

# Reduced Differentiation Potential of Primary *MyoD*<sup>-/-</sup> Myogenic Cells Derived from Adult Skeletal Muscle

Luc A. Sabourin, Adele Girgis-Gabardo, Patrick Seale, Atsushi Asakura, and Michael A. Rudnicki

Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

**Abstract.** To gain insight into the regeneration deficit of *MyoD*<sup>-/-</sup> muscle, we investigated the growth and differentiation of cultured *MyoD*<sup>-/-</sup> myogenic cells. Primary *MyoD*<sup>-/-</sup> myogenic cells exhibited a stellate morphology distinct from the compact morphology of wild-type myoblasts, and expressed c-met, a receptor tyrosine kinase expressed in satellite cells. However, *MyoD*<sup>-/-</sup> myogenic cells did not express desmin, an intermediate filament protein typically expressed in cultured myoblasts in vitro and myogenic precursor cells in vivo. Northern analysis indicated that proliferating *MyoD*<sup>-/-</sup> myogenic cells expressed fourfold higher levels of *Myf-5* and sixfold higher levels of *PEA3*, an ETS-domain transcription factor expressed in newly activated satellite cells. Under conditions that normally induce differentiation, *MyoD*<sup>-/-</sup> cells continued to proliferate and with delayed kinetics yielded

reduced numbers of predominantly mononuclear myocytes. Northern analysis revealed delayed induction of *myogenin*, *MRF4*, and other differentiation-specific markers although *p21* was upregulated normally. Expression of *M-cadherin* mRNA was severely decreased whereas expression of *IGF-1* was markedly increased in *MyoD*<sup>-/-</sup> myogenic cells. Mixing of lacZ-labeled *MyoD*<sup>-/-</sup> cells and wild-type myoblasts revealed a strict autonomy in differentiation potential. Transfection of a *MyoD*-expression cassette restored cytomorphology and rescued the differentiation deficit. We interpret these data to suggest that *MyoD*<sup>-/-</sup> myogenic cells represent an intermediate stage between a quiescent satellite cell and a myogenic precursor cell.

**Key words:** MyoD • myogenic regulatory factor • satellite cell • differentiation • proliferation

**T**HE myogenic regulatory factors (MRFs)<sup>1</sup> form a group of four basic helix-loop-helix transcription factors consisting of MyoD, Myf-5, myogenin, and MRF4. The MRFs have been demonstrated to play pivotal roles in the determination and differentiation of myogenic precursors into mature skeletal muscle (Weintraub et al., 1991; Rudnicki and Jaenisch, 1995). Gene targeting experiments have provided much insight into understanding MRF function in vivo. The introduction of null mutations in all four factors into the germline of mice has revealed the existence of a hierarchical relationship among the MRFs and has defined two functional groups of MRFs (Braun et al., 1992; Rudnicki et al., 1992, 1993; Nabeshima et al., 1993; Patapoutian et al., 1995; Zhang et al., 1995). The primary MRFs, MyoD and Myf-5, appear to be required for myogenic determination, whereas the secondary MRFs, myogenin and MRF4, are required later in the

developmental program as differentiation factors (Megeny and Rudnicki, 1995; Rudnicki and Jaenisch, 1995).

Satellite cells, the stem cells of adult skeletal muscle, reside beneath the basal lamina of adult skeletal muscle closely juxtaposed against muscle fibers. Satellite cells arise around day 17 of development and are believed to represent a unique myoblast lineage distinct from embryonic and fetal lineages (Cossu et al., 1985; Bischoff, 1994). Satellite cells make up 2–7% of the nuclei associated with a particular fiber and the proportion varies with age and particular muscle group. Satellite cells are normally mitotically quiescent, but are activated (i.e., initiate multiple rounds of proliferation) in response to stress induced by weight bearing or other trauma such as injury. The descendants of the activated satellite cells, myogenic precursor cells, undergo multiple rounds of division prior to fusing to existing or new fibers. Satellite cells appear to form a population of stem cells that are distinct from their daughter myogenic precursor cells as defined by biological and biochemical criteria (Bischoff, 1994). The total number of satellite cells in muscle remains relatively constant, suggesting that a capacity for self-renewal in the satellite cell compartment maintains the population of quiescent cells (Bischoff, 1994).

Address correspondence to Dr. Michael Rudnicki, Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada L8S 4K1. Tel.: (905) 525-9140. Fax: (905) 521-2995. E-mail: rudnicki@mcmaster.ca

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; MHC, myosin heavy chain; MRF, myogenic regulatory factors.

Quiescent satellite cells express no detectable MRFs. Upon activation and entrance into the cell cycle, MyoD is rapidly upregulated concomitantly with proliferating cell nuclear antigen, a marker for cell proliferation, whereas myogenin is expressed last during the time associated with fusion and differentiation (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994). Analysis of gene expression by RT-PCR of individual satellite cells in cultured intact muscle fibers at times after their activation substantiates that quiescent satellite cells express no detectable MRFs but do express *c-met*, a receptor tyrosine kinase (Cornelison and Wold, 1997). Moreover, these experiments reveal that activated satellite cells first express either *Myf-5* or *MyoD* followed soon after by coexpression of *Myf-5* and *MyoD*. After proliferation, *myogenin* and *MRF4* are expressed in cells beginning their differentiation program (Cornelison and Wold, 1997).

The regeneration deficit observed in *MyoD*<sup>-/-</sup> muscle strongly supports the assertion that MyoD plays an essential role in regulating the satellite cell myogenic program (Megeny et al., 1996). Muscle regeneration is severely deficient in *MyoD*<sup>-/-</sup> mice and compound mutant mice lacking both *MyoD* and *dystrophin* (designated *mdx:MyoD*<sup>-/-</sup>) exhibit severe myopathy leading to premature death (Megeny et al., 1996). Muscle regeneration in *MyoD*<sup>-/-</sup> muscle is characterized by an almost complete absence of proliferative myogenic precursor cells. However, electron microscopic examination of *MyoD*-deficient muscle reveals increased numbers of satellite cells and the number of primary myoblasts recovered is increased 2.5-fold in *MyoD*<sup>-/-</sup> muscle and 13-fold in *mdx:MyoD*<sup>-/-</sup> muscle. Taken together, our data suggest a model in which upregulation of *MyoD* is required for satellite cells to enter the myogenic precursor cell proliferative phase that precedes terminal differentiation. In the absence of *MyoD*, myogenic stem cells undergo an apparent increase in numbers as a consequence of an increased propensity for self-renewal rather than progression through the myogenic differentiation program (Megeny et al., 1996).

To investigate the function of *MyoD* during the course of satellite cell activation, we established primary myogenic cultures from wild-type and *MyoD*-deficient adult muscle. Importantly, these experiments were performed with newly established low-passage primary cultures. Our analysis strongly supports the hypothesis that *MyoD* is required for satellite cells to progress efficiently through the myogenic precursor cell developmental program. Moreover, our data suggest that *MyoD*<sup>-/-</sup> myogenic cells represent an intermediate developmental stage between quiescent satellite cells and myogenic precursor cells.

## Materials and Methods

### Isolation of Primary Myoblasts, Cell Culture, and Immunohistochemistry

Satellite cell-derived primary myoblasts were isolated from adult lower hindlimb muscle from 2–3-mo-old mice as described previously (Megeny et al., 1996), with the exception that hepatocyte growth factor (10 ng/ml; R&D Systems Inc.) and heparin (5 ng/ml; Sigma Chemical Co.) were included in the growth medium for the first 48 h after the plating of the final cell preparation, and supplemented with FGF2 (2.5 ng/ml FGF2) thereafter. The primary cultures were maintained on collagen-coated dishes in

Ham's F10 (GIBCO BRL) supplemented with 20% FCS, 200 U/ml penicillin, 200 µg/ml streptomycin, and 0.002% Fungizone (GIBCO BRL). The medium was changed daily and cultures were routinely passaged 1:3 as they reached 60–70% confluence. To maintain the primary characteristics of the cells, all experiments were performed using cultures that had undergone between four and seven passages. Differentiation medium consisted of DME supplemented with 5% horse serum and antibiotics as described above (GIBCO BRL).

The extent of culture purity and differentiation was determined by subjecting the purified myoblasts to c-met, desmin, and myosin heavy chain (MHC) immunostaining. Briefly, myoblasts in growth medium were fixed in 4% paraformaldehyde in PBS (PFA), stained with anti-c-met antibody SP260 (Santa Cruz Biotechnology), and antidesmin antibody DE-U-10 (DAKO). MHC expression was detected by fixing differentiated cultures with 90% methanol and staining with MF20 mAb (Bader et al., 1982). Immunostaining was similarly performed with anti-β-catenin antibody sc-1496 (Santa Cruz Biotechnology) and anti-M-cadherin antibody sc-6470 (Santa Cruz Biotechnology). Immunostaining with anti-c-met and anti-β-catenin antibodies were detected with fluorescein-conjugated anti-goat antibodies (Sigma Chemical Co.), and photographed on a Zeiss Axiophot microscope equipped with a UV source and FITC detection filters. Desmin, M-cadherin, and MHC staining was visualized using a HRP-coupled secondary antibody (Bio-Rad Laboratories) in PBS containing 0.6 mg/ml diaminobezidine (Sigma Chemical Co.).

