MyoD is required for myogenic stem cell function in adult skeletal muscle

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To investigate the function of MyoD in adult skeletal muscle, we interbred MyoD mutant mice with mdx mice, a model for Duchenne and Becker muscular dystrophy. Mice lacking both MyoD and dystrophin displayed a marked increase in severity of myopathy leading to premature death, suggesting a role for MyoD in muscle regeneration. Examination of MyoD mutant muscle revealed elevated numbers of myogenic cells; however, myoblasts derived from these cells displayed normal differentiation potential in vitro. Following injury, MyoD mutant muscle was severely deficient in regenerative ability, and we observed a striking reduction in the in vivo proliferation of myogenic cells during regeneration. Therefore, we propose that the failure of MyoD-deficient muscle to regenerate efficiently is not caused by a reduction in numbers of satellite cells, the stem cells of adult skeletal muscle, but results from an increased propensity for stem-cell self-renewal rather than progression through the myogenic program.

[Key Words: MyoD; satellite cell; regeneration; Mdx; muscular dystrophy]

Received February 27, 1996; revised version accepted April 8, 1996.

The myogenic regulatory factors (MRFs) form a group of basic helix-loop-helix (bHLH) transcription factors consisting of MyoD, myogenin, Myf-5, and MRF4. The MRFs are expressed exclusively in skeletal muscle, and forcing their expression in a wide range of cultured cells induces the skeletal muscle differentiation program. Therefore, these transcription factors were postulated to have a master regulatory role in the development of the skeletal muscle lineage (for review, see Weimbrau et al. 1991).

Gene targeting has clearly elucidated the roles of the MRFs during embryogenesis. The introduction of null mutations in Myf-5, MyoD, myogenin, and MRF4 into the germ line of mice has revealed the hierarchical relationships existing among the MRFs and established that functional redundancy is a feature of the MRF regulatory network (for review, see Rudnicki and Jaenisch 1995; Megeney and Rudnicki 1995). The MRF family can be divided into two functional groups: The primary MRFs, MyoD and Myf-5, are required for the determination of skeletal myoblasts; and the secondary MRFs, myogenin and MRF4, act later in the program, likely as differentiation factors.

Mice lacking a functional MyoD gene were found to have no overt abnormalities in skeletal muscle but expressed about four-fold higher levels of Myf-5 (Rudnicki et al. 1992). Newborn mice deficient for both MyoD and Myf-5 are totally devoid of skeletal myoblasts and muscle (Rudnicki et al. 1993). Mice lacking myogenin die at birth because of a virtual absence of myofibers, however, normal numbers of MyoD-expressing myoblasts are present (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). In contrast, mice lacking MRF4 are viable with seemingly normal skeletal muscle but display a four-fold increase in myogenin (Patapoutian et al. 1995; Zhang et al. 1995).

Vertebrate skeletal muscle is derived from cells in the prechordal and somitic mesoderm that give rise to committed myogenic cells, which become the skeletal muscle of the head, trunk, and limbs (for review, see Buckingham 1992; Miller 1992, 1994). The different myofiber and myogenic constituents of muscle have been postulated to be formed from different lineages of myogenic cells (Miller 1992; Bischoff 1994). In the mouse, primary myofibers develop first at 8.5 days of gestation, followed by secondary myofibers around day 14. Satellite cells, the stem cells of adult skeletal muscle, arise around day 17 of development as a unique myogenic cell lineage (Bischoff 1994).

Satellite cells lie beneath the basal lamina of adult skeletal muscle closely juxtaposed to the myofiber sarcolemma. Satellite cells make up 2%-7% of the nuclei associated with a particular fiber, and the proportion varies with age and muscle group. These adult muscle stem cells are normally mitotically quiescent but are activated (i.e., enter the cell cycle) in response to stress induced by weight-bearing or other trauma, such as injury.
The descendants of the activated satellite cells, called myogenic precursor cells [mpc], undergo multiple rounds of division before fusing to existing myofibers or forming new myofibers, leading to repair and/or hypertrophy of the damaged or stressed muscle fibers, respectively. Satellite cells form a stable population, suggesting that self-renewal in the stem-cell compartment maintains the pool of quiescent satellite cells. Moreover, satellite cells and their descendants have a very large potential for proliferation, perhaps as much as 80 doublings, as revealed by experiments in which muscle was subjected to repetitive cycles of injury-induced regeneration (for review, see Grounds and Yablonka-Reuveni 1993; Bischoff 1994).

The expression pattern of MRFs following satellite-cell activation and during mpc proliferation and differentiation appears to be distinct from the program manifested during the embryonic development of skeletal muscle. Quiescent satellite cells express no detectable MRFs. Upon activation and entrance into the cell cycle, MyoD is rapidly up-regulated concomitant with expression of proliferating cell nuclear antigen [PCNA], a marker for cell proliferation (e.g., Bravo et al. 1987). Myf-5 and MRF4 are expressed at intermediate times when PCNA and MyoD expression are declining. Myogenin is expressed last during the time associated with fusion and differentiation [Smith et al. 1994; Yablonka-Reuveni and Rivera 1994]. This expression of MyoD early after activation is suggestive of an important role for MyoD in satellite cell function.

