoblasts plated on different substrates for 1 h. Data are presented as mean ± SD from four separate experiments, each performed in duplicate.

themselves (Campbell, 1995). The novel form of muscular dystrophy which we describe here differs from the others, including those caused by α7 integrin deficiencies, in having no obvious connection with laminins. α5β1 has no affinity for laminins and is believed to be specific for fibronectin. The α5-null myoblasts show defects in adherence and survival on fibronectin substrates but behave normally on laminin (Fig. 7).

The fact that the muscle defect of the α5-chimeric mice is visible at a very early age (embryonic and postnatal life) and is more attenuated later in life might be due to the high expression, and probable importance, of α5 in embryonic and postnatal muscle followed by later downregulation (McDonald et al., 1995). In vitro data show that overexpression of α5β1 in myoblasts promotes proliferation and inhibits differentiation, suggesting a proliferative function of this integrin (Sastry et al., 1996). It is possible that, in the chimeras, α5 is particularly important when a high rate of proliferation is occurring. However, since our mice are chimeras and the muscle fiber is a fusion of α5-null and wild-type cells we cannot exclude the possibility that the defect is partially rescued by the presence of wild-type cells. Another possible reason for amelioration of the phenotype in later life could be gradual replacement of α5-null cells by wild type during regeneration. Consistent with this possibility is the appearance of ring fibers in the older muscles, indicating some fiber regeneration.

We favor the hypothesis that the dystrophy arises from defects in the myofibers themselves, as discussed above. In particular, the time of onset during fetal life corresponds with the period when α5β1 is known to be strongly expressed in muscle cells and the parallels with the muscle defects seen in *Drosophila* integrin mutants are suggestive. However, we cannot rule out the possibility that defects or deficits in other α5-null cells, such as interstitial fibroblasts, neurons or Schwann cells or vascular endothelial cells, could contribute to the phenotype observed. We do know that α5-null fibroblasts can assemble FN matrix and migrate and adhere normally (Yang and Hynes, 1996) and proliferate normally in vitro (Goh, K.L., and R.O Hynes, unpublished data) which argues against a causal defect in fibroblasts without eliminating that possibility.

The reasons for the degenerative changes observed in the muscles deficient in α5β1 remain unclear, as indeed is the case for other muscular dystrophies. Several possible explanations can be imagined. As mentioned above, if α5β1 (and α7β1) are important for maintaining mechanical connections between the myotubes and adjacent structures (e.g., tendons), disruption of the weakened linkage under contraction is a likely initiating cause. Perhaps less likely in this case is a general weakening of the cell surface structure comprising submembranous cytoskeleton connected to the basal lamina. Another possibility is that the apoptosis (Fig. 5) could be a causative event rather than a



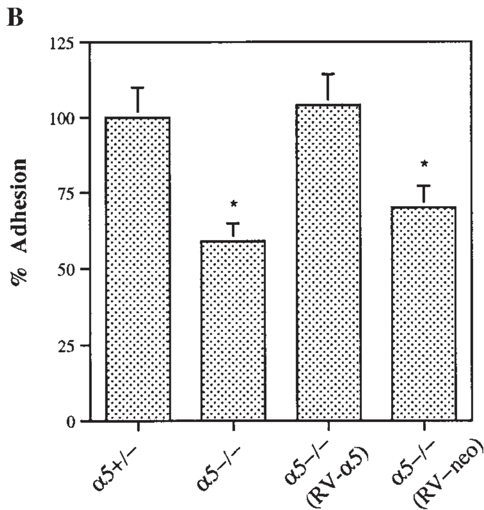


Figure 8. Phenotypic rescue of α5-deficient myoblasts by retrovirus-mediated gene transfer of the α5 gene (α5 integrin expression is shown in Fig. 7 *A*). (*A*) α5 −/− cells into which α5 has been introduced by retrovirus-mediated gene transfer (α5 −/− (RV- α5)) were plated on fibronectin for 1 h. Unlike the α5 −/− cells (see *B*), the α5 −/− (RV-α5) cells adhered well to fibronectin and were indistinguishable from the control cells (α5 +/−). (*B*) Quantitative adhesion assay demonstrates that expression of α5 integrin by retrovirus-mediated gene transfer (α5 −/− (RV- α5) cells) restores normal adhesion to FN in the α5 −/− cells. Infection of α5 −/− cells with a control retrovirus expressing only the neo gene (α5 −/− (RV-neo) cells) did not restore normal adherence.

secondary consequence. Precedent exists for cells' being dependent on specific integrin-matrix adherence for cell survival (Zhang et al., 1995) and such dependences include dependence on α5β1–fibronectin interactions (Zhang et al., 1995). However, our in vitro data somewhat argue against this idea without ruling it out. The α5-null myoblasts do indeed show increased apoptosis when plated on fibronectin but do not when plated on laminin (Fig. 9). Myotubes are surrounded by a basal lamina rich in laminin, although also containing fibronectin. Thus, although it is possible that adherence to fibronectin via α5β1 is specifically necessary for myotube survival, it seems more likely that any such requirement for attachment to basal lamina is satisfied by connection to laminin via α7β1 or via dystroglycans. Whatever the detailed cause–effect relationships leading to fiber degeneration and muscular defects in the mice, the results reported here reveal a novel form of muscular dystrophy.

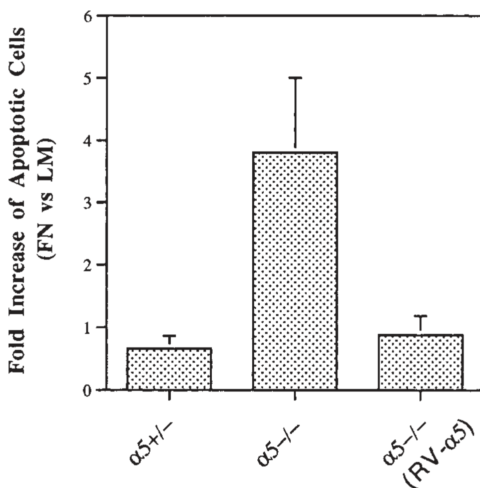


Figure 9. Myoblast survival on fibronectin versus laminin. Apoptosis in α5 +/−, α5 −/−, and α5 −/− (RV-α5) cells plated on FN or LM was determined by annexin staining (see Materials and Methods). Data are presented as percentages of apoptotic cells on FN compared with that on LM for each cell type. Whereas the percentage of apoptosis in α5 +/− and α5 −/− (RV-α5) were similar on the two substrates, there was increased apoptosis of α5 −/− cells on fibronectin compared with laminin.

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