Developmental Biology **224,** 122–137 (2000)
doi:10.1006/dbio.2000.9682, available online at http://www.idealibrary.com on IDEAL®

MyoD$^{-/-}$ Satellite Cells in Single Fiber Culture Are Differentiation Defective and MRF4 Deficient

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MyoD-deficient mice are without obvious deleterious muscle phenotype during embryogenesis and fetal development, and adults in the laboratory have grossly normal skeletal muscle and life span. However, a previous study showed that in the context of muscle degeneration on a mdx (dystrophin null) genetic background, animals lacking MyoD have a greatly intensified disease phenotype leading to lethality not otherwise seen in mdx mice. Here we have examined MyoD$^{-/-}$ adult muscle fibers and their associated satellite cells in single myofiber cultures and describe major phenotypic differences found at the tissue, cellular, and molecular levels. The steady-state number of satellite cells on freshly isolated MyoD$^{-/-}$ fibers was elevated and abnormal branched fiber morphologies were observed, the latter suggesting chronic muscle regeneration *in vivo*. Single-cell RNA coexpression analyses were performed for c-met, m-cadherin, and the four myogenic regulatory factors (MRFs.) Most mutant satellite cells entered the cell cycle and upregulated expression of myf5, both characteristic early steps in satellite cell maturation. However, they later failed to normally upregulate MRF4, displayed a major deficit in m-cadherin expression, and showed a significant diminution in myogenin-positive status compared with wildtype. MyoD$^{-/-}$ satellite cells formed unusual aggregate structures, failed to fuse efficiently, and showed greater than 90% reduction in differentiation efficiency relative to wildtype. A further survey of RNAs encoding regulators of growth and differentiation, cell cycle progression, and cell signaling revealed similar or identical expression profiles for most genes as well as several noteworthy differences. Among these, GDF8 and Msx1 were identified as potentially important regulators of the quiescent state whose expression profile differs between mutant and wildtype. Considered together, these data suggest that activated MyoD$^{-/-}$ satellite cells assume a phenotype that resembles in some ways a developmentally "stalled" cell compared to wildtype. However, the MyoD$^{-/-}$ cells are not merely developmentally immature, as they also display novel molecular and cellular characteristics that differ from any observed in wild-type muscle precursor counterparts of any stage.

INTRODUCTION

Skeletal muscle is formed by myoblast fusion during fetal and neonatal development in mice. Myonuclei that have fused into muscle fibers are terminally postmitotic and therefore unable to participate in repair of damaged muscle; this function is fulfilled by satellite cells (Bischoff, 1994; Schultz and McCormick, 1994; Yablonka-Reuveni, 1995). Several naturally occurring instances are known in humans in which satellite cells fail to fully regenerate damaged tissue. In terminal phases of neuromuscular wasting diseases such as Duchenne's muscular dystrophy, satellite cells which have previously mediated recovery from acute episodes of muscle degeneration become depleted, contributing to severe muscle atrophy and ultimately to death (Bischoff, 1994). In the muscle hypotrophy characteristic of aging, it is also suggested that the previously competent satellite cell pool has somehow lost some of its regenerative capacity.

The mouse model of Duchenne's muscular dystrophy, the dystrophin-deficient mdx mouse, shows a less severe phenotype than do human patients. This difference has been attributed to more active and persistent satellite cell activity although the small mass, short life span, and

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0012-1606/00 $35.00

quadrapedal gait of the mouse may also contribute to the attenuation of the mdx phenotype. In any case, these mice experience intense muscle necrosis and vigorous regeneration from 21 days to 6 months of age, by which time they return to a grossly normal phenotype (Bulfield *et al.*, 1984; DiMario *et al.*, 1989). A mouse mutant which has more subtle deficiencies in muscle regeneration is the targeted disruption of mouse MyoD (Rudnicki *et al.*, 1992). While they have been characterized as having no macroscopic degenerative phenotype under normal conditions, when bred into a dystrophin-deficient background the additional lack of MyoD exacerbates the mdx phenotype and leads to premature death. In addition, when challenged by acute muscle injury during adulthood, MyoD$^{-/-}$ mice show significant deficiencies in regeneration (Megeney *et al.*, 1996). Specifically, MyoD-deficient satellite cells from crushed muscle become activated but are unable to properly execute the entire regeneration program. The gross phenotype is that few replacement myofibers are formed compared with wildtype, with the majority of satellite cells remaining mononucleate within the damaged area. Based on this phenotype, Megeny *et al.* (1996) proposed that, in the absence of MyoD, some essential step in myogenic progression was blocked, leading to a population of activated satellite cells which, instead of terminally differentiating, returns to a quiescent state.

Here we have examined MyoD$^{-/-}$ adult muscle and satellite cells in the context of individual fiber cultures for morphological, cellular, and molecular phenotypes. MyoD-deficient satellite cells were present in excess over wildtype in experimentally undamaged muscle tissue and displayed aberrant morphology during later phases of proliferation and differentiation in culture. They also exhibited major differences in myogenic gene expression and efficiency of terminal differentiation compared to wildtype satellite cells, including failure to correctly upregulate myogenic regulatory factor (MRF) 4, myogenin, and m-cadherin. These data suggest a molecular basis for the differentiation-defective phenotype. Additional data on expression of a panel of regulatory genes of interest in muscle suggests that MyoD$^{-/-}$ satellite cells also display neomorphic characteristics that differ from their wildtype counterparts at any stage examined.

MATERIALS AND METHODS

MyoD homozygous mutant female adult mice (>100 days of age) were imported from the Rudnicki colony and were in a mixed Balb/cJ:129/Sv genetic background. Muscle fibers from these and from MyoD$^{+/+}$ adult female mice (C57B/6 × DBA/2 F1; Jackson) were prepared and cultured as described previously (Cornelison and Wold, 1997). Single-satellite cells were harvested with a patch-clamp pipette and their cellular mRNA was reverse transcribed as described previously (Cornelison and Wold, 1997). Single cells were assayed for coexpression of c-met, m-cadherin, myf-5, myogenin, and MRF4 using the PCR conditions described previously (Cornelison and Wold, 1997). Total percentages of wildtype and MyoD$^{-/-}$ satellite cells in each of 16 possible coexpression states were analyzed for significance by two-way correlation using Fisher's exact test; $P \leq 0.05$ was considered to be significant. Analysis was performed in StatView (Abacus). Cell pools were also assayed for expression of a larger panel of regulatory genes (see Table 1 for primer sets). The amount of input RNA for each gene tested for the gene survey was 1/30 of a 30-cell pool. Otherwise, all conditions used were as in Cornelison and Wold (1997). To establish a minimal sensitivity level and to verify primer efficacy, RNA samples representing approximately one cell equivalent from whole embryos, C2C12 or MM14 myoblasts and myocytes, or EC cells were assayed and shown to score reproducibly positive with a band of expected size was used.

We have recently determined that the c-met primers (designated in Table 1 as c-met-A) used previously for RT-PCR (Andermarcher *et al.*, 1995), single-cell RT-PCR (Cornelison and Wold, 1997), and in this paper do not cross an intron. As a result these primers can generate a PCR band from genomic DNA that is indistinguishable from an mRNA template band. However, we believe that this does not affect the conclusions presented here for several reasons: (1) We have found by empiric tests that c-met-A primers do not amplify successfully from genomic DNA in our single-cell samples. When individual wild-type satellite cells ($n > 60$) were harvested and treated as usual, except that reverse transcriptase was omitted from the postharvest incubation, no DNA band was detected using c-met-A primers when amplified as usual (unpublished data). (2) Cells that do not express c-met RNA or protein by other criteria such as skin fibroblasts or muscle-derived fibroblasts have all scored uniformly negative for c-met in the single-cell assay using this primer set (Cornelison and Wold, 1997, and unpublished data). Apparently, substantially larger amounts of DNA than $2N$ are needed for successful amplification using the c-met-A primer set. (3) The assignment of cell identity as a satellite cell does not depend on their c-met molecular status, because they were picked based on their physical location on individual myofibers, which meets a classical satellite cell definition.

New mouse c-met primers sets have been written and tested, and are included in Table 1 (primer set c-met-B). They have been verified to cross at least one intron and have been successful in priming amplification of c-met RNA from single-cell amounts of satellite RNA. However, we caution that the c-met-B primer set has not been fully tested in a multiplex single-cell format, so we cannot assert that they are compatible with primers for m-cadherin and the four MRFs as is the c-met-A primer set.

Immune staining was performed on MyoD$^{+/+}$ and mutant satellite cell cultures grown 7 days in growth medium (DMEM + 10% horse serum + 5% chick embryo extract + antibiotic/antimycotic), after which myofibers were removed and the remaining adherent myocytes were cultured for 7 days in differentiation medium (DMEM + 2% horse serum). Cells were fixed for 20 min in ice-cold methanol/acetone at −20°C and dried for 24 h, then rehydrated in PBS and stained with anti-myosin heavy chain (MF-20; Developmental Studies Hybridoma Bank) and detected with anti-mouse HRP and DAB; alternatively, they were stained with MF-20 and anti-c-met (Santa Cruz) or MF-20 and anti-m-cadherin (Santa Cruz) and detected with donkey anti-mouse FITC and donkey anti-rabbit TRITC (Jackson). Hoechst 33347 was added to the final wash after staining to visualize nuclei.