### Differentiation Time Course and Growth Rate Measurements

Growth rate analysis was determined by [<sup>3</sup>H]thymidine incorporation of three independent isolates of wild-type and *MyoD*<sup>-/-</sup> cultures seeded in 24-well plates at 10<sup>4</sup> cells per well in growth medium (each in triplicate). For day 0, the cells were cultured 24 h in growth medium before addition of 2 µCi of [<sup>3</sup>H]thymidine for 2 h. Incorporation of [<sup>3</sup>H]thymidine was normalized to protein concentration as determined by Bradford assay. The remaining wells were exposed to differentiation medium and labeled with [<sup>3</sup>H]thymidine for 2 h on sequential days.

To assay the differentiation potential of wild-type and *MyoD*<sup>-/-</sup> cultures, 10<sup>5</sup> low passage cells were seeded into 35-mm dishes in growth medium and cultured for an additional 24 h (day 0) before addition of differentiation medium. On subsequent days (1–5), the cultures were fixed and immunostained for MHC with antibody MF20. To establish the differentiation potential of the cultures, at least 1,000 nuclei from MF20-positive cells were counted from several random fields. The percentage of differentiated cells was calculated as: (nuclei within MF20-stained myocytes/total number of nuclei) × 100; or the fusion index calculated as: (MF20-stained myocytes containing ≥2 nuclei/total number of nuclei) × 100. 5-bromo-2'-deoxyuridine (BrdU) incorporation assays on cultured cells were performed (cell proliferation kit; Amersham Pharmacia Biotech). All experiments were performed in triplicate on three independent wild-type and *MyoD*<sup>-/-</sup> isolates.

### Northern and Western Analysis

To analyze the expression of the MRFs and of differentiation-specific markers, total RNA from low passage cells in growth (day 0) or differentiation medium (days 1–5) was isolated (Birnbom, 1988) and subjected to Northern analysis (Maniatis et al., 1982). Replicate filters were sequentially hybridized to MRF-specific cDNAs as well as α-cardiac and α-skeletal actin, and acetylcholine receptor δ subunit probes (Rudnicki et al., 1993). Dr. Paul Hastings (McGill University) kindly provided the M-cadherin probe. The *Musk* cDNA probe was obtained by RT-PCR of C2C12 RNA. The *β-catenin* and *PEA3* probes were kindly provided by Drs. Rolf Kemler (Max Planck Institute) and John A. Hassell (McMaster University), respectively. Western analysis with rabbit anti-Myf-5 antibody C-20 (Santa Cruz Biotechnology), mouse anti-MyoD antibody 5A8 (PharMingen), and mouse antimyogenin antibody F5D (Developmental Studies Hybridoma Bank) was performed on extracts prepared from cultures in growth medium as described previously (LeCouter et al., 1996).

### Mixing of LacZ-expressing MyoD<sup>-/-</sup> and Wild-Type Cultures

Early passage *MyoD*<sup>-/-</sup> cultures were lipofected with a 1:10 ratio of PGK-LacZ-MAR and PGK-Puro plasmids, and stable transformants were pooled after 10 d of selection in 2 µg/ml puromycin. Plasmid PGK-LacZ-

MAR contains the phosphoglycerate kinase 1 promoter expressing nls-lacZ and a chicken lysozyme matrix attachment region to confer high level site-independent expression (Phi-Van and Stratling, 1996). Primary cells were plated with an initial density of  $10^5$  cells per 60-mm well, in ratios of 1:0, 1:4, 4:1, and 0:1 of *MyoD*<sup>-/-</sup> to wild-type cells. Duplicate cultures were grown overnight in growth medium before exposing to differentiation medium for 5 d. Wells were washed with PBS, fixed in 2% formaldehyde/0.4% glutaraldehyde followed by X-Gal staining. Cells were post-fixed with 90% methanol for 7 min, rinsed with PBS and 0.3% Triton X, before immunostaining with antibody MF-20 as described above.

### Transfection with MyoD Plasmid and Generation of Stable MyoD+ Pools

Low passage subconfluent cultures of *MyoD*<sup>-/-</sup> cells were transfected with pEMSV-MyoD/PGK-Puro or PGK-Puro alone by lipofectamine (GIBCO BRL), according to the manufacturer's instructions. The *MyoD* expression plasmid carries the murine *MyoD* cDNA driven by the EMSV promoter and enhancer, as well as a puromycin resistance cassette. The cultures were refed 24 h after transfection and daily with growth medium containing 2  $\mu$ g/ml puromycin (Sigma Chemical Co.) for 10 d. The resulting colonies (>200) were pooled and expanded for further analysis. MyoD expression was evaluated by Western blot analysis (LeCouter et al., 1996) using anti-MyoD mAb 5A8 (PharMingen). The differentiation and fusion potentials of MyoD-expressing pools were assayed as described above.

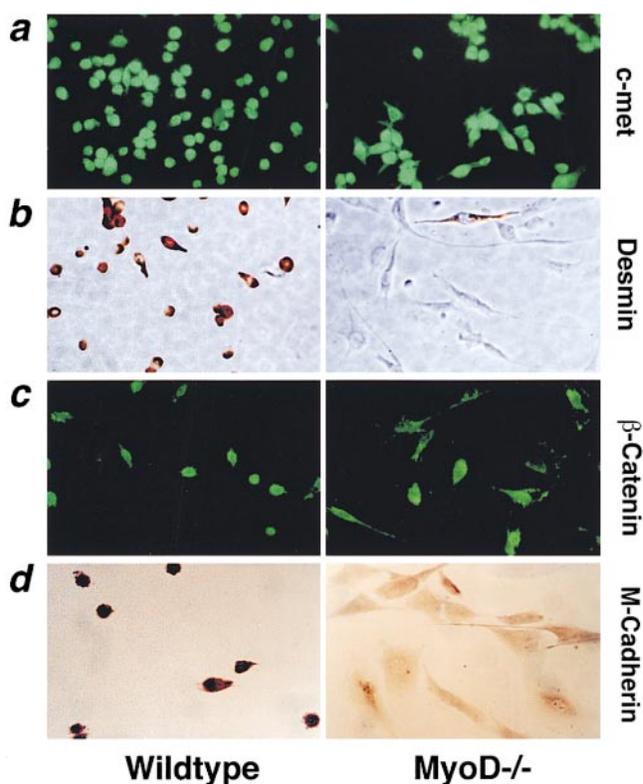
## Results

### Altered Cellular Phenotype of MyoD<sup>-/-</sup> Myogenic Cells

To gain insight into the role of *MyoD* in satellite cell activation, low passage primary cultures were isolated from 2–3-mo-old wild-type and *MyoD*<sup>-/-</sup> mice to facilitate the generation of highly purified satellite cell-derived cultures and preclude the inclusion of neonatal myoblasts. Cultures were grown for 48 h in the presence of hepatocyte growth factor and thereafter in medium supplemented with FGF2 to allow the rapid recovery of high numbers of low passage primary myoblasts as described previously (Allen et al., 1995).

As suggested by our previous observations (Megeny et al., 1996), *MyoD*<sup>-/-</sup> cultures displayed a stellate flattened morphology with an enlarged cytoplasm and extended cytosolic processes. By contrast, wild-type cells were highly refractile under phase-contrast microscopy and displayed the rounded morphology and small compact cytoplasm characteristic of primary myoblasts (see Figs. 1 and 8).

Quiescent and activated satellite cells in vivo express the receptor tyrosine kinase c-met as do cultured myoblasts (Allen et al., 1995; Cornelison and Wold, 1997). Proliferating myogenic precursor cells in vivo and myoblasts in vitro express the intermediate filament desmin. However, satellite cells do not express desmin (George-Weinstein et al., 1993). Therefore, primary cultures were immunostained with antibody reactive with c-met and desmin to assess their developmental status. Virtually 100% of the primary cells derived from both wild-type and *MyoD*<sup>-/-</sup> animals expressed high levels of c-met as detected by indirect immunofluorescence. Fibroblast cell lines did not express c-met (not shown). Therefore, the isolation procedure generated highly purified cultures of myogenic cells (Fig. 1 a). In contrast to the uniform expression of c-met, only  $6.2 \pm 5.4\%$  of *MyoD*<sup>-/-</sup> myogenic cells expressed the myoblast marker desmin, whereas  $89 \pm 4.2\%$  of wild-type



