Myogenin can substitute for Myf5 in promoting myogenesis but less efficiently

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SUMMARY

The myogenic basic Helix-Loop-Helix transcription factors, including Myf5, MyoD, myogenin (myg) and MRF4, play important roles in skeletal muscle development. The phenotypes of mutant mice deficient in either gene are different, suggesting that each gene may have a unique function in vivo. We previously showed that targeting myogenin into the Myf5 locus (Myf5myg-ki) rescued the rib cage truncation in the Myf5-null mutant, hence demonstrating functional redundancy between Myf5 and myogenin in skeletal morphogenesis. Here we present the results of crossing myogenin knock-in (myg-ki) mice with either MyoD-null or myogenin-null mutants. The Myf5myg-ki allele rescued early myogenesis, but Myf5(myg-ki/myg-ki);MyoD(−/−) mutant mice died immediately after birth owing to reduced muscle formation. Therefore, myogenin, expressed from the Myf5 locus, is not able to completely replace the function of Myf5 in muscle development although it is capable of determining and/or maintaining myogenic lineage. Myf5(myg-ki/myg-ki);myg(−/−) mutant mice displayed the same phenotype as myg(−/−) mutants. This indicates that the earlier expression of myogenin cannot promote myogenic terminal differentiation, which is normally initiated by the endogenous myogenin. Thus, our results are consistent with the notion that Myf5 and myogenin are functionally interchangeable in determining myogenic lineage and assuring normal rib formation. Our experiment revealed, however, that some aspects of myogenesis may be unique to a given myogenic factor and are due to either different regulatory sequences that control their temporal and spatial expression or different functional protein domains.

Key words: Myf5, myogenin, MyoD, knock-in, redundancy, myogenesis, mouse

INTRODUCTION

During vertebrate embryogenesis, somites are formed from the segmental mesoderm in a rostral-to-caudal direction. The ventral part of the somite becomes the sclerotome, which differentiates into skeletal structures, such as the vertebrae and ribs. Simultaneously, the dorsal part develops into the dermatome, which contributes to dermis, and the myotome, which differentiates into skeletal muscle (Sassoon, 1992). Skeletal muscle formation involves two steps: (1) commitment of mesodermal progenitors to myoblasts, and (2) terminal differentiation of committed skeletal myoblasts into myotubes. Both steps are regulated by the myogenic basic Helix-Loop-Helix (bHLH) transcription factors, including Myf5, MyoD, myogenin and MRF4 (Rudnicki and Jaenisch, 1995; Olson and Klein, 1994; Buckingham, 1994). In tissue culture, Myf5 and MyoD are expressed in proliferating myoblasts, and myogenin and MRF4 are only detected in differentiating myoblasts or myotubes. However, forced expression of each of these myogenic bHLH genes can convert non-muscle cells into myoblasts and eventually into myotubes (Weintraub et al., 1991; Olson, 1990). In addition, embryonic stem cells deficient in both Myf5 and MyoD are able to differentiate into skeletal muscle (P. Schegelsberg, P. Klemm and R. Jaenisch, unpublished observations). These observations suggest that all of the myogenic bHLH factors are capable of regulating both steps of myogenesis and therefore may be functionally interchangeable.

It is known that the axial muscle and the limb muscle are derived from the dorsal-medial half and the ventral-lateral half of the somite, respectively (Christ et al., 1978; Ordahl and Le Douarin, 1992). Also, it has been shown that all four myogenic bHLH genes have different expression patterns in early mouse embryos. Myf5 is expressed in the somites at embryonic day 8.0 (E8.0) (Ott et al., 1991), followed by myogenin at day E8.5, MRF4 at day E9.0 and MyoD at day E10.5 (Sassoon et al., 1989; Bober et al., 1991; Hinterberger et al., 1991). Moreover, some of the factors are expressed in different compartments of the myotome. While Myf5 and MyoD are initially expressed in the dorsal-medial half and the ventral-lateral half of the myotome, respectively, myogenin and MRF4 are expressed in the whole myotome (Smith et al., 1994). The distinct expression pattern of each gene implies they may play different roles during skeletal muscle development. Indeed, the phenotypes of mice deficient in each of the four myogenic bHLH genes suggest that they function at different points in the myogenic pathway. MyoD-null mice generated by homologous recombination were viable with apparently normal skeletal muscle development (Rudnicki et al., 1992), although a recent study indicated that the adult
Materials and Methods

Animal breeding

Myf5(myg-ki/myg-ki) mice were crossed with MyoD(+/−) mice to generate Myf5(+/myg-ki);MyoD(+−), Myf5(myg-ki/myg-ki);MyoD(−/−) and Myf5(+/myg-ki);MyoD(−/−) were recovered by interbreeding Myf5(+/myg-ki);MyoD(+/−) double heterozygotes.