The viability and apparently normal skeletal muscle of MyoD mutant mice [Rudnicki et al. 1992], and the early expression of MyoD following satellite cell activation [Smith et al. 1994; Yablonka-Reuveni and Rivera 1994], lead us to consider the hypothesis that MyoD may have some role in the postnatal growth and regeneration of skeletal muscle. To investigate the function of MyoD in adult skeletal muscle, we interbred MyoD mutant mice with mdx mice carrying a loss-of-function mutation in the dystrophin gene, and performed injury-induced regeneration experiments on muscle in MyoD-deficient animals. Our data clearly establish a role for MyoD in adult muscle and reveal novel insights into the genetic regulation of muscle stem-cell function.

Results
Mice lacking MyoD and dystrophin exhibit increased myopathy

To determine whether MyoD might have a role in skeletal muscle regeneration, we interbred MyoD-deficient mice [Rudnicki et al. 1992] with the mdx mouse strain as an in vivo bioassay for skeletal muscle regeneration. The mdx mice carry a loss-of-function point mutation in the X-linked dystrophin gene and are therefore an animal model for human Duchenne and Becker muscular dystrophy [Sicinski et al. 1989; Ahn and Kunkel 1993]. The mdx mice display extensive necrosis of muscle fibers by 2 weeks of age, but unlike humans, maintain skeletal muscle integrity due to a high muscle regenerative capacity. This regeneration results in the formation of additional muscle fibers, which leads to a significant level of muscle hypertrophy. Hypertrophy in mdx muscle is characterized by a 25% increase in the number of fibers, a four-fold increase in the distribution of the fiber calibers, centrally located nuclei within 70%-80% of all fibers, and a 1.7-fold increase in muscle mass [Anderson et al. 1987; Carnwath and Shotton 1987; Coulton et al. 1988; e.g., see Fig. 2, below; Table 1]. If MyoD is required for efficient regeneration of skeletal muscle, mice lacking MyoD [designated mdx:MyoD(-/-)] should display more extensive myopathic or dystrophic changes in skeletal muscle.

As predicted, mutant mdx:MyoD(-/-) mice uniformly displayed several phenotypic traits indicative of a marked increase in the penetrance of the disease [Fig. 1]. By 3 to 5 months of age, mdx:MyoD(-/-) mice developed a profound dorsal-ventral curvature of the spine [Fig. 1, cf. C and D], similar to the lordosis and kyphosis of patients with Duchenne muscular dystrophy, and an abnormal waddling gait characterized by weight bearing on the hocks [Fig. 1, compare A and B]. The animals became progressively less active, with concomitant weight loss, before premature death at ~12 months of age. In contrast, mdx mice display virtually normal external appearance and normal murine life spans of 1.5–2 years [Coulton et al. 1988].

Examination of skeletal muscle from the lumbar region of MyoD(-/-) mice revealed an ~17% reduction in the cross-sectional area of back muscle compared with BALB/c [hereafter referred to as wild type] back muscle [Fig. 2A,B]. Moreover, MyoD(-/-) tibialis anterior (TA) muscle was significantly reduced in mass relative to wild-type TA by 32% [P<0.05], whereas MyoD(-/-) soleus (SL) muscle was reduced by 8% [Table 1]. These data suggest that MyoD has a role in the postnatal growth of skeletal muscle.

The mdx phenotype is characterized by an ~1.7-fold increase in the mass of individual skeletal muscles compared with wild-type mice [Anderson et al. 1987; Carnwath and Shotton 1987; Coulton et al. 1988; e.g., see Fig. 2 and Table 1]. Importantly, the hypertrophic response is

| Table 1. Mean muscle mass from tibialis anterior and soleus hindlimb muscles from 4-month-old animals of the different genotypes |
|---|---|---|
| Mouse strain | TA muscle [mg] | SL muscle [mg] |
| Wild type | 59.3 ± 1.4 | 10.1 ± 0.5 |
| MyoD(-/-) | 40.0 ± 1.7 | 9.3 ± 0.5 |
| mdx | 95.9 ± 5.3 | 17.9 ± 1.6 |
| mdx:MyoD(-/-) | 45.8 ± 4.2 | 9.6 ± 0.6 |

These data reveal the relative hypertrophy in mdx muscle and relative hypotrophy in MyoD(-/-) and mdx:MyoD(-/-) muscle. The values above represent the mean and standard error of the mean of muscle weights from five animals [n = 10].
Impaired regeneration of MyoD(−/−) skeletal muscle

Wild-type skeletal muscle typically undergoes several stages following injury leading to complete repair of the lesion. Within 24 hr, damaged fibers become necrotic and begin to degenerate, and large numbers of infiltrating eosinophils, neutrophils, and macrophages accumulate and remove debris from the area of damage. Satellite cell-derived mpc cells invade the site, proliferate, and fuse to each other as they differentiate to form new muscle fibers within pre-existing basal lamina. By 2 weeks, every fiber is continuous and fiber calibers are comparable with undamaged muscle (for review, see Grounds and Yablonka-Reuveni 1993).