TABLE 1
Primer Sets Used in Gene Survey

Gene	Forward primers	Reverse primers	Product MW
myf-5	OF 5′ TGC CAT CCG CTA CAT TGA GAG 3′	OR 5′ CCG GGG TAG CAG GCT GTG AGT TG 3′	353
	IF 5′ GAG GGA ACA GGT GGA GAA CTA TTA 3′	IR 5′ CGC TGG TCG CTG GAG AG 3′	293
MyoD	OF 5′ GCC CGC GCT CCA ACT GCT CTG AT 3′	OR 5′ CCT ACG GTG GTG CGC CCT CTG C 3′	397
	IF 5′ CCC CGG CGG CAG AAT GGC TAC G 3′	IR 5′ GGT CTG GGT TCC CTG TTC TGT GT 3′	234
myogenin	OF 5′ GGG CCC CTG GAA GAA AAG 3′	OR 5′ AGG AGG CGC TGT GGG AGT T 3′	364
	IF 5′ CCG TGG GCA TGT AAG GTG TG 3′	IR 5′ TAG GCG CTC AAT GTA CTG GAT GG 3′	198
MRF4	OF 5′ CTG CGC GAA AGG AGG AGA CTA AAG 3′	OR 5′ ATG GAA GAA AGG CGC TGA AGA CTG 3′	367
	IF 5′ TGC GGA GTG CCA TCA GC 3′	IR 5′ CTC CTC CTT CCT TAG CAG TTA TCA 3′	215
MEF2A	OF 5′ CAG TCT TGG AAT GAA CAG TCG GAA ACC 3′	OR 5′ TCA CTG CAG TAA CAC AAA GAA CAA ACA T 3′	758
	IF 5′ GCC CAA AGG ATA AGC AGT TCT CAA G 3′	IR 5′ AGT ATT AGC AGG TCG GCC AAG CAC A 3′	554
MEF2B	OF 5′ CCA ACG CGG ATA TCC TTC AGA CAC TTA 3′	OR 5′ CCC TCG GGC ACC AAC CAG 3′	404
	IF 5′ GCC CGG GGG TCG AAA GTG AG 3′	IR 5′ GCC CGG GGG TCG AAA GTG AG 3′	236
MEF2C	OF 5′ AAC ACG GGG ACT ATG GGG AGA AAA 3′	OR 5′ TAT GGC TGG ACA CTG GGA TGG TAA 3′	469
	IF 5′ GAG TAC AAC GAG CCG CAC GAG A 3′	IR 5′ TAT GGC TGG ACA CTG GGA TGG TAA 3′	247
MEF2D	OF 5′ CCC CCA GCA GCC AGC ACT ACA 3′	OR 5′ GAC TTG GGG ACA CTG GTT CTG ACT TGA 3′	779
	IF 5′ CCC CTG GCC TCC TCC CTG TG 3′	IR 5′ CTG CTG TGG CTG TGG CTG TGG TAA 3′	485
MEF2D-MS	OF 5′ GAC AAG TAC CGG CGG GCC AGT GAG GAG 3′	OR 5′ CTG GGG TGG TGA GCG AGT GGG TAG AC 3′	589
	IF 5′ CAG TGC CCG TGT CCA ATC AGA GC 3′	IR 5′ GTG GGT GGG TGG GGG CGG AGA CTT 3′	338
Id-1	OF 5′ GCC CCA GAA CCG CAA AGT GA 3′	OR 5′ TGA TTA ACC CCC TCC CCA AAG TCT 3′	429
	IF 5′ GCA TGT GTT CCA GCC GAC GAT 3′	IR 5′ CCC TGG GGA ACC GAG AGC AC 3′	116
Id-2	OF 5′ CCC AGC ATC CCC CAG AAC AAG A 3′	OR 5′ CCA GGC CGG AGA ACG ACA CC 3′	453
	IF 5′ ACG CGC CTG ACC ACC CTG AAC 3′	IR 5′ TCT CCT GGT GAA ATG GCT GAT AAC AAA A 3′	219
Id-3	OF 5′ GCG CCT GCG GGA ACT GG 3′	OR 5′ CAG GGT GGG GAC AGA GTG ACG 3′	480
	IF 5′ GGT GGA AAT CCT GCA GCG TGT CAT AG 3′	IR 5′ TCG AGG CGT TGA GTT CAG GGT AAG TG 3′	329
Id-4	OF 5′ CGC CGC GGA CCC CAC TCA 3′	OR 5′ CAG CGG TCA TAA AAG AAG AAA CGA AAG A 3′	365
	IF 5′ GGA CCC CAC TCA CCG CGC TCA ACA CTG 3′	IR 5′ TTA ATT TCT GCT CTG GCC CTC CCT TCC T 3′	161
m-twist	OF 5′ CCC TCG GAC AAG CTG AGC AAG AT 3′	OR 5′ ACA AAC GAG TGT TCA GAC TTC TAT CAG 3′	485
	IF 5′ CGG CCAGGT ACA TCG ACT TCC 3′	IR 5′ GGT CTG CTG CTG CCC CTC TGG GAA TCT 3′	402
HGF	OF 5′ TTG GCC ATG AAT TTG ACC TC 3′	OR 5′ ACA TCA GTC TCA TTC ACA GC 3′	558
	IF 5′ ACG GTA TCC ATC ACT AAG AGT GGC 3′	IR 5′ CGC ACG TTT TAA TTG CAC AAT ACT CCC 3′	450
c-met-A[a]	OF 5′ GAA TGT CGT CCT ACA CGG CC 3′	OR 5′ CAC TAC ACA GTC AGG ACA CTG C 3′	370
	IF 5′ GAA GGT ATC CGA ATT CAA GAC CGG 3′	IR 5′ GAA CAT GCA GTG GAC CTC AGA CTG 3′	249
c-met-B[b]	OF 5′ TAA CAA GCA TTT CTC CGA GGT 3′	OR 5′ ACA CCC ACT TCA TGC ACA TCT 3′	376
	IF 5′ GCT GGA GGC ACC TTA CTC ACT 3′	IR 5′ GCT AAC CGA ATT CAG GGT CTT 3′	306
FGF-1	OF 5′ CAC CGT GGA TGG GAC AAG GGA CAG 3′	OR 5′ CAC CGG GAG GGG CAG AAA CAA GAT 3′	325
	IF 5′ GCG GAA AGT GCG GGC GAA GTG 3′	IR 5′ TCC GAG GAC CGC GCT TAC AG 3′	226
FGF-2	OF 5′ CGG CAT CAC CTC GCT TCC 3′	OR 5′ CTT CTG TCC AGG TCC CGT TTT G 3′	418
	IF 5′ CGG CTT CTT CCT GCG CAT CCA TCC 3′	IR 5′ AGC CAG CAG CCG TCC ATC TTC CTT CAT 3′	169
FGF-4	OF 5′ GAC CGC CGC ACC CAA CGG 3′	OR 5′ TCA TGG TAG GCG ACA CTC 3′	500
	IF 5′ GAC CGC CGC ACC CAA CGG 3′	IR 5′ TCA TGG TAG GCG ACA CTC 3′	500
FGF-5	OF 5′ GGC AGC CGG GGC AGA AGT AG 3′	OR 5′ CTG GGT AGG AAG TGG GTG GAG ACG 3′	521
	IF 5′ TCG TCT TCT GCC TCC TCA CCA 3′	IR 5′ CCA CGT ACC ACT CTC GGC CTG TCT TT 3′	406
FGF-6	OF 5′ CGC TGC AGG CTC TCG TCT TCT TAG 3′	OR 5′ GCC CCG CTT TAC CCG TCC TAT 3′	514
	IF 5′ TGG TGG GCA TTA AGC GAC AGC GGA GAC 3′	IR 5′ CGT AGG CGT TGT AGT TGT TTG GAA GGA G 3′	278
FGF-7	OF 5′ ACG GAT CCT GCC AAC TCT GCT CTA CA 3′	OR 5′ TCT TTC TTC GTT TTC TTC CCT TTG ACA 3′	525
	IF 5′ CGG AGC AAA CGG CTA CGA GTG TG 3′	IR 5′ CTT TGC ATA GAG TTT CCC TTC CTT GTT CA 3′	281
FGF-8	OF 5′ ATC AAC GCC ATG GCA GAA GAC 3′	OR 5′ CGT GAA GGG CGG GTA GTT GAG G 3′	384
	IF 5′ CAT TGT GGA GAC CGA TAC TTT TG 3′	IR 5′ AGG CTC TGC TCG GTG GTG TGG TG 3′	309
FGF-10	OF 5′ GGG CTG CTG TTG CTG CTT CT 3′	OR 5′ TTG GAT CGT CAT GGG GAG GA 3′	574
	IF 5′ GGA AGG CAT GTG CGG AGC TA 3′	IR 5′ CAT TTG CCT GCC ATT GTG CT 3′	339
FGFR-1	OR 5′ GGG ATG TGG GGC TGG AAG TGC 3′	OR 5′ CAG GCC TAC GGT TTG GTT TGG TGT 3′	454
	IF 5′ ATG GGG TGC AGC TGG TGG AGA G 3′	IR 5′ CAG GCC TAC GGT TTG GTT TGG TGT 3′	246
FGFR-2	OF 5′ CGG CGT GGC GAC TGC TCT CC 3′	OR 5′ AGG CGG CTG TGC TGC TGT TGC 3′	419
	IF 5′ CGC CGG CTG CTA CCC AAG GAA T 3′	IR 5′ TGC TGC TGC TGC TGC TGC TGT G 3′	154
FGFR-3	OF 5′ CGC GGC GCT GCT TGA GG 3′	OR 5′ GCA TCT TCG TGG GAG GCA TTT AGC 3′	347
	IF 5′ TGG TCC AGA GCA GCG AGT TGT 3′	IR 5′ GGC CAC CAG ACC TGT ACC ATC CTT 3′	175
FGFR-4	OF 5′ CGT GGA CAA CAG CAA CCC 3′	OR 5′ AGC CAC GGT GCT GGT TTG 3′	460
	IF 5′ CGT GGA CAA CAG CAA CCC 3′	IR 5′ AGC CAC GGT GCT GGT TTG 3′	460