Similarly, Myf5(+/myg-ki);myg(+−) double heterozygotes were produced by crossing Myf5(myg-ki/myg-ki) mice with myg(+/−) mice. Subsequently, those double heterozygotes were interbred to generate Myf5(myg-ki/myg-ki);myg(+/−) and Myf5(+/myg-ki);myg(−/−).

The genotyping for each gene was performed as described (Rudnicki et al., 1992; Wang et al., 1996; Hasty et al., 1993).

RNA isolation and RT-PCR

RNA was isolated (Chomczynski and Sacchi, 1987) from the carcasses of newborn mice, in which the internal organs and hearts were removed. The PCR primers used for detecting muscle-specific genes were described previously, and a different quantitative PCR condition was used for each set of primers (Zhang et al., 1995; Patapoutian et al., 1995) and tested to ensure the amplification was in a linear range. The ribosomal RPL7 was used for normalizing the RT-PCR. The PCR products were then loaded on 1% agarose gel for Southern blot analysis by various probes.

Skeletal preparation and histological analysis

Newborn pups were skinned, eviscerated and stained with alizarin red and alcian blue (Zhang et al., 1995). For histological analysis, newborn mice were fixed in 4% paraformaldehyde, dehydrated with ethanol and embedded in paraffin. Tissue sections were stained with both hematoxylin and eosin. Immunofluorescence was performed on frozen sections with a monoclonal antibody against striated muscle-specific actinin (1:400 dilution; mouse IgG1; Sigma).

Immunostaining of primary myoblast cultures

Primary myoblasts were isolated from both wild-type and the mutant neonates and cultured according to Freshney (1987). To induce myogenic differentiation, 2% horse serum in DMEM was used. Immunostaining was performed by anti-skeletal myosin heavy chain (MF-20) and FITC-conjugated antibody to mouse IgG on fixed muscle cells.

Results

Myf5myg-ki;MyoD-null mice die perinatally

Double heterozygous mutant mice carrying Myf5(+/myg-ki) and MyoD(+/−) were generated by crossing mice carrying the Myf5(myg-ki/myg-ki) mutation to MyoD-null mutants and double heterozygous mutants were interbred. Nine different genotypes were expected among the offspring from the double heterozygous mutant intercrossing, with 6.25% and 12.5% expected to be Myf5(myg-ki/myg-ki);MyoD(+/−) and Myf5(+/myg-ki);MyoD(+/−), respectively. As shown in Table 1, all genotypes were found at the expected frequency after weaning except Myf5(myg-ki/myg-ki);MyoD(+/−) and Myf5(+/myg-ki);MyoD(+/−). To investigate lack of both mutant classes, pups were delivered by Cesarean section at term (day E18.5) or killed immediately after birth. Mice carrying all nine genotypes were observed from either the Myf5(+/myg-ki);MyoD(+/−) intercross or the cross between Myf5(myg-ki/myg-ki);MyoD(+/−) and Myf5(+/myg-ki);MyoD(+/−), approximately at the expected frequency (Table 2A,B). This observation suggests that the combination
of either two Myf5<sup>myg-ki</sup> alleles, or one Myf5 plus one Myf5<sup>myg-ki</sup> allele, was unable to support the postnatal survival of MyoD-null mutants.

Myf5<sup>myg-ki</sup>/+;MyoD(+/-) newborn mice initially appeared alive but quickly became immobile and cyanotic. They were relatively smaller (80% of their wild-type litter mates) and displayed arched spines. Dissection of these mutant pups revealed obvious skeletal muscle reduction in trunks and limbs. Slightly deformed rib cages were observed in skeletons prepared from those pups (Fig. 1). Mutant mice carrying Myf5<sup>+/myg-ki</sup>;MyoD(</-) genotype appeared normal immediately after they were born. They had normal breathing and movements initially, but their respiration gradually became intermittent. Eventually the mutants became cyanotic and died. Some pups survived for a few hours postnatally. Newborn intercrosses of MyoD-null mutants.

Skeletons prepared from those pups displayed normal rib cage formation (Fig. 1). These results indicated that the Myf5<sup>myg-ki</sup> allele contributes little to postnatal viability when compared to Myf5(<+/->);MyoD(<+->) pups (Rudnicki et al., 1993).