The increased myopathy of skeletal muscle from mdx:MyoD(−/−) mice suggests a marked decrease in their regenerative ability. Nevertheless, some regenerative potential persists, as evidenced by the presence of centrally located nuclei in mdx:MyoD(−/−) muscle fibers. Taken together, these data are consistent with the hypothesis that MyoD has an important role in the embryonic formation, postnatal survival, or function of satellite cells.

Figure 1. Increased penetrance of the mdx phenotype in mdx:MyoD(−/−) mice. [A] The mdx mouse appears morphologically normal compared with wild-type mice. [B] In contrast, the mdx:MyoD(−/−) mouse displays a severe dorsal–ventral curvature of the spine and increased myopathy characterized by an abnormal waddling gait and weight-bearing on the hocks. x-Ray radiographic visualization revealed abnormal curvature of the spine (arrowhead) of mdx:MyoD(−/−) mice [D], compared with mdx mice [C], and MyoD(−/−) mice [E]. All animals shown are 5 months of age.

Reduced significantly or absent in mdx:MyoD(−/−) skeletal muscle. For example, examination of skeletal muscle from the lumbar region of mdx:MyoD(−/−) mice revealed an ~35% reduction in the cross-sectional area of back muscle compared with mdx back muscle (Fig. 2C,D). A similar reduction was observed in the cross-sectional area of the lower leg containing TA, gastrocnemius, SL, and extensor digitorum longus muscles [not shown]. In addition, the weight of TA muscle from 4-month-old mdx:MyoD(−/−) mice was reduced significantly by 52% (P<0.05), relative to 4-month-old mdx TA muscle, and SL muscle was reduced significantly by 46% (P<0.05) [Table 1]. The diaphragm is the only muscle in mdx mice that undergoes extensive dystrophic changes caused by repetitive work-related injury [Stedman et al. 1991]. Examination of the mdx:MyoD(−/−) diaphragm revealed extreme severity in the dystrophic changes relative to the mdx diaphragm, resulting in a marked reduction in muscle mass and thickness [not shown].

Muscle fibers, which have undergone a cycle of degeneration–regeneration, typically display centrally rather than peripherally located nuclei [for review, see Grounds and Yablonka-Reuveni 1993]. Light microscopic examination of hematoxylin–eosin (HE)-stained thin sections of skeletal muscle from mdx:MyoD(−/−) mice revealed the widespread presence of centrally located nuclei in muscle fibers, suggesting that these fibers have undergone at least some regeneration (Fig. 2, cf. G and H).

All mdx:MyoD(−/−) mice from the first and subsequent generations displayed the same degree of phenotype suggesting that the absence of MyoD is the cause of the phenotype. Furthermore, the BALB/c strain from which the MyoD(−/−) mice are derived, when interbred with mdx mice, did not generate the phenotype observed in mdx:MyoD(−/−) mice. Therefore, we conclude that it is the lack of MyoD, and not some other second-site modifier gene, that causes the defect in regeneration.

The increased myopathy of skeletal muscle from mdx:MyoD(−/−) mice suggests a marked decrease in their regenerative ability. Nevertheless, some regenerative potential persists, as evidenced by the presence of centrally located nuclei in mdx:MyoD(−/−) muscle fibers. Taken together, these data are consistent with the hypothesis that MyoD has an important role in the embryonic formation, postnatal survival, or function of satellite cells.
Figure 2. Attenuated hypertrophy and increased myopathy in \textit{mdx}:MyoD\textsuperscript{(-/-)} skeletal muscle. HE-stained sections through the lumbar region of the lower back in wild-type (A,E], MyoD\textsuperscript{(-/-)} (B,F], mdx (C,G], and mdx:MyoD\textsuperscript{(-/-)} (D,H] mice. MyoD-deficient mice exhibited a reduced cross-sectional area of muscle relative to wild-type (cf. A and B] and mdx mice (cf. C and D]. Higher magnification of these sections revealed increased numbers of interstitial mononuclear cells and reduced fiber calibers in mdx:MyoD\textsuperscript{(-/-)} muscle (cf. G and H]. Note the presence of centrally located nuclei in mdx:MyoD\textsuperscript{(-/-)} fibers, suggesting that some degree of regeneration has occurred (arrows).

[e.g., Mitchell et al. 1992]. Like MyoD\textsuperscript{(-/-)} muscle, mdx:MyoD\textsuperscript{(-/-)} muscle was similarly deficient in regenerative proficiency following injury [not shown].

These results strongly support the hypothesis that the defect in skeletal muscle regeneration in MyoD\textsuperscript{(-/-)} mice is at the level of satellite cell formation, survival, or function. Furthermore, these data support the contention that the increased severity of the phenotype observed in mdx:MyoD\textsuperscript{(-/-)} mice is a consequence of decreased regenerative ability.

