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Overlapping functions of the myogenic bHLH genes *MRF4* and *MyoD* revealed in double mutant mice

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SUMMARY

The myogenic basic helix-loop-helix (bHLH) genes – *MyoD*, *Myf5*, *myogenin* and *MRF4* – exhibit distinct, but overlapping expression patterns during development of the skeletal muscle lineage and loss-of-function mutations in these genes result in different effects on muscle development. MyoD and Myf5 have been shown to act early in the myogenic lineage to establish myoblast identity, whereas myogenin acts later to control myoblast differentiation. In mice lacking myogenin, there is a severe deficiency of skeletal muscle, but some residual muscle fibers are present in mutant mice at birth. Mice lacking MRF4 are viable and have skeletal muscle, but they upregulate myogenin expression, which could potentially compensate for the absence of MRF4. Previous studies in which *Myf5* and *MRF4* null mutations were combined suggested that these genes do not share overlapping myogenic functions in vivo. To determine whether the functions of MRF4 might overlap with those of myogenin or MyoD, we generated double mutant mice lacking MRF4 and either myogenin or MyoD. *MRF4/myogenin* double mutant mice contained a comparable number of residual muscle fibers to mice lacking myogenin alone and myoblasts from those double mutant mice formed differentiated multinucleated myotubes in vitro as efficiently as wild-type myoblasts, indicating that neither myogenin nor MRF4 is absolutely essential for myoblast differentiation. Whereas mice lacking either MRF4 or MyoD were viable and did not show defects in muscle development, *MRF4/MyoD* double mutants displayed a severe muscle deficiency similar to that in *myogenin* mutants. Myogenin was expressed in *MRF4/MyoD* double mutants, indicating that myogenin is insufficient to support normal myogenesis in vivo. These results reveal unanticipated compensatory roles for MRF4 and MyoD in the muscle differentiation pathway and suggest that a threshold level of myogenic bHLH factors is required to activate muscle structural genes, with this level normally being achieved by combinations of multiple myogenic bHLH factors.

Key words: Skeletal muscle, Myogenesis, MyoD, Myf5, Myogenein, MRF4, mouse

INTRODUCTION

Skeletal muscle has become a paradigm for understanding the mechanisms that control cell specification and differentiation during embryogenesis. The formation of skeletal muscle during mouse embryogenesis is controlled by four basic helix-loop-helix (bHLH) transcription factors – MyoD, Myf5, myogenin, and MRF4 – which are expressed in overlapping, but distinct patterns during development of the skeletal muscle lineage. These factors auto- and cross-regulate their expression and collaborate with members of the myocyte enhancer factor-2 (MEF2) family of MADS-box transcription factors to directly activate the transcription of muscle-specific genes at multiple points in the myogenic pathway (reviewed in Ludolf and Konieczny, 1995: Molkentin and Olson, 1996; Yun and Wold, 1996; Rudnicki and Jaenisch, 1995).

Myf5 is the first of the myogenic bHLH genes to be expressed during mouse embryogenesis, with transcripts appearing in the rostral dermomyotomes at embryonic day 8.0 (E8; Ott et al., 1991). Myogenin and MyoD are expressed in the myotome beginning at E8.5 and 10.0, respectively (Sassoon et al., 1989). MRF4 is expressed transiently in the myotome between E9 and E11.5 and is subsequently downregulated until later in development when it is expressed in differentiated muscle fibers (Hinterberger et al., 1991; Bober et al., 1991). The preferential expression of MRF4 in adult muscle fibers and the upregulation of MRF4 expression during differentiation of muscle cell lines in culture has suggested that MRF4 controls a late step in muscle development, similar to myogenin.

Gene knockout experiments have shown that MyoD and Myf5 play redundant roles in establishing myoblast identity; deletion of either gene alone has no effect on muscle

development, whereas deletion of both genes results in the complete absence of skeletal myoblasts (Rudnicki et al., 1992, 1993; Braun et al., 1992). In contrast, myogenin acts later to control myoblast differentiation (Hasty et al., 1993; Nabeshima et al., 1993). In mice lacking myogenin, myoblasts are specified and primary muscle fibers are formed, but secondary myogenesis fails to occur, resulting in a severe deficiency of skeletal muscle at birth (Venuti et al., 1995). There are, however, residual muscle fibers in *myogenin*-mutant neonates, suggesting the existence of a myogenin-independent pathway for muscle differentiation. Mice bearing compound null alleles of *myogenin* and *MyoD* or *myogenin* and *Myf5* do not show an enhancement of the muscle deficits seen in *myogenin*-mutant mice (Rawls et al., 1995). The functions of MRF4 have been more difficult to determine. Mice lacking MRF4 develop normal skeletal muscle and show about a four-fold increase in expression of myogenin (Braun et al., 1995; Patapoutain et al., 1995; Zhang et al., 1995), which raises the possibility that myogenin may compensate for the function of MRF4. MRF4 is also upregulated in the residual muscle fibers that form in *myogenin* mutant mice (Rawls et al., 1995), but whether MRF4 supports the differentiation of these residual muscle fibers has not been determined.

In simplest terms, the phenotypes of myogenic bHLH gene knockout mice have led to a two-step model for muscle development in which MyoD and Myf5 act first to establish the lineage and myogenin acts later to control terminal differentiation. It has generally been assumed that MRF4 must also have a late function in the myogenic pathway that potentially overlaps with that of myogenin, but there has been no direct evidence to support this conclusion.

In addition to their roles in skeletal muscle development, Myf5, myogenin and MRF4 have been shown to influence rib development, which has been attributed to an influence of the developing myotome on the adjacent sclerotome, from which the ribs are derived (Grass et al., 1996; Floss et al., 1996). In *Myf5*-null mice, the distal regions of the ribs fail to form and in *myogenin*-null mice, the ribs are malformed (Braun et al., 1992; Hasty et al., 1993). Three different null mutations have been introduced into the *MRF4* gene, with remarkably different effects on rib development (reviewed in Olson et al., 1996). The *MRF4* and *Myf5* genes are linked in a head-to-tail orientation, separated by about 8.5 kb, in the mouse genome. The most severe *MRF4* mutant allele resulted in a phenocopy of the rib defects associated with the *Myf5* null mutation due to a *cis*-effect that extinguished *Myf5* expression (Braun et al., 1995; Floss et al., 1996). Skeletal muscle development occurred relatively normally in these *MRF4* mutant mice, demonstrating that MyoD and myogenin are sufficient to support muscle formation. Mice homozygous for the least severe *MRF4* allele were viable and exhibited only minor rib defects (Zhang et al., 1995), which were also ascribed to a *cis*-effect on *Myf5* expression (Yoon et al., 1997). The disruption of *Myf5* expression with these various *MRF4* alleles is due to *Myf5* regulatory elements within or close to the *MRF4* gene (Yoon et al., 1997; P. Rigby, personal communication).