TABLE 1—*Continued*

Gene	Forward primers	Reverse primers	Product MW
IGF-1	OF 5′ CTG GCG CTC TGC TTG CTC ACC TTC 3′	OR 5′ TCT TGT TTC CTG CAC TTC CTC TAC TTG T 3′	346
	IF 5′ GTG TGG ACC GAG GGG CTT TTA CTT CAA 3′	IR 5′ TGG CGC TGG GCA CGG ATA GAG 3′	183
IGF-2	OF 5′ AGC CGT GGC ATC GTG GAA GAG T 3′	OR 5′ GCA TGG GGG TGG GTA AGG AGA AA 3′	497
	IF 5′ CCC CGC CAA GTC CGA GAG G 3′	IR 5′ GCG GGG TCT TTG GGT GGT AAC A 3′	243
IGF-1 R	OF 5′ GTG GGC CCG GCA TTG ACA TC 3′	OR 5′ TGC AGG CTC GCT TCC CAC AC 3′	576
	IF 5′ TTC CCC AAG CTC ACA GTC ATC ACC 3′	IR 5′ CAT TCC TTT GGG GGC TTG TTC C 3′	311
IGF-2 R	OF 5′ CCT TCG GGA CCC CAG CAC AC 3′	OR 5′ TTC TCT TGA CAA ACA GCA GCC TCT T 3′	504
	IF 5′ TGA AGG GGA ACC AGG CAT TTG AC 3′	IR 5′ TGT GGC AGG CAT ACT CGG TGA T 3′	249
PDGF α	OF 5′ CCG GGA CCT CCA GCG ACT CTT G 3′	OR 5′ GGC CGG CTC TATCTC ACC TCA CAT CT 3′	482
	IF 5′ GAC CCC ACA TCG GCC AAC TTC C 3′	IR 5′ AGG TCG CAC ATG CAC ACT CCA G 3′	214
PDGF β	OF 5′ CTG CTG GGC GCT CTT CCT TCC T 3′	OR 5′ TGG GCT TCT TTC GCA CAA TCT CAA TCT T 3′	482
	IF 5′ CGA CCA CTC CAT CCG CTC CTT TGA 3′	IR 5′ CGT CTT GCA CTC GGC GAT TAC AGC 3′	196
PDGF α R	OF 5′ CGG TAT GAA ATT CGC TGG AGG GTT ATC G 3′	OR 5′ CTT CGG CTT CTC TGG GTG TTG GCT CAT 3′	447
	IF 5′ GGA CCC CAT GCA GTT GCC TTA CGA 3′	IR 5′ GAG CAT CTT CAC AGC CAC CTT CAT TAC 3′	163
PDGF β R	OF 5′ ACC AAA GGA GGG CCC ATC TAC ATC ATC A 3′	OR 5′ CGA GCC AGG CCG AAG TCA CAG 3′	533
	IF 5′ TGG ACT ACC TGC ACC GGA ACA AAC A 3′	IR 5′ GGT AGC TGA AGC CCA CGA GGT C 3′	357
cyclin A	OF 5′ TGA GAC CCT GCA TTT GGC TGT GAA CT 3′	OR 5′ TTG TGG CGC TTT GAG GTA GGT CTG GTG A 3′	523
	IF 5′ CCC CCA GAA GTA GCA GAG TTT GTG TA 3′	IR 5′ TCC AGC AAT GAG TGA AGG CAG GTA 3′	258
cyclin D1	OF 5′ CCT GTG CTG CGA AGT GGA GA 3′	OR 5′ CTG GCA TTT TGG AGA GGA AGT GT 3′	494
	IF 5′ AAG TGC GTG CAG AAG GAG ATT GTG 3′	IR 5′ TCG GGC CGG ATA GAG TTG TCA GT 3′	269
cyclin D2	OF 5′ TGG CCG CAG TCA CCC CTC AC 3′	OR 5′ TCT CTT GCC GCC CGA ATG G 3′	446
	IF 5′ GCT CTG TGC GCT ACC GAC TTC AAG 3′	IR 5′ TTG GAT CCG GCG TTA TGC TGC TCT 3′	257
cyclin D3	OF 5′ TTC CAG TGC GTG CAA AAG GA 3′	OR 5′ CTC GCA GGC AGT CCA CTT CA 3′	592
	IF CGC TGC GAG GAG GAT GTC TT 3′	IR 5′ CTG GGC ATG CTT TTT GAC CA 3′	336
cyclin E	OF 5′ TTG TGT CCT GGC TGA ATG TCT ATG TCC 3′	OR 5′ CTG CTC GCT GCT CTG CCT TCT TAC T 3′	486
	IF 5′ TGT CCT CGC TGC TTC TGC TTT GTA TCA T 3′	IR 5′ GGC TTT CTT TGC TTG GGC TTT GTC C 3′	244
cdk-2	OF 5′ GAA ATT CAT GGA TGC CTC TGC TCT CAC 3′	OR 5′ GAA ATC CGC TTG TTG GGG TCA TAG T 3′	564
	IF 5′ CTT TCT GCC ATT CTC ACC GTG TCC TTC A 3′	IR 5′ TTT GCT AAA ATC TTG CCG AGC CCA CTT G 3′	404
cdk-4	OF 5′ AAG CCC GAG ATC CCC ACA GT 3′	OR 5′ GGG CTC GGA AGG CAG AGA TT 3′	804
	IF 5′ AAG GAG GCT GGA GGC CTT TG 3′	IR 5′ CGT AAC CAC CAC AGG CGT GA 3′	352
cdk-5	OF 5′ CAG GCT GGA TGA TGA CGA TGA GG 3′	OR 5′ TAG CAG CGG ACG GGG ATA CCA A 3′	369
	IF 5′ CAG CTG CAA TGG TGA CCT GGA CCC TGA GAT 3′	IR 5′ CAG CCA ATT TCA ACT CCC CAT TCC TGT TTA 3′	155
p15	OR 5′ ACC CCA CCC CGC CTA TTT GTC TC 3′	OR 5′ ACT TGC CCA GCT TGT ACG GAA CCA G 3′	555
	IF 5′ ACC CGG CGT GAG TCG TCT GTG C 3′	IR 5′ CGC CCT TGT CCC CGG TCT GTG G 3′	178
p16	OF 5′ TTG GGC GGG CAC TGA ATC T 3′	OR 5′ AGG CAT CGC GCA CAT CCA 3′	361
	IF 5′ CGC CCA ACG CCC CGA ACT CTT 3′	IR 5′ GTG CAC CGG GCG GGA GAA GG 3′	140
p18	OF 5′ CTG CGA AGG ACC TGA CTC TGA A 3′	OR 5′ GGC TCC CCC AAC CCC ATT T 3′	556
	IF 5′ GCC GAG CCT TGG GGG AAC G 3′	IR 5′ CCA AGT CGA AGG CGG TGT C 3′	427
p19	OF 5′ AAG GTG CCA GCC CCA ATG TCC AAG ATG C 3′	OR 5′ CCA AAA GGG GTG AGG AAA AAC AAA TGA G 3′	499
	IF 5′ CCT AGC TCC TGA ATC TGA TCT CCA CCA C 3′	IR 5′ AAC TGC TCC TCC CAC TCC CTT CTT CAA T 3′	234
p21	OF 5′ TGT GGA CAT CAC CCG TGA CC 3′	OR 5′ GGA GAG GGC AGG CAG CGT AT 3′	793
	IF 5′ TGG TGG AAA AGC ACC TGC AA 3′	IR 5′ GGG ACC CAG GGC TCA GGT AG 3′	344
p27	OF 5′ AAA TCT CTT CGG CCC GGT CAA TCA 3′	OR 5′ CTC CAC AGT GCC AGC GTT CG 3′	466
	IF 5′ AGC ACT GCC GGG ATA TGG AAG AA 3′	IR 5′ GCG CGG GGG CCT GTA GTA GAA C 3′	140
p57	OF 5′ CCG CAG GAG CCG TCC ATC AC 3′	OR 5′ AGC CGG CGG CCC AGA ACG 3′	415
	IF 5′ CGA CGT GTA CCT CCG CAG CAG AAC 3′	IR 5′ GCG GTA GAA GGC GGG CAC AGA CT 3′	271
Pax-3	OF 5′ GCC AGG GCC GAG TCA ACC AG 3′	OR 5′ GAT CCG CCT CCT CCT CTT CTC CTT 3′	414
	IF 5′ GCC TCT GCC CAA CCA TA 3′	IR 5′ TTC CTC AAT TTT CTT CTC CA 3′	214
GDF-8	OF 5′ GAG CGC CTC CAC TCC GGG AAC TGA T 3′	OR 5′ CCT GGT CCT GGG AAG GTT ACA GCA AGA T 3′	487
	IF 5′ CAC GCT ACC ACG GAA ACA ATC ATT ACC A 3′	IR 5′ GCC TGG GCT CAT GTC AAG TTT CAG AGA T 3′	264
Msx-1	OF 5′ GCC GAA AGC CCC GAG AAA CTA 3′	OR 5′ CAG GAC CGC CAA GAG GAA AAG AG 3′	379
	IF 5′ GGA CGC CTT TCA CCA CAG C 3′	IR 5′ TCG CGG CCA TCT TCA GC 3′	201

Note. OF, outside forward; OR, outside reverse; IF, inside forward; IR, inside reverse. In some cases it was necessary to use the same primer for both outside and inside reactions, in which cases the identical primers and fragment sizes are listed.

[a] Recent reevaluation established that the primer set identified above as c-met-A that was used previously (Cornelison and Wold, 1997) and in this work does not cross an intron border (see Materials and Methods). As discussed under Materials and Methods, this does not significantly affect conclusions in this work.

[b] A new c-met primer set designated c-met-B does cross intronic sequence and can successfully amplify c-met by RT-PCR from single-cell quantities of RNA.

RESULTS

MyoD-deficient adult muscle has abnormalities characteristic of chronic repair. Muscle fibers derived from MyoD$^{-/-}$ adult mice (>100 days of age) showed several unanticipated differences from wildtype. In particular, some MyoD-deficient fibers (about 1%) were forked or showed "sprouts," morphologies associated with muscle fibers undergoing chronic regeneration, such as from patients with Duchenne's and Becker's muscular dystrophies (Bradley, 1978) (Fig. 1a). This fiber phenotype is consistent with the regeneration deficit observed in MyoD$^{-/-}$ muscle (Megeney *et al.*, 1996). Additionally, we noted that myonuclei in MyoD$^{-/-}$ fibers displayed a consistent shape difference relative to myonuclei in MyoD$^{+/+}$ myofibers: while wild-type fibers, especially when freshly harvested, have uniformly distributed and regularly shaped elongate myonuclei, myonuclei of mutant fibers varied greatly in distribution, size, and shape (Fig. 1b). Nuclei of some MyoD$^{-/-}$ satellite cells also developed further aberrant morphology after several days in fiber culture, becoming teardrop-shaped with a distinct point on one end (not shown). The significance of these differences in morphology is unclear, but they would seem to support the idea of a widespread steady-state difference in adult muscle fibers of MyoD$^{-/-}$ mice.

MyoD$^{-/-}$ satellite cells are initially present in excess in fibers explanted from undamaged muscle. At the time of fiber harvest, while the number of myonuclei/mm fiber is comparable between wild-type and MyoD$^{-/-}$ myofibers, satellite cells on MyoD$^{-/-}$ fibers were present in excess relative to wildtype (Fig. 2a). Elevation in the steady-state satellite cell number from MyoD$^{-/-}$ muscle that had not been experimentally injured would be consistent with stimulation by chronic damage followed by inefficient differentiation of activated satellite cells. Satellite cells were identified in these experiments by the criteria of c-met positivity and/or their physical position relative to the myofibers. Previously we showed that in isolated fiber cultures, the c-met receptor is expressed in all satellite cells from the earliest time of isolation until after their fusion into new myotubes (Cornelison and Wold, 1997). This did not, however, ensure that c-met would be expressed similarly in a MyoD$^{-/-}$ genetic background. This was tested directly and we found that all cells selected/identified as satellite cells by the criterion of their physical association with isolated myofibers also expressed c-met mRNA or protein, depending on the assay used. Muscle-derived fibroblasts from MyoD$^{-/-}$ fiber cultures, like wild-type muscle-derived fibroblasts, were negative for c-met expression.