Skeletal muscle formation in mice deficient for MyoD but carrying the Myf5<sup>myg-ki</sup> allele

To investigate muscle-specific gene expression in mutant mice carrying Myf5<sup>myg-ki</sup>/MyoD(<+->) intercrosses. The newborn Myf5<sup>myg-ki</sup>/MyoD(<+->) pups expressed all the skeletal muscle-specific markers tested, including myosin light chain, fetal-myosin heavy chain, muscle creatine kinase, troponin T and alpha-skeletal actin (Fig. 2A). However, the levels of expression were reduced to approximately 10% of wild type. Similar reduction in the expression of muscle-specific genes was also observed in the E9.5 mutant embryo (data not shown). These results indicate that the Myf5<sup>myg-ki</sup> allele is able to promote myogenesis and activate the downstream muscle-specific genes.

However, Myf5<sup>myg-ki</sup> is less potent than Myf5 in promoting myogenesis. Myf5<sup>+</+/-</sup>;MyoD(<+->) mutant mice died shortly after birth despite their relatively normal appearance, Fig. 2A shows that the expression levels of muscle-specific genes in those pups were generally about 50-60% of the wild-type litter mates. This suggested that the muscle formation promoted by the Myf5<sup>myg-ki</sup> allele was much less than that by the Myf5 allele.

The expression of the myogenic bHLH genes was also analyzed in mutant mice using RT-PCR. Both myogenin and MRF4 were expressed in Myf5<sup>myg-ki</sup>/MyoD(<+->) mice, but the levels of expression were only about 10% of that of wild type (Fig. 2B). This suggests that myogenin expressed from the Myf5 locus is able to activate the endogenous myogenin and subsequently MRF4. The expression levels of myogenin and MRF4 in Myf5<sup>+</+/-</sup>;MyoD(<+->) were only half of that of wild type (Fig. 2B), a similar reduction as seen for muscle-specific gene

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**Table 1. Offspring of Myf5(+/ki);MyoD(+/-) intercrosses**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency</th>
<th>Predicted %</th>
<th>Observed % (No.)*</th>
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<td>21 (12)</td>
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*Data from 58 of 3-week-old mice.

**Table 2(A). Offspring of Myf5(+/ki);MyoD(+/-) intercrosses**

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<td>21 (8)</td>
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<td>5 (2)</td>
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*Data from 38 newborn mice.

**Table 2(B). Offspring of Myf5(+/ki);MyoD(+/-) and Myf5(+/ki);MyoD(+/-) crosses**

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<td>14 (3)</td>
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<td>MyoD&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<td>9 (2)</td>
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<td>MyoD&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>12.5</td>
<td>18 (4)</td>
</tr>
<tr>
<td>Myf5&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>MyoD&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>25</td>
<td>27 (6)</td>
</tr>
<tr>
<td>Myf5&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>MyoD&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>12.5</td>
<td>18 (6)</td>
</tr>
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**Data from 22 newborn mice.**

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**Fig. 1.** Myf5<sup>myg-ki</sup> and MyoD double mutant mice show defects in spines. Both wild-type and mutant newborns were stained with alizarin red and alcian blue to show bones and cartilage. (A) Wild-type skeleton, (B) Myf5(+/myg-ki);MyoD(<+->) and (C) Myf5(myg-ki/myg-ki);MyoD(<+->).
expression (Fig. 2A). These results suggest the Myf5<sup>myg-ki</sup> allele induces reduced levels of downstream bHLH genes or muscle-specific genes as compared to the Myf5 wild-type allele.

To assess the morphology of skeletal muscle in mutant mice, Harris’ hematoxylin and eosin (HE)-stained tissue sections were prepared from Myf5<sup>(myg-ki/myg-ki);MyoD(-/-)</sup> mutants, as well as wild-type litter mates at newborn stage. In Myf5<sup>(myg-ki/myg-ki);MyoD(-/-)</sup> mutants, apparently normal muscle fibers with normal density of nuclei were found throughout the body. However, the numbers of muscle fibers and nuclei were greatly reduced, and the reduction was more predominant in the back and limbs than in the intercostal area between ribs and the diaphragm (Fig. 3; Table 3). Also, immunofluorescent staining on tissue sections using a monoclonal antibody against striated muscle-specific actinin showed reduced numbers of skeletal muscle fibers, although they were normally stained in the mutant mice (Fig. 4A). Similarly, immunostaining of primary cultured muscle cells from both wild type and mutants revealed comparable levels of muscle-specific proteins (Fig. 4B). These findings indicate that myogenin, expressed from the Myf5 locus, is able to activate the whole process of myogenesis but the numbers of muscle fibers and nuclei are reduced as compared to myogenesis promoted by Myf5. In Myf5<sup>(+/myg-ki);MyoD(-/-)</sup> mutants, HE staining revealed about 50-60% muscle fiber formation as compared to wild type (data not shown; Rudnicki et al., 1993), which is consistent with the finding by RT-PCR. This implies that the Myf5 allele played a quantitatively more important role in muscle formation, whereas the Myf5<sup>myg-ki</sup> allele was much less efficient.