To further define the regulatory relationships between *MRF4* and the other myogenic bHLH factors, we combined the *MRF4* null mutation from our laboratory (Zhang et al., 1995), which does not affect viability, with null mutations in *myogenin* and *MyoD*. Here we report that mice bearing null mutations in *MRF4* and *myogenin* contain a comparable number of residual muscle fibers to *myogenin*-null mice and myoblasts from mice of the single and double mutant genotypes are able to differentiate in culture. These results demonstrate that myogenin and MRF4 do not play compensatory roles in the control of myoblast differentiation. We also show that, although mice bearing null mutations in *MyoD* or *MRF4* are viable, combination of these two null mutations results in a severe skeletal muscle deficiency. These results suggest that MRF4 and MyoD have overlapping functions in the muscle differentiation pathway and support a model in which a threshold level of expression of myogenic bHLH factors is required to trigger the differentiation program.

MATERIALS AND METHODS

Intercrosses and genotyping

The *myogenin* mutant mice were described previously (Hasty et al., 1993). The *MRF4* mutant mice used for this study have also been described (Zhang et al., 1995). These mice harbor an *MRF4* null allele and are fully viable as homozygotes. The *MyoD* mutant mice were a generous gift from Dr M. Rudnicki (McMaster University) and have been previously described (Rudnicki et al., 1992). The mutations were maintained in a C57Bl6 background.

Mice carrying mutations in *myogenin*, *MyoD* and *MRF4* were identified by Southern blot analysis. Genomic DNA was isolated as described previously (Zhang et al., 1995). Briefly, tissue from the tail was digested in lysis buffer (10 mM Tris, pH 8.0, 25 mM EDTA, 100 mM NaCl, 1% SDS, 0.2 mg/ml proteinase K) at 55°C overnight, followed by removal of protein by phenol/chloroform extraction and ethanol precipitation. DNA was digested with restriction endonucleases, separated on a 0.8% agarose gel, and transferred to Zeta-probe GT membranes. The presence of the mutant and wild-type alleles was determined by probing the membrane with a gene-specific ^{32}P-labeled DNA fragment, followed by autoradiography. The *myogenin* mutant allele was detected as described by Rawls et al. (1995). Genomic DNA was digested with *Sac*I and the membrane was probed with a 350 bp *Sma*I-*Kpn*I fragment of the *myogenin* promoter (Edmondson et al., 1992). The *MyoD* mutant allele was detected by digesting DNA with *Xba*I and probing with a 700 bp fragment from the 5′ end of the MyoD cDNA. The *MRF4* mutant and wild-type alleles were distinguished by digestion of genomic DNA with *Kpn*I and probing with a 300 bp fragment from the first exon of *Myf5*.

Histology and immunostaining

The preparation, sectioning, and staining of embryonic muscle was performed using standard procedures. Briefly, neonatal mice were skinned and eviscerated, followed by fixation in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C. Neonates were embedded in paraffin after a stepwise dehydration with progressively higher concentrations of ethanol and two changes of xylene. 7 μm transverse sections were stained with hematoxylin and eosin. Immunohistochemistry on paraffin-embedded thin sections was performed with the mouse embryonic myosin heavy chain (MHC) antibody (Sigma) as described previously (Rawls et al., 1995). Cartilage and bone were stained using the method described by McLeod (1980).

Cell culture and immunostaining

Primary myoblast cultures were prepared from the limbs of wild-type and mutant neonates as described previously (Rawls et al., 1995). To induce differentiation, cultures were transferred from growth medium to Dulbecco's modified Eagle's Medium (DMEM) with 2% horse serum.

MHC expression was detected by immunostaining as described

(Rawls et al., 1995), using an anti-embryonic MHC antibody (Sigma) and a biotinylated goat anti-mouse antibody (Sigma). Following removal of unbound antibody, an avidin-conjugated FITC-labeled secondary was used and MHC-expressing cells were detected by immunofluorescence.

RNA isolation and reverse transcription-PCR

Total cellular RNA was isolated from the carcasses of wild-type and mutant mice, using the TRIzol method (Gibco, BRL), as described (Rawls et al., 1995), following removal of the head and internal organs. Northern blot analysis was performed using standard techniques. Acetylcholine receptor (AChR)-δ transcripts were detected using a full-length cDNA (provided by J. Sanes, Washington University School of Medicine). Muscle creatine kinase (MCK), MHC, α-skeletal actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were detected using partial EST cDNA clones (Genome Systems).

To detect muscle-specific transcripts by RT-PCR, the method described by Munsterberg et al. (1995) and modified by Rawls et al. (1995) was used. Total RNA was used as a template for reverse transcription using Mo-MuLV reverse transcriptase (Gibco, BRL) and a random hexamer primer. A typical reaction included 1 μg of RNA in 50 mM Tris (pH8.3), 75 mM KCl, 3 mM $MgCl_2$, 3.3 mM dithiothreitol, 0.5 mM of each dNTP, 200 ng random hexamer primer, RNase inhibitor, and 200 U of reverse transcriptase in a total volume of 30 μl, incubated at 42°C for 1 hour. A typical PCR reaction contained, 2 μl of the RT reaction in 10 mM Tris (pH7.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 100 ng of each primer, 2.5 U Taq polymerase (Boehringer-Mannheim Biochemicals) and 0.1 μl of [α-^{32}P]dCTP (3000 Ci/mmol) (NEN) in a 50 μl total volume. A typical temperature profile included DNA strand melting at 95°C for 1 minute, primer annealing at 62°C for 1 minute, followed by polymerization at 72°C for 30 seconds. The number of cycles required to generate a PCR product during linear amplification was determined for each primer pair. PCR products were separated on a 6% polyacrylamide gel and visualized with a phosphoimager. Primers for detecting muscle specific transcripts and sizes of PCR products were: MyoD (Hannon et al., 1992, with minor modifications), 5′-GCAGGCTCTGCTGCGCGACC-3′ and 5′-TGCAGTCGATCTCTCAAAGCACC-3′, with a 370 bp PCR product; Myf5 (Hannon et al., 1992) 5′-TGTATCCCCTCACCAGAGGAT-3′ and 5′-GGCTGTAATAGTTCTCCACCTGTT-3′, with a 379 bp PCR product; MRF4 (Patapoutian et al., 1995) 5′-CTACATTGAGCG-TCTACAGGACC-3′ and 5′-CTGAAGACTGCTGGAGGCTG-3′, with a 235 bp PCR product; myogenin (Edmonson et al., 1989) 5′-TGGAGCTGTATGAGACATCCC-3′ (nt −45 to −65) and 5′-TGGACAATGCTCAGGGGTCCC-3′ (nt −229 to −209), with a 184 bp PCR product. To ensure that equivalent amounts of total RNA were used in different samples, template primers specific to the ribosomal protein L7, which is not affected by myogenesis (Hollenberg et al., 1993), 5′-GGAGCTCATCTATGAGAAGGC-3′ (nt −231 to −251) and 5′-AAGACGAAGGAGCTGCAGAAC-3′ (nt −432 to −412), with a 202 bp PCR product, were used.