**Enumeration of satellite cells and satellite cell-derived myoblasts**

The increased severity of \textit{mdx} phenotype in the mdx:MyoD\textsuperscript{(-/-)} mouse, and the limited regeneration of skeletal muscle from MyoD\textsuperscript{(-/-)} mice following injury, indicated that MyoD has a unique role in satellite cell biology. One possibility was that MyoD-deficient muscle contained reduced numbers of stem cells. To address this question, we assessed whether quiescent satellite cells were present by electron microscopic examination of sectioned skeletal muscle and enumerated the total number of cells with myogenic potential following culture of primary cells derived from skeletal muscle. Unexpectedly, these experiments provided evidence for markedly increased numbers of myogenic stem cells in MyoD\textsuperscript{(-/-)} and mdx:MyoD\textsuperscript{(-/-)} muscle.

Quiescent satellite cells can be reliably identified by electron microscopy by their morphological appearance and location external to the muscle-fiber sarcolemma and beneath the basal lamina encasing the fiber (Schultz 1976). Satellite cells comprise \~32% of sublaminal nuclei in newborn mice, and this proportion declines to \<5% by 8 weeks of age [for review, see Bischoff 1994]. TA muscle from 4-month old wild-type, MyoD\textsuperscript{(-/-)},...
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**Figure 3.** Impaired regeneration of MyoD(-/-) skeletal muscle. Longitudinal sections of HE-stained TA muscle sampled 4.5 days [A,B], and 2 weeks following crush-induced injury [C,D]. [A] Wild-type muscle 4.5 days following injury displayed a robust regenerative capacity with well-advanced myofibrogenesis, as evidenced by centrally located nuclei [arrows], in addition to putative mononuclear mpc [arrowhead]. [B] MyoD(-/-) muscle 4.5 days following injury exhibited markedly reduced levels of regeneration with many mononuclear cells present [arrowheads], as well as small myotubes [arrows]. [C] Wild-type muscle 2 weeks following injury, appeared similar to undamaged skeletal muscle except for the presence of centrally located nuclei [arrows] and reduced fiber calibers. [D] MyoD(-/-) muscle 2 weeks following injury exhibited many small caliber myotubes [arrows] and unusually high numbers of mononuclear cells [arrowheads].

**Figure 4.** Satellite cells in MyoD(-/-) skeletal muscle. Electron microscopic examination of sections revealed the presence of morphologically normal satellite cells in TA muscle of all genotypes (Fig. 4). mdx, and mdx:MyoD(-/-) mice were processed for transmission electron microscopic examination. Analysis of these sections clearly revealed the presence of morphologically normal satellite cells in TA muscle of all genotypes (Fig. 4).

Electron microscopy also allowed an approximation of the proportion of quiescent satellite cells in vivo as determined by counting the ratio of sublaminar mononuclear cells to intrafibrillar nuclei in representative fields (Table 2). Satellite cells made up 2.9% of the sublaminar nuclei in MyoD(-/-) TA muscle, 3.3% in mdx TA muscle, and 4.4% in wild-type TA muscle (Table 2). These values are typically considered within the normal range (Schultz 1976; Bischoff 1994). In contrast, we observed a 2.5-fold increase in the proportion of satellite cells in mdx:MyoD(-/-) TA muscle, where satellite cells made up 8.3% of sublaminar nuclei (Table 2). Because of the limited sample size, this experiment can only reveal trends rather than significant differences. However, these data suggest that quiescent satellite cells, as identified by morphology and position, are present in normal proportions in MyoD(-/-) muscle and in elevated proportions in mdx:MyoD(-/-) muscle.

Electron microscopy only allows the identification of satellite cells when they are located between the sarcolemma and basal lamina, and not outside this position (Grounds 1991). To quantitate the relative proportions of muscle stem cells between animals of different genotypes, populations of myoblasts were cultured in vitro from primary cultures derived from wild-type, MyoD(-/-), mdx, and mdx:MyoD(-/-) hindlimb muscle. Muscles were weighed, minced, dispersed enzymatically, filtered, and plated onto collagen-coated dishes in medium supplemented with fetal calf serum and basic fibroblast growth factor [bFGF] (after Rando and Blau 1994). After 4 days of culture, total cell yield [myoblasts plus fibroblasts] per milligram of muscle was determined, and the relative proportion of myoblasts assessed by indirect immunochemical staining using monospecific antisera DE-U-10, reactive to desmin (see Table 2). [mn] Muscle cell nucleus; [sn] satellite cell nucleus; Bar, 1 μM.
Table 2. MyoD(-/-) muscle contains elevated numbers of myogenic cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent satellite cells in vivo</th>
<th>Total number cells/mg wet weight</th>
<th>Percent cells desmin +ve</th>
<th>Total number myoblasts/mg wet weight</th>
<th>Percent nuclei MHC +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>4.4</td>
<td>1531 ± 174</td>
<td>22.4 ± 1.8</td>
<td>343</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>MyoD(-/-)</td>
<td>2.9</td>
<td>3356 ± 599</td>
<td>18.5 ± 1.3</td>
<td>621</td>
<td>11.0 ± 1.8</td>
</tr>
<tr>
<td>mdx</td>
<td>3.3</td>
<td>1506 ± 174</td>
<td>4.8 ± 0.7</td>
<td>72</td>
<td>N.D.</td>
</tr>
<tr>
<td>mdx:MyoD(-/-)</td>
<td>8.3</td>
<td>8314 ± 1509</td>
<td>11.7 ± 1.4</td>
<td>972</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The values represent the mean and standard error of the mean for five independent experiments ([n > 200]. (N.D.) Not determined.

