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Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size

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Although the mechanisms regulating the formation of embryonic skeletal muscle in vertebrates are well characterized, less is known about postnatal muscle formation even though the largest increases in skeletal muscle mass occur after birth. Adult muscle stem cells (satellite cells) appear to recapitulate the events that occur in embryonic myoblasts. In particular, the myogenic basic helix-loop-helix factors, which have crucial functions in embryonic muscle development, are assumed to have similar roles in postnatal muscle formation. Here, we test this assumption by determining the role of the myogenic regulator myogenin in postnatal life. Because *Myog*-null mice die at birth, we generated mice with floxed alleles of *Myog* and mated them to transgenic mice expressing Cre recombinase to delete *Myog* before and after embryonic muscle development. Removing myogenin before embryonic muscle development resulted in myofiber deficiencies identical to those observed in *Myog*-null mice. However, mice in which *Myog* was deleted following embryonic muscle development had normal skeletal muscle, except for modest alterations in the levels of transcripts encoding Mrf4 (Myf6) and Myod1 (MyoD). Notably, *Myog*-deleted mice were 30% smaller than control mice, suggesting that the absence of myogenin disrupted general body growth. Our results suggest that postnatal skeletal muscle growth is controlled by mechanisms distinct from those occurring in embryonic muscle development and uncover an unsuspected non-cell autonomous role for myogenin in the regulation of tissue growth.

KEY WORDS: Skeletal muscle growth, Myogenic bHLH transcription factors, Myogenin, Conditional knockout mice

INTRODUCTION

Following birth, skeletal muscles grow dramatically, a process that continues until adulthood. In rodents, skeletal muscle mass increases 50-fold or more and skeletal muscle growth can contribute as much as 50% of the added mass of the organism as it reaches maturity (Allen et al., 1979). Despite the obvious importance of skeletal muscle, relatively little is known about the events that govern its growth following birth.

In perinatal (embryonic day 18.5 until birth) and postnatal life, skeletal muscles grow through hypertrophy – the accretion of muscle protein within growing fibers – and through the action of a population of muscle stem cells, the satellite cells, which arise from embryonic somitic progenitors and reside underneath the basal lamina of the myofiber. At birth, satellite cells, which have yet to exit the cell cycle, account for ~30% of the nuclei in rodent limb muscle (Cardasis and Cooper, 1975). During the first few weeks of life, satellite cells fuse to growing fibers so that the cells eventually account for at least 50% of the nuclei inside the fiber. As adulthood is reached, muscle growth declines and the residual satellite cell population accounts for only 6% of the nuclei in limb muscles (Cardasis and Cooper, 1975). The residual satellite cells become quiescent until receptor-mediated signaling triggered by exercise or wounding causes them to re-enter the cell cycle, proliferate and differentiate into muscle (Seale et al., 2001; Charge and Rudnicki, 2004). Thus, postembryonic skeletal muscle formation takes place in two phases: a rapid, intense growth phase that occurs in perinatal and early postnatal life, followed by a phase of muscle maintenance and repair adapted for later periods of life.

Some progress has been made in identifying transcription factors that are essential for perinatal and postnatal skeletal muscle growth. Pax7, a paired-box transcription factor, is expressed in satellite cells and is required for their specification (Seale et al., 2000). More recent studies have shown that Pax3, a close relative of Pax7, is also involved in postnatal muscle growth and Pax3/Pax7-positive progenitor cells appear to be the source of postnatal satellite cells (Relaix et al., 2005; Kassar-Duchossoy et al., 2005). Serum response factor (SRF), a transcription factor that controls the transcription of muscle genes by interacting with Mrtfa, a member of the myocardin family of transcriptional co-activators, is also required for perinatal and postnatal muscle growth (Li et al., 2005).

The mechanisms by which satellite cells are activated in adult muscle maintenance and repair are thought to be similar to those underlying the specification and differentiation of embryonic myoblasts (Buckingham, 2001; Seale et al., 2001). In the embryo, initial muscle development is controlled by the myogenic basic helix-loop-helix (bHLH) transcription factors in conjunction with the Mef2 MADS-box family of transcription factors (Yun and Wold, 1996). Myod1, Myf5, Mrf4 (Myf6 – Mouse Genome Informatics), myogenin and Mef2 isoforms are all expressed in activated satellite cells in temporal patterns analogous to those seen in embryonic development (Cornelison and Wold, 1997). Among the myogenic bHLH factors, only Myod1 has been implicated in skeletal muscle formation in postnatal life. Satellite cells isolated from *Myod1*-knockout mice fail to fuse and double knockout mice harboring mutations in the genes encoding Myod1 and dystrophin develop skeletal myopathies in postnatal life that are not seen in mice lacking only one of the genes (Megeney et al., 1996; Sabourin et al., 1999; Cornelison et al., 2000).

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If postnatal muscle growth recapitulates the regulatory events that occur during embryonic myogenesis, then we would expect the myogenic bHLH factors to function after birth as they do during embryonic myoblast specification and differentiation. Myf5, Mrf4 and Myod1 would therefore be predicted to have functions in specifying postnatal myoblasts with Myf5 and Mrf4 acting upstream of Myod1, whereas myogenin, Mrf4, and Myod1 would be crucial for the subsequent differentiation of those myoblasts into myocytes and myofibers (Yun and Wold, 1996; Penn et al., 2004; Kassar-Duchossoy et al., 2004; Tapscott, 2005). Single-, double- and triple-knockout mice have been created that harbor null mutations in up to three of the four myogenic bHLH regulatory genes (reviewed by Pownall et al., 2002), and analysis of embryonic skeletal muscle development in these mice has revealed complex relationships among the four factors (Rawls et al., 1998; Valdez et al., 2000; Kassar-Duchossoy et al., 2004). For example, Myf5 or Myod1 define independent myogenic compartments during embryonic development but these are not revealed in single *Myf5* or *Myod1* knockout mice (Kablar et al., 2003). The conventional model in which Myf5 and Myod1 act together to specify myogenic fate requires revision in light of recent studies indicating that Mrf4 is also necessary for myoblast specification (Kassar-Duchossoy et al., 2004).

Notably, of the single-knockout mice, only *Myog*-null mice exhibit severe skeletal muscle deficiencies, thereby demonstrating that myogenin is absolutely required for embryonic muscle differentiation (Hasty et al., 1993; Nabeshima et al., 1993; Venuti et al., 1995). Unlike the other myogenic bHLH factors, myogenin has no redundant or compensatory mechanisms to replace its function.

Given its essential role in embryogenesis, myogenin might be expected to have an analogous function in satellite cells during postnatal muscle growth. To test this hypothesis, we generated floxed alleles of *Myog* and used Cre-recombinase mouse lines to delete *Myog* before and after embryonic muscle development. Our results suggest that myogenin-independent mechanisms can compensate for the loss of myogenin and that these mechanisms are likely to be crucial in regulating postnatal skeletal muscle growth.

MATERIALS AND METHODS

Gene targeting

A 3.8 kb genomic fragment containing a region of *Myog* from position –1770 to +2078 (transcriptional start site +1) was inserted into the *Eco*RI site of pBluescript (Stratagene, La Jolla, CA). A floxed neomycin cassette (Bi et al., 1999) was inserted into the first intron at a unique *Bam*HI site at position +620 for positive selection. A *loxP* sequence was inserted into a unique *Bsm*BI site at position –303 in the promoter region of *Myog*. A second construct was generated with a modified *loxP* sequence inserted at position +40 in the 5′ untranslated region (UTR) of *Myog*. The modified *loxP* sequence is recognized by Cre recombinase but does not form a hairpin loop and therefore should not interfere with translational initiation (Sauer, 1998). For negative selection, the MC1TK cassette (McMahon and Bradley, 1990) was inserted into the *Sal*I site of pBluescript.