In situ hybridization

In situ hybridization to detect myogenin transcripts in thin sections was performed using ^{32}P-labeled myogenin cDNA probe as described previously (Venuti et al., 1995).

RESULTS

Muscle development in *MRF4/myogenin*-null mice

Our initial interest was to determine whether MRF4 shared overlapping functions with myogenin. If so, then it might be expected that combining the mutations in these genes would result in phenotypes more severe than with either mutant gene alone. We were particularly interested in determining whether MRF4, which is expressed at high levels in the residual muscle fibers in *myogenin*-null mice (Rawls et al., 1995), was responsible for the differentiation of this subpopulation of skeletal muscle cells. We therefore intercrossed mice heterozygous for null mutations in *MRF4* and *myogenin* and examined the effects on musculoskeletal development. Mice arising from these intercrosses were genotyped at birth or within a few days thereafter. Nine potential genotypes are expected from the double heterozygous intercross, with the double null phenotype being obtained at a frequency of 6.25%. The *MRF4*(+/–)/*myogenin*(+/–) intercross yielded neonates of all nine genotypes at approximately the predicted Mendelian frequencies (Table 1). Only the offspring bearing the homozygous *myogenin* null mutation were nonviable; all others survived to adulthood, appeared normal, and were fertile. We also intercrossed *MRF4*(–/–)/*myogenin*(+/–) mice and obtained offspring of the predicted genotypes (Table 2). Thus, a single wild-type *myogenin* allele was sufficient to support normal development in the absence of *MRF4*, indicating that the absence of MRF4 does not sensitize the animal to the level of myogenin.

To determine the consequences of the combined null mutations on muscle formation, we analyzed histological sections of muscle from wild-type and double mutant neonates. As reported previously, the presumptive muscle-forming regions of *myogenin*(–/–) mice are populated primarily by unfused myoblasts, but contain residual skeletal muscle fibers (Fig. 1A,B) that express MRF4 (Rawls et al., 1995). In contrast, the skeletal muscle of *MRF4*-null mice appears normal (Braun et al., 1995; Patapoutain et al., 1995; Zhang et al., 1995). Mice bearing homozygous null mutations in *MRF4* and *myogenin* contained a comparable number of residual muscle fibers to *myogenin*-null mice (Fig. 1C). These findings demonstrate that MRF4 does not support differentiation of the residual fibers in *myogenin* mutant mice and suggests that

Table 1. Genotypes of offspring from *MRF4 (+/–); myogenin (+/–)* intercrosses

	Genotype								
	1	2	3	4	5	6	7*	8*	9*
MRF4	+/+	+/+	+/–	+/–	–/–	–/–	–/–	+/–	+/+
Myogenin	+/+	+/–	+/+	+/–	+/+	+/–	–/–	–/–	–/–
Observed no.	12	23	24	50	12	23	12	23	13
Predicted %	6.25	12.5	12.5	25.0	6.25	12.5	6.25	12.5	6.25
Observed %	6.28	12.0	12.6	26.2	6.28	12.0	6.28	12.0	7.0

*Denotes genotypes that were lethal at birth.

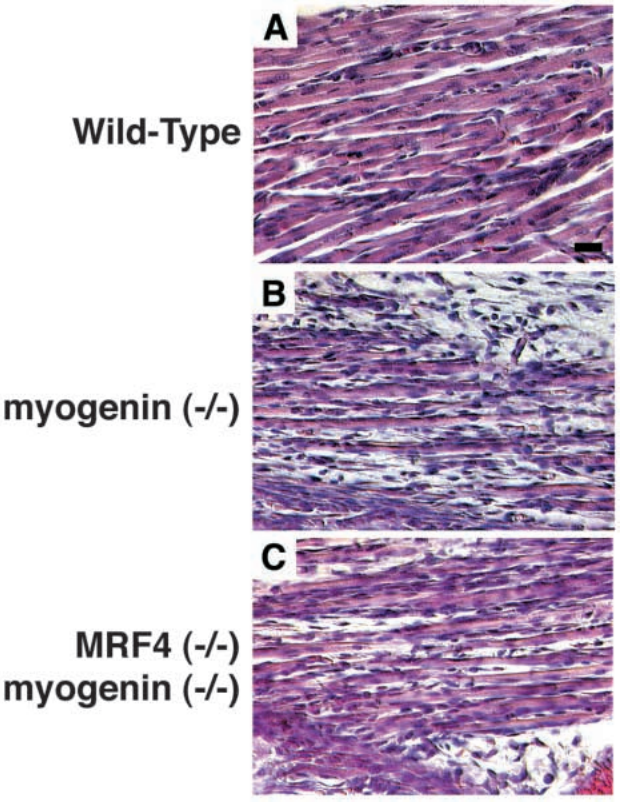

Fig. 1. *Myogenin(–/–)* and *myogenin(–/–)/MRF4(–/–)* neonates contain comparable numbers of residual muscle fibers. Thin sections were cut through the hind limbs of wild-type (A), *myogenin*(–/–) (B), and *MRF4*(–/–)/*myogenin*(–/–) (C) neonates and were stained with H & E. There was a deficiency in muscle fibers in mice of the two mutant genotypes, but comparable numbers of residual fibers were seen. Bar in A, 38 μm.