The Myf5<sup>myg-ki</sup> allele does not rescue the phenotype of myogenin-null mice
To ask whether myogenin expressed from the Myf5 locus is functional in later myogenic terminal differentiation in the absence of the endogenous myogenin, double heterozygotes were produced by crossing Myf5<sup>(myg-ki/myg-ki)</sup> mice with myg<sup>(+/-)</sup> mutants, and the double heterozygotes were intercrossed to generate mutant mice that carried various genotypes.
Neither Myf5(myg-ki/myg-ki);myg(−/−) nor Myf5(+/myg-ki);myg(−/−) mutants were found among 3-week-old pups, but were recovered by Cesarean section at birth. Newborn mice carrying either genotype appeared very similar to myg(−/−) mutants (data not shown). To assess skeletal muscle formation in both Myf5(myg-ki/myg-ki);myg(−/−) and Myf5(+/myg-ki);myg(−/−) mutants, HE-stained tissue sections were prepared from the mutants as well as from pups carrying wild-type Myf5 but deficient for myogenin (Hasty et al., 1993; Nabeshima et al., 1993). A similar reduction in muscle fiber density was observed in all three mutants, and similar numbers of myoblasts were seen in areas normally occupied by muscle fibers (Fig. 5). These data suggest that earlier expressed myogenin did not rescue the phenotype of myg(−/−) mutants, thus implying that the function of the endogenous myogenin cannot be substituted by ectopically expressed myogenin.

**DISCUSSION**

We have shown that both Myf5(myg-ki/myg-ki);MyoD(−/−) and Myf5(+/myg-ki);MyoD(−/−) mutants die shortly after birth, due to reduced skeletal muscle formation. These results suggest that Myf5<sup>myg-ki</sup> is unable to completely replace the function of Myf5 in skeletal muscle formation. Nevertheless, both RT-PCR and histological studies revealed normal skeletal muscle formation, although at a quantitatively reduced level. We have also shown that the phenotype of Myf5(myg-ki/myg-ki);myg(−/−) mutant mice essentially resembles that of myg(−/−) mutant (Hasty et al., 1993; Nabeshima et al., 1993), indicating that earlier expression of myogenin cannot promote differentiation of myoblasts into myotubes. Our investigation is relevant to the question of functional redundancy of the myogenic bHLH factors during skeletal muscle development.

**Table 3. Myf5(ki/ki);MyoD(−/−) mutant has reduced numbers of muscle fibers**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diaphragm</th>
<th>Intercostal</th>
<th>Back</th>
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<tr>
<td>Wild type</td>
<td>9.5±0.5</td>
<td>20.5±1.6</td>
<td>18.5±1.9</td>
</tr>
<tr>
<td>Myf5(ki/ki);MyoD(−/−)</td>
<td>2.8±0.4</td>
<td>9.4±1.4</td>
<td>2.9±0.6</td>
</tr>
</tbody>
</table>

*The numbers of muscle fibers were counted in HE-stained sections of wild-type and mutant newborn mice, and shown with the mean and the standard deviation.