"The proportion of satellite cells in TA muscle as determined by electron microscopy ([n > 200].

"After 4 days in culture, the total number of cells recovered per mg of muscle was determined by monodispersing the cultures and counting cells in a hemocytometer.

"The proportion of myoblasts was determined by immunodetection with anti-desmin antibody DE-U-10.

"The relative number of cells with myogenic potential per mg wet weight was determined by multiplying the percent [%] by the total number of cells recovered [\( n \)].

"The proportion of differentiated myocytes was determined by immunodetection with anti-myosin heavy chain antibody MF20.

Fig. 5. Desmin is an intermediate filament protein expressed in mpc in vivo, and myoblasts in vitro, but not satellite cells (George-Weinstein et al. 1993; Bischoff 1994).

Immunohistological analysis revealed that the percentage of myoblasts observed in 4-day cultures derived from wild-type skeletal muscle was 22.4%, whereas the percentage of myoblasts in cultures from MyoD(-/-) muscle was 18.5%. However, because the total yield of cells per milligram was increased 2.2-fold in the MyoD(-/-) muscle relative to wild-type muscle ([P<0.05], the number of myogenic cells recovered per milligram of muscle was therefore increased 1.8-fold in MyoD(-/-) muscle relative to wild-type muscle (Table 2). The percentage of myoblasts observed in cultures from mdx muscle was 4.8%, whereas the percentage of myoblasts in cultures from mdx:MyoD(-/-) muscle was 11.7%. Similarly, because the total number of cells per muscle weight was increased 5.5-fold in the mdx:MyoD(-/-) muscle relative to mdx muscle ([P<0.05], the number of myogenic cells recovered per milligram was therefore increased 13-fold in mdx:MyoD(-/-) muscle relative to mdx muscle (Table 2). Interestingly, wild-type and MyoD mutant myoblasts differed both in morphological appearance and intensity of anti-desmin antibody labeling. As described elsewhere (e.g., Ontell et al. 1992), >90% of the desmin-labeled myoblasts from wild-type muscle were typically spherical, phase dark, and appeared to express high levels of desmin (Fig. 5A). In contrast, the majority of myoblasts from MyoD(-/-) muscle displayed a flat stellate morphology and expressed lower levels of desmin (Fig. 5B).

The differentiation potential of myoblasts derived from primary cultures from wild-type and MyoD(-/-) muscle was also investigated. Cultured myoblasts were expanded under growth conditions for 1 week and trans-
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ferred to differentiation medium for a second week. Differen-
tiated myocytes were detected using indirect immu-
nochemistry with mouse monoclonal antibody MF20
reactive with striated muscle myosin heavy chain [Bader
et al. 1982]. MyoD mutant myocytes were morpho-
logically indistinguishable from wild-type myocytes
(Fig. 5, cf. C and D). The proportion of nuclei within
immunoreactive myocytes was found to be 11.0% in
MyoD(-/-) cultures and 6.5% in wild-type cultures
(Table 2). The increased proportions of differentiated
MyoD-deficient myocytes observed in vitro [P<0.05]
suggests that desmin may be expressed below the limit
of detection in some portion of MyoD mutant myo-
blasts, hence, the numbers of myogenic cells per milli-
gram in MyoD(-/-) muscle may represent an underes-
timate (Table 2).

The increased numbers of myoblasts recovered from
MyoD(-/-) muscle raised the possibility that myo-
blasts lacking MyoD may display increased rates of pro-
liferation. To determine the doubling time of myoblasts
in vitro, we measured the increase in numbers of
desmin-staining cells over a period of 8 days. Impor-
tantly, we observed the in vitro doubling time of myo-
blasts derived from MyoD(-/-) muscle to be about
three-fold longer than wild-type myoblasts. Furth-
more, purified populations of MyoD(-/-) myoblasts in-
corporated three-fold less tritiated thymidine. These ob-
servations support the notion that MyoD has an impor-
tant role in positively inducing myoblast proliferation.
Furthermore, these data suggest that we have underesti-
mated the numbers of myoblasts isolated from
MyoD(-/-) muscle by at least two-fold.

In vivo proliferation of MyoD(-/-) myogenic cells

Skeletal muscle from mdx:MyoD(-/-) mice relative to
mdx mice contained a 2.5-fold increase in numbers of
satellite cells by morphology and position (Fig. 4, Table
2), and at least a 13-fold increase in numbers of myogenic
cells per milligram of muscle (Fig. 5; Table 2). If MyoD(-/-) mpc proliferate normally in vivo, we
should observe a 13-fold increase in the proportion of
cells undergoing cell division in mdx:MyoD(-/-) muscle
relative to mdx muscle. Therefore, we examined cel-