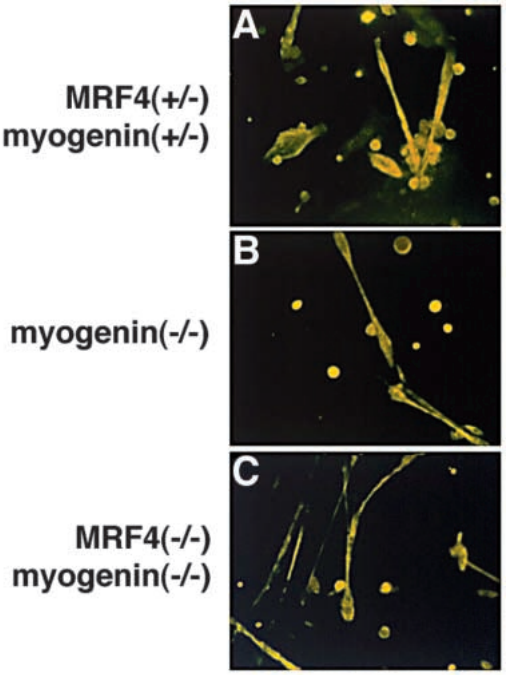

Fig. 2. Differentiation of *myogenin/MRF4* double mutant myoblasts in vitro. Primary myoblasts were isolated from hind limbs of mice of the indicated genotypes. After culturing, as described in Materials and Methods, cells were stained with anti-MHC antibody. Cells of the three genotypes differentiated equivalently.

MyoD and/or Myf5 may be able to activate myogenesis in the absence of myogenin and MRF4.

Myoblasts from *MRF4/myogenin* double mutants differentiate in vitro

Although myogenin is required for normal myoblast differentiation in vivo (Nabeshima et al., 1993; Rawls et al., 1995), myoblasts from *myogenin*-null mice differentiate normally in vitro. Because MRF4 is upregulated when *myogenin*-null myoblasts differentiate (Rawls et al., 1995), we sought to determine whether MRF4 regulated differentiation of these cells. Myoblasts were isolated from the hindlimbs of wild-type and mutant neonates and were cultured in the presence of low serum to promote differentiation. Under these conditions, wild-type and mutant myoblasts formed multinucleated myotubes and stained for MHC (Fig. 2). We detected no difference in the rate or extent of fusion of wild-type, *myogenin*-null, or *myogenin/MRF4* mutant myoblasts. These results demonstrate that myogenesis can occur normally in vitro in the absence of MRF4 and myogenin.

Muscle development in *MRF4/MyoD*-null mice

We also combined the *MyoD* and *MRF4* null mutations to test for possible overlapping functions of these genes. Because mice homozygous for null mutations in either gene alone are viable and fertile, we intercrossed *MRF4* and *MyoD* homozygous mutants to obtain *MRF4*/*MyoD* double heterozygotes, which were used to obtain the compound null mutants. Mice heterozygous for null mutations in either gene in the background of the other homozygous null allele were viable, whereas *MRF4*/*MyoD* double null mice did not survive beyond birth. We therefore established a population of mice of the genotype *MRF4*(–/–)/*MyoD*(+/–), which were bred to obtain double null offspring. Mutant neonates were obtained at the predicted mendelian frequency from *MRF4*(–/–)/*MyoD*(+/–) intercrosses (Table 3) and from double heterozygous intercrosses (data not shown).

MRF4/*MyoD* double mutants were grossly indistinguishable from wild-type up to about E14.5 (not shown), after which time they showed curvature of the spine, an apparent lack of skeletal muscle, and an accumulation of fat at the apex of the neck (Fig. 3A). At a gross level, this phenotype was identical to the *myogenin* mutant phenotype (Hasty et al., 1993; Nabeshima et al., 1993). All *MRF4*/*MyoD* double mutants died within minutes after birth, apparently due to an inability to breathe.

To determine whether the *MRF4*/*MyoD* double mutation resulted in muscle defects, we examined H&E sections of various muscle-forming regions of double mutants at E16.5. Wild-type fetuses contain well-developed skeletal muscle fibers at this stage. In contrast, the double mutants showed a severe muscle deficiency, with only residual muscle fibers surrounded by mononucleated cells (Fig. 3B-G). The residual muscle fibers in the double mutants contained centrally located nuclei, in contrast to normal muscle at this stage, in which the nuclei are peripherally located. Staining of muscle sections

with anti-MHC antibody also demonstrated a dramatic reduction in differentiated muscle cells in the double mutants (Fig. 3H,I). At a histological level, the muscle defects in *MRF4/MyoD* mutants were indistinguishable from those in *myogenin* mutants and were clearly distinct from the defects described in *Myf5/MyoD* mutants, in which myoblasts are completely lacking (Rudnicki et al., 1993). The muscle defects in the *MRF4*/*MyoD* mutants suggested that MRF4 and MyoD may play at least partially redundant roles in the regulation of myoblast differentiation.

Muscle gene expression in *MRF4/myogenin* and *MRF4/MyoD* double mutants

To further characterize the nature of muscle defects in *MRF4/MyoD* double mutants, we analyzed the expression of a series of muscle-specific mRNAs in neonates of different mutant genotypes. Expression of most muscle transcripts, such as MCK, MHC and α-skeletal actin, was reduced in *myogenin* mutants, but was relatively unaffected in mice lacking either *MRF4* or *MyoD* alone (Fig. 4A). In *MRF4/myogenin* double mutants, the above transcripts were expressed at levels comparable to those of *myogenin* mutants. The low level of muscle gene expression in *myogenin* and *myogenin/MRF4* mutants reflects the presence of residual muscle fibers in these mice. Consistent with the severe muscle deficiency in *MRF4*/*MyoD* double mutants, expression of markers of muscle differentiation was reduced in offspring of this genotype to levels comparable to those in *myogenin* mutants (Fig. 4A). GAPDH mRNA, which is expression independently of the state of muscle development, was measured as a control for RNA integrity and equal loading on the gel.

Previously, we reported that the δ-subunit of the (ACh) receptor, unlike other muscle-specific gene products, was not dependent on myogenin for expression (Hasty et al., 1993). Similarly, ACh receptor δ-subunit transcripts were unaffected in *MRF4* or *MyoD* mutants. However, in *MRF4/myogenin* and *MRF4/MyoD* double mutants, this transcript was downregulated (Fig. 4A). This suggests some level of redundancy between MRF4 and myogenin or MyoD in regulation of ACh receptor δ-subunit expression.