Generation of embryonic stem cells and mouse lines

The targeting constructs were electroporated into AB1 embryonic stem (ES) cells (provided by Richard Behringer, The University of Texas M. D. Anderson Cancer Center) and positive colonies were selected using G418 for neomycin resistance and fialuridine (FIAU) for thymidine kinase sensitivity. To identify targeted ES cells, Southern blot analysis was performed with DNA from individual colonies following enzymatic digestion with *Bam*HI and *Hin*dIII, and targeted DNA was detected as described by Vivian et al. (Vivian et al., 1999). Correctly targeted ES cells were amplified and injected into C57BL/6 blastocysts. The resulting chimeric mice (a mixture of 129 and C57BL/6 strains) were mated to C57BL/6 mice to establish mice harboring the floxed *Myog* allele in their germline. Both the *loxP* promoter construct and the *loxP* 5′ UTR construct yielded germline mice; four lines were established for the promoter construct and one line for the 5′ UTR construct. In this study, we describe results obtained with the promoter construct, although the 5′ UTR construct gave similar results (data not shown). The *loxP* promoter construct is referred to hereafter as $Myog^{flox}$.

Mice harboring the $Myog^{flox}$ allele were mated either to *CMV-Cre* transgenic mice, which express active Cre recombinase in the single-cell zygote (Arango et al., 1999) or to $CAGGCre\text{-}ER^{TM}$ transgenic mice, which ubiquitously express a conditional Cre recombinase that is activated by intraperitoneal injection of tamoxifen (Hayashi and McMahon, 2002). The deleted allele is referred to as $Myog^{flox\Delta}$. The genotypes of the mice harboring the $Myog^{flox}$ allele and either the *CMV-Cre* or $CAGGCre\text{-}ER^{TM}$ transgene were determined by polymerase chain reaction (PCR) using primers described by Ovchinnikov et al. (Ovchinnikov et al., 2000) or by Southern blot hybridization of yolk sac DNA as described by Rawls et al. (Rawls et al., 1995). Two milligrams of tamoxifen was injected intraperitoneally into pregnant females at either E14.5 or E17.5 of pregnancy (Hayashi and McMahon, 2002). All mouse experiments were conducted according to the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. The procedures for use of mice in this study were approved by The University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Histology and immunostaining

Dissected skinned skeletal muscle tissue from hindlimbs, diaphragms and tongues was prepared for paraffin embedding by fixing in 0.2% gluteraldehyde and 2% formaldehyde in phosphate-buffered saline (PBS) for 1 hour, rinsing three times in PBS and fixing in 3.7% formaldehyde overnight. The tissue was then washed in increasing concentrations (50%, 75%, and 100%) of isopropanol for 2 hours before embedding in Paraplast (Structure Probe, West Chester, PA). The embedded tissue was sectioned into 6 μm slices and stained with Hematoxylin and Eosin. For frozen sections, dissected, skinned muscle tissue was incubated overnight in 30% sucrose and 0.1 M sodium phosphate buffer (pH 7.2) at 4°C. The tissue was then embedded in OCT (Sakura, Tokyo, Japan) and rapidly frozen with an ethanol/dry ice mix. The frozen tissue was sectioned into 10 μm slices that were then fixed in 4% paraformaldehyde.

Antibody staining was performed using the HistoMouse-Max kit (Zymed, San Francisco, CA) with a 1:50 dilution of anti-myogenin antibody M-225 (Santa Cruz Biotechnology, Santa Cruz, CA) for embryonic skeletal muscle or a 1:250 dilution of anti-myogenin antibody F5D (NeoMarkers, Fremont, CA) for adult skeletal muscle.

Quantitative PCR and reverse-transcriptase PCR

To quantify the extent of Cre recombinase-mediated deletion, dissected tail or hindlimb muscle was flash-frozen in liquid nitrogen. Genomic DNA with the genotype $Myog^{flox/flox}$;$CAGGCre\text{-}ER^{TM}$/+ or $Myog^{+/+}$;$CAGGCre\text{-}ER^{TM}$/+ was isolated and used as a template for quantitative PCR using the iCycler iQ system and iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA). Cre recombinase-mediated deletion was intended to remove the first exon of $Myog^{flox}$ so that *Myog* primers corresponding to sequences in the first exon of *Myog* would amplify fragments specifically representing undeleted $Myog^{flox}$. As a control, *Myf5* primers were used to amplify fragments representing the first exon of *Myf5*, which was assumed to be unaltered in the genomic DNA. The primer sequences used for this study are described below. The ratio of undeleted $Myog^{flox}$ to *Myf5* was normalized to the same ratio obtained from genomic DNA of wild-type mice; this value was the relative fraction of *Myog* remaining in the genomic DNA.

For reverse-transcriptase (RT)-PCR of the myogenic regulatory factors, RNA was isolated from dissected muscle tissue using TRI Reagent (Molecular Research Center, Cincinnati, OH). Ten micrograms of RNA was treated with DNase (Invitrogen, Carlsbad, CA), and 1 μg was used as a template for the RT reaction using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Each PCR experiment was performed with one tenth of the RT product using the iCycler iQ system and iQ SYBR Green SuperMix (BioRad).

To determine the transcript levels of *Igf1*, *Igf2* and *Igfbp2*, RNA was isolated from a 30 μm section of hindlimb with skin and fat removed. RNA was isolated using an RNeasy fibrous tissue kit (Qiagen, Valencia, CA). Two micrograms of RNA were used as a template for the RT reaction using Superscript as above. Each PCR reaction was performed with one-fiftieth of the RT product using the Applied Biosystems 7500 Fast real-time PCR system and SYBR Green Master Mix (Applied Biosystems, Foster City, CA).

The oligonucleotide primer sequences for RT-PCR were as follows: iL7 (forward), 5′-CAAGTTATCTTCCCCACGAGGT-3′; iL7 (reverse), 5′-TTCATCCGTCTAATAAGCCTGTTT-3′; iMyf5 (forward), 5′-TGAGCTGGCTCTTCAGGACA-3′; iMyf5 (reverse), 5′-ACGTGATAGATAAGTCTGGAGCTG-3′; iMyod1 (forward), 5′-TTCCTTTCCTCATAGCACAGGG-3′; iMyod1 (reverse), 5′-GGCTCCAGAAAGTGACAAACAAA-3′; iMrf4 (forward), 5′-CTCAGCCTCCAGCAGTCTTC-3′; iMrf4 (reverse), 5′-GGGTGAAGAATGTTCCAAATGCT-3′; Mygi (5′), 5′-CCTGGAAGAAAAGGGACTGGG-3′; Mygi (3′), 5′-TCATTCACTTTCTTGAGCCTGC-3′; Mck (lower), 5′-GATGGGATCAAACAGGTCCTTG-3′; Mck (upper), 5′-CAATAAGCTTCGCGATAAGGAG-3′; Igf1a (forward), 5′-CGGAGCTAGCAATACCCTTC-3′; Igf1a (reverse), 5′-CTGACCTCTCTTCCCTGAGC-3′; Igf2a (forward), 5′-TGTGACCAGGCTGCTAGTTC-3′; Igf2a (reverse), 5′-ACCATGTGGACAGGTGCTTA-3′; Igfbp2c (forward), 5′-CTCTACTCCCTGCACATCCC-3′; Igfbp2c (reverse), 5′-TCCGTTCAGAGACATCTTGC-3′.