Figure 6. Reduced cellular proliferation
during regeneration of MyoD(-/-) muscle.
Samples were prepared for immunohis-
tochemical staining with an antibody reac-
tive to PCNA 24 hr following injury to de-
tect satellite-cell activation (A,B). In
surviving areas immediately adjacent the
site of injury, ~10% of peripherally located
nuclei expressed PCNA in both mdx [A]
and mdx:MyoD(-/-) muscle [B] even
though mdx:MyoD(-/-) muscle contains
13-fold higher numbers of myogenic cells
(see Table 2). Mice were injected with tri-
tiated thymidine 3.5 days following injury,
and samples were prepared for autoradiog-
raphy 24 hr later (C,D). TA muscle from
mdx mice (C) displayed abundant nuclei
decorated heavily with silver grains,
whereas TA muscle from mdx:MyoD(-/-)
mice (D) displayed only rare nuclei decorated
lightly with silver grains.
midine 3 days after injury, and tissue samples were prepared for autoradiography 24 hr later (n = 5). This approach allows detection of mpc replication during injury-induced muscle regeneration (e.g., Anderson et al. 1987; McGeachie and Grounds 1990). Four and a half days following injury, mdx TA muscle displayed abundant nuclei decorated heavily with silver grains, indicating that high numbers of mpc cells were cycling following injection of the labeled thymidine (Fig. 6C). Furthermore, in situ hybridization of companion sections revealed that 30% of cells expressing myogenin were heavily labeled (data not shown). In contrast, mdx:MyoD(−/−) TA muscle displayed only low numbers of nuclei decorated lightly with silver grains and no cells decorated heavily with silver grains, indicating that the majority of myogenic cells had not passed through S phase (Fig. 6D). Only rare myogenin-expressing cells were observed, and only 12% of these were labeled lightly (data not shown).

One possible explanation for the apparent decrease in mpc proliferation during regeneration in MyoD(−/−) muscle, was that MyoD mutant mpc may undergo programmed cell death. We therefore employed the terminal transferase-mediated dUTP–biotin nick end labeling (TUNEL) in situ staining technique (Gavrieli et al. 1992) to determine whether regenerating MyoD(−/−) muscle contained elevated numbers of apoptotic nuclei. We observed only very low numbers of apoptotic nuclei at similar frequencies in all the muscle genotypes (data not shown). Therefore, we conclude that MyoD-deficient myogenic cells do not inappropriately undergo apoptosis.

MyoD-deficient satellite cells appear to exhibit decreased rates of activation following trauma, and their mpc daughter cells display a markedly reduced proliferative potential in vivo as assessed by tritiated thymidine incorporation. Paradoxically, however, we observed a marked increase in the total number of cells with myogenic potential (Table 2). We interpret these data to suggest that the apparent increase in numbers of myogenic cells is a consequence of increased muscle stem-cell self-renewal because of an altered propensity for self-renewal rather than progression through the developmental program.

Discussion

Mice lacking MyoD are viable and display no obvious defects in function, morphology, or gene expression in skeletal muscle (Rudnicki et al. 1992). Interbreeding of MyoD and Myf-5 mutant mice clearly established that these factors functionally substitute for one another during embryonic development, because in the absence of both factors, no myoblasts or myofibers are formed (Rudnicki et al. 1993). The apparently normal phenotype of MyoD mutant mice and the early expression of MyoD following satellite-cell activation (Yablonka-Reuveni and Rivera 1994; Smith et al. 1994) led us to hypothesize that MyoD may have an important role in the postnatal growth and regeneration of skeletal muscle.

In this study we show that adult mice lacking MyoD display marked deficits in satellite cell function. MyoD(−/−) mice interbred with mdx mice exhibited increased penetrance of the mdx phenotype characterized by reduced muscle hypertrophy and increased myopathy leading to premature death (Figs. 1 and 2). Consistent with this observation, skeletal muscle regeneration in MyoD(−/−) mice was strikingly impaired following application of an injury to the TA muscle (Fig. 3). Electron microscopic examination of MyoD-deficient muscle revealed the presence of morphologically normal satellite cells (Fig. 4), and enumeration of myoblasts from primary cultures revealed elevated levels of cells with myogenic potential in MyoD(−/−) and mdx:MyoD(−/−) muscle (Fig. 5, Table 2). Moreover, cultured MyoD(−/−) myoblasts displayed reduced rates of proliferation. Furthermore, cellular proliferation during regeneration of mdx:MyoD(−/−) muscle is dramatically reduced (Fig. 6). Therefore, we conclude that MyoD has a unique and novel role in satellite cells and that Myf-5 does not substitute for this function. Taken together, our results have important implications for understanding the genetic mechanisms that regulate the skeletal-muscle stem-cell program in postnatal growth and regeneration.

Satellite cells have been postulated to represent a compartment of self-renewing stem cells distinct from their proliferating mpc descendants that form a transient population following satellite cell activation. For example, whereas mpc express desmin, α7-integrin, neural cell adhesion molecule (NCAM), and MRFS, satellite cells do not (for review, see Bischoff 1994). We propose that MyoD expression is required in vivo for stem cells to enter the mpc proliferative phase that precedes terminal differentiation (Fig. 7). According to this model, the apparent increase in numbers of myogenic cells in MyoD-deficient muscle is caused by an increased propensity for satellite cell self-renewal rather than progression through the developmental program. Consistent with this hypothesis, our data indicate that MyoD(−/−) muscle contains increased numbers of cells with myogenic potential (Table 2) and that these cells exhibit reduced proliferation both in vitro and during injury-induced muscle regeneration (Fig. 6).