Transcripts encoding the four myogenic bHLH factors were also measured in neonates of the different genotypes by semi-

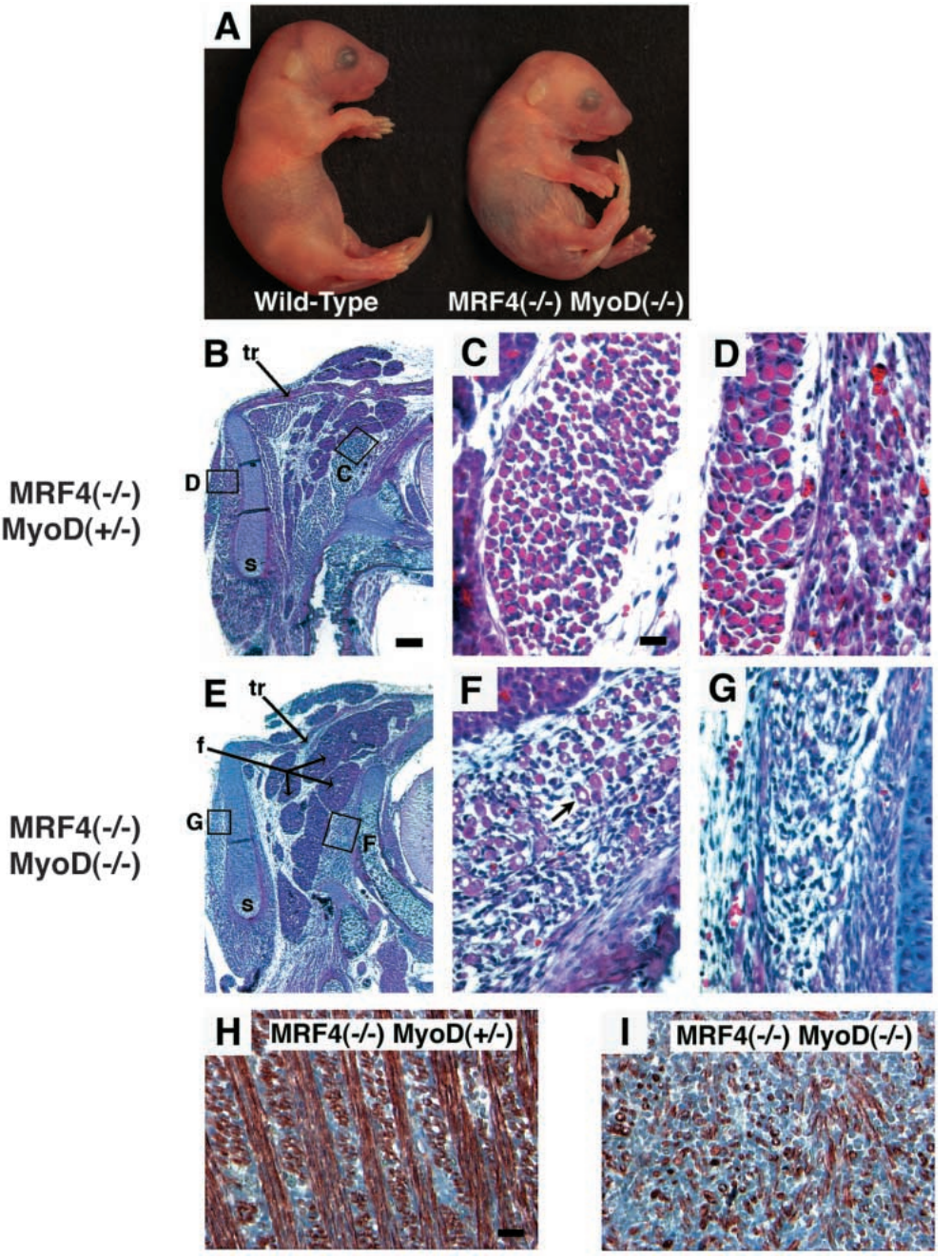

Fig. 3. Muscle defects in *MRF4/MyoD* double mutants. (A) Wild-type (left) and *MRF4/MyoD* double mutant (right) neonates. Note the reduction in body mass, severe kyphosis, and thickening of the neck in the mutant. (B,E) show H&E-stained sections through the upper thoracic region of wild-type and *MRF4/MyoD* double mutants, respectively, at E16.5. C,D and F,G show enlargements of the regions indicated by boxes in B and E, respectively. The arrows in E point to brown fat deposits (f) at the back of the neck. Arrow in F points to residual muscle fibers. (H, I) The intrinsic and extrinsic muscles of the tongue stained with anti-MHC antibody. s, scapula; tr, trapezius muscle. Bars, 96 μm in B and E, 9 μm in C,D,F and G, and 24 μm in H and I.

Table 2. Genotypes of offspring from *MRF4 (–/–); myogenin (+/–)* intercrosses

	Genotype		
	1	2	3*
MRF4	–/–	–/–	–/–
Myogenin	+/+	+/–	–/–
Observed no.	25	50	22
Observed %	25.8	51.5	22.7
Predicted %	25.0	50.0	25.0

*Denotes genotypes that were lethal at birth.

quantitative RT-PCR (Fig. 4B). As reported previously (Zhang et al., 1995), myogenin mRNA was upregulated several-fold in *MRF4* mutants. Myogenin expression was unaffected in *MyoD* mutants and in *MRF4/MyoD* double mutants. Thus, despite the similarity in muscle defects in *MRF4/MyoD* and *myogenin* mutants, these defects cannot be attributed to a lack of myogenin expression in *MRF4/MyoD* mutants. In *MRF4/myogenin* mutants, MyoD expression was not significantly affected, but surprisingly, Myf5 mRNA was not expressed above background levels. Since Myf5 expression was unaffected in neonates lacking either MRF4 or myogenin alone, these results suggest that MRF4 and myogenin play redundant roles in maintenance of Myf5 expression. L7 transcripts, which are expressed constitutively, were used as an internal control.

Detection of myogenin transcripts in presumptive muscle-forming regions by in situ hybridization

To further investigate whether the unexpected muscle deficits in *MRF4/MyoD* mutants might arise from a lack of myogenin expression, we examined myogenin mRNA expression by in situ hybridization to the muscle-forming regions of wild-type and *MRF4/MyoD* mutants at E17.5. As shown in Fig. 5, myogenin transcripts were detected throughout the muscle-forming regions of the trunk and limbs. Although the double mutant lacked the well-defined muscle groups seen in wild-type embryos, myogenin transcripts were expressed throughout the presumptive muscle-forming regions of the mutants. These results are consistent with the RT-PCR experiments, which showed that myogenin was expressed at near normal levels in *MRF4/MyoD* double mutants, despite the fact that muscle differentiation was severely impaired in these mutants.