Measurement of fiber diameters and body weights

Muscle fiber diameters were calculated by measuring the narrowest diameter of the fiber with the ruler function of Adobe Photoshop software 7.0.1 (Adobe, San Jose, CA). Four sections from hindlimbs containing 10 fibers per section were analyzed from a single 10-week-old mouse for each genotype. Mice were weighed 10 days after birth and weekly thereafter. Differences between genotype groups in weights at 6 weeks were analyzed using a two-tailed Student's *t* test with significance set at $P<0.05$.

Statistical analyses

The results were analyzed using a two-sided Wilcoxon rank-sum test (normal approximation with continuity correction: significance set at $P<0.05$). Statistical analyses were performed by Carla Warneke (Department of Biostatistics and Applied Mathematics, The University of Texas M. D. Anderson Cancer Center).

RESULTS

Generation of mice carrying the *Myog*^{flox} allele

We used the Cre-*loxP* system to delete *Myog* before and after embryonic muscle development (Sauer, 1998). A *loxP* site was inserted into the promoter region of *Myog* and a neomycin cassette with flanking *loxP* sites was inserted into the first intron to generate targeted ES cells containing the *Myog*^{flox} allele (Fig. 1A,B). Four *Myog*$^{flox/+}$ heterozygous mouse lines were established for further investigation; mice containing the *Myog*^{flox} allele are available upon request.

To determine whether functional myogenin protein was expressed from the *Myog*^{flox} allele, *Myog*$^{flox/+}$ mice were mated to mice heterozygous for a null allele of *Myog* (*Myog*$^{+/-}$) to generate mice with a *Myog*$^{flox/-}$ genotype (Fig. 1C). These mice were viable and fertile, in contrast to the neonatal lethality seen in *Myog*$^{-/-}$ mice (Hasty et al., 1993). Thus, the *Myog*^{flox} allele was fully functional, despite the presence of a *loxP* site in its promoter and a floxed neomycin cassette in its first intron.

We next determined whether *Myog*^{flox} behaves as a null allele following Cre recombinase-mediated deletion. To do this, we mated *Myog*$^{flox/flox}$ mice to transgenic mice expressing *CMV-Cre* to generate a floxed allele, *Myog*^{floxΔ}, that lacked the first exon of *Myog* (Fig. 2A). *CMV-Cre* mice first express Cre recombinase in the zygote, several days before the formation of skeletal muscle (Arango et al., 1999). As expected, *Myog*$^{floxΔ/floxΔ}$ mice died as neonates and had

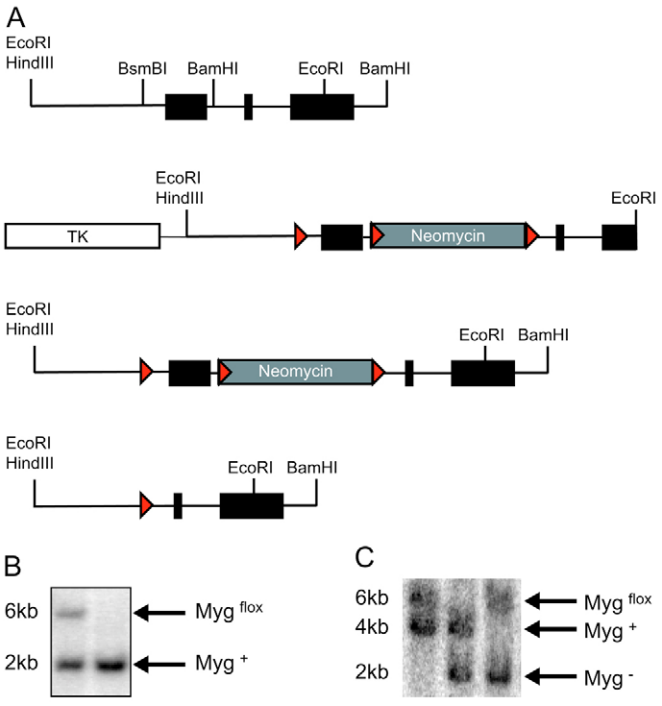

Fig. 1. Generation of the *Myog*^{flox} allele, targeted ES cells and heterozygous mice. (**A**) The top line shows the myogenin locus with its three exons (black rectangles). The second line shows the targeting construct. The region of homologous DNA (thicker line) spans 3.8 kb and is flanked by *Eco*RI sites. Also shown are *loxP* sites (red triangles) and a neomycin cassette placed into the *Bam*HI site in the first intron (gray rectangle). A thymidine kinase (TK) cassette (white rectangle) lies upstream of the homology region. The next two lines depict the targeted *Myog* locus before and after Cre recombinase-mediated deletion of the first exon and neomycin cassette. (**B**) Southern genome hybridization of ES cell DNA digested with *Bam*HI and *Hin*dIII, confirming proper targeting. A 3′ probe derived from the third exon immediately 3′ to the *Eco*RI site outside of the homology region was used in the hybridization analysis. The 2 kb band shown on the figure represents the wild-type *Bam*HI fragment bounding the second and third exons (*Myog*$^{+}$). The 6 kb band represents the targeted *Bam*HI-*Hin*dIII fragment (*Myog*^{flox}) containing the neomycin cassette. The left and right lanes show DNA from targeted and wild-type ES cell lines, respectively. (**C**) Southern genome hybridization of tail DNA digested with *Eco*RI and hybridized with a *Myog* cDNA probe. Tail DNA was obtained from P10 pups resulting from mating *Myog*$^{+/-}$ and *Myog*$^{+/flox}$ mice. The left, idle and right lanes show DNA from *Myog*$^{+/flox}$, *Myog*$^{+/-}$ and *Myog*$^{flox/-}$ mice, respectively. The 2 kb band representing the *Myog*$^{-}$ allele is the result of a *Eco*RI site in the neomycin cassette that is not present in the neomycin cassette of the *Myog*^{flox} allele. The origins of the 4 kb and 6 kb bands associated with the wild-type and *Myog*^{flox} alleles, respectively, are shown in A.

skeletal muscle deficiencies resembling those of *Myog*$^{-/-}$ mice (Fig. 2B,C). Diaphragms from E18.5 *Myog*$^{floxΔ/floxΔ}$ embryos were abnormally thin and lacked myofibers, closely resembling diaphragms from E18.5 *Myog*$^{-/-}$ embryos (Fig. 2B). Hindlimbs from E18.5 *Myog*$^{floxΔ/floxΔ}$ embryos also showed skeletal muscle abnormalities and, as expected, myogenin protein was not detectable (Fig. 2C). We also observed a two- to threefold increase in *Myod1* expression using quantitative RT-PCR (data not shown); this upregulation of *Myod1* was not observed previously in *Myog*$^{-/-}$ embryos (Hasty et al., 1993; Venuti et al., 1995). In contrast to *Myog*$^{floxΔ/floxΔ}$ embryos, corresponding hindlimbs from *Myog*$^{floxΔ/+}$