**Fig. 4. The expression of muscle-specific proteins in muscle cells of both wild-type and mutant newborn mice.** (A). Immunofluorescence was performed on frozen sagittal sections through the diaphragm. Myf5(myg-ki/myg-ki);MyoD(−/−) mutant with reduced numbers of skeletal muscles in the diaphragm (right) showing normal staining as in wild type (left). (B). Myoblasts were isolated from newborn mice with indicated genotypes and cultured in growth medium. In vitro differentiation was induced by culturing myoblasts in differentiation medium for 3 days, and the cells subsequently showed expression of muscle-specific proteins with anti-MHC antibody. Magnification: (A,B) ×400.
muscle reduction in double homozygotes. First, the expression level of Myf5<sup>myg-ki</sup> may not be as high as that of Myf5. This is unlikely because the only alteration in the Myf5<sup>myg-ki</sup> locus was the replacement of coding sequences of Myf5 exon 1 by myogenin cDNA, leaving all other regulatory elements intact. However, RNA stability may be different. Second, translation from the Myf5<sup>myg-ki</sup> transcript may be delayed or the myogenin protein may have a higher turnover rate than Myf5. Previous studies have shown that during embryogenesis myogenin protein was, indeed, detected at least one day later than myogenin transcripts (Cusella-De Angelis et al., 1992; Smith et al., 1994). Since either Myf5 or Myf5<sup>myg-ki</sup> was hardly detected in early mouse embryo by Western blot (data not shown), we have no direct evidence indicating whether the protein level of Myf5<sup>myg-ki</sup> was lower than that of Myf5 in early myogenic cells. Nevertheless, we do know that the level of the myogenic bHLH factors is important in determining the number of cells being recruited into myogenic lineage. This has been suggested by the previous study in which Myf5(+/−);MyoD(−/−) mutants produced only half as much muscle as Myf5(+/+);MyoD(−/−) (Rudnicki et al., 1993). Third, myogenin may be intrinsically less potent than Myf5 or MyoD in determining myogenic lineage. Consistent with this hypothesis, a recent study showed that myogenin has only 10% of the activity of either MyoD or Myf5 in activating transcription of muscle-specific genes in repressive chromatin (Gerber et al., 1997). The transcriptional activation domains of the myogenic bHLH factors are very divergent (Olson, 1990; Weintraub et al., 1991) and they may determine the efficiency of the different factors to promote the different steps of myogenesis. It was shown that two highly conserved regions in the transcriptional activation domains shared by MyoD and Myf5 may be responsible for their greater potency in myogenic lineage determination as compared to that of myogenin (Gerber et al., 1997). In the future, switching the transcriptional activation domains among the myogenic bHLH factors would be informative for understanding the roles of those domains in myogenesis.

The importance of the expression specificity of Myf5 and myogenin in determining the unique role played by the respective gene was further corroborated by the fact that the Myf5<sup>myg-ki</sup> allele did not rescue the phenotype of myogenin-null mice. Our study supports the notion that the temporal expression of each myogenic bHLH gene may be important in determining its unique role in the myogenic pathway. Moreover, our results imply that the total level of the myogenic bHLH factors may be critical in determining the different state of muscle differentiation. A certain level of the myogenic bHLH factors may be necessary for myoblast determination or maintaining myoblasts in an active proliferating state and a much higher level of the same factors may be needed for terminal differentiation. We propose that, in vivo, one of the functions of Myf5 or MyoD is to greatly increase the level of total myogenic bHLH factors by activating myogenin, which then promotes myogenic terminal differentiation. In other words, the level of total myogenic bHLH factors, rather than an intrinsic specificity of each protein, may be important in determining whether myoblasts undergo terminal differentiation or not. Consistent with this is that myoblasts lacking myogenin were able to undergo terminal differentiation in vitro (Nabeshima et al., 1993; Rawls et al., 1995). Alternatively, the myogenic bHLH proteins

![Fig. 5. Myf5<sup>myg-ki</sup> does not rescue myogenic differentiation in the myogenin knock-out mouse. Hematoxylin and eosin staining was performed on the sections through diaphragms (A,C,E) and intercostal muscles (B,D,F) from myg(−/−) mutant (A and B). Myf5(myg-ki/myg-ki);myg(−/−) (C,D) and Myf5(+/myg-ki);myg(−/−) (E,F). The arrows point to the diaphragms or intercostal areas. The numbers of muscle fibers in those mutants are largely reduced as compared to wild-type mice (Fig. 3A). Magnification: (A-F) ×200.](image-url)
may have some intrinsic differences in promoting different steps of myogenesis, as was proposed by others (Gerber et al., 1997).

Our results are consistent with a model of hierarchical regulation during skeletal muscle development. Each myogenic bHLH gene may have distinct regulatory elements in its promoter, which respond to specific upstream activators. During embryogenesis, the promoters of Myf5 and MyoD may be turned on by some as yet unidentified upstream transcription factors, whereas those of myogenin and MRF4 may respond to the myogenic bHLH factors. Consistent with this model, the important binding site for the myogenic bHLH factors (E-box) has not been identified in either the Myf5 or MyoD promoter (Patapoutian et al., 1993; Askura et al., 1995; Goldhamer et al., 1995). In contrast, E-boxes present in the myogenin and MRF4 promoters are known to be essential for their proper activation (Cheng et al 1995; Yee and Rigby, 1995; Naidu et al., 1995; Black et al., 1995). The availability of more mouse mutations in myogenic genes will allow us to finally define the regulatory network operating in skeletal muscle development.

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