Threshold levels of total myogenic bHLH factors are required for normal myogenesis

If there is a critical threshold of myogenic bHLH factor expression necessary to trigger the differentiation program, and myogenin, MyoD and MRF4 each contribute to achieving this threshold, then one might predict that reducing the level of myogenin by one-half would sensitize the animal to the level of MyoD and MRF4, and result in a muscle deficit in mice carrying a single copy of *myogenin* and *MyoD* in the absence of *MRF4*. We tested this possibility by generating mice of the genotype *myogenin*(+/–); *MyoD*(+/–); *MRF4*(–/–). These mice appeared normal, were viable, and fertile. Thus, a single *myogenin* and *MyoD* gene are necessary and sufficient to support muscle differentiation in the absence of *MRF4*. However, when the myogenic bHLH genotype was reduced to: *myogenin(+/–)*; *MyoD(–/–)*; *MRF4(–/–)* myogenesis appeared to be arrested at the level observed in the *myogenin* null mutants.

Table 3. Genotypes of offspring from *MRF4 (–/–); MyoD (+/–)* intercrosses

	Genotype		
	1	2	3*
MRF4	–/–	–/–	–/–
MyoD	+/+	+/–	–/–
Observed no.	8	14	6
Observed %	29	50	21
Predicted %	25	50	25

*Denotes genotypes that were lethal at birth.

Rib defects in *MRF4/myogenin* and *MRF4/MyoD* mutant mice

Because the *Myf5*, *myogenin* and *MRF4* mutations result in rib defects, it was of interest to determine whether these rib anomalies were exacerbated by the double null mutations. As reported previously (Zhang et al., 1995), the distal portions of the ribs in our *MRF4*-null mice show mild anomalies that include bifurcations, fusions and supernumerary processes (Fig. 6). *Myogenin*-null mice also show deformations in the rib cage and the absence of intersternebral cartilage (Hasty et al., 1993). When the *MRF4* and *myogenin* mutations were combined, we observed an exaggerated rib phenotype in which the distal portions of the ribs failed to reach the sternum and the sternebral bodies were severely malformed. The upper region of the sternum of double mutants was also bifurcated. The rib defects in the *MRF4/myogenin* double mutants appeared to be more severe than the combined defects of the individual *MRF4* and *myogenin* null mutations. *MRF4*/*MyoD* double mutants showed rib defects indistinguishable from those of *MRF4* mutant mice (Fig. 6). These results suggest that Myf5 expression is not dramatically altered in these mice or the rib defects would have been expected to be more severe.

DISCUSSION

The generation of mice bearing mutations in different pairs of the myogenic bHLH genes has been a powerful means of revealing shared and unique functions of these genes and placing them in the skeletal myogenic pathway. Previous studies demonstrated that MyoD and Myf5 play overlapping roles in myoblast specification (Rudnicki et al., 1993), whereas myogenin plays a unique role in differentiation (Hasty et al., 1993; Nabeshima et al., 1993). Combining the *myogenin* null mutation with null mutations in either *MyoD* or *Myf5* failed to reveal novel phenotypes that were not exhibited with null mutations in the individual genes (Rawls et al., 1995). These results were consistent with a model in which the in vivo functions of myogenin are distinct from those of MyoD and Myf5.

Based on the preferential expression of MRF4 in mature muscle fibers (Rhodes and Konieczny, 1989), the upregulation of myogenin in *MRF4* mutant mice (Zhang et al., 1995), and the expression of MRF4 in residual differentiated muscle fibers in *myogenin* mutant mice (Rawls et al., 1995), it has been suggested that MRF4 plays a role in late stages of muscle development that may overlap with the functions of myogenin.

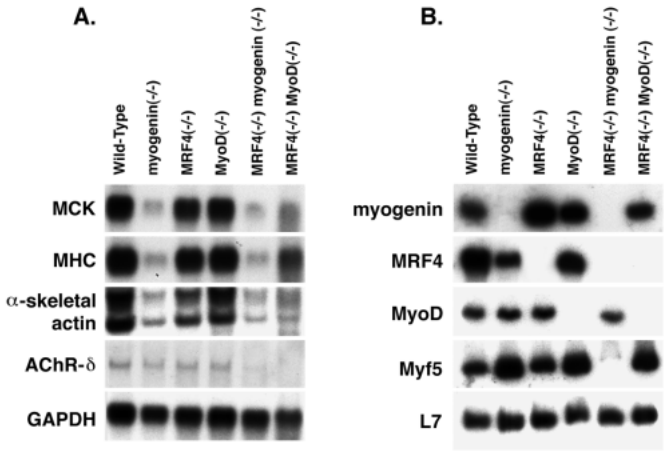

Fig. 4. Analysis of muscle transcripts in wild-type and mutant neonates. Total RNA was isolated from the carcasses of wild-type and mutant neonates as described in Materials and Methods. (A) Northern blots of indicated muscle transcripts. (B) RT-PCR products for each myogenic bHLH transcript and L7 as a loading control.

However, the results of the present study demonstrate that some muscle fibers do form in *MRF4/myogenin* mutant mice, indicating that neither of these genes is essential for activation of the differentiation program. Rather, our results suggest unexpected overlapping roles for MRF4 and MyoD in the control of myoblast differentiation in the embryo.

Myoblast differentiation in the absence of myogenin and MRF4

The presence of residual differentiated muscle fibers in *myogenin* mutant mice reveals the existence of a myogenin-independent pathway for myoblast differentiation in vivo. The finding that residual fibers are also present in *myogenin*/*MRF4* double mutants indicates that MyoD or Myf5, in addition to their role in myoblast specification, are also capable of activating differentiation in a subset of myoblasts in vivo. Because residual fibers are also found in *myogenin*/*MyoD* and *myogenin*/*Myf5* double mutants (Rawls et al., 1995), it appears that no single myogenic factor is essential for differentiation although myogenin is clearly essential for the differentiation of most myoblasts. In addition, because Myf5 is not significantly expressed in *MRF4/myogenin* double mutant neonates, MyoD alone appears to control differentiation of at least a subset of myoblasts. These findings support the notion that any of the myogenic bHLH factors can activate the muscle differentiation program under the appropriate conditions.