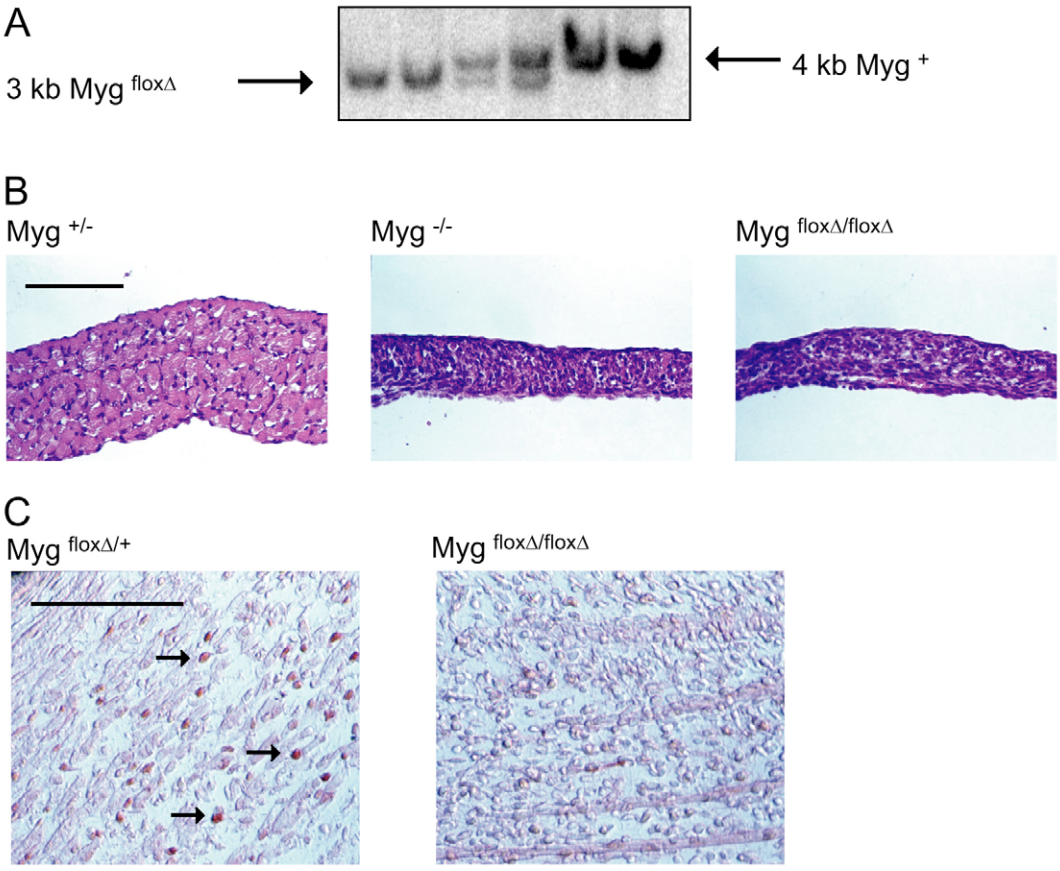

Fig. 2. Deletion of floxed *Myog* sequences using *CMV-cre* and analysis of embryonic skeletal muscle at E18.5. (**A**) Southern genome hybridization of yolk-sac DNA digested with *Eco*RI from embryos resulting from a *Myog*$^{flox/+}$*;CMV-cre/+* intercross and probed with *Myog* cDNA. Deletion of the floxed myogenin sequences produced a 3 kb *Eco*RI fragment representing the *Myog*$^{flox\Delta}$ allele. The 4 kb *Eco*RI fragment represents the wild-type allele (*Myog*$^{+}$). Lanes 1 and 2 show DNA from *Myog*$^{flox\Delta/flox\Delta}$ embryos; lanes 3 and 4 show *Myog*$^{flox\Delta/+}$ embryos; and lanes 5 and 6 show wild-type embryos. (**B**) Diaphragms of E18.5 *Myog*$^{+/-}$, *Myog*$^{-/-}$ and *Myog*$^{flox\Delta/flox\Delta}$ embryos. The genotype is shown above each image. Scale bar: 100 μm. (**C**) Hindlimb sections from E18.5 *Myog*$^{flox\Delta/+}$ and *Myog*$^{flox\Delta/flox\Delta}$ embryos immunostained with polyclonal M225 anti-myogenin antibody. Arrows indicate positively stained cells. Scale bar: 100 μm.

embryos showed normal skeletal muscle in the hindlimbs and efficient expression of myogenin (Fig. 2C). These results demonstrate that the *Myog*^{flox} allele can be effectively deleted using the Cre-*loxP* system.

Generation of mice lacking myogenin in postnatal life

Mice with the genotype *Myog*$^{flox/flox}$*;+/+* were mated to *Myog*$^{flox/flox}$*;CAGGCre-ER*^{TM}*/+* mice and pregnant females resulting from the cross were injected with tamoxifen at E15.5 or E17.5 days of pregnancy to activate Cre recombinase. In this breeding scheme, half of the F1 embryos were expected to inherit Cre recombinase and half were not. In mouse development, primary myogenesis occurs between E12.5 and E14.5, and is a time when primary fibers form and serve as a scaffold for secondary myogenesis. The latter process involves extensive myoblast fusion and further fiber formation. E15.5 and E17.5 were chosen for tamoxifen injections because myogenin expression begins at E9.5, secondary myogenesis begins at E15.5, and the majority of embryonic muscle development has occurred by E17.5. Efficient Cre recombinase-mediated deletion should therefore result in a lack of myogenin in postnatal life, provided that the half-life of myogenin is relatively short. Pregnant females injected at E15.5 or E17.5 days were allowed to give birth, and the genotypes of the pups in the resulting litters were determined at postnatal day 10 (P10). The Cre recombinase-mediated deletion was highly efficient and we were able to readily detect the deleted *Myog* allele by quantitative PCR genotyping from tail DNA (described below).

If the *Myog*$^{flox\Delta/flox\Delta}$ mice were fully viable, a 1:1 ratio of *Myog*$^{flox/flox}$ pups to *Myog*$^{flox\Delta/flox\Delta}$ pups would be expected. However, after either day of tamoxifen injection, we consistently found a ratio of 2:1 in the offspring from *Myog*$^{flox/flox}$*;+/+* × *Myog*$^{flox/flox}$*;CAGGCre-ER*^{TM}*/+* matings (Fig. 3A,B). These results indicated that 50% of the *Myog*$^{flox/flox}$ mice – those that inherited the *CAGGCre-ER*^{TM} transgene and therefore had a *Myog*$^{flox\Delta/flox\Delta}$ genotype – were dying before P10. It is not clear why these mice did not survive as the *Myog*$^{flox\Delta/flox\Delta}$ mice that did survive were fully viable, although by P10 they were noticeably smaller than their *Myog*$^{flox/flox}$ littermates (see below). *Myog*$^{flox/flox}$ and *Myog*$^{flox\Delta/flox\Delta}$ embryos were found at 1:1 ratios at E18.5 (data not shown), suggesting that 50% of the *Myog*$^{flox\Delta/flox\Delta}$ mice died immediately before birth or early in postnatal life. The reduced viability associated with *Myog*$^{flox\Delta/flox\Delta}$ mice did not appear to be attributable to defects in skeletal muscle growth (see below) and we did not find any gender bias in the surviving mice. A possible explanation for the 2:1 ratio was that a dominant modifier gene in the mixed

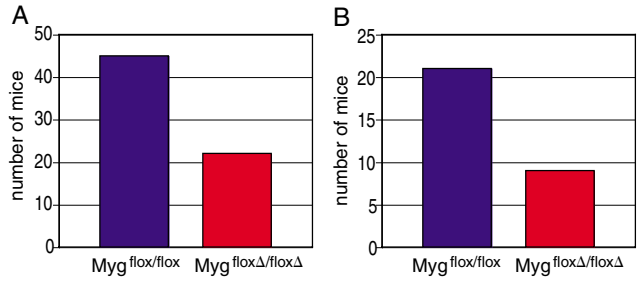

Fig. 3. Perinatal lethality associated with *Myog*$^{floxΔ/floxΔ}$ mice. Mice with the genotype *Myog*$^{flox/flox}$;+/+ were mated to *Myog*$^{flox/flox}$;*CAGGCre-ER*^{TM}/+ mice and pregnant females resulting from the cross were injected with tamoxifen at E15.5 or E17.5 days of pregnancy. (**A**,**B**) Histograms show the number of mice surviving to P10 after tamoxifen injection of pregnant females at E17.5 (A) or E15.5 (B). Genotypes are shown below each figure.