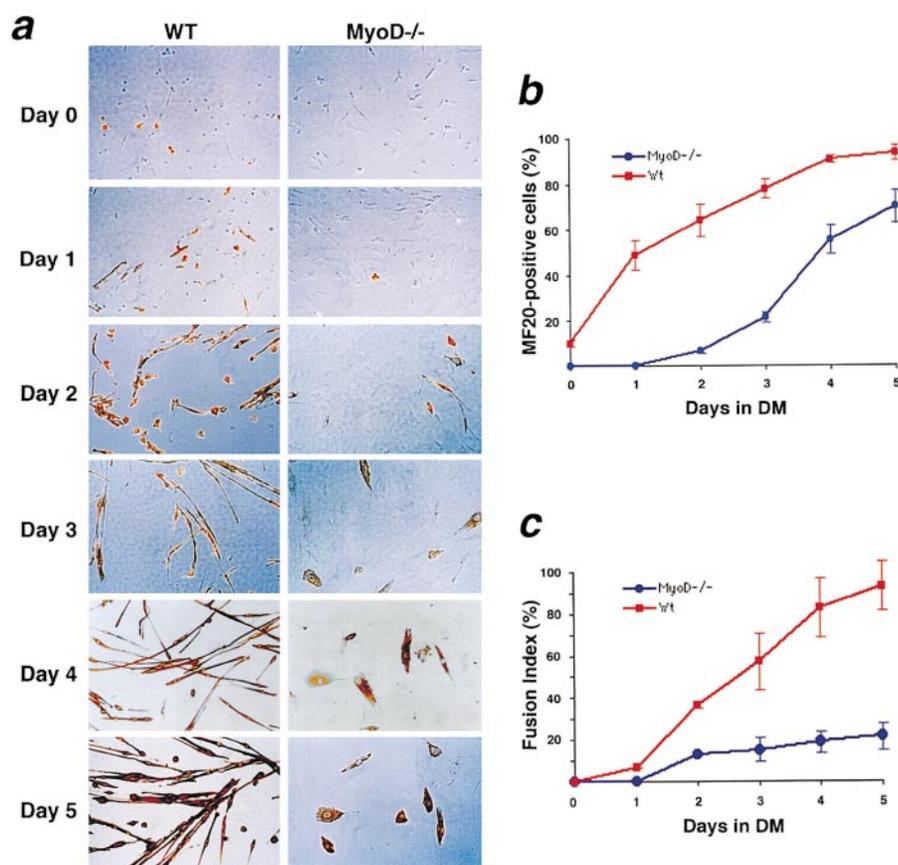
**Figure 1.** Primary *MyoD*<sup>-/-</sup> myogenic cells exhibit a premyoblastic cellular phenotype. (a) Immunostaining revealed that both wild-type (Wildtype) and *MyoD*-deficient myogenic cells (*MyoD*<sup>-/-</sup>) expressed the c-met receptor tyrosine kinase, a marker for satellite cells and proliferating myoblasts. (b) Immunohistochemical analysis for desmin expression revealed a marked reduction in the proportion of cells in *MyoD*<sup>-/-</sup> cultures expressed desmin relative to wild-type cultures. (c) Normal level and localization of  $\beta$ -catenin in *MyoD*<sup>-/-</sup> cells as revealed by immunofluorescence. (d) Immunohistochemical detection of M-cadherin revealed low level expression of M-cadherin in *MyoD*<sup>-/-</sup> cells relative to primary wild-type myoblasts.

cells expressed desmin (Fig. 1 b). These data are consistent with the notion that *MyoD*<sup>-/-</sup> myogenic cells represent an intermediate developmental stage between a satellite cell and myogenic precursor cells.

### Reduced Differentiation Potential of MyoD<sup>-/-</sup> Myogenic Cultures

To evaluate the differentiation potential of the *MyoD*<sup>-/-</sup> myogenic cultures the extent of myogenic differentiation was assessed at the cellular level by immunostaining cultures fixed at daily intervals after mitogen withdrawal with antibody MF20 reactive with MHC (Fig. 2 a). Importantly, this analysis was performed on three independently isolated wild-type and *MyoD*<sup>-/-</sup> primary myogenic cultures. After MF20 immunostaining, the proportion of MHC-positive cells and the fusion index of both wild-type and *MyoD*<sup>-/-</sup> cultures were assessed (Fig. 2, b and c).

Under growth conditions, *MyoD*<sup>-/-</sup> myogenic cells exhibited about a 100-fold reduction in the rate of spontaneous differentiation ( $0.10 \pm 0.14\%$ ) compared to wild-type



cells ( $10 \pm 1.4\%$ ) (see day 0 in Fig. 2, a and b). Consistent with this observation, *MyoD*<sup>-/-</sup> cultures displayed a severe defect in their ability to differentiate and form multinucleated myotubes after mitogen withdrawal (Fig. 2 a). Differentiated wild-type myocytes displayed the typical elongated and multinucleated morphology, whereas differentiated *MyoD*<sup>-/-</sup> myocytes were primarily mononuclear and retained a fibroblastic stellate cytomorphology (Fig. 2 a).

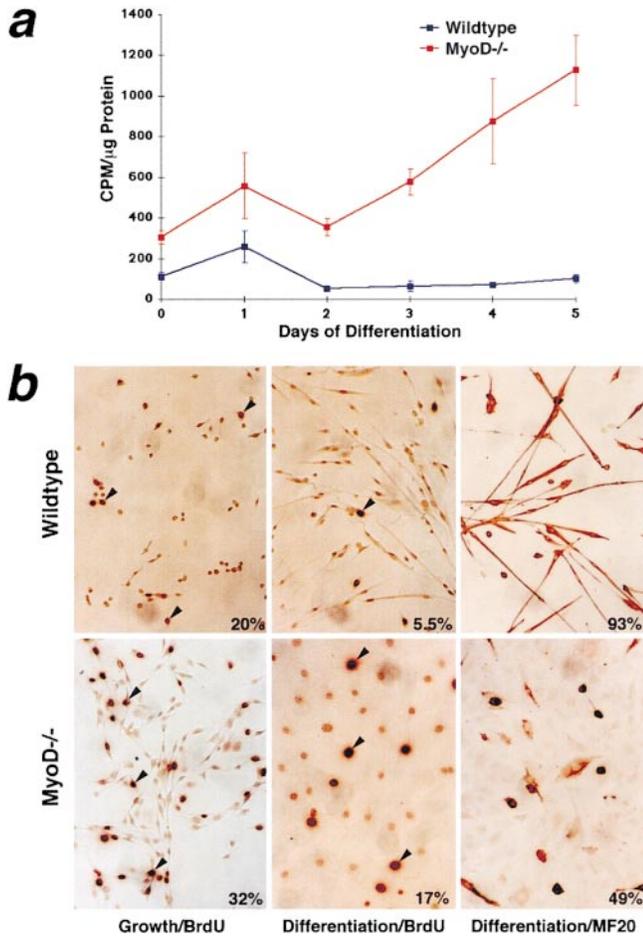
About 50% of the cells in wild-type cultures after 24 h in differentiation medium had begun to undergo terminal differentiation as assessed by MHC immunostaining (Fig. 2, a and b). The number of differentiated wild-type myocytes continued to accumulate in a linear manner reaching  $\sim 94\%$  5 d after serum withdrawal (Fig. 2, a and b). By contrast, 24 h after mitogen withdrawal, the number of differentiated myocytes in *MyoD*<sup>-/-</sup> cultures remained below the limit of detection (Fig. 2, a and b). The number of differentiated *MyoD*<sup>-/-</sup> myocytes began to accumulate after 3 d and reached  $\sim 70\%$  5 d after serum withdrawal (Fig. 2, a and b).

Enumeration of differentiated myocytes containing two or more nuclei in these cultures (i.e., fusion index) revealed a marked reduction in fusion capacity in *MyoD*<sup>-/-</sup> cultures (Fig. 2 c). The fusion index of differentiated wild-

type cultures was  $\sim 90\%$  5 d of differentiation with an average of  $4.6 \pm 0.3$  nuclei per myocyte. By contrast, by day 5 only about 15% of *MyoD*<sup>-/-</sup> myocytes contained 2 nuclei, with an average of  $1.2 \pm 0.2$  nuclei per myocyte (Fig. 2, a and c).

The rate of cell-cycle withdrawal after induction of differentiation was assessed by measuring [<sup>3</sup>H]thymidine incorporation at daily intervals after transfer into differentiation medium (Fig. 3 a). In growth medium, [<sup>3</sup>H]thymidine labeling experiments revealed that *MyoD*<sup>-/-</sup> cells exhibited an apparent twofold higher growth rate than wild-type cells (Fig. 3 a). Direct cell count experiments in growth medium revealed that numbers of primary *MyoD*<sup>-/-</sup> cells accumulated  $\sim 1.5$ -fold faster than wild-type cells (not shown). Moreover, *MyoD*<sup>-/-</sup> cells displayed a 1.6-fold increase in mitotic index as determined by BrdU incorporation (Fig. 3 b). However, this increase in apparent growth can be partly accounted for by the reduced rate of spontaneous differentiation of mutant ( $0.10 \pm 0.14\%$ ) versus wild-type cells ( $10.0 \pm 1.4\%$ ) (see day 0, Fig. 2, a and b). In addition, interpretation of these results is also confounded by other potential variables: differences in the proportion of cells temporarily withdrawn from the cell-cycle, differences in rates of apoptosis, and differences in cell-cycle kinetics. Therefore, additional

**Figure 2.** Reduced differentiation potential of *MyoD*<sup>-/-</sup> myogenic cells. (a) Differentiated myocytes were detected by immunostaining with antibody MF20 reactive with MHC. Incubation of wild-type (WT) myoblast cultures in differentiation medium resulted in a rapid increase in MHC synthesis and formation of elongated multinucleated myotubes. By contrast *MyoD*-deficient cells (*MyoD*<sup>-/-</sup>) differentiated with reduced kinetics and failed to form multinucleated elongated myotubes. Note the 100-fold reduced rate of spontaneous differentiation observed in *MyoD*<sup>-/-</sup> cultures under growth conditions (day 0). Days correspond to the time spent in differentiation medium before staining, whereas day 0 represents cultures in growth medium. (b) Percent MF20 positive cells was determined by enumeration of MHC expressing differentiated myocytes by immunostaining with antibody MF20. Note the delayed and reduced kinetics of differentiation in the absence of MyoD. (c) Calculation of fusion indices as percent cells containing two or more nuclei within a differentiated myocyte confirmed that *MyoD*<sup>-/-</sup> myoblasts were severely deficient in fusion capacity with the majority of differentiated myocytes containing a single nuclei. The error bars represent the standard error of the mean from three independently derived primary cultures.



**Figure 3.** Enhanced proliferative potential of primary *MyoD*<sup>-/-</sup> myogenic cells in growth and differentiation. (a) Analysis of [<sup>3</sup>H]thymidine incorporation during differentiation revealed that *MyoD*<sup>-/-</sup> myogenic cells continued to synthesize DNA after mitogen withdrawal. Incorporation was normalized to protein concentration. The error bars represent the standard error of the mean for three different isolates. (b) Immunodetection of BrdU incorporation in *MyoD*<sup>-/-</sup> cultures revealed continued DNA synthesis after mitogen withdrawal. Differentiation was assessed by immunostaining with antibody MF20. Taken together, these data indicate that *MyoD*<sup>-/-</sup> myogenic cells inefficiently withdraw from the cell cycle under differentiation promoting conditions.

analyses are required to determine whether primary *MyoD*<sup>-/-</sup> myogenic cells exhibit altered cell-cycle kinetics relative to primary wild-type myoblasts.

Wild-type myoblasts and *MyoD*<sup>-/-</sup> myogenic cells both exhibited about a twofold increase in the rate of [<sup>3</sup>H]thymidine incorporation 24 h after mitogen withdrawal (Fig. 3 a). This apparent increase in DNA synthesis may reflect a characteristic of primary cells analogous to the transient increase in cell proliferation observed after the IGF-I treatment of myoblasts under culture conditions that induce differentiation (Engert et al., 1996). After the first day of mitogen withdrawal, wild-type myoblasts exhibit a rapid withdrawal from the cell cycle as evidenced by the low levels of [<sup>3</sup>H]thymidine incorporation (Fig. 3 a). By contrast, the rate that *MyoD*<sup>-/-</sup> myogenic cells incor-

porated [<sup>3</sup>H]thymidine continued to increase during the 5 d after transfer into differentiation medium. Similar results were obtained using three independent preparations of low passage primary myogenic cultures.

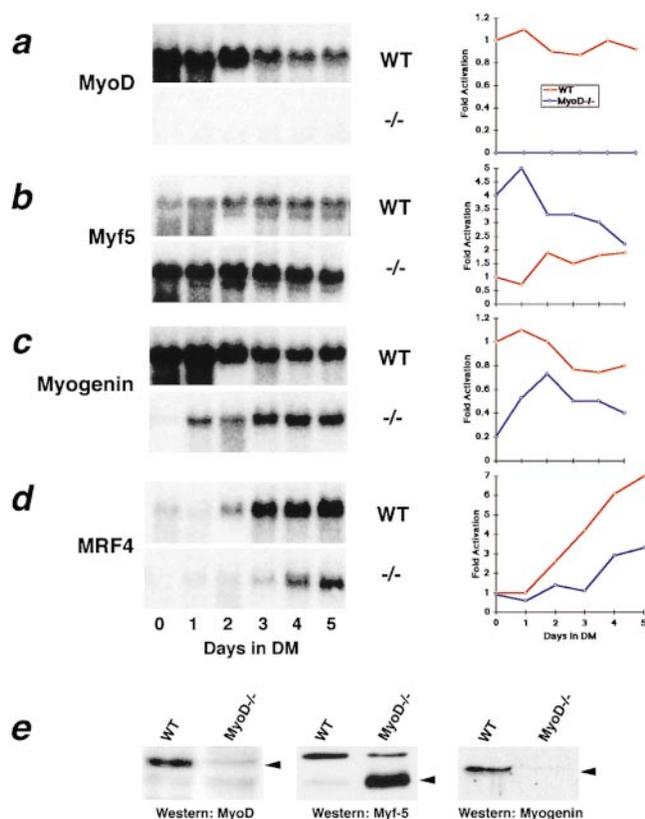
Taken together, these experiments suggested that *MyoD*<sup>-/-</sup> myogenic cells displayed continued proliferation under conditions of low mitogens that normally induce cell-cycle withdrawal and terminal differentiation of wild-type myoblasts. To investigate cell proliferation under conditions of growth and differentiation, cultured cells were exposed to BrdU for 4 h followed by immunodetection of nuclear localized BrdU incorporated during DNA synthesis (Fig. 3 b). Wild-type primary myoblasts in growth medium exhibited a 20% rate of BrdU incorporation, whereas *MyoD*<sup>-/-</sup> myogenic cells exhibited a 32% rate of labeling. After 5 d of differentiation, 93% of nuclei in wild-type cultures expressed MHC and 5.5% were labeled by BrdU. In contrast, after 5 d of differentiation, 49% of cells in *MyoD*<sup>-/-</sup> cultures expressed MHC and 17% were labeled by BrdU (see Fig. 2 b). Interestingly, subconfluent cultures of *MyoD*<sup>-/-</sup> cells in differentiation medium exhibited up to 20% rates of BrdU incorporation, whereas confluent cultures exhibited as low as 5% BrdU labeling (not shown).

Considered together, these data indicate that *MyoD*<sup>-/-</sup> myogenic cells continue to proliferate under low-mitogen conditions that normally induce terminal differentiation of wild-type myoblasts and suggest that *MyoD*<sup>-/-</sup> cells exhibit contact inhibition of growth during differentiation. The observation that *MyoD*<sup>-/-</sup> cells exhibit an enhanced proliferative potential under conditions that normally induce differentiation strongly supports the notion that *MyoD*<sup>-/-</sup> myogenic cells exhibit an increased propensity for self-renewal rather than progression through the differentiation program.

### Analysis of Muscle-specific Gene Expression

To gain further insight into the differentiation defect in *MyoD*<sup>-/-</sup> primary cultures, total RNA was prepared from both wild-type and *MyoD*<sup>-/-</sup> cultures in growth medium and at daily intervals after induction of differentiation. Northern blot analysis was performed using a panel of muscle-specific probes.

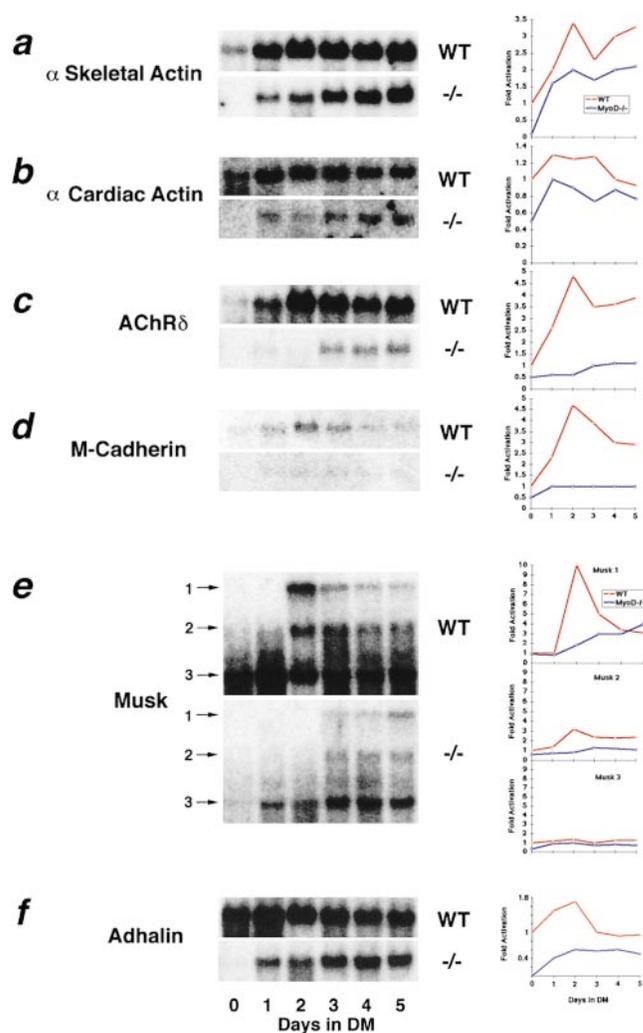
The expression of the MRFs was investigated to elucidate the regulatory relationships and the potential for functional compensation in the absence of MyoD. Northern analysis of the expression pattern of the four MRFs confirmed that MyoD was expressed at high levels in wild-type myoblasts and was absent in the *MyoD*-deficient myogenic cells. Furthermore, densitometric analysis and normalization to 18S rRNA revealed that *MyoD* was somewhat downregulated during the differentiation of wild-type cells (Fig. 4 a). Previously, we observed a 3.5-fold increase in *Myf-5* mRNA in newborn and adult muscles in vivo (Rudnicki et al., 1993). We similarly observed a fourfold increase in *Myf-5* mRNA in growing *MyoD*<sup>-/-</sup> myogenic cells relative to wild-type myoblasts, supporting the hypothesis that MyoD negatively regulates *Myf-5* expression (Fig. 4 b). In wild-type cells, *Myf-5* mRNA levels decreased about twofold after differentiation, whereas in *MyoD*<sup>-/-</sup> cultures, *Myf-5* mRNA levels were upregu-



**Figure 4.** Upregulation of *Myf-5* and delayed expression of *myogenin* and *MRF4* in *MyoD*<sup>-/-</sup> myogenic cells. Northern analysis revealed an absence of *MyoD* mRNA (a) in *MyoD*<sup>-/-</sup> cells (-/-), and a fourfold upregulation of *Myf-5* mRNA (b) relative to wild-type myoblasts (WT). Upon differentiation, *myogenin* expression was upregulated and reduced (c). Similarly, *MRF4* expression was delayed about 1 d in *MyoD*<sup>-/-</sup> cultures after transfer to differentiation medium (d). Day 0 denotes samples isolated from cells in growth medium. RNA samples were prepared 1, 2, 3, 4, and 5 d after the transfer of the cells to differentiation medium (DM). The fold activation in arbitrary units for each mRNA species is shown graphically beside each Northern blot. Fold activation was measured by densitometry and was normalized to 18S rRNA. (e) Western analysis of lysates prepared from wild-type or *MyoD*<sup>-/-</sup> cultures in growth medium with anti-Myf-5, anti-MyoD, and antimyogenin antibodies.

lated about twofold after mitogen withdrawal (Fig. 4 b). In addition, consistent with the observed 100-fold reduction in the rate of spontaneous differentiation, *MyoD*<sup>-/-</sup> myogenic cells expressed fivefold lower levels of *myogenin* mRNA in growth medium relative to wild-type myoblasts (Fig. 4 c). After the induction of differentiation, the relative levels of *myogenin* mRNA in *MyoD*<sup>-/-</sup> cells remained significantly reduced relative to wild-type cultures (Fig. 4 c). In wild-type cells, the level of *MRF4* mRNA remained unchanged until day 2 of differentiation and was thereafter upregulated about sevenfold. By contrast, *MRF4* mRNA was upregulated in differentiating *MyoD*<sup>-/-</sup> cultures to levels approximately two- to threefold lower than that of wild-type cells (Fig. 4 d).

Western analysis of MRF protein expression in lysates of primary cultures in growth medium confirmed the absence of



**Figure 5.** Reduced expression of differentiation-specific markers in *MyoD*<sup>-/-</sup> myogenic cells. Northern analysis of α-skeletal (a) and α-cardiac actin (b) mRNA levels revealed reduced and delayed kinetics of induction upon differentiation of *MyoD*<sup>-/-</sup> cultures. Similarly, acetylcholine receptor δ subunit (AChR δ) (c) and *M-cadherin* mRNA levels were found to be reduced (d). A marked reduction in the expression of *Musk* (e) and *adhalin* (f) mRNAs was also observed. The numbered arrows for *Musk* correspond to the specific isoforms quantitated on the associated graphs. The fold activation in arbitrary units for each mRNA species is shown graphically beside each Northern blot. Fold activation was measured by densitometry and was normalized to 18S rRNA.

MyoD protein in *MyoD*<sup>-/-</sup> cells (Fig. 4 e). Interestingly, Myf-5 protein was upregulated >10-fold in *MyoD*<sup>-/-</sup> cells, suggesting that posttranscriptional mechanisms may contribute to this increase (see Fig. 4, b and e for comparison). As suggested by the Northern analysis (Fig. 4 c), myogenin protein was absent in *MyoD*<sup>-/-</sup> cells in growth medium, whereas low levels were detected in wild-type myoblasts (Fig. 4 e).

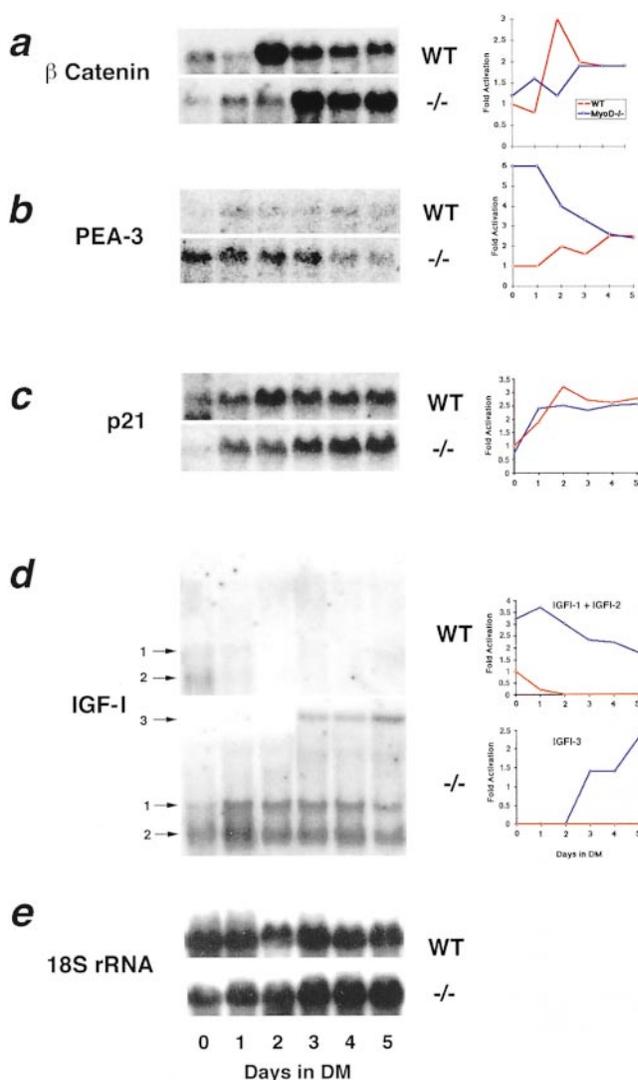
To assess the differentiation kinetics at the level of gene expression, Northern analysis was performed with a panel of muscle-specific markers. Analysis of mRNA levels for differentiation-specific markers revealed a pattern consis-

tent with overall delayed kinetics of differentiation in *MyoD*<sup>-/-</sup> myogenic cells. For example, low levels of  $\alpha$ -skeletal and  $\alpha$ -cardiac actin mRNAs were detected in wild-type cells in growth medium and these increased rapidly after mitogen withdrawal. By contrast, *MyoD*<sup>-/-</sup> cells in growth medium expressed no detectable sarcomeric actin mRNA (Fig. 5, a and b). After induction of differentiation  $\alpha$ -skeletal actin mRNA increased about threefold in wild-type cells, whereas in *MyoD*<sup>-/-</sup> cells the levels increased  $\sim$ 65% of wild-type levels by day 5 (Fig. 5 a). Although lower than that of  $\alpha$ -skeletal actin, the levels of  $\alpha$ -cardiac actin were found to increase in a similar pattern (Fig. 5 b). Cultured *MyoD*<sup>-/-</sup> myogenic cells displayed a twofold reduction in levels of acetylcholine receptor  $\delta$  subunit (AChR  $\delta$ ) mRNA in growth medium. After the induction of differentiation, a 5-fold increase was observed in wild-type cultures compared to an  $\sim$ 2-fold increase in *MyoD*<sup>-/-</sup> cells (Fig. 5 c) for a net 10-fold relative difference.

The protein coding for M-cadherin, a muscle-specific adhesion molecule, has been suggested to be expressed in quiescent satellite cells as well as to play an important role during myoblast differentiation and fusion (Irintchev et al., 1994; Pouliot et al., 1994; Zeschnig et al., 1995). However, RT-PCR analysis only detects *M-cadherin* expression in a small number of quiescent satellite cells suggesting that M-cadherin may not be useful as a marker for satellite cells (Cornelison and Wold, 1997). In wild-type cells, *M-cadherin* mRNA was detected at low levels in growth medium, *M-cadherin* increased fivefold by day 2 of differentiation, and was subsequently downregulated (Fig. 5 d). By contrast, *MyoD*<sup>-/-</sup> myogenic cells in growth medium expressed no detectable *M-cadherin* mRNA. However, low levels were detectable after mitogen withdrawal. Immunohistochemical detection of M-cadherin on cells in growth medium confirmed the reduced level of protein detectable on *MyoD*<sup>-/-</sup> cells relative to wild-type myoblasts (Fig. 1 d). The reduced levels of *M-cadherin* expression observed in *MyoD*<sup>-/-</sup> myogenic cells may account in part for the differentiation deficiency as M-cadherin appears to be required for efficient myogenic differentiation and fusion (Zeschnig et al., 1995).

The receptor tyrosine kinase Musk has been suggested to be expressed in activated satellite cells (DeChiara et al., 1996), and therefore may provide an additional marker for early myogenic cells. Northern blot analysis with a *Musk*-specific probe revealed the expression of three distinct isoforms in differentiating myogenic cells (Fig. 6 a). In wild-type cultures in growth medium, the large mRNA (isoform 1) was not expressed, the midsize mRNA (isoform 2) was expressed at intermediate levels, and the small mRNA (isoform 3) was expressed at higher levels. Induction of differentiation of wild-type cultures resulted in upregulation of isoforms 1 and 2, but little change in isoform 3. By contrast, *MyoD*<sup>-/-</sup> myogenic cells in growth medium expressed no detectable expression of *Musk* mRNA isoforms 1 and 2, and low levels of isoform 3. After induction of differentiation of *MyoD*<sup>-/-</sup> cultures, delayed upregulation of isoforms 1 and 2 was observed. Therefore, these data suggest that *Musk* is upregulated in a differentiation-dependent manner during muscle regeneration.

Adhalin, a dystrophin-associated protein also known as



**Figure 6.** Northern analysis of growth-associated gene products. (a) Primary wild-type myoblasts expressed abundant  $\beta$ -catenin under growth conditions. These levels increased threefold after 2 d of differentiation and subsequently decreased. In *MyoD*<sup>-/-</sup> cultures,  $\beta$ -catenin levels were found to continuously increase to levels that were comparable to that of wild-type cells by day 5 of differentiation. (b) Wild-type myoblasts during growth expressed very low levels of *PEA3* mRNA, and these levels increased about twofold by day 5 of differentiation. Growing *MyoD*<sup>-/-</sup> cells displayed sixfold higher levels of *PEA3* mRNA, which declined steadily to wild-type levels by day 4 of differentiation. (c) No significant differences were observed in *p21* mRNA levels between wild-type and *MyoD*<sup>-/-</sup> cells. (d) Wild-type myoblasts in growth conditions expressed low levels of *IGF-I* mRNA and these levels were rapidly extinguished after mitogen withdrawal. Mutant *MyoD*<sup>-/-</sup> myogenic cells expressed over threefold higher levels of the small *IGF-I* mRNA isoforms (1 and 2) and the 7-kb pre-*IGF-I* mRNA (isoform 3) was rapidly upregulated after 3 d of differentiation. The numbered arrows adjacent to *IGF-I* denote specific isoforms depicted on the corresponding graph. (e) Expression levels as determined by densitometry were normalized to 18S rRNA. Differentiation and graphical representation of the fold activation is as described in Fig. 5.

$\alpha$ -sarcoglycan, is upregulated during myoblast differentiation and is required for fully functional myofibers (Roberds et al., 1994; Liu et al., 1997). Human loss-of-function mutations in adhalin results in limb girdle childhood autosomal recessive muscular dystrophy (SCARMD) (Roberds et al., 1994). The delayed differentiation in the limb girdles evident in *MyoD*<sup>-/-</sup> embryos (Kablar et al., 1997) raised the possibility that adhalin may represent a *MyoD* target gene. Northern analysis revealed that adhalin is completely absent in growing *MyoD*<sup>-/-</sup> cells (Fig. 5 f). At the onset of differentiation, *adhalin* was slightly downregulated in wild-type cells, whereas it steadily increased in *MyoD*<sup>-/-</sup> cultures reaching ~50% of wild-type levels (Fig. 6 b). Therefore, these data substantiate the delayed differentiation of *MyoD*<sup>-/-</sup> myocytes, but do not elucidate whether adhalin is specifically upregulated in the *MyoD*-induced embryonic lineage that gives rise to hypaxial musculature.

### Analysis of Growth-associated Gene Products

To investigate the continued proliferation of *MyoD*<sup>-/-</sup> cells in differentiation medium, we examined the mRNA expression levels of several growth-associated proteins that have been demonstrated to play important roles in the control of myoblast differentiation. The plakoglobin-related protein,  $\beta$ -catenin (Butz et al., 1992), is believed to play important roles in cellular growth and morphogenesis in response to cellular adhesion and Wnt signaling (Miller and Moon, 1996; Barth et al., 1997). Primary wild-type myoblasts expressed abundant  $\beta$ -catenin mRNA under growth conditions. These levels increased threefold after 2 d of differentiation but subsequently decreased (Fig. 6 a). In *MyoD*<sup>-/-</sup> cultures,  $\beta$ -catenin mRNA levels were found to continuously increase and to stabilize at levels that were comparable to that of wild-type cells by day 5 of differentiation (Fig. 6 a). Detection of  $\beta$ -catenin protein by immunofluorescence revealed a similar nuclear cytoplasmic distribution in wild-type and mutant myogenic cells (Fig. 1 c). Therefore, these data do not support a role for  $\beta$ -catenin in the differentiation deficiency evident in *MyoD*<sup>-/-</sup> myogenic cells.

The *PEA3* gene product is upregulated in activated satellite cells in vivo, and has been suggested to be important for myoblast fusion in vitro (Taylor et al., 1997). Wild-type primary myoblasts in growth medium expressed very low levels of *PEA3* mRNA. However *PEA3* levels increased about twofold by day 5 of differentiation (Fig. 6 b). By contrast, *MyoD*<sup>-/-</sup> cells in growth medium displayed sixfold higher levels of *PEA3* mRNA, which declined steadily to wild-type levels by day 4 of differentiation (Fig. 6 b). However, this increased level of *PEA3* was not associated with enhanced differentiation (see above). Nevertheless, because *PEA3* is expressed in activated satellite cells (Taylor et al., 1997), these data are consistent with the notion that primary *MyoD*<sup>-/-</sup> myogenic cells represent an intermediate stage between a satellite cell and a myogenic precursor cell.

The p53-inducible cyclin-dependent kinase inhibitor p21/WAF1 arrests proliferating cells when ectopically expressed (el-Deiry et al., 1993; Harper et al., 1993) and is upregulated by MyoD during the differentiation of C2C12

myocytes (Guo et al., 1995; Halevy et al., 1995). Moreover, forced overexpression of p21 in myoblasts drives induction of differentiation-specific genes (Skapek et al., 1995). Unexpectedly, we observed no significant differences in *p21* mRNA levels between wild-type and *MyoD*<sup>-/-</sup> cells, in growth medium or during differentiation (Fig. 6 c). Therefore, the normal induction of *p21* in *MyoD*<sup>-/-</sup> myogenic cells after mitogen withdrawal suggests that *p21* induction is not MyoD-dependent and that p21 requires MyoD to positively stimulate differentiation.

The insulin-like growth factor, IGF-I, stimulates the proliferation and inhibits differentiation of cultured myoblasts (Quinn and Roh, 1993; Ewton et al., 1994; Engert et al., 1996). To assess whether IGF-I expression was altered in the absence of MyoD, we analyzed expression levels in primary myogenic cultures. Wild-type myoblasts in growth conditions expressed low levels of *IGF-I* mRNA and these levels were rapidly extinguished after mitogen withdrawal (Fig. 6 d). By contrast, *MyoD*<sup>-/-</sup> myogenic cells expressed over threefold higher levels of the small *IGF-I* mRNA isoforms 1 and 2 (Yamori et al., 1991) and these remained constant after transfer of the cells to differentiation medium (Fig. 6 d). Interestingly, the 7-kb pre-*IGF-I* mRNA (isoform 3) was not expressed under growth conditions but was rapidly upregulated after 3 d of differentiation. These observations strongly suggest that MyoD negatively regulates IGF-I expression and raises the possibility that MyoD is required for the repression of IGF-I expression during normal myogenic differentiation.

### The *MyoD* Mutant Cellular Phenotype Is a Cell Autonomous Deficit

The reduced fusion and continued proliferation of *MyoD*<sup>-/-</sup> myogenic cells under conditions that normally promote cell-cycle withdrawal and terminal differentiation can be hypothesized to be a consequence of cell autonomous attributes. For example, the marked reduction in M-cadherin expression (Figs. 1 d and 6 b) and the overexpression of IGF-I (Fig. 6 d) in *MyoD*<sup>-/-</sup> myogenic cells could both act to inhibit differentiation. Alternatively, *MyoD*<sup>-/-</sup> cells may have a unique developmental identity that precludes participation in the myogenic precursor cell differentiation program. To explore these possibilities, we mixed different proportions of wild-type myoblasts with lacZ-expressing *MyoD*<sup>-/-</sup> myogenic cells, and induced differentiation by culturing the cells in 5% horse serum for 5 d (Fig. 7). Importantly, PGK-lacZ expression is unaffected by terminal differentiation in transfected wild-type myoblasts (not shown). Strikingly, lacZ-labeled nuclei were never detected within any myotubes containing more than two nuclei (Fig. 7, b and c). Conversely, the differentiation of wild-type myocytes was completely unaffected by the presence of high numbers of *MyoD*<sup>-/-</sup> myogenic cells (compare Fig. 7, b and c, with Fig. 7 d). Taken together, these data support the notion that Myf-5 expression may define a distinct cell identity in the satellite cell developmental program.

Our analysis suggested that in satellite cell-derived myogenic cell lineage, important aspects of cytomorphology, differentiation, and ultimately fusion of mononuclear cells into myotubes are highly dependent on MyoD activ-

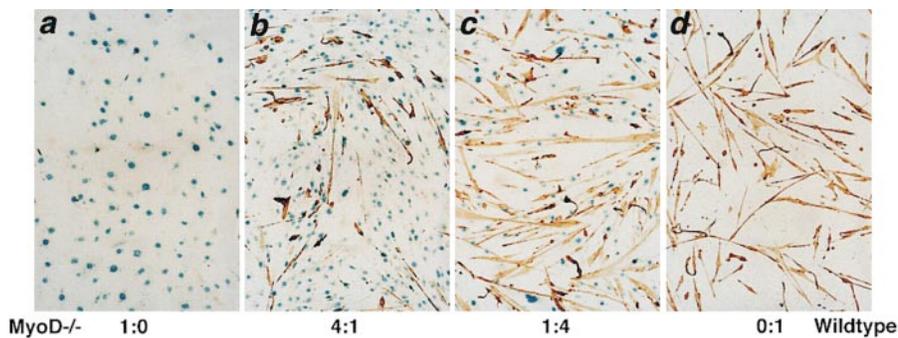


Figure 7. Strict cell autonomy in differentiated cocultures of lacZ-expressing *MyoD*<sup>-/-</sup> myogenic cells and wild-type myoblasts. Primary cells were plated in ratios of 1:0 (a), 1:4 (b), 4:1 (c), and 0:1 (d) of *MyoD*<sup>-/-</sup> to wild-type cells. After 5 d of differentiation, cells were fixed, and stained for  $\beta$ -galactosidase and with antibody MF20 reactive with MHC. Note the complete absence of lacZ-labeled nuclei in myotubes containing greater than two nuclei and the normal differentiation of wild-type myotubes.