Why does myogenin play such an important role in myoblast differentiation in vivo, whereas in culture, *myogenin*-null myoblasts differentiate apparently normally? The abilities of the myogenic bHLH factors to initiate myogenesis is known to be exquisitely sensitive to extracellular influences such as peptide growth factors, extracellular matrix molecules, and cell-cell interactions (reviewed in Olson, 1993). Insulin-like growth factors, for example, stimulate myogenesis, whereas type β-transforming growth factor (TGF-β) and fibroblast growth factor (FGF) inhibit myogenesis by blocking the expression and transcriptional activity of the myogenic factors. TGF-β1 and FGF can also collaborate to induce myogenesis in somites (Stern et al., 1997). Recently, a TGF-β related factor termed myostatin has also been shown to be a potential inhibitor of skeletal myogenesis, acting as an autocrine factor (McPherron et al., 1997). By creating chimeric mice containing wild-type and *myogenin*-mutant myoblasts, we have shown previously that *myogenin*-mutant myoblasts can readily fuse with wild-type myoblasts (Myer et al. 1997). These results imply that myogenin controls the expression of one or more extracellular proteins required to initiate the muscle differentiation program. *Myogenin*-mutant myoblasts apparently can respond to these extracellular factors, but cannot generate them. It is possible that the extracellular environment is more permissive for activation of the differentiation program in vitro and the myogenin-dependent extracellular signal(s) can be bypassed.

Muscle defects in *MRF4/MyoD* double mutants

A surprising result from this study was that, while neither MyoD nor MRF4 alone is required for muscle development, *MRF4*/*MyoD* double mutants exhibited severe skeletal muscle defects, similar to those seen in *myogenin* mutants. Since myogenin and Myf5 were expressed in these double mutants, these findings suggest that the overall concentration of myogenic bHLH factors may need to achieve a critical threshold for myoblast differentiation to occur. In the absence of MRF4 and MyoD, myogenic bHLH protein levels are insufficient to trigger the differentiation program. Alternatively, the severe muscle deficiency in *MRF4/MyoD* double mutants could also indicate that MRF4 shares a specific myogenic function with MyoD that cannot be compensated for by myogenin or Myf5. If the latter explanation is correct, it might be expected that the residual fibers present in the *myogenin*-mutants would be distinct from those in the *MRF4/MyoD* mutants.

If myogenin is a stronger activator of the muscle differentiation program than either MyoD or MRF4, but all three factors contribute to achieving a threshold level of myogenic bHLH protein expression required for initiating myogenesis, reducing the level of myogenin expression in a *myogenin(+/–)* background might sensitize the animal to the level of MyoD and MRF4 and result in muscle deficits when animals were homozygous for one of these mutant alleles and heterozygous for the other. However, we found that *myogenin*(+/–); *MyoD*(+/–); *MRF4*(–/–) mice were normal. Thus, the level of bHLH protein expression from a single *myogenin* and *MyoD* allele is sufficient to support normal muscle development, whereas the level of expression from two *myogenin* alleles in the absence of *MyoD* and *MRF4* is not.

A potential complication in interpreting phenotypes of *MRF4* mutants is that Myf5 levels are affected in *cis* by the *MRF4* mutation (Olson et al. 1996). Thus, it is conceivable that the *MRF4/MyoD* double mutant phenotype could reflect a reduction in Myf5 expression, as well. While we cannot rule out this possibility, we feel it is unlikely to account for the specific block to myoblast differentiation in *MRF4/MyoD* double mutants for several reasons. First, in our *MRF4* mutant mice, there is only a transient reduction in Myf5 expression at E10.5, but by a day later, Myf5 expression is normal (Zhang et al., 1995; Yoon et al., 1997) and muscle development is unaffected. The muscle phenotype in the *MRF4*/*MyoD* double mutants is not observed until the late fetal and neonatal period, which is several days later than the transient reduction in Myf5 expression. Second, since MyoD and Myf5 have overlapping functions in myoblast specification, if the severe muscle

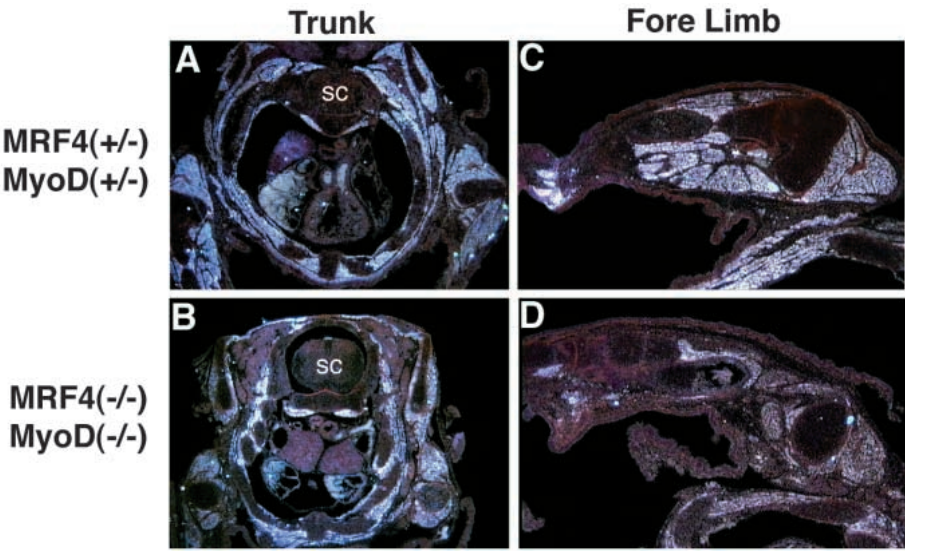

Fig. 5. Detection of myogenin transcripts by in situ hybridization at E17.5. Myogenin transcripts were detected by in situ hybridization to sections from the trunk (A,B) and fore limbs (C,D) of *MyoD*(+/–); *MRF4*(+/–) (A,C) and *MyoD*(–/–); *MRF4*(–/–) (B,D) offspring at E17.5. Myogenin appeared to be expressed at comparable levels in mice of the two genotypes, but *MyoD*(–/–); *MRF4*(–/–) mice lacked well-developed skeletal muscle fibers. sc, spinal cord.

defects in *MyoD/MRF4* double mutants arose from a reduction in Myf5 expression, we would expect to observe a reduction in myoblasts in the double mutants, but that is not the case. Instead, the muscle defects in the *MRF4/MyoD* mutants are remarkably similar to those of *myogenin* mutants, with unfused myoblasts populating the presumptive muscle-forming regions of the animals. Finally, because the severity of rib defects in *MRF4* mutants provides a sensitive indicator of the level of Myf5 expression (Yoon et al., 1997), the rib and muscle defects might be expected to parallel each other in severity if both arose from a reduction in Myf5 expression. However, the rib defects in the *MRF4*/*MyoD* double mutant are much less severe than the muscle defects. The rib defects in *MRF4*/*MyoD* double mutants are also much less severe than in *MRF4*(–/–)/*Myf5*(+/–) mutants, in which the level of *Myf5* expression is half that of wild-type, suggesting that the transient reduction in *Myf5* expression associated with our *MRF4* mutant allele is relatively minor.