129/C57BL/6 genetic background was inherited by 50% of the offspring and enhanced a lethal phenotype in *Myog*$^{floxΔ/floxΔ}$ mice. We have not yet pursued the possibility of a modifier gene, and the basis for the lethality observed in some of the *Myog*$^{floxΔ/floxΔ}$ mice remains unclear.

The extent of genomic deletion of myogenin was determined by quantitative PCR of tail DNA from the surviving P10 pups produced in the *Myog*$^{flox/flox}$;+/+ × *Myog*$^{flox/flox}$;*CAGGCre-ER*^{TM}/+ crosses. We observed efficient cre-recombinase-mediated deletion of *Myog* in the genomes of *Myog*$^{flox/flox}$ mice that inherited the *CAGGCre-ER*^{TM} transgene. Genomic DNA from these pups contained only 3-20% of the floxed *Myog* sequence, whereas the corresponding genomic DNA from *Myog*$^{flox/flox}$ littermates that did not inherit the *CAGGCre-ER*^{TM} transgene contained 80-120% of the floxed *Myog* sequence (Fig. 4A). The detectable residual *Myog* DNA that was seen in *Myog*$^{floxΔ/floxΔ}$ genomes was probably an overestimate because the high cycle threshold values for quantitative PCR with

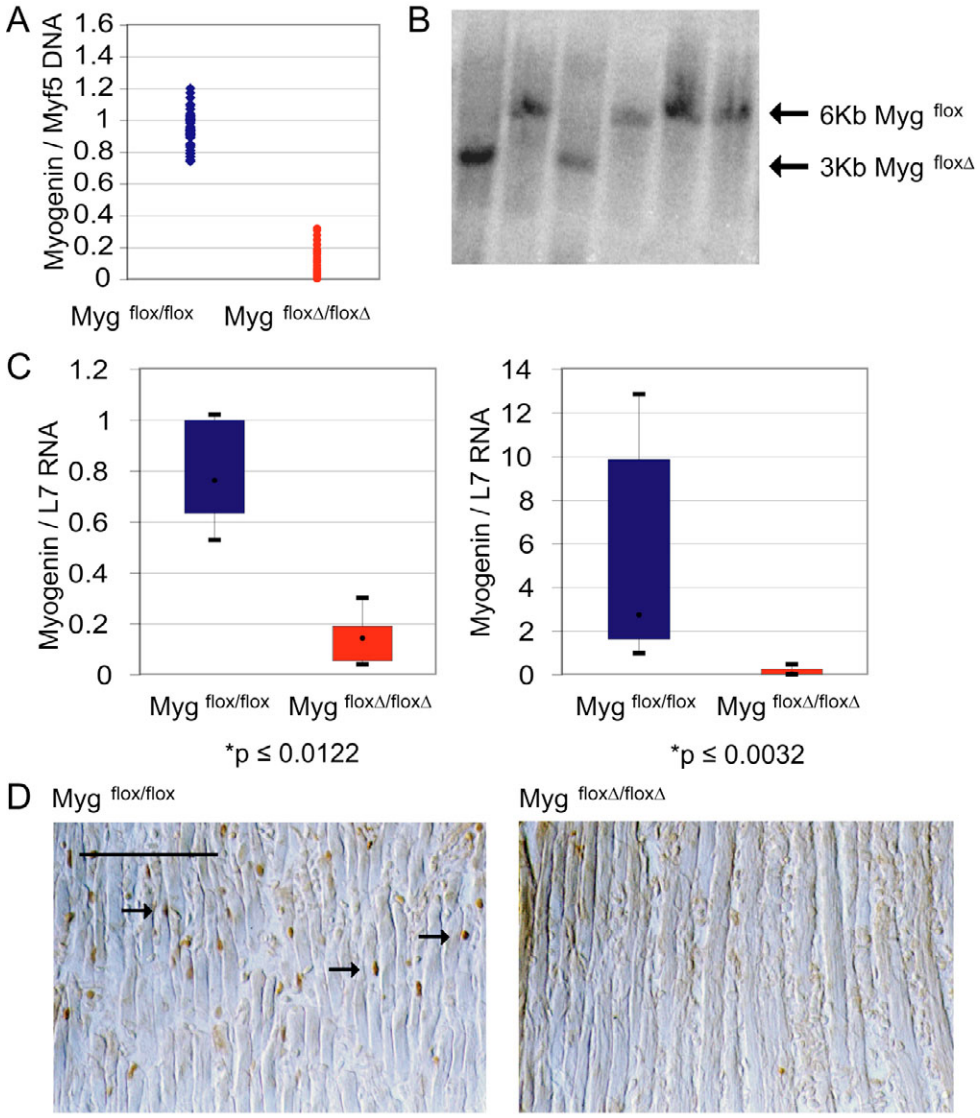

Fig. 4. Deletion of *Myog* and attenuation of myogenin transcripts and protein in *Myog*$^{floxΔ/floxΔ}$ mice. (**A**) Quantitative PCR of tail DNA to determine the extent of deletion of floxed *Myog* sequences in *Myog*$^{flox/flox}$ and *Myog*$^{floxΔ/floxΔ}$ mice at P10. The ratio of *Myog* to *Myf5* genomic DNA from one *Myog*$^{flox/flox}$ mouse was arbitrarily set to 1 and all other ratios from both *Myog*$^{flox/flox}$ and *Myog*$^{floxΔ/floxΔ}$ genotypes were normalized to that value. (**B**) Southern genome hybridization with *Eco*RI-digested DNA extracted from the hindlimb skeletal muscle of 10-week-old mice from a *Myog*$^{flox/flox}$;+/+ × *Myog*$^{flox/flox}$;*CAGGCre-ER*^{TM}/+ cross and probed with *Myog* cDNA. The 6 kb and 3 kb bands represent the *Myog*^{flox} and *Myog*^{floxΔ} alleles, respectively, as depicted in Fig. 1. The skeletal DNA in lanes 1 and 3 contained very little of the *Myog*^{flox} allele and therefore represents a *Myog*$^{floxΔ/floxΔ}$ genotype, while the skeletal DNA in lanes 2, 4, 5 and 6 contained no *Myog*^{floxΔ} allele and therefore represents a *Myog*$^{flox/flox}$ genotype. (**C**) Quantitative RT-PCR to determine the levels of *Myog* transcript expression in hindlimb skeletal muscle from mice at 3 days (right panel) and 2 weeks (left panel) of age. The genotypes are shown below the histograms. The ratio of *Myog* to ribosomal protein L7 RNA for one RNA sample with a *Myog*$^{flox/flox}$ genotype was arbitrarily set to 1, and all other ratios from both *Myog*$^{flox/flox}$ and *Myog*$^{floxΔ/floxΔ}$ genotypes were normalized to that value. The median value for each genotype is shown as a black dot. Asterisks indicate significant differences between the two genotypes. The arrow bars indicate one s.d. from the median. (**D**) Hindlimb sections from *Myog*$^{flox/flox}$ (right panel) and *Myog*$^{floxΔ/floxΔ}$ (left panel) mice at 3 days of age immunostained with the monoclonal F5D anti-myogenin antibody. Arrows indicate positively stained cells. Scale bar: 100 μm.