The kinetics of entry into the first cell cycle were similar for MyoD$^{-/-}$ and wild-type satellite cells after several days of fiber culture, as indicated by the onset of BrdU labeling for S phase. However, in a preparation in which we scored adherent colonies from MyoD$^{-/-}$ cultures, the number of cells was substantially reduced compared with wildtype, even though the number of satellite cells per fiber at the time of dissection had been severalfold higher. We therefore tested for apoptosis at 48 and 96 h of culture, as measured by TdT labeling (ApopTag Direct, Oncor), and found virtually none in the MyoD$^{-/-}$ adherent colonies or on fiber cultures; this concurs with previous *in vivo* results obtained by Megeney *et al.* (1996). Thus there is an apparent paradox between the satellite census upon fiber isolation and the cell number at the end of the cultures. As shown below, a simple resolution is suggested by the inefficient differentiation that is characteristic of mutant cells in culture. If similar behavior occurs *in vivo* it could easily elevate the steady-state number of undifferentiated muscle precursors in the presence of chronic low level injury, even if the absolute rate of cell division is lower than in wildtype. In contrast, wild-type cells might be activated less frequently and, when activated, differentiate more efficiently, even if that differentiation is preceded by more rapid division. The net effect could easily be a smaller steady-state pool of precursor cells relative to the mutant. Other factors might also play a role. Only a fraction of mutant cells might continue to proliferate in the cultures beyond the first round, or there may be genotype-dependent differences in cell adhesion to the culture substrate that could alter the census at late times.

MyoD$^{-/-}$ satellite cells aggregate abnormally in fiber explant cultures. MyoD$^{-/-}$ satellite cells resident on single explanted myofibers became activated (as determined by emergence from under the basal lamina of the host fiber and an increase in cytoplasm) within the first 24 h after harvest, in a manner indistinguishable from that of wildtype. While MyoD-deficient satellite cells appeared identical to wildtype cells at 24 h (Figs. 3a and 3b), by 48 h in culture they were enlarged compared to wildtype and had begun to accumulate in abnormal aggregates (Figs. 3c and 3d), and by 96 h after harvest, many mutant satellite cells were contained in very large, multicellular calluses adherent to the fibers (Figs. 3e and 3f). We have never observed such aggregates on wild-type fibers. At 96 h, a few rare MyoD$^{-/-}$ satellite cells were observed in depressions in the host fiber, and in some cases they were partially enclosed by the fiber sarcolemma. None of these were observed at earlier time points nor have they ever been seen on wild-type fibers. RNA analysis on these rare cells confirmed that they are c-met- and MRF-positive, and thus they were included in the cell sets chosen for both individual and pool analysis. An intriguing possibility is that these cells have failed to execute the myogenic program and are in the act of returning to the sublaminal position and status of resting satellite cells.

MyoD-deficient satellite cells from isolated fiber cultures are differentiation defective. Satellite cells isolated from MyoD-deficient muscle that had become adherent to the culture plate during the first week of fiber culture were subsequently cultured for 1 week in differentiation medium and assayed for expression of the differentiated muscle marker myosin heavy chain (MHC). Compared to wild-type cultures, very few multinucleate myotubes could be found;

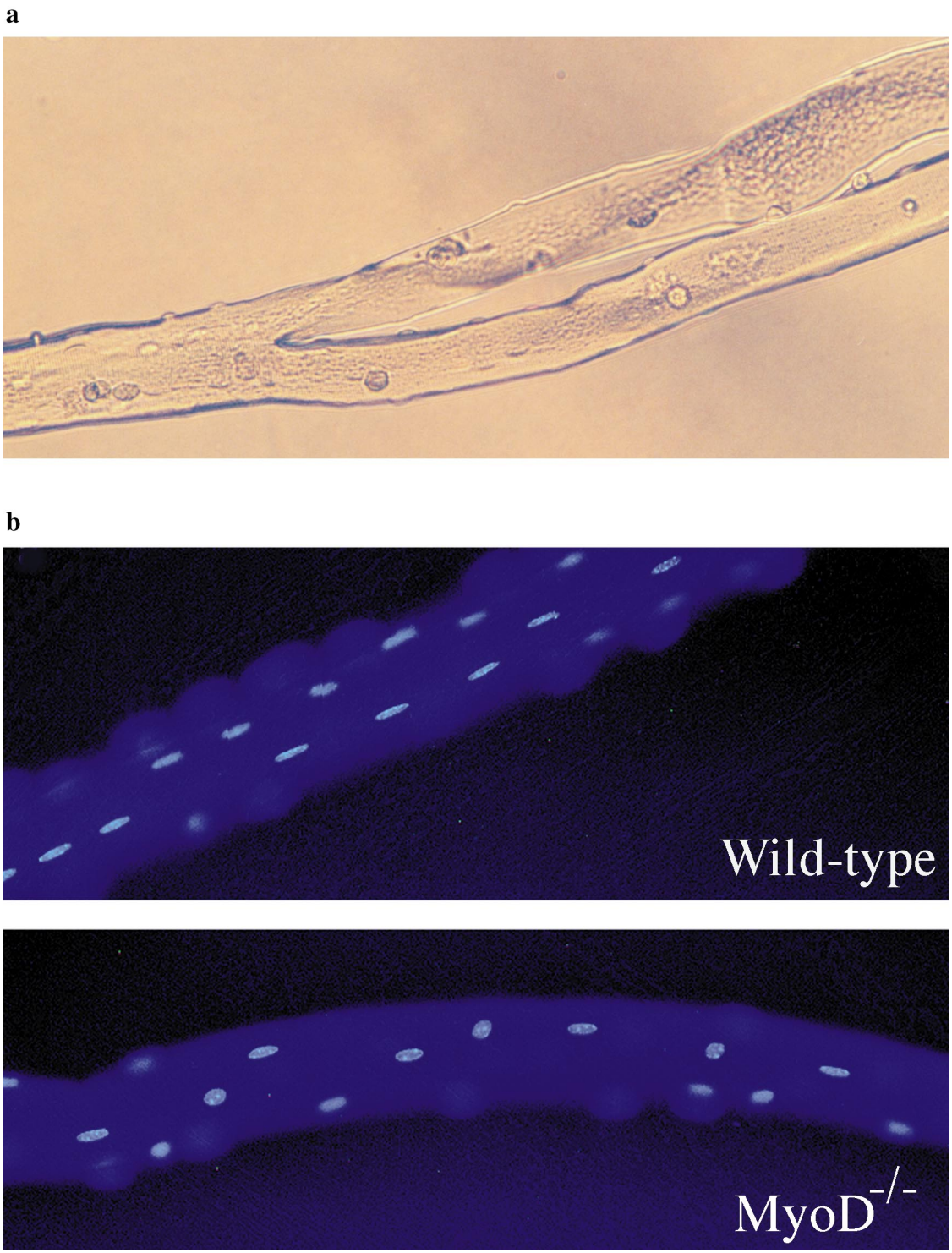


FIG. 1. (a) MyoD-null myofiber with a fork. MyoD-null fibers which forked and rejoined, and fibers with small sprouts, were also observed. (b) Comparison of myonuclear morphology between MyoD$^{+/+}$ and MyoD$^{-/-}$ myofibers.

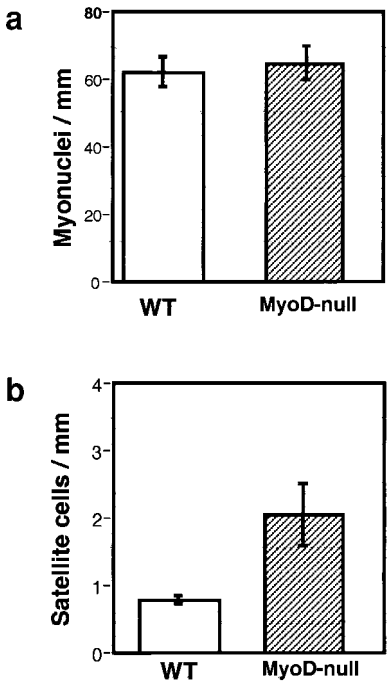


FIG. 2. Histograms comparing myonuclei per millimeter of myofiber at the time of fiber harvest. (a) Satellite nuclei per millimeter of fiber; (b) satellite cell nuclei per 100 myofiber nuclei. Measurements were made from three different animals of each genotype by counting the number of c-met-positive cells per unit fiber and the number of myofiber nuclei per unit fiber as visualized by DAPI staining.

those rare multinucleate "myotube" structures that did form were abnormal in morphology (i.e., stunted, having very few nuclei, or very little cytoplasm, as in Fig. 4b). MHC-positive cells from MyoD$^{-/-}$ cultures tended to be small, rounded, and mononucleate (Fig. 4b). A more quantitative comparison of cell morphologies found that 17% of MyoD$^{-/-}$, MHC-positive cells were elongated and bipolar, compared with 95% of wild-type MHC-positive cells. When these cultures were costained for MHC and the satellite cell marker c-met, whose expression is also typically retained on newly fused myotubes (Tatsumi *et al.*, 1998; Cornelison and Wold, unpublished results), only 11% of c-met-positive MyoD$^{-/-}$ satellite cells were MHC positive, while 86% of wild-type c-met-positive cells were MHC positive (Fig. 5). Thus, the fraction of satellite cells that differentiated by the molecular criterion of MHC expression was eightfold lower in the mutant, and those MyoD$^{-/-}$ cells that did become MHC positive were morphologically abnormal myocytes that fused with each other only rarely.

MyoD$^{-/-}$ satellite cells are defective in upregulating MRF4 and m-cadherin. When single satellite cells from MyoD$^{-/-}$ animals were analyzed for coexpression of c-met, m-cadherin, myf5, myogenin, and MRF4 mRNAs over the first 4 days in culture, several departures from the wild-type program were evident (Fig. 6; Table 2). The first temporal distinction was relatively subtle, but potentially illuminating in the context of chronic regeneration which may occur in MyoD$^{-/-}$ muscle: several MyoD-deficient satellite cells scored positive for myogenin RNA at either 0 or 24 h. This is in contrast to wildtype, in which myogenin positives were first detected at 48 h. A simple explanation is that these early myogenin positives represent a subpopulation of satellite cells that were present and already activated in MyoD$^{-/-}$ muscle prior to its harvest. This would be consistent with other phenotypic evidence of chronic damage and repair. However, it is also theoretically possible that these cells were activated *de novo* by the dissection and then upregulated myogenin on an accelerated time course compared with wildtype.

At 24 h, a similar fraction from each genotype scored negative for all four MRFs: 30% MyoD$^{-/-}$ versus 27% wildtype. The fractions positive for myf5 were also similar: 61% MyoD$^{-/-}$, 53% wildtype. The remaining 20% from wildtype were positive for MyoD alone. At this time point, a striking difference in m-cadherin expression began to emerge with only 3% of MyoD$^{-/-}$ satellite cells scoring positive for m-cadherin RNA compared with 24% m-cadherin-positive wild-type cells.