Downregulation of Myf5 expression in *MRF4/myogenin* double mutants

Our results also reveal a previously unknown dependence of Myf5 expression on MRF4 and myogenin. In mice lacking either myogenin or MRF4, Myf5 expression at birth was unaffected, whereas in *MRF4/myogenin* double mutants, Myf5 expression was downregulated to background levels at this stage. These findings suggest that MRF4 and myogenin play redundant roles in the maintenance of Myf5 expression during the period of muscle fiber maturation. In contrast, the early expression of Myf5 in the myotome must be independent of MRF4 and myogenin because Myf5 is expressed before MRF4 and myogenin (Sassoon et al., 1989; Bober et al., 1991; Hinterberger et al., 1991; Ott et al., 1991). In addition, if Myf5 was dependent on MRF4 and myogenin for expression in the early myotome, we would expect the ribs to be missing in the *MRF4/myogenin* double mutant, as is characteristic of the *Myf5* mutant, but this is not the case.

Enhancers that control *Myf5* expression have been identified within the region between the *Myf5* and *MRF4* genes (Patapoutian et al., 1993), within the body of the *MRF4* gene (Yoon et al., 1997), as well as a region more than 45 kb upstream and within 500 kb downstream of the *Myf5* gene (Zweigerdt et al., 1997). However, none of these enhancers appears to direct the late expression of the *Myf5* gene in differentiated muscle fibers, a time when Myf5 is predicted from our results to fall under myogenin and MRF4 control.

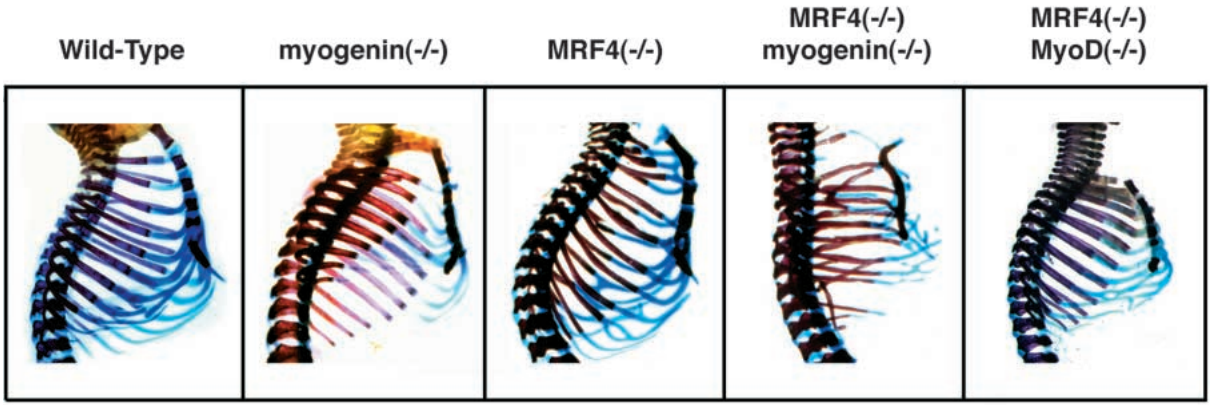

Fig. 6. Ribs and vertebrae in wild-type and mutant neonates. Wild-type and mutant neonates were fixed and stained for bone and cartilage with alizarin red and alcian blue, respectively.

When the regulatory region for late expression is identified, it will be of interest to determine whether it is a direct target for myogenin and MRF4 activation.

Unique expression versus unique functions of the myogenic bHLH factors

A central question in skeletal myogenesis is whether the different myogenic bHLH factors in vertebrates have evolved specialized functions or whether the different myogenic phenotypes that result from inactivation of these genes reflect their distinct expression patterns. Collectively, the results of in vivo and in vitro studies suggest that both the levels of expression and unique functional activities of the individual factors are important for precisely orchestrating muscle determination and differentiation during embryogenesis.

In transfection assays in which the individual factors are over-expressed, the different factors show similar myogenic activities, although subtle differences in their abilities to transactivate certain muscle promoters have been reported (Yutzey et al., 1990; Brennan et al., 1990; Chakraborty et al., 1991). Recent gene replacement studies suggest that certain of the myogenic bHLH genes can partially compensate for one another's functions in vivo. For example, Jaenisch and colleagues created mice in which the *Myf5* gene was replaced with the *myogenin* coding region (Wang et al., 1996). When these *myogenin* knock-in mice were bred to homozygosity, the expression of *myogenin* in place of *Myf5* was found to rescue the rib defects normally associated with the *Myf5* mutation, suggesting that the functions of Myf5 and myogenin were at least partially interchangeable. When this *myogenin* knock-in allele was bred into a *MyoD*-null background, it could also support the early functions of Myf5 in myoblast specification (Wang and Jaenisch, 1997). However, when this knock-in allele was introduced into a *myogenin* mutant background, it could not support normal muscle development in the absence of myogenin, probably because expression from the *Myf5* locus declines during the period of secondary myogenesis, making the overall level of myogenic bHLH factors too low to support differentiation.

Expression of a transgene in which *MRF4* is controlled by the *myogenin* promoter in a *myogenin*-null background also results in partial rescue of muscle differentiation (Zhu and Miller, 1997), suggesting that MRF4 has the ability to partially substitute for myogenin's functions if it is expressed in the same temporospatial pattern as myogenin. These conclusions are also consistent with the finding that myoblasts from the various mutants can differentiate in culture with no apparent requirement for any specific myogenic bHLH factor.

While it seems clear that the different myogenic factors can compensate, at least partially, for each other's functions and that their unique expression patterns contribute to their roles in muscle development, what remains unclear is precisely how these factors control the series of events required for myoblast determination and differentiation. There must be distinct sets of target genes activated by these factors during each of these stages in the myogenic pathway. Elucidation of the mechanism that allows the myogenic factors to discriminate between these different sets of target genes is an important question for the future.

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