DNA from *Myog*$^{flox\Delta/flox\Delta}$ mice tended to inflate the final calculation. However, even if 20% of the cells in *Myog*$^{flox\Delta/flox\Delta}$ mice contained genomes with the *Myog* gene intact, this would still result in a *Myog*-null phenotype. We showed in a previous study that chimeric embryos containing mixtures of 60% wild-type and 40% *Myog*$^{-/-}$ myoblasts do not survive past birth and have skeletal muscle defects identical to those seen in *Myog*$^{-/-}$ embryos (Myer et al., 1997). Additionally, when *Myog* expression in embryos containing a low-expressing *Myog* allele is less than 50% but greater than 25% of the expression in *Myog*$^{+/+}$ control embryos, the extent of skeletal muscle deficiencies is the same as that seen in *Myog*$^{-/-}$ mice (Vivian et al., 1999).

To demonstrate that the floxed *Myog* sequence was deleted from the genomes of skeletal muscle cells, DNA was extracted from the hindlimbs of *Myog*$^{flox\Delta/flox\Delta}$ mice at 10 weeks after birth and the extent of Cre-mediated deletion was determined by Southern blot genome hybridization. Only low levels of the floxed *Myog* sequence were detected in hindlimb DNA (Fig. 4B), as was the case with the tail DNA. Moreover, the expression of *Myog* transcripts *Myog*$^{flox\Delta/flox\Delta}$ hindlimbs was one-fifth (Fig. 4C, left panel) and one-fiftieth (Fig. 4C, right panel) of that of control littermates at 3 days and 2 weeks after birth.

Myogenin protein expression was also greatly attenuated in *Myog*$^{flox\Delta/flox\Delta}$ mice but reduced expression was not seen immediately. When tamoxifen was injected into pregnant females at E15.5 and the levels of myogenin expression determined 3 days later, expression levels were the same in embryos that inherited the *CAGGCre-ER*^{TM} transgene as those in embryos that did not (data not shown). Tail DNA from embryos that inherited the *CAGGCre-ER*^{TM} transgene had only 6% of the floxed *Myog* sequence compared with control embryos (data not shown). However, when tamoxifen was injected at E17.5 and the levels of myogenin expression determined 5 days later (P3), we were unable to detect myogenin expression in sections of hindlimb muscle from *Myog*$^{flox\Delta/flox\Delta}$ pups (Fig. 4D, right panel), although control *Myog*$^{flox/flox}$ littermates showed robust expression in myofiber nuclei (Fig. 4D, left panel). In this case, genomic *Myog* in the *Myog*$^{flox\Delta/flox\Delta}$ mice was 19% of the level in control mice. These results indicate that sometime between 3 to 5 days after *Myog* was deleted, myogenin protein was largely depleted from the skeletal muscle of *Myog*$^{flox\Delta/flox\Delta}$ mice.

Normal skeletal muscle in postnatal *Myog*$^{flox\Delta/flox\Delta}$ mice

Skeletal muscle of *Myog*$^{flox\Delta/flox\Delta}$ mice and their *Myog*$^{flox/flox}$ littermates was examined for any anatomical and histological abnormalities that might be associated with the absence of myogenin. Muscle from hindlimbs (Fig. 5A-D) and tongues (Fig. 5E,F) of 10-week-old mice was histologically normal regardless of whether the mice had inherited the *CAGGCre-ER*^{TM} transgene. Nuclei were correctly positioned at the periphery of the myofibers and muscle striations appeared grossly normal. Skeletal muscle from diaphragms was also normal with one exception: the diaphragms from one pair of *Myog*$^{flox\Delta/flox\Delta}$ littermates were notably thinner than their *Myog*$^{flox/flox}$ counterparts (Fig. 5G,H) and resembled the muscle-deficient diaphragms observed in *Myog*$^{-/-}$ embryos. It was difficult to reconcile this diaphragm defect with the overt behavior of the mice; they breathed normally and moved about in a normal manner. This was the only instance in which muscle abnormalities were seen and all other *Myog*$^{flox\Delta/flox\Delta}$ mice examined (n=20) had normal musculature in their diaphragms (Fig. 5I,J).

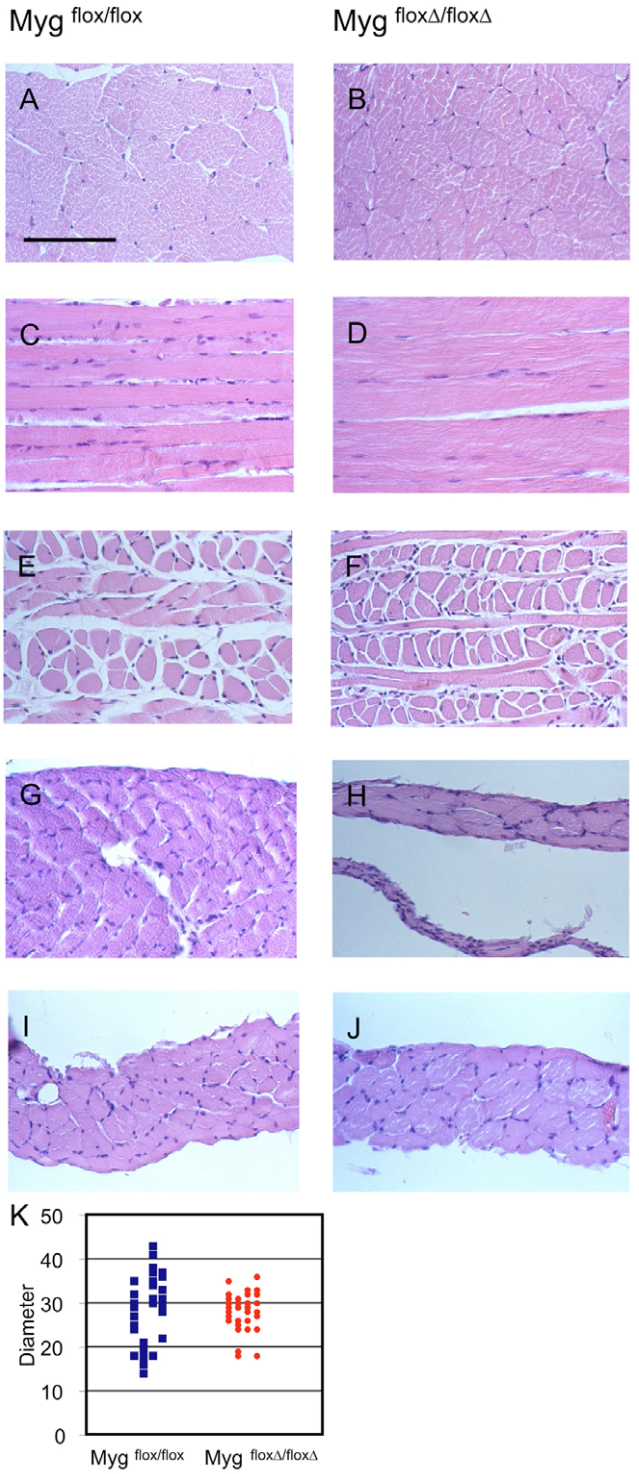

Fig. 5. Skeletal muscle from *Myog*$^{flox/flox}$ and *Myog*$^{flox\Delta/flox\Delta}$ mice at 10 weeks of age. (**A-D**) Cross-sections (A,B) and longitudinal sections (C,D) of hindlimbs. (**E**,**F**) Sections through tongues. (**G-J**) Sections through diaphragms. One *Myog*$^{flox\Delta/flox\Delta}$ mouse has a thin diaphragm (H). Thin diaphragms were found in only a single pair of mice from a single litter. All other *Myog*$^{flox\Delta/flox\Delta}$ mice had normal diaphragms (J). Scale bar: 100 μm. (**K**) Identical myofiber diameters were observed in *Myog*$^{flox/flox}$ and *Myog*$^{flox\Delta/flox\Delta}$ mice. Myofiber diameters were determined from cross-sections of hindlimbs of mice at 10 weeks of age. Each column represents the diameters determined for individual myofibers from a single hindlimb section (squares). The genotypes are shown below the graph.