The requirement for MyoD in the adult muscle stem-cell program may be direct, for example, MyoD up-regulation in activated satellite cells is required for expression of genes essential for normal mpc proliferation and differentiation. Alternatively, the requirement for MyoD may be indirect, for example, MyoD may be required in myofibers for the expression of growth factors essential for activating satellite cells or stimulating mpc proliferation. Although our experiments cannot discriminate between these two possibilities, the observations that MyoD is expressed at very low levels in adult myofibers, and is strongly up-regulated only in satellite cells following denervation, injury, or other trauma (e.g., Fuchrbauer and Westphal 1992; Bischoff 1994; Smith et al. 1994; Yablonka-Reuveni and Rivera 1994), strongly supports the notion that the requirement is cell autonomous. Transplantation of MyoD(−/−) myoblasts into γ-irradi-
Wildtype expression of MyoD with PCNA suggests that MyoD has
with the expression of Myf-5 and MyoD in proliferating
quired requirement for MyoD is cell autonomous.
ated wild-type muscle should clarify whether the re-
 requirement for MyoD is cell autonomous.
MyoD is expressed early during the program as satel-
nite cells enter the cell cycle and become mpc, Myf-5 and
MRF4 are expressed at intermediate times, and myoge-
nin is expressed on terminal differentiation [Smith et al.
1994; Yablonka-Reuveni and Rivera 1994]. The coex-
pression of MyoD with PCNA suggests that MyoD has
an early role in the satellite cell developmental program.
Such a role appears inconsistent with the well-established
capacity of MyoD to arrest the proliferation of cultured
cells [e.g., see Olson 1992] but is consistent with the
expression of Myf-5 and MyoD in proliferating mpc during embryogenesis and in proliferating myoblast
cell lines [for review, see Rudnicki and Jaenisch 1995].

Several possible roles can be hypothesized. [1] The in-
duction of MyoD is required for the specification of mpc
from satellite cells, [2] expression of MyoD positively
regulates mpc proliferation, and [3] MyoD expression
acts to counter the proliferation induced by activation
and leads to differentiation. Clearly, identification of the
genes that are regulated by MyoD in this context should
elucidate the function of MyoD in satellite cells.

Satellite cells are postulated to arise from a unique cell
population that forms late in development and is sepa-
rate from the lineages believed to give rise to primary
and secondary myofibers [Cossu and Molinaro 1987;
Stockdale 1992]. Our results indicate that MyoD mutant
muscle contains morphologically normal satellite cells in
calculated numbers as evidenced by their recovery in
cell culture (Fig. 4; Table 2). Therefore, these data sug-
gest that MyoD is not required for the determination of
the satellite-cell lineage during embryonic development.
Potentially, other MRFs, for example, Myf-5, could be
considered candidates for such a role, but at this time the
ge genetic mechanisms that determine the embryonic or-
igin of satellite cells remain unknown.

Relatively little is known of the pathways regulating the
quiescence, activation, and proliferation of satellite
cells. Clearly, our data indicate that induction of MyoD
expression is required for appropriate satellite cell func-
tion. Transgenic and transfection analysis of MyoD reg-
ulatory sequences has indicated that at least two enhanc-
ers are involved in regulating MyoD transcription. Be-
tween -18 and -22 kb upstream from the transcription
start site there is a strong enhancer that is capable of
directing appropriate muscle-specific expression [Gold-
hamer et al. 1992] and has a conserved core sequence of
258 bp that is functional even after mutation of E-boxes
Goldhamer et al. 1995]. A second, less robust MyoD
enhancer between -4.73 and -5.39 kb upstream from the
transcription start site [Tapscott et al. 1992] can also
direct appropriate myotomal-specific expression
Asakura et al. 1995]. Whether either of the two MyoD
denhancers is required for appropriate induction of MyoD
following satellite cell activation remains to be deter-
mined. However, such an analysis should elucidate the
regulatory pathways involved in satellite cell activation.

The interbreeding of mdx and MyoD mutant mice has
revealed a role for MyoD in the satellite cell program and
has provided an experimental animal model that more
closely resembles Duchenne and Becker muscular dys-
trophy. Except in the diaphragm, mdx mice do not dis-
play the muscle fiber loss and extensive interstitial fi-
brosis observed in humans [Stedman et al. 1991]. More-
over, unlike mdx muscle, human dystrophin deficiency
leads to severe reduction in satellite cell populations and
mpc proliferative potential [Webster and Blau 1990].
Therefore, the reduced proliferative potential of myo-
genic cells and the increased myopathic phenotype sug-
gests that the mdx:MyoD(-/-) mice will be a useful
system for a wide variety of studies relevant to the un-
derstanding of different aspects of muscular dystrophy.
For example, physiological studies of mdx:MyoD(-/-)
skeletal muscle, determination of isometric twitch ki-
netics, metabolic perturbations, and so forth, will allow
insight into the progressive degenerative consequences
produced as a result of impaired satellite cell function.
Importantly, the mdx:MyoD(+/-) mice should be a
useful model for testing the impact of different viral or
DNA gene transfer, myoblast transfer, or pharmacologic
treatment modalities. Clearly, the more severe pheno-
type evident in \textit{mdx}:\textit{MyoD}(−/−) mice will allow a
more relevant testing of the biological efficacy of these
different approaches.