After 48 h in culture the expression patterns diverged further. By this time, the majority of wild-type cells had progressed to a more developmentally advanced state that included detectable expression of myogenin, MRF4, and m-cadherin RNA, while MyoD$^{-/-}$ cells were mainly accumulating in the no-MRF or myf5-only expression states that are typical of wild-type cells at earlier time points: 21% of mutant cells scored negative for all MRFs compared with only 11% of wild-type cells. Further evidence of their relative immaturity is that only 23% of mutant satellites scored positive for myogenin, while 62% of wild-type cells were positive. Myogenin expression is generally associated with imminent execution of differentiation in other skeletal myogenic settings. Thus, by this time most wild-type satellites had transited through the MyoD^{+}, myf5^{+}, and MyoD^{+}myf5^{+} RNA expression states, leaving just 3, 3, and 14%, respectively, in those compartments. In the mutant, the equivalent myf5-only expression state comprised 56% of the cells. Moreover, in the wild-type cultures approximately half of the myogenin-positive cells had also begun to express MRF4, while no MRF4 positives were detected in MyoD mutants. It was also evident by 48 h that m-cadherin RNA expression was severely affected in MyoD$^{-/-}$ satellite cells. No MyoD $^{-/-}$ satellite cells scored m-cadherin RNA positive at this time, compared with over 80% m-cadherin-positive wild-type cells. Additionally, while all myogenin-positive wild-type cells coexpressed m-cadherin at this time, this expression relationship was uncoupled in the mutant, with several MyoD$^{-/-}$ cells scoring positive for myogenin but negative for m-cadherin.

At 96 h in culture, the MyoD$^{-/-}$ population had diverged still further from wildtype in the direction of an immature phenotype with respect to MRFs. All MyoD$^{-/-}$ satellite

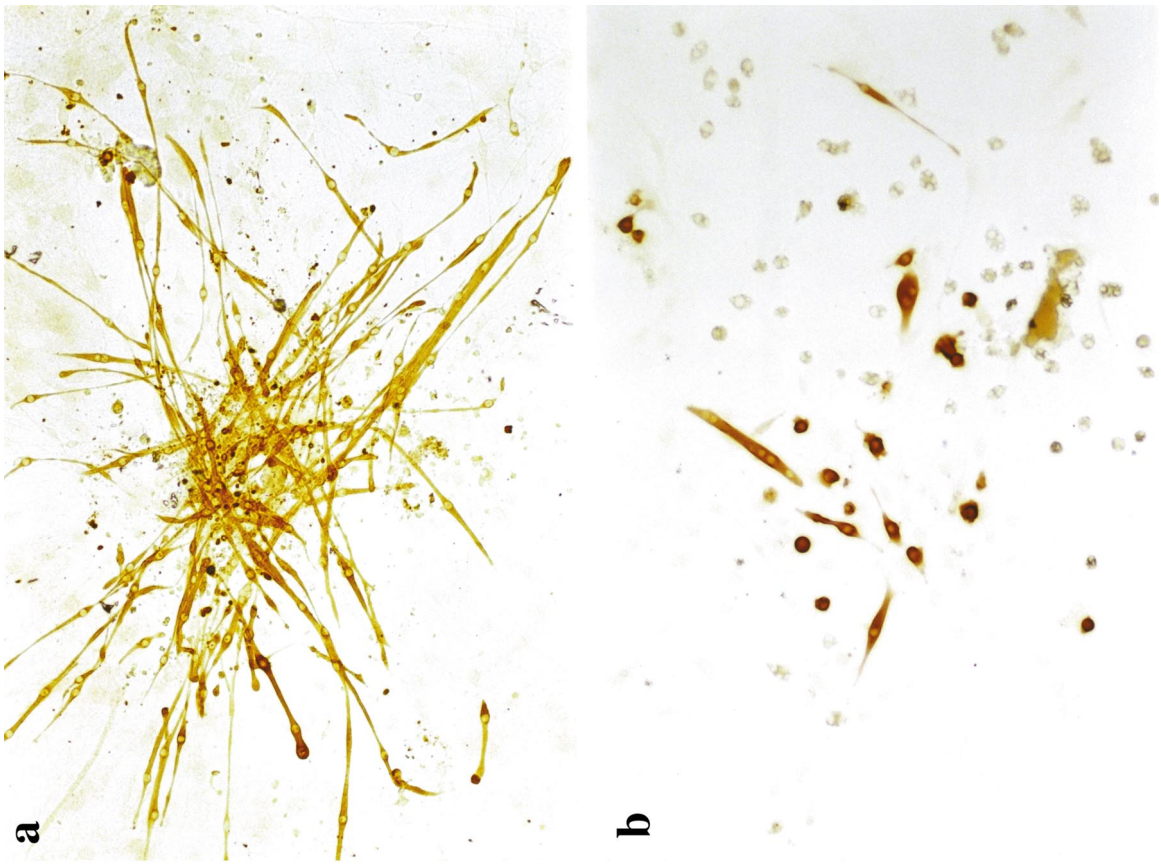
cells still scored negative for MRF4 compared with nearly one-half positive in wildtype, a significantly smaller fraction scored positive for myogenin (22% MyoD$^{-/-}$ versus 67% for wildtype), and the fraction scoring negative for all four MRFs remained at about a third in contrast to a negligible value for wildtype. While a few more MyoD$^{-/-}$ cells in this period expressed m-cadherin, it was far from being universally expressed, as it would be in wild-type cells. To test whether MRF4 expression in MyoD mutant fiber cultures is simply on a slightly delayed time course, additional mutant cells were assayed at 8 and 12 days after fiber harvest. Even at these late time points, no MRF4 expression was detected in a sample of 60 individual cells. We conclude that the reduction in myogenin positives and absence of MRF4 positives in the MyoD$^{-/-}$ satellites is not the result of a subtle time delay. Nevertheless, these results do not entirely preclude MRF4 expression at very late times, especially under differentiation-promoting conditions. Preliminary data identified a few rare MRF4-positive cells in cultures maintained in differentiation medium for 3 to 5 weeks. From this we provisionally infer that either rare stochastic events generate MRF4 positives over time or that prolonged exposure to differentiation-promoting conditions can ultimately, if inefficiently, induce MRF4 expression in MyoD-deficient cells.

Given the extreme decrease in MyoD$^{-/-}$ satellite cells which express m-cadherin, the previously suggested roles of m-cadherin in mediating myoblast fusion and subsequent cytodifferentiation (Zeschnigk *et al.*, 1995), and the failure of fusion of most MyoD$^{-/-}$ cells that finally express MHC, it seemed possible that the rare mutant myotubes observed were selectively derived from the small population of m-cadherin-expressing (and therefore fusion-favored) mononucleate myocytes. This hypothesis was investigated by examining m-cadherin and myosin heavy chain protein distribution in differentiation cultures from MyoD$^{-/-}$ muscle. As expected, in control wild-type-differentiation cultures, virtually all mononucleate cells (myocytes) coimmunostained positive for MHC and m-cadherin, while more mature MHC-positive myotubes that had formed during the differentiation culture had mainly become m-cadherin negative. In MyoD$^{-/-}$ differentiation cultures, m-cadherin immunoreactivity was rare, as anticipated from the RNA data. When m-cadherin staining was observed, it was most often detected selectively at points of contact between two MHC-positive mononucleate myocytes (Fig. 7a), consistent with the notion that these may be cells caught in the act of aligning for subsequent fusion. The very rare, more elongated myotubes that could be found in MyoD$^{-/-}$ cultures did not express m-cadherin, which is the same result obtained for large, mature myotubes in wild-type cultures. Among the many MHC-negative cells in the MyoD$^{-/-}$ cultures (by far the majority of cells), none scored positive for m-cadherin. Very occasionally, a phenotype that may be intermediate between aligned but unfused myocytes and fused mature myotubes was observed. It consisted of m-cadherin immunostaining distributed uniformly over the surface of multinucleate (but small, only two to four nuclei), MHC-positive, nonelongated "myobag" structures (Fig. 7b). MyoD$^{-/-}$ myocytes with m-cadherin localized to a zone of apposition between cells may therefore be kinetic intermediates in an m-cadherin-dependent fusion process.

An expanded molecular portrait of satellite cells. Expression of a substantial panel of additional genes was surveyed by using RNA from 30-cell pools of MyoD$^{-/-}$ satellite cells and compared to data from similar wild-type cell pools (Table 3). Our intent with this broader gene survey was to identify those genes within interesting functional groups that are expressed in at least some satellite cells, to gain a rough appreciation of their expression over the course of activation and differentiation in isolated fiber culture, and to see which ones vary substantially in MyoD$^{-/-}$ cultures. The classes of genes tested were chosen based on their involvement in activation, proliferation, and differentiation and included growth factors and growth factor receptors of the IGF, FGF, PDGF, GDF, and HGF families; various negative regulators of muscle differentiation, including the four Id family members, twist, Msx1, and positive regulators of cell cycle progression; positive-acting coregulators of myogenesis such as MEF2s; and the negative regulators of cell cycle progression. These were tested for expression from pools of individually picked satellite cells (three or more separate determinations from 30-cell pools per time point).

Sensitivity and interpretation of pool assays. The pool assay is generally quite sensitive to the presence of the target RNA, but it is inherently probabilistic and sensitivity will not be identical for all genes. Detection level is a combination of absolute assay sensitivity, including the efficiency of reverse transcription, statistical sampling from a mixed cell population, and RNA levels expressed within positive cells. For each gene, an operational minimum detection level was established by testing cDNA that represented approximately one cell equivalent from either embryo or tissue culture sources and demonstrating it to be reproducibly positive. Although quite sensitive, this assay ultimately delivers a "yes" or "no" readout that will tend to obscure quantitative differences between low, modest, and high levels of the target RNA per cell. In pooled samples, it will sometimes mask differences between a subset of cells expressing a given gene and homogeneous expression by all cells. Finally, in unusual cases of genes with RNA splice isoforms unknown to us, the differentially spliced transcripts might be missed, detected in only one form, or summed together, depending on primer selection (the example of known MEF2D isoforms is in Tables 1 and 3). For cases for which we have both pool data and extensive single-cell data, such as the MRFs, the pool assay scored mainly positive when 30% or more of the cells in a population were individually positive. We also found that the determinations are in good agreement with immunostaining, such as for myogenin with the 5FD antibody.