The growth of myofibers in perinatal and postnatal life is associated with increases in fiber diameter, number of fibers and density of fiber nuclei (Allen et al., 1979). It was therefore possible that the loss of myogenin would result in a reduction of myofiber diameter without affecting muscle histology per se. However, this proved not to be the case. The diameters of myofibers from hindlimbs of $Myog^{flox/flox}$ and $Myog^{floxΔ/floxΔ}$ mice were indistinguishable from each other (Fig. 5K). Moreover, the $Myog^{floxΔ/floxΔ}$ mice were as active as $Myog^{flox/flox}$ control mice and showed no signs of fatigue or inability to perform routine tasks, including, running, jumping and grasping. These results indicate that the absence of myogenin did not alter postnatal skeletal muscle growth or function.

Expression of muscle-specific genes in $Myog^{floxΔ/floxΔ}$ mice

The lack of noticeable skeletal muscle defects in adult $Myog^{floxΔ/floxΔ}$ mice was unexpected as deleting myogenin in the germline or zygote leads to severe muscle deficiencies in embryogenesis and causes neonatal death (Hasty et al., 1993; Nabeshima et al., 1993). It is possible that the loss of myogenin in early postnatal life led to an upregulation of *Mrf4*, *Myod1* or *Myf5*. If so, abnormally high levels of these related transcription factors might functionally compensate for the loss of myogenin. Using quantitative RT-PCR, we determined the levels of expression of *Mrf4*, *Myod1* and *Myf5*, and the control, *Mck*, in the hindlimbs of $Myog^{flox/flox}$ and $Myog^{floxΔ/floxΔ}$ mice at 3 days and 2 weeks after birth. *Mck* expression is greatly attenuated in skeletal muscle of $Myog^{-/-}$ embryos (Hasty et al., 1993; Rawls et al., 1998) and is thought to be a direct target of myogenin (Chakraborty et al., 1991). However, there were no significant differences in *Mck* expression between $Myog^{flox/flox}$ and $Myog^{floxΔ/floxΔ}$ mice (Fig. 6). This result implies that other myogenic bHLH factors had replaced myogenin in maintaining *Mck* expression in postnatal life.

Mrf4 expression is also strongly downreglated in $Myog^{-/-}$ mice (Hasty et al., 1993), but we observed less than twofold downregulation of *Mrf4* expression in $Myog^{floxΔ/floxΔ}$ mice compared with *Mrf4* expression in $Myog^{flox/flox}$ control mice (Fig. 6). The lack of notable downregulation of *Mck* and *Mrf4* expression indicates that the loss of myogenin did not have a major impact on skeletal muscle gene expression in postnatal life.

Previous studies have shown that *Myod1* and *Myf5* expression levels are not significantly different in $Myog^{-/-}$ mice compared with wild-type mice (Hasty et al., 1993; Venuti et al., 1995). We found a twofold and 1.5-fold upregulation in *Myod1* expression in the skeletal muscle of $Myog^{floxΔ/floxΔ}$ mice at 3 days and 2 weeks after birth, respectively, compared with $Myog^{flox/flox}$ control mice (Fig. 6). Although the differences in *Myod1* expression between $Myog^{floxΔ/floxΔ}$ mice and $Myog^{flox/flox}$ mice were significant, the extent of the upregulation was probably too low to contribute to functional compensation. In a previous study, we showed that overexpressing *Myod1* from a strong constitutive promoter was not sufficient to restore normal skeletal muscle differentiation in *Myog*-null ES cells (Myer et al., 2001), a conclusion consistent with in vitro studies showing that Myod1 preferentially activates early, and myogenin late, muscle genes (Bergstrom et al., 2002; Penn et al., 2004). Fig. 6 also shows that *Myf5* expression was not significantly different in $Myog^{floxΔ/floxΔ}$ and $Myog^{flox/flox}$ mice. This expression analysis shows that the absence of myogenin in postnatal life did not markedly upregulate the expression of any of the myogenic bHLH factors and suggests that these factors are unlikely to compensate for the absence of myogenin. *Mef2c* is also expressed in postnatal skeletal muscle (Wang et al., 2001) but *Mef2c* expression was not significantly affected by the loss of myogenin (data not shown).

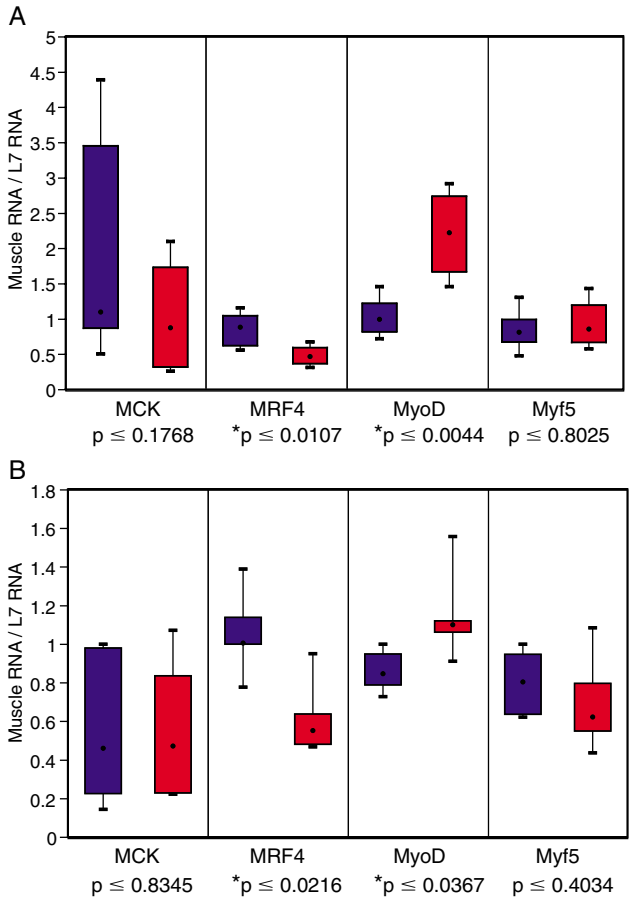

Fig. 6. Skeletal muscle gene expression of *Mck*, *Mrf4*, *Myod1* and *Myf5* in $Myog^{flox/flox}$ and $Myog^{floxΔ/floxΔ}$ mice. Quantitative RT-PCR was performed on RNA prepared from hindlimb muscle at 3 days (**A**) and 2 weeks (**B**) of age. In each case, the ratio of myogenin to ribosomal protein L7 RNA for one RNA sample with a $Myog^{flox/flox}$ genotype was arbitrarily set to 1, and all other ratios from both $Myog^{flox/flox}$ and $Myog^{floxΔ/floxΔ}$ genotypes were normalized to that value. The median value for each genotype is shown as a black dot. Asterisks indicate significant differences between the two genotypes. Error bars indicate one s.d. from the median.