Materials and methods

\textit{Induced regeneration of skeletal muscle}

To induce regeneration of skeletal muscle, the TA muscle was
subjected to a single freeze–crush injury as described by
McGeachie and Grounds (1990). Briefly, mice were anesthetized
through an intraperitoneal injection of Avertin (0.016 ml of
2.5% avertin/g body weight). An incision was made (frontal
portion of lower hind limb), knee joint to ankle joint; exposing
the TA muscle and a pair of serrated forceps (jaw width 2.5 mm),
cooled in liquid nitrogen, were used to apply a crush injury to
the TA. The contralateral limb was used as an undamaged con-
trol.

\textit{Cell culture and immunodetection of desmin and myosin}

Satellite cell-derived myoblasts were isolated from the skeletal
muscle of the lower hindlimb of adult mice as described previ-
ously (Rando and Blau 1994). Growth medium consisted of
Ham’s F-10 (GIBCO BRL) supplemented with 20% fetal calf
serum (GIBCO BRL), 2.5 \text{ng/ml} of bFGF (Boehringer Mann-
heim), 200 Units/ml of penicillin (GIBCO BRL), 200 \text{µg/ml}
of streptomycin (GIBCO BRL), and 0.002% Fungizone (GIBCO
BRL). Differentiation medium consisted of Dulbecco’s modified
Eagle medium (DMEM) (GIBCO BRL) supplemented with 2%
horse serum (GIBCO BRL) and antibiotics listed above. Growth
medium was changed twice every day, and differentiation me-
dium every second day.

Cultured myoblasts were detected with mouse anti-desmin
antibody DE-U-10 (Sigma, St. Louis, MO) after 4 days of culture.
Differentiated myocytes were detected with mouse anti-myo-
sin heavy chain antibody MF20 (Developmental Studies Hybr-
doma Bank, Iowa City, IA) in primary cells cultured for 7 days in
growth medium and then switched to differentiation medium
for 7 days. Briefly, immunohistochemistry was performed as follows.
Culture dishes rinsed in PBS were fixed in 90\% methanol for
5 min at \text{-20} °C, rinsed three times in PBS containing 5\% skim
milk powder, incubated with primary antibody (1:10 dilution)
for 1 hr, and rinsed three times with PBS. Culture dishes were
then incubated with secondary antibody [goat-anti-mouse IgG
HRP conjugate [Bio-Rad] diluted 1:1000] for 1 hr, rinsed three
times in PBS, and developed with 0.6 mg/ml of diamobenzi-
dine (Sigma) for 20 min. Nuclei were counterstained with Gill’s
hematoxylin.

\textit{Histological analysis}

TA muscle was prepared for electron microscopy by fixation in
2\% glutaraldehyde/0.1 M cacodylate (pH 7.4), for 2 hr at 4 °C and
processed using standard procedures as described previously (Ka-
blar 1995). Randomly chosen fields for each section were viewed
and assessed with a Jeol 1200 EX Biosystem transmission elec-
tron microscope. TA muscle was prepared for immunohisto-
chemistry by overnight fixation in 4\% paraformaldehyde at 4 °C.
Immunohistochemistry, as described previously (Rudnicki et al.
1992) was performed on paraffin-embedded TA sections with
mouse monoclonal PC10 antibody reactive with PCNA (Dako-
patts). For HE staining, muscles were fixed for 1 week in 10\%
formalin and prepared as described previously (Bancroft and
Stevens 1990).

Detection of cell proliferation by tritiated thymidine incor-
poration in vivo was performed as described previously (And-
erson et al. 1987). Mice were intraperitoneally injected 3.5 days
following injury with tritiated thymidine (Amersham) at a dose
of 2.0 \text{µCi/g} body weight. Frozen sections were prepared 4.5
days following injury for autoradiography. Sections were fixed
in 10\% formalin for 10 min, rinsed, dipped in K3 emulsion
[Ilford], and exposed for 6 weeks at 4 °C. Slides were developed
in D-19 (Kodak, Rochester, NY), fixed, and counterstained in Go-
morri’s trichrome stain.

\textit{Acknowledgments}

We thank John Hassell, William Muller, Lucy Sabourin, and Jim
Smiley for critical comments on the manuscript. This work was
supported by grants to M.A.R. from the Medical Research Coun-
cil of Canada, and the Muscular Dystrophy Association, and to
J.E.A. from the P.H.T. Thorlakson Foundation and the Manitoba
Health Research Council. L.A.M. held a Postdoctoral Fellow-
ship from the Natural Science and Engineering Research Coun-
cil of Canada and is currently a Postdoctoral Fellow of the Med-
ical Research Council of Canada. K.L.G. held a combined Post-
doc toral Fellowship from the Muscular Dystrophy Association of
Canada and Medical Research Council of Canada. M.A.R. is a
Research Scientist of the National Cancer Institute of Canada,
and is a member of the Canadian Genetic Disease Network of
Excellence.

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