A general observation was that despite considerable mor-



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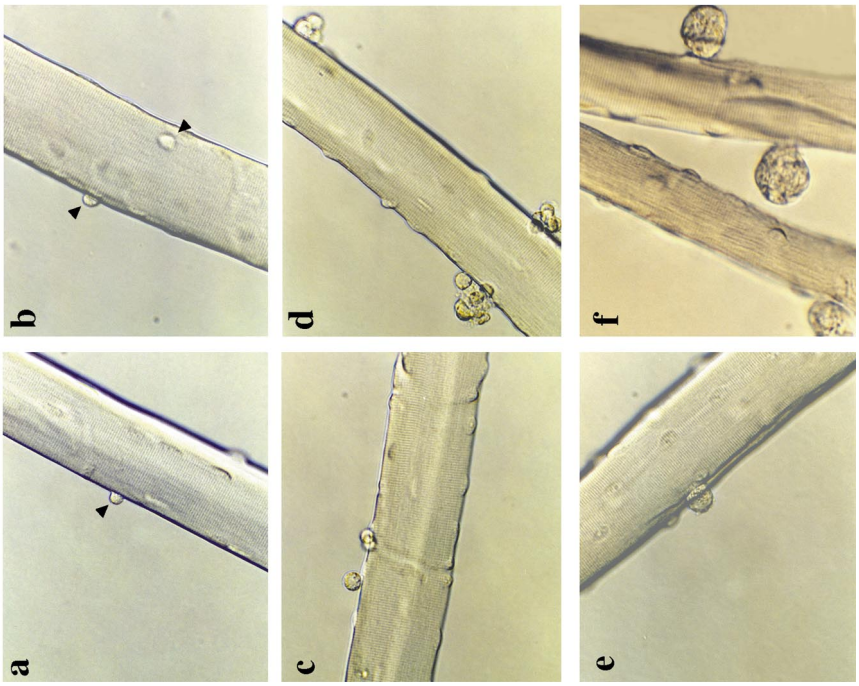

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FIG. 3. Wildtype (a, c, e) and MyoD$^{-/-}$ (b, d, f) satellite cells on myofibers at 24, 48, and 96 h in culture. Arrowheads in a and b point to individual satellite cells. Note the abnormal clustering of mutant cells at 48 h and formation of large multicellular "calluses" at 96 h in culture.

FIG. 4. Immunostaining for myosin heavy chain (MHC) on colonies derived from adherent satellite cells. Fibers and their associated satellite cells were cultured for 7 days in growth medium, followed by removal of parental myofibers and culture for additional 7 days in differentiation medium. (a) In wild-type cultures, most cells had become MHC^{+} and the majority of these are in multinucleate myotubes. (b) In cultures of MyoD$^{-/-}$ cells most cells are not MHC positive, and the majority of those that are MHC^{+} cells are round, mononucleate myocytes, while those myotubes which do form are abnormally stunted and have few myonuclei.

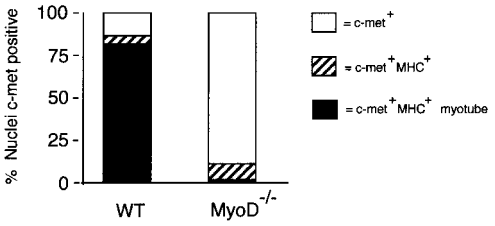


FIG. 5. Comparison of the percentage of c-met-expressing cells that coexpress MHC and c-met (hatched area) or coexpress MHC and have a myotube morphology (shaded area) in wild-type and MyoD$^{-/-}$ satellite cell cultures.

phological and developmental differences between wild-type and mutant satellite cells (described above), there were relatively few substantive differences in the survey expression profiles. However, among the RNAs that did differ substantially, we found several classes of variations: some appeared later in the mutant or not all while others were detected earlier or scored positive in the mutant and were consistently negative or delayed in wildtype. This overall observation also implies, perhaps not surprisingly, that changes in the mutant satellite cells must be more diverse and far-reaching than the mere absence of transcripts from genes regulated directly and positively by MyoD.

RNAs that scored negatively at some or all time points in mutant samples compared with positive scores in companion wild-type samples were MRF4, m-cadherin, twist, and cdk5. MRF4 and m-cadherin may help to explain the differentiation defect in MyoD mutants, but the meaning of the twist and cdk5 observations is not obvious. Twist can act as an inhibitor of myogenesis in cultured cells (Hebrok *et al.*, 1994, 1997; Hamamori *et al.*, 1997; Spicer *et al.*, 1996) and its absence from the differentiation-defective mutant was therefore unexpected. We examined it in more detail using the single-cell format together with simultaneous measurement of the MRFs. Surprisingly, twist expression in wild-type single cells appeared randomly distributed relative to MRF expression type, leaving its meaning mysterious (Cornelison and Wold, unpublished).

A simple mechanism that would further explain the differentiation-defective phenotype would be elevated expression of negative regulators of myogenesis in MyoD$^{-/-}$ satellite cells such as Id family members or cyclins. This notion received no support from the expression survey in which both genotypes gave similar data.

Genes that scored positive or were consistently stronger at some time points in MyoD$^{-/-}$ cells relative to wildtype included the cdk inhibitors (CDIs) p19, p21, and p27 and the MEF family factor MEF2A. The CDIs are modulators of cyclin-dependent kinases and their strong upregulation in wild-type myogenic cells has heretofore been associated with cell cycle arrest and the onset of muscle differentiation. p27 and p21 were reported to be targets of myogenin in tissue culture cells (Skapek *et al.*, 1995), and the same may be true in satellite cells in which myogenin is expressed in a subset of cells at an earlier time point (though in a smaller fraction of cells at later times). p19 was reproducibly detected in mutant pools at all times but was never de-

TABLE 2
Quantitative Analysis of Expression States

				Wildtype				MyoD-null			
MyoD	myf5	myg	MRF4	0 (%)	24 (%)	48 (%)	96 (%)	0 (%)	24 (%)	48 (%)	96 (%)
−	−	−	−	92	27	11	2	95	**30**	21	**30**
+	−	−	−	4	20	3	2	0	0	0	0
−	+	−	−	4	14	3	0	0	**61**	**56**	**47**
−	−	+	−	0	0	5	4	**5**	3	2	3
−	−	−	+	0	0	0	0	0	0	0	0
+	+	−	−	0	39	14	28	0	**0**	**0**	**0**
+	−	+	−	0	0	0	0	0	0	0	0
+	−	−	+	0	0	0	0	0	0	0	0
−	+	+	−	0	0	0	0	0	**6**	**21**	**20**
−	+	−	+	0	0	0	0	0	0	0	0
−	−	+	+	0	0	1	2	0	0	0	0
+	+	+	−	0	0	32	20	0	0	**0**	**0**
+	+	−	+	0	0	0	0	0	0	0	0
+	−	+	+	0	0	0	0	0	0	0	0
−	+	+	+	0	0	0	0	0	0	0	0
+	+	+	+	0	0	30	41	0	0	**0**	**0**

Note. Percentage occupancy for each possible coexpression state in single satellite cells from MyoD$^{+/+}$ or MyoD$^{-/-}$ fiber cultures. Expression state occupancy values for MyoD$^{-/-}$ samples with statistically significant differences relative to wildtype appear in boldface.

TABLE 3

Gene Expression Measured for Pools of Satellite Cells from either MyoD$^{+/+}$ or MyoD$^{-/-}$ Fiber-Associated Satellite Cells

	Wildtype				MyoD$^{-/-}$			
Hours:	0	24	48	96	0	24	48	96
MyoD	0/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3
Myf5	0/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3
Myogenin	0/3	0/3	3/3	3/3	0/3	1/5	3/3	3/3
MRF4	*0/3*	*0/3*	*3/3*	*3/3*	*0/3*	*0/3*	*0/3*	*0/3*
MEF2A	*0/3*	*1/5*	*2/4*	*1/4*	*0/3*	*5/5*	*3/3*	*4/4*
MEF2B	0/3	0/4	0/3	0/3	0/3	0/6	0/4	0/5
MEF2C	0/3	1/4	0/3	1/4	0/3	1/5	0/3	2/5
MEF2D	1/4	1/4	1/4	1/4	0/3	0/4	1/3	1/4
MEF2D-MS	0/3	1/3	0/3	3/3	0/3	0/4	1/5	1/4
Id1	1/4	2/5	4/7	4/6	0/3	4/5	2/3	4/5
Id2	0/3	4/7	4/7	4/6	0/3	2/8	5/7	5/6
Id3	0/3	1/5	3/5	2/4	0/3	1/7	2/5	2/5
Id4	0/3	0/5	0/4	1/4	0/3	0/7	1/5	0/5
Twist	*0/3*	*2/5*	*3/3*	*3/3*	*0/3*	*0/7*	*0/5*	*0/5*
FGFR1	3/5	3/3	3/4	3/3	3/4	2/5	3/3	3/4
FGFR2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
FGFR3	0/3	0/3	0/3	0/3	0/3	0/5	0/3	0/4
FGFR4	2/4	3/3	3/3	3/4	2/4	2/4	1/4	2/4
IGF1R	1/4	2/5	0/3	3/4	0/3	2/5	1/4	2/5
IGF2R	0/3	2/4	1/4	1/4	0/3	2/5	1/4	3/5
PDGFaR	1/5	2/5	0/3	0/3	0/3	2/5	0/4	3/5
PDGFbR	0/3	0/7	1/6	2/5	0/3	0/6	0/7	1/5
CycA	0/3	4/4	3/3	3/4	0/3	6/6	3/3	4/5
CycD1	0/3	2/5	0/3	0/4	0/3	1/5	2/4	1/4
CycD2	0/3	0/7	1/6	0/6	0/3	1/9	0/8	1/5
CycD3	0/3	3/3	3/3	3/3	0/3	2/4	3/3	3/4
CycE	0/3	0/4	0/4	0/3	0/3	0/4	0/4	0/3
P16	0/3	0/4	0/3	0/3	0/3	0/3	1/4	0/3
P18	0/3	0/4	1/4	2/4	0/3	0/4	1/4	1/5
P19	*0/3*	*0/4*	*0/3*	*0/3*	*3/4*	*1/4*	*2/4*	*2/4*
P21	*0/3*	*0/3*	*0/3*	*0/3*	*0/3*	*2/3*	*2/4*	*1/4*
P27	*0/3*	*1/5*	*1/4*	*2/4*	*2/4*	*2/5*	*3/4*	*3/5*
P57	0/3	0/4	3/4	0/3	0/3	0/4	0/3	0/4
Cdk2	0/3	0/3	0/3	2/4	0/3	1/5	3/3	0/4
Cdk4	0/3	3/3	3/4	3/3	0/3	3/3	3/4	3/4
Cdk5	*0/3*	*0/3*	*3/5*	*1/4*	*0/3*	*0/3*	*0/3*	*0/3*
HGF/SF	0/3	3/3	2/4	3/3	0/3	3/3	1/4	1/4
FGF1	0/3	0/3	0/3	0/3	0/3	1/6	0/6	0/4
FGF2	0/3	1/4	0/3	0/3	0/3	0/4	0/3	1/4
FGF4	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
FGF5	0/3	0/4	0/3	0/3	0/3	0/3	0/3	0/3
FGF6	0/3	0/4	0/3	0/3	0/3	0/3	0/3	0/3
FGF7	0/3	0/4	0/3	0/3	0/3	0/3	0/3	0/3
FGF8	0/3	0/4	0/3	0/3	0/3	0/3	0/3	0/3
FGF10	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
IGF1	0/3	0/4	0/3	0/3	0/3	0/4	0/4	0/3
IGF2	0/3	0/4	0/3	3/4	0/3	0/4	0/4	1/4
PDGFa	*0/3*	*0/4*	*1/4*	*0/4*	*0/3*	*4/4*	*1/5*	*1/4*
PDGFb	0/3	0/3	1/4	0/3	0/3	0/4	0/3	0/3
Pax3	0/3	0/4	0/3	0/3	0/3	0/3	0/3	0/3
GDF-8	*3/4*	*0/3*	*0/3*	*0/3*	*3/3*	*0/3*	*0/3*	*2/4*
Msx-1	*3/4*	*0/3*	*0/3*	*0/3*	*2/3*	*0/3*	*0/3*	*2/4*

Note. Samples of 1 cell equivalent from pools of 30 individually picked cells were assayed for individual genes. In this assay, the frequency of positives is a function of the percentage of cells within a population expressing a given gene, transcript number per cell, and the efficiency of reverse transcription and PCR on a gene-by-gene basis. Genes in italic highlight differences discussed in the text.

tected in wild-type satellite cells, suggesting that the mutant cells differ, at least with respect to this gene, from any stage of wildtype that we have assayed.