Small size of $Myog^{floxΔ/floxΔ}$ mice

Despite the normal histological appearance of their skeletal muscle, $Myg^{floxΔ/floxΔ}$ mice were uniformly smaller than $Myog^{flox/flox}$ mice. Fig. 7A shows a representative image of $Myog^{floxΔ/floxΔ}$ and $Myog^{flox/flox}$ littermates at 12 weeks after birth. The $Myog^{floxΔ/floxΔ}$ mouse weighed 30% less than its $Myog^{flox/flox}$ littermate, although it was not thinner or wasted, but rather proportionally smaller. To show that the smaller size of $Myog^{floxΔ/floxΔ}$ mice was the result of a slower growth rate, littermates produced from a pregnant female injected with tamoxifen at E17.5, were weighed from 1.5 to 9 weeks after birth. Throughout this time, $Myog^{floxΔ/floxΔ}$ mice weighed less than their $Myog^{flox/flox}$ littermates (Fig. 7B). Moreover, the smaller size and decreased weight was not specific to this litter, as the weights of 6-week-old $Myog^{floxΔ/floxΔ}$ and $Myog^{flox/flox}$ mice from multiple $Myog^{flox/flox}$;+/+ ×

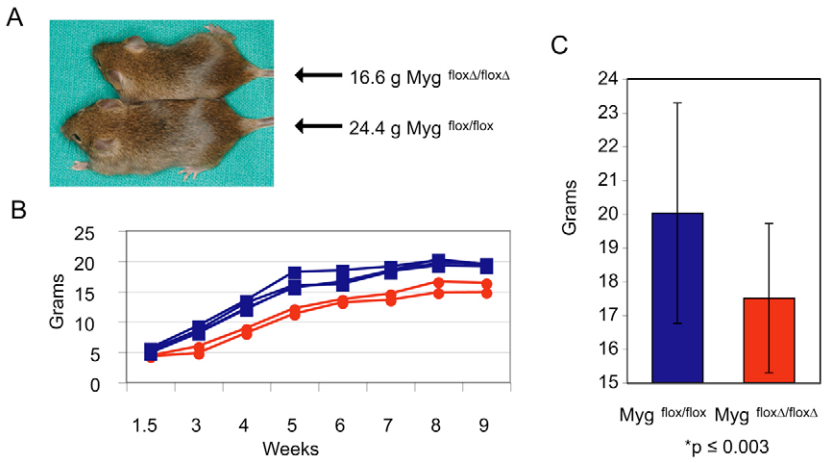

Fig. 7. Body size and weight differences between *Myog*$^{flox/flox}$ and *Myog*$^{flox\Delta/flox\Delta}$ mice during postnatal growth. (**A**) Image of *Myog*$^{flox/flox}$ and *Myog*$^{flox\Delta/flox\Delta}$ littermates at 12 weeks of age. (**B**) Growth curves for two litters of *Myog*$^{flox/flox}$ and *Myog*$^{flox\Delta/flox\Delta}$ mice from 1.5 to 9 weeks of age. Each data point represents one individual mouse. (**C**) Range in body weights for the two genotypes at 12 weeks of age. Asterisk indicates significant mean weight difference between the two genotypes. Error bars indicate one s.d. from the mean.

Myog$^{flox/flox}$;*CAGGCre-ER*^{TM}/+ matings were significantly different (Fig. 7C). The mean weight for *Myog*$^{flox/flox}$ mice was 20.0 g, and for *Myog*$^{flox\Delta/flox\Delta}$ mice, 17.5 g.

From birth until adulthood, *Myog*$^{flox\Delta/flox\Delta}$ mice ate and drank with the same regularity as their *Myog*$^{flox/flox}$ littermates. We have not established whether *Myog*$^{flox\Delta/flox\Delta}$ mice consumed the same number of calories as the wild-type control mice, but they showed no signs of loss of appetite and they were not forcibly excluded from food or water sources by their larger littermates. Collectively, these results show that the absence of myogenin in postnatal life had an unexpected consequence on normal body growth. The smaller size of *Myg*$^{flox\Delta/flox\Delta}$ mice was clearly a secondary effect of the absence of myogenin because *Myog* expression is restricted to skeletal muscle and muscle precursors throughout life (Cheng et al., 1993; Cheng et al., 1995).

Igf1 is expressed in skeletal muscle and has been broadly implicated in skeletal muscle growth, hypertrophy and regeneration through a calcineurin-mediated pathway (Musaro et al., 1999; Grounds, 2002). IGFs, IGF receptors and IGF-binding proteins (IGFBPs) regulate the growth of many tissues (Baker et al., 1993; Liu et al., 1993; Peng et al., 2003; Fisher et al., 2005). IGFBP2, which is required for IGF1 and IGF2 activity, is expressed in limb mesoderm and has been recently shown regulate long bone growth in chicks (Fisher et al., 2005). Upregulation of *Igfbp2* or downregulation of *Igf1* and *Igf2* might therefore indirectly regulate body size by affecting bone growth. If myogenin regulated the expression of the genes encoding these factors, skeletal muscle from *Myog*-depleted mice might have altered expression levels compared with wild-type levels. However, quantitative RT-PCR with RNA isolated from hindlimb tissue of P14 *Myog*-depleted mice showed no evidence for a significant change in the expression of *Igf1*, *Igf2* and *Igfbp2* when compared with expression in the corresponding tissues of wild-type mice (Table 1).

Table 1. Expression levels of *Igf1*, *Igf2* and *Igfbp2* are not significantly altered in the absence of myogenin

Gene	Fold change of *Myog*$^{flox\Delta/flox\Delta}$ versus *Myog*$^{flox/flox}$*
Igf1	1.06±0.16
Igf2	0.84±0.12
Igfbp2	0.86±0.13

*Calculated as $2^{-(\Delta\Delta CT)}$ for each gene. For this comparison, a P14 *Myog*$^{flox/flox}$ mouse weighing 9.1 grams and a P14 *Myog*$^{flox\Delta/flox\Delta}$ mouse weighing 5.7 grams were used. Mean expression levels were normalized using Rpl7 and final values calculated as ΔCt *Myog*$^{flox\Delta/flox\Delta}$ minus ΔCt *Myog*$^{flox/flox}$. Values are fold change ($2^{-(\Delta\Delta CT)}$)±s.d. of six PCR replicates.

DISCUSSION

Myogenin is not essential for postnatal skeletal muscle growth

In this study, we used mice genetically engineered to contain a conditional Cre recombinase-expressing transgene and a floxed *Myog* allele in their genomes to assess the in vivo consequences of removing myogenin after embryonic skeletal muscle development but before postnatal skeletal muscle growth. Activating Cre recombinase at E15.5 or E17.5 resulted in an 80-97% reduction of floxed *Myog* sequences in genomic DNA and, by 3 days after birth, a corresponding attenuation of *Myog* transcripts and protein. From P3 until maturity, skeletal muscle mass in mice increases concomitant with increased body mass and can represent as much as 50% of the total added mass (Allen et al., 1979). This crucial growth period should be exceptionally sensitive to disruptions in any events that regulate skeletal muscle growth. Unexpectedly, we found that myogenin, an essential regulator of embryonic skeletal muscle development, was dispensable for skeletal muscle growth in postnatal life.

Because *myogenin* is expressed exclusively in skeletal muscle and its progenitors, its deletion from the genomes of non-skeletal muscle cell types should not be relevant. However, Cre recombination did not result in the complete elimination of myogenin from all genomes, implying that while most skeletal muscle cells in *Myog*$^{flox\Delta/flox\Delta}$ mice were *Myog*-null, a few were wild type. However, the *Myog*$^{flox\Delta/flox\Delta}$ mice that we chose to analyze for skeletal muscle abnormalities had more than 85% of their floxed myogenin sequences deleted from their genomes, and, in most cases, more than 95%. We previously showed that chimeric mice composed of 40% or more *Myog*-deleted myoblasts cannot support skeletal muscle development (Myer