ity and cannot be substituted for by other MRFs. To determine whether the observed phenotypic differences between *MyoD*<sup>-/-</sup> and wild-type primary myogenic cells were attributable to MyoD, a *MyoD*-expression plasmid was introduced into low passage cells and stable pools of transfectants were analyzed. Western blot analysis indicated that transfected *MyoD*<sup>-/-</sup> cells (termed *MyoD*<sup>+</sup>) expressed the exogenous MyoD protein (Fig. 8 a). By densitometry, the levels were found to be approximately threefold lower than in wild-type cells but similar to MyoD levels in C2C12 myoblasts. As expected, untransfected or PGK-Puro transfected *MyoD*<sup>-/-</sup> cells did not express MyoD protein (Fig. 8 a). In growth medium, pools of stable *MyoD*<sup>+</sup> cells displayed an almost complete reversion of the fibroblastlike phenotype and exhibited a rounded compact cytomorphology similar to wild-type cells (Fig. 8 b). Transfer of *MyoD*<sup>+</sup> cultures into differentiation medium resulted in increased numbers of MF20-positive differentiated myocytes that displayed the elongated bipolar multinucleated myotube morphology typical of wild-type myocytes (Fig. 8 b). After 3 d in low serum medium, the *MyoD*<sup>+</sup> pools displayed fusion indices that were comparable to wild-type cultures and approximately fivefold higher than *MyoD*<sup>-/-</sup> cells (Fig. 8 c). Differentiated *MyoD*<sup>+</sup> myocytes contained  $2.5 \pm 0.4$  nuclei on average, differentiated wild-type myocytes contained  $2.7 \pm 0.1$  nuclei on average, and differentiated *MyoD*<sup>-/-</sup> myocytes contained only single nuclei (Fig. 8 c). Lastly, transfection of *MyoD*<sup>-/-</sup> cells with a *Myf-5* expression plasmid did not rescue cytomorphology or differentiation potential (not shown). Taken together, these observations suggest that expression of MyoD is necessary and sufficient to re-establish the progression of *MyoD*<sup>-/-</sup> myogenic cells through the differentiation program.

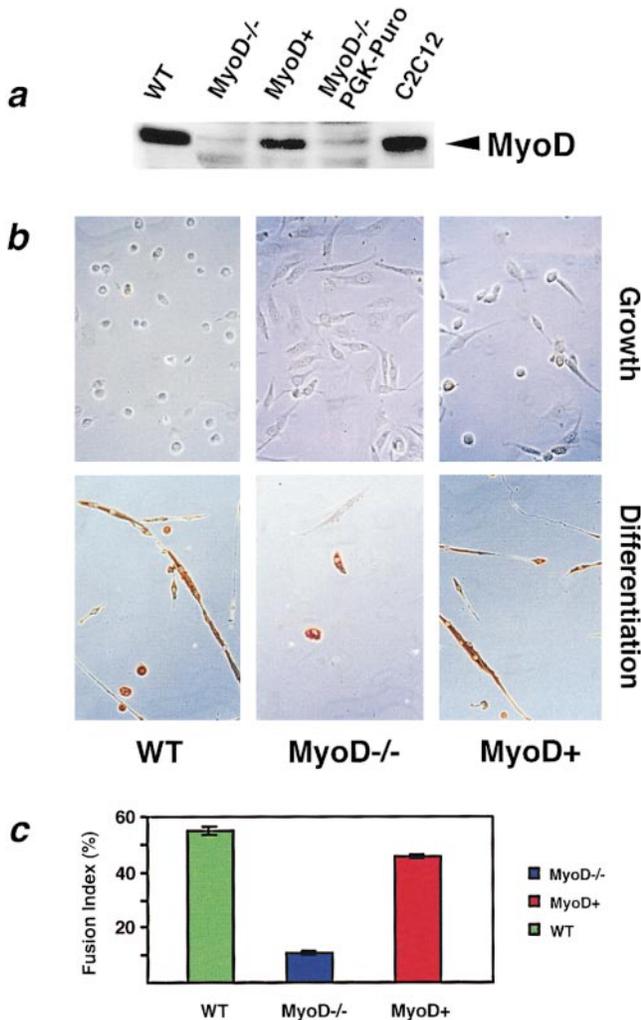
## Discussion

Analysis of muscle regeneration in *MyoD*<sup>-/-</sup> mice led to the suggestion that MyoD is required for satellite cells to efficiently give rise to proliferative myogenic precursor cells (Megeney et al., 1996). To characterize the phenotype of *MyoD*-deficient myoblasts and to gain insight into the role of *MyoD* in the activation and differentiation of satellite cells, an in-depth characterization of *MyoD*<sup>-/-</sup> primary myoblast cultures was undertaken. The establishment and propagation of stable cell lines can potentially result in aneuploidy as well as the introduction of addi-

tional mutations, which are necessary for immortalization and continuous proliferation in culture. To avoid such anomalies, all of our analyses were carried out using newly established low-passage primary myoblast cultures. Our characterization of early passage primary cultures reported here differs from the analysis of later passage *MyoD*<sup>-/-</sup> cultures (>15 doublings) that exhibited decreased growth, readily formed multinucleated myotubes, and grew in an FGF2-independent manner (Megeney et al., 1996; data not shown). Therefore, these results underscore the importance of characterizing early passage (<10 doublings) primary cultures before any adaptation to growth in tissue culture conditions.

Primary *MyoD*<sup>-/-</sup> myogenic cells exhibited a fibroblastlike cytomorphology, and expressed *c-met* and high levels of *PEA3* and *IGF-I*, but did not express desmin, *M-cadherin*, or *Musk*. Transfer of primary *MyoD*<sup>-/-</sup> cells into differentiation medium resulted in formation of reduced numbers of mononuclear myocytes and delayed induction of differentiation-specific markers. Mixing experiments revealed that *MyoD*<sup>-/-</sup> cells did not influence the differentiation of wild-type myocytes and did not fuse with differentiating myotubes. Forced expression of *MyoD* rescued both the cytomorphology and the differentiation deficit. Taken together, these data suggest that *Myf-5* expressing cells represent an intermediate between a quiescent satellite cell and a proliferating myogenic precursor cell.

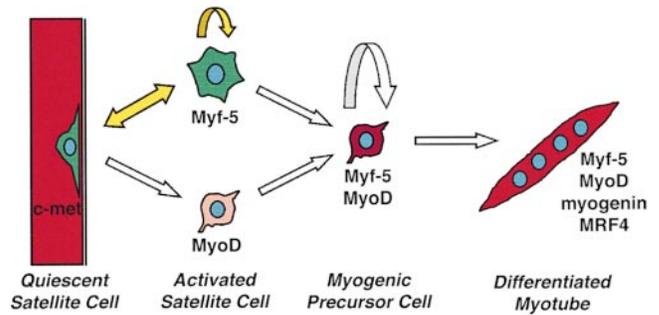
Northern blot analysis revealed that *MyoD*<sup>-/-</sup> myogenic cells expressed fourfold higher levels of *Myf-5* mRNA and Western analysis revealed an even greater increase in Myf-5 protein. Similar findings are observed for the in vivo expression of *Myf-5* in the muscle of *MyoD*-deficient mice (Rudnicki et al., 1992). Interestingly, delayed muscle differentiation has also been reported during limb development of *MyoD*<sup>-/-</sup> embryos in which Myf-5 expressing myogenic processors arrive in the limb but differentiate with markedly delayed kinetics (Kablar et al., 1997). Embryos lacking *MyoD* display normal development of trunk musculature in the body proper, whereas muscle development in limb buds and branchial arches is delayed by  $\sim 2.5$  d. In contrast, embryos lacking *Myf-5* display normal muscle development in limb buds and branchial arches, but exhibit a marked delay in development of trunk muscles. Although *MyoD*-mutant embryos exhibit delayed development of limb musculature, the migration of Pax-3-expressing cells into the limb buds and subsequent in-



**Figure 8.** Rescue of the differentiation deficiency of *MyoD*<sup>-/-</sup> myogenic cells by forced expression of MyoD. (a) Western blot analysis revealed the expression of MyoD protein in wild-type cells (WT) and no expression in *MyoD*<sup>-/-</sup> cultures or *MyoD*<sup>-/-</sup> cells transfected with a selectable vector alone (PGK-Puro). However, *MyoD*-transfected pools of *MyoD*<sup>-/-</sup> cells (*MyoD*<sup>+</sup>) expressed readily detectable MyoD protein as did C2C12 myoblasts. (b) In growth medium, *MyoD*<sup>+</sup> cells exhibited a refractile compact morphology typical of wild-type primary myoblasts and distinct from the stellate fibroblastlike morphology of *MyoD*<sup>-/-</sup> cells (growth). *MyoD*<sup>+</sup> cultures exposed to differentiation medium for 3 d exhibited increased numbers of differentiated myocytes as detected with antiserum MF20 reactive with MHC, and restored formation of elongated bipolar multinucleated myotubes (day 3/MF20). (c) Determination of fusion index indicated a fivefold increase in the fusion potential of *MyoD*<sup>+</sup> cells relative to untransfected *MyoD*<sup>-/-</sup> cells and similar to wild-type (WT) cultures. Fusion indices were calculated as described in Fig. 4.

duction of Myf-5 in myogenic precursors occurs normally. These data, together with the observed regeneration deficit in *MyoD*<sup>-/-</sup> muscle, indicate that *MyoD* and *Myf-5* cannot fully substitute for each other during embryogenesis and in satellite cells, and suggest that *Myf-5* and *MyoD* activate discrete subsets of target genes that differentially define myogenic cell identity.

Forced expression of *MyoD* in a variety of cell lines in-



**Figure 9.** Role of *MyoD* in regulating satellite cell function. RT-PCR analysis of single cells on cultured myofibers reveals quiescent satellite cells express *c-met* but no detectable MRFs. Activated satellite cells first express *Myf-5* or *MyoD* before coexpressing both *Myf-5* and *MyoD*, and progressing through their normal developmental program leading to terminal differentiation (Cornelison and Wold, 1997). In the absence of *MyoD*, satellite cells appear to exhibit a propensity for self-renewal rather than progression through the differentiation program (Megeny et al., 1996). Therefore, expression of *Myf-5* alone may allow self-renewal of satellite cells either before returning to quiescence (yellow arrows) or upregulating *MyoD* and formation of proliferative myogenic precursor cells (mpc) (white arrows). The potential and replicative capacity of cells expressing *MyoD* alone is unknown; however, these cells likely irreversibly progress through the myogenic program. Taken together, we interpret these data to suggest that *MyoD*<sup>-/-</sup> myogenic cells represent an intermediate stage between myogenic stem cell and a myogenic precursor cell.

duces growth arrest even in the absence of differentiation (Olson, 1992). Consistent with this, myogenic cells lacking *MyoD* displayed inefficient withdrawal from the cell cycle in response to low mitogens. However, the cell-cycle inhibitor p21 was upregulated to similar levels in wild-type and *MyoD*<sup>-/-</sup> cells in growth medium and following induction of differentiation. In C2C12 myoblasts, expression of p21 appears to be directly induced by *MyoD* upon cell-cycle arrest and terminal differentiation (Guo et al., 1995; Halevy et al., 1995; Skapek et al., 1995). Moreover, forced expression of p21 arrests proliferating cells (el-Deiry et al., 1993; Harper et al., 1993), and induces the terminal differentiation of C2C12 myoblasts (Skapek et al., 1995). Therefore, the relationship between cell-cycle control and differentiation appears to be uncoupled in primary *MyoD*<sup>-/-</sup> myogenic cells. Interestingly, depending on context, p21 expression can either arrest cells via inhibiting cdk activity, or promote cell division by acting as a cdk4/cyclin D1 assembly factor (LaBaer et al., 1997). Future characterization of cell cycle control in mutant cells should elucidate this aspect of the *MyoD*<sup>-/-</sup> myogenic cell phenotype.

The *PEA3* transcription factor was observed to be expressed at about sixfold higher levels in *MyoD*-deficient myoblasts under growth conditions. The ETS-domain transcription factor *PEA3* (Xin et al., 1992) is rapidly induced after muscle damage and forced expression of *PEA3* stimulates myogenesis in vitro when overexpressed in satellite cell-derived cultured myoblasts (Peterson and Houle, 1997; Taylor et al., 1997). These data led to the suggestion that *PEA3* is an important regulator of activated satellite cell function (Taylor et al., 1997). The lack of cor-

relation between increased expression of *PEA3* and differentiation potential in *MyoD*<sup>-/-</sup> myogenic cells supports the assertion that *MyoD*<sup>-/-</sup> myogenic cells have an identity distinct from wild-type myoblasts. Interestingly, overexpression of *PEA3* is correlated with increased metastatic potential of mammary adenocarcinomas (Trimble et al., 1993). Moreover, overexpression of *PEA3* in cultured cells directly induces the upregulation of a subset of matrix metalloproteases (Hassell, J.A., personal communication). After muscle damage, activated satellite cells readily cross the basal lamina and are capable of migrating from surviving to damaged areas (Hughes and Blau, 1990; Phillips et al., 1990). By contrast, cultured primary myoblasts injected into muscle exhibit a very poor ability to migrate away from the injection site (Fan et al., 1996; Gussoni et al., 1997). Therefore, it will be of interest to determine whether *MyoD*<sup>-/-</sup> cells are more invasive than their wild-type counterpart.

M-cadherin had been suggested to be expressed in satellite cells (Irintchev et al., 1994). However, recent RT-PCR analysis indicates that a small minority of satellite cells express *M-cadherin* mRNA (Cornelison and Wold, 1997). Consistent with this we observed markedly reduced expression of M-cadherin in *MyoD*<sup>-/-</sup> myogenic cells relative to wild-type cells (see Figs. 1 d and 5 b). This observation raises the possibility that *MyoD* directly or indirectly regulates the expression of surface adhesion molecules involved in fusion and differentiation. Interestingly, incubation of antagonistic M-cadherin peptides or antisense RNA inhibits both myoblast fusion and cell-cycle withdrawal in conditions that normally promote differentiation (Zeschnigk et al., 1995). Therefore, these data suggest that cell-cycle withdrawal during terminal differentiation also involves cell-cell interactions.

Cellular adhesion is clearly linked to regulation of proliferation, migration, and differentiation. For example, expression of dominant negative cadherin inhibits proliferation and stimulates terminal differentiation of human epidermal keratinocytes (Zhu and Watt, 1996). Moreover, integrin and cadherin synergistically inhibit migration and promote the aggregation of myoblasts (Huttenlocher et al., 1998). Forced overexpression of an effector of adhesion mediated signaling integrin-linked kinase (ILK) in intestinal epithelial cells and mammary epithelial cells induces the activity of G1/S cyclin/Cdks, downregulates E-cadherin expression, induces nuclear translocation of  $\beta$ -catenin, and results in increased invasiveness (Radeva et al., 1997; Novak et al., 1998). Therefore, examination of integrin and cadherin function in *MyoD*<sup>-/-</sup> myogenic cells should elucidate the role of adhesion in the control of proliferation and migration in early myogenic precursors.

High level expression of IGF-I in L6 myoblasts stimulates proliferation and inhibits differentiation, whereas lower levels of IGF-I stimulate both proliferation and differentiation (Quinn and Roh, 1993; Ewton et al., 1994; Lefaucheur and Sebille, 1995; Engert et al., 1996). A striking feature of *MyoD*<sup>-/-</sup> cells was the relatively high expression of *IGF-I* mRNA and its continued upregulation during differentiation. In contrast, wild-type myoblasts expressed lower levels of *IGF-I* in growth conditions and these levels decreased during differentiation. It is interesting to speculate that increased *IGF-I* expression in

*MyoD*<sup>-/-</sup> cultures contributes to the observed differentiation delay. As *IGF-I* levels rapidly decrease in wild-type cultures induced to differentiate, an interesting hypothesis is that *MyoD* is required to downregulate *IGF-I* at the onset of differentiation. In *MyoD*<sup>-/-</sup> myogenic cells, continued *IGF-I* expression could result in an autocrine loop that acts to promote proliferation. However, the presence of *MyoD*<sup>-/-</sup> myogenic cells did not inhibit the differentiation of wild-type primary myoblasts raising the possibility that expression of other components of the IGF-I signaling pathway are downregulated in wild-type myoblasts. These data further underscore the assertion that *MyoD*<sup>-/-</sup> myogenic cells are distinct from wild-type myoblasts.

Continuous myoblast cell lines lacking *MyoD* exhibit somewhat similar traits in comparison to primary *MyoD*<sup>-/-</sup> myogenic cells. For example, C2C12 cells expressing antisense *MyoD* RNA, display increased *Myf-5* expression, decreased *IGF-II* expression, and are defective in differentiation (Montarras et al., 1996). The brain tumor-derived BC3H1 myoblast cell line (Taubman et al., 1989) expresses *Myf-5* but does not express *MyoD*, and exhibits a differentiation deficit with reduced ability to form multinucleated myotubes (Brennan et al., 1990). However, unlike primary *MyoD*-deficient myogenic cells, BC3H1 myocytes in differentiation-inducing medium exhibit normal upregulation of myogenin together with normal induction of subsets of MHC isoforms and other differentiation-specific markers (Taubman et al., 1989; Miller, 1990; Brennan et al., 1990). Forced expression of *myogenin* in BC3H1 cells is unable to rescue the differentiation deficit, whereas forced expression of *MyoD* confers competency for myogenic differentiation (Brennan et al., 1990). Forced expression of a functional *MyoD* protein in *MyoD*-deficient cells was sufficient to revert the *MyoD*<sup>-/-</sup> cytomorphology and rescue the differentiation defect as evidenced by a dramatic increase in fusion index of *MyoD*<sup>+</sup> cells. Therefore, these data support the assertion that lack of *MyoD* results in a cell-autonomous deficit in the satellite cell differentiation program. Taken together, the parallels observed between primary *MyoD*<sup>-/-</sup> myocytes and continuous myoblast cell lines deficient in *MyoD* support a unique set of functions for *MyoD* that cannot be substituted for by *Myf-5*.

The muscle regeneration deficit in *MyoD*<sup>-/-</sup> muscle suggests that expression of *MyoD* is required for satellite cells to efficiently form differentiation-competent myogenic precursor cells (Megeny et al., 1996). RT-PCR analysis reveals that activated satellite cells first express either *Myf-5* alone or *MyoD* alone, before coexpressing *Myf-5* and *MyoD*, and subsequently progressing through the myogenic program (Cornelison and Wold, 1997). Our analysis of the phenotype of primary *MyoD*<sup>-/-</sup> myogenic cells can be interpreted to suggest that *MyoD*<sup>-/-</sup> myogenic cells represent an intermediate stage between a quiescent satellite cell and a myogenic precursor cell. Together, these data suggest the hypothesis that expression of *Myf-5* alone may define an intermediate developmental stage that provides a mechanism for satellite cell self-renewal. In this model, activated satellite cells expressing only *Myf-5* could undergo cell division and either return to quiescence by downregulating *Myf-5*, or alternatively upregulating *MyoD* and progressing through the myogenic program (see Fig. 9). Clearly, further analysis of the devel-

opmental potential and phenotype of primary *MyoD*<sup>-/-</sup> myogenic cells may present a unique opportunity to investigate the early myogenic program of satellite cells.

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