Finally, the expression of GDF-8 and Msx1-1 at time zero and their reappearance at 96 h in MyoD$^{-/-}$ pools is intriguing on two levels. First there are obvious functional implications for these genes as possible regulators of the quiescent state of wild-type satellite cells (see Discussion). Second, the observed reexpression of both RNAs in MyoD-deficient fiber cultures, but not in wildtype, supports the idea that some MyoD$^{-/-}$ cells might be returning to a quiescent state after several days of culture.

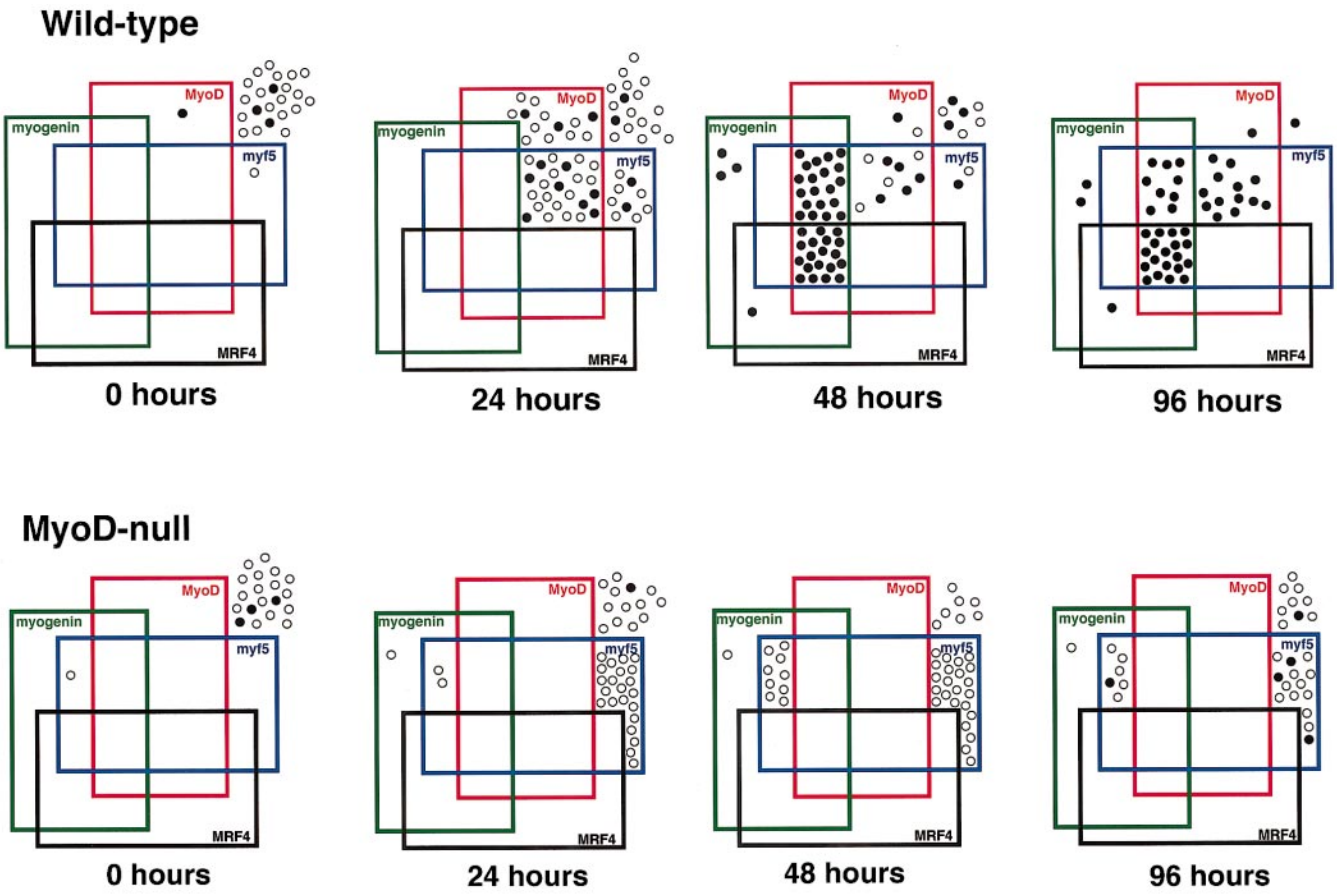
DISCUSSION

It has become increasingly clear in the past few years that distinct skeletal muscle sublineages, virtually all of which originate from paraxial mesoderm and eventually differentiate into similarly functioning mature muscle tissue, nevertheless use the core group of myogenic regulatory factors differently. In the developing myotome, for example, different populations of presumptive myoblasts destined to give rise to epaxial and hypaxial sublineages are preferentially dependent on either MyoD or myf5 for myogenic commitment and progression (Braun *et al.*, 1992; Cossu *et al.*, 1996; Rudnicki *et al.*, 1992, 1993; Tajbakhsh *et al.*, 1997; Kablar *et al.*, 1997). Another example from the embryo is the head muscle lineage, which displays clear differences from trunk and limb sublineages in MRF expression patterns and in the repertoire of muscle differentiation genes (Patapoutian, 1996). Considerably less is known about satellite cell development and its regulation. The lineage history of satellite cells remains very uncertain, and relatively little is known about how their expression is regulated in adult satellite cells or what each contributes functionally during regeneration. In this work we have examined the genetic requirements for MyoD for successful satellite cell myogenesis in isolated fiber explants, and our findings are schematized in Fig. 8. A central conclusion is that there is a strong epistasis relationship between MyoD and MRF4 in satellite cells that has not been observed in embryonic and fetal myogenesis. A weaker relationship between MyoD$^{-/-}$ status and myogenin expression was also indicated. Further molecular and cellular consequences associated with the MyoD deficit included a dramatic differentiation deficiency, failure of myocyte fusion, significantly lowered m-cadherin expression, and differences in RNA expression patterns for several potentially important regulators of growth, differentiation, and fusion together with multiple morphological differences in adult muscle tissue *in vivo*. The latter adult muscle phenotypes suggest chronic low level muscle injury and repair in the mutant.

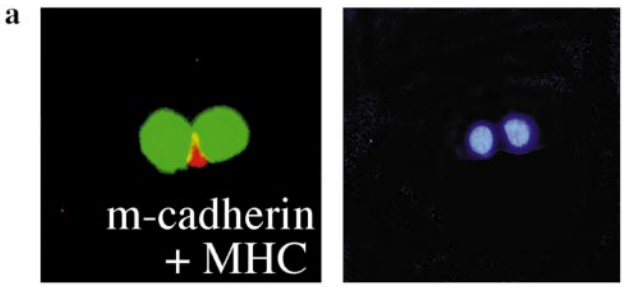
Previous analyses of single and double knockout mutations in the MRFs have shown that embryonic and fetal skeletal myogenesis depends on expression of multiple MRF family members in various combinations for different sublineages (reviewed in Molkentin and Olson, 1996; Yun and Wold, 1996). MyoD and myf5 apparently act in a lineage-preferential fashion to determine myogenic precursor status. Their roles in execution of differentiation are less certain, but myogenin is needed for efficient differentiation in a majority of muscle *in vivo*, and MRF4 on its own appears largely dispensable for differentiation in all settings examined thus far. In adult muscle, our previous study of MRF family coexpression in single wild-type satellite cells suggested that, once activated, an individual satellite could begin by expressing either MyoD or myf5, followed by coexpression of both genes prior to expression of myogenin and later MRF4 (Cornelison and Wold, 1997). That picture is very similar to what has been observed in embryonic lineages and it suggested that, as in the myotome, expression of either MyoD or myf5 might be sufficient for phenotypically complete myogenesis. Consistent with this we found here that a majority of MyoD$^{-/-}$ satellite cells appear to be efficiently activated in fiber cultures, based on cell morphology, on the kinetics of myf5 upregulation, and on the proportion of myf5-positive cells in the population. However, the subsequent failure of later stages in myogenic progression now suggests that there is a nonredundant requirement for MyoD in the satellite cell program.

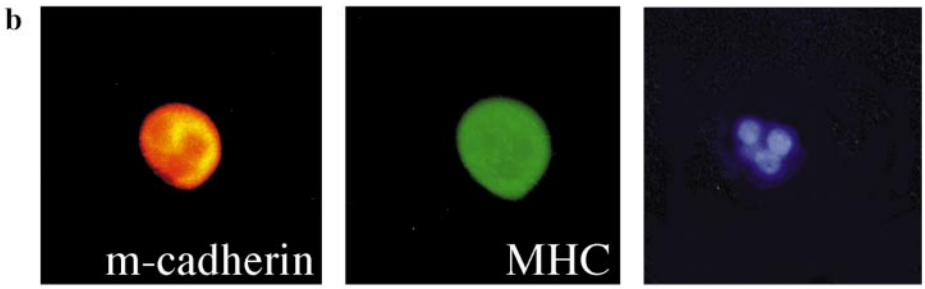
The expanded gene survey also identified several differences, even at time zero, between mutant and wild-type cells, which suggests an earlier and more pervasive influence of MyoD than simply facilitating differentiation. Although the pool survey is subject to caveats of interpretation that come from mixed cell populations and from detection thresholds (see discussion of these under Results), these data indicate that there are already differences that distinguish MyoD$^{-/-}$ satellite cells from wildtype, even in quiescence. Since quiescent satellite cells express no detectable MRF RNA, differences we see at this time suggest changes in cell lineage history or pleiotropic effects of the MyoD deficiency on adult muscle tissue.

Differences between satellite myogenesis and embryonic and fetal myogenesis. Among the genes studied in single-cell detail, the most striking genotype-specific effect was on MRF4. This differs significantly from the effect of MyoD mutation at earlier times in development. At birth MyoD$^{-/-}$ mice not only are phenotypically normal, but they also show levels of expression of myf5, myogenin, and MRF4 RNA that are indistinguishable from wildtype (Rudnicki *et al.*, 1992) In contrast, by 96 h in culture, a large fraction of wild-type satellite cells coexpress all four MRFs, but no MRF4 positives were detected in MyoD$^{-/-}$ cells, and myogenin positives were less frequent. This argues for an absolute and nonredundant requirement for MyoD to support proper MRF4 expression in activated satellite cells in addition to other epistasis relationships within the MRF family (Fig. 8a). The molecular mechanism responsible for this epistasis relationship is uncertain. The simplest hypothesis is that MyoD is required as a positive transcription



6 ○ = c-met^{+}m-cadherin^{-} ● = c-met^{+}m-cadherin^{+}





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factor to act on a satellite-cell-specific MRF4 enhancer. However, there is no direct evidence for this mechanism as yet, and the molecular pathway could be a more indirect one, including possible contributions from reduced myogenin.

Although the dependence of MRF4 on MyoD has not been observed at other times in muscle development, the impact on differentiation of the absence of both MRF gene products has a strong precedent. It was recently shown that animals doubly null for MRF4 and MyoD have a massive differentiation-defective phenotype *in vivo* that is similar to a myogenin null, including failure of most muscle differentiation and perinatal death (Rawls *et al.*, 1998). Thus the differentiation deficit observed for MyoD$^{-/-}$ satellite cells that fail to properly upregulate MRF4 is a second example of requirement for this particular pair of MRF family regulators for efficient myocyte differentiation and myotube fusion. In the satellite cells, this differentiation deficit may be further exacerbated by reduced myogenin.

An important general conclusion from this study was that the distinction between transcriptional regulatory pathways in satellite cell myogenesis versus fetal or embryonic myogenesis is not limited to the effects of MyoD on other MRFs. For example, the enhancer–transactivator interaction between Pax-3 and c-met, which is required for c-met expression in embryonic limb muscle precursors (Bladt *et al.*, 1995; Epstein *et al.*, 1996), is apparently unnecessary in limb muscle satellite cells. Thus, while c-met is robustly and constitutively expressed, satellites of both genotypes lacked Pax-3 RNA at detection limits well below levels easily detected in the embryo (Table 3 and extensive single-cell data not shown). The likely explanation is that the signaling pathways and transcription factors that drive c-met expression in satellites are different from those in migratory limb muscle precursor cells.

Myostatin and Msx1. An intriguing new finding was expression of myostatin (GDF-8) and Msx1 at time zero in satellite cells of both genotypes. At the latest time point (96 h) they were again detectable, but only in MyoD mutants. Both genes have interesting functional implications for establishing or maintaining a quiescent satellite state. Myostatin is a TGFβ superfamily factor that apparently functions to limit muscle tissue growth (McPherron *et al.*, 1997). Its expression at time zero in satellite cultures may be part of maintaining the arrested state and regulating the transition to the activated state. Msx1 (Hox7) is a transcriptional regulator (reviewed in Davidson, 1995), and during embryogenesis it is expressed in migratory muscle precursors of developing the limbs (Robert *et al.*, 1989). In transfection assays it is a potent transcriptional repressor (Catron *et al.*, 1996) and inhibitor of MRF expression and activity (Song *et al.*, 1992). Similar functions in satellite cells would be consistent with their precursor status. In the MyoD$^{-/-}$ fibers, some cells began to reexpress myostatin and Msx1 at the latest times studied. An attractive interpretation is that this reexpression marks a return to a quiescent phenotype by some cells on MyoD$^{-/-}$ fibers.

A developmentally "stalled" phenotype? Our results highlight the dependence of MRF4 expression on MyoD in satellite cells and suggest that the muscle differentiation defect in MyoD-deficient fiber cultures likely results from a joint deficit in MyoD and MRF4 activities. A concurrent study of growth and differentiation characteristics of low-passage-cultured *MyoD*$^{-}$ myogenic cells from younger animals revealed a differentiation deficit similar in many respects to that described here for adult muscle satellite cells (Sabourin *et al.*, 1999). These phenotypes differ interestingly from those of high-passage myoblasts studied in a previous work (Megeney *et al.*, 1996). Taken at face value, the contrast implies that extended mass culture of activated muscle precursor cells from MyoD$^{-/-}$ animals promotes a phenotypic shift to differentiation competence. Properties shared between our fiber culture satellite phenotype and the low-passage mass phenotype included delayed or reduced expression of myogenin, MRF4, and other differentiation markers; reduced levels of m-cadherin; and ultimately reduced cytodifferentiation and fusion, and many of these properties were also reported recently by Yablonka-Reuveni *et al.* (1999), working with fiber cultures. A model proposed by Sabourin *et al.* (1999) is that MyoD$^{-/-}$ muscle precursor cells become activated but are then "stalled" in a developmentally intermediate state that resembles early stages of wild-type muscle precursor cells which is followed by return of some cells to a quiescent phenotype. Some new support for the idea of return to the quiescent state is provided by our observations on myostatin and Msx1. However, in this work, we also found that the resemblance to immature activated wild-type cells is only partial. Thus, the MyoD$^{-/-}$ fiber satellite phenotype also included novel cellular (Fig. 3) and molecular (Table 3) characteristics that

FIG. 6. Summary representation of the combinatorial expression states of c-met, m-cadherin, MyoD, myf5, myogenin, and MRF4 in wild-type and MyoD$^{-/-}$ satellite cells during the first 4 days in fiber culture. Note that in the MyoD$^{-/-}$ cells there are some precocious myogenin positives at 0 and 24 h, complete absence of MRF4 positives at 48 and 96 h, and a general decrease of m-cadherin positives coupled with failure of myogenin expression to correlate with m-cadherin expression.

FIG. 7. Coimmunostaining of myosin heavy chain (green) and m-cadherin (red) proteins in differentiated cultures of adherent MyoD$^{-/-}$ satellite cells. (a) Among the relatively rare m-cadherin-positive cells, most reactivity is observed at the contact zone between two apposed mononucleate MHC^{+} myocytes which are in contact with each other but are not fused. (b) More rarely, expression more similar to that of most wild-type cells is seen, in which m-cadherin is more uniformly distributed. The yellow color in the m-cadherin image is an artifact of the TRITC filter set used and indicates more intense fluorescence than the red color.

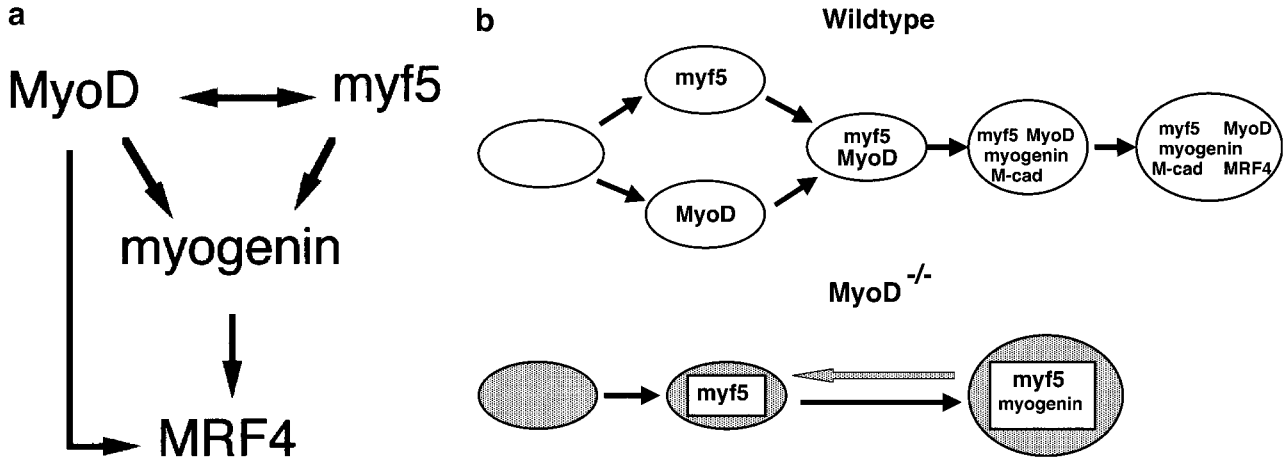

FIG. 8. (a) Genetic hierarchy for MRFs suggested by data presented here and by prior genetic analyses. The gray arrow indicates a relationship between MyoD and MRF4 which, thus far, appears to be specific for satellite cells. (b) Comparative summary models for MRF expression state progression in satellite cells from MyoD$^{+/+}$ and MyoD$^{-/-}$ mice. Gray reverse arrow indicates pathway suggested by the expression patterns but for which there is no direct evidence. In the MyoD$^{-/-}$ model, cells are shown shaded to indicate that they differ in additional phenotypic characteristics compared to wild-type cells expressing no MRFs, myf5 only, or myf5/myogenin. These differences include m-cadherin expression which is shown explicitly together with other differences (see Figs. 3, 4, and 5, Table 3, and text for further discussion).

were not observed for wild-type cultures at any stage tested. Taken together, the data from both studies argue that MyoD$^{-/-}$ muscle precursors, as a population, have novel, neomorphic properties combined with some properties of wild-type satellite cells in the early stages of activation (Fig. 8).

Possible implications for aging, exercise, and human dystrophies. At the level of the intact animal, our results indicate that even under the relatively sedentary conditions of standard research animal husbandry, there is ongoing regeneration in the muscles of MyoD$^{-/-}$ mice. MyoD may therefore be a candidate to cause a recessive muscular dystrophy in humans. Also, the results indicate that studies on the effects of aging and exercise challenge in MyoD$^{-/-}$ animals are warranted.

ACKNOWLEDGMENTS

This work was supported by grants from the Muscular Dystrophy Association and NIH NIAMS to B.J.W., grants from the Muscular Dystrophy Association and the NIH to B.B.O., grants from the NIH and the Muscular Dystrophy Association to M.A.R., and an ARCS Fellowship to D.D.W.C. M.A.R. is a Research Scientist of the National Cancer Institute of Canada and a member of the Canadian Genetic Disease Network of Excellence. D.D.W.C and B.B.O. thank Dr. Russell Moore for the use of his patch-clamp equipment and Dr. Marie Csete for her invaluable assistance.

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Received for publication January 5, 1999
Revised February 25, 2000
Accepted February 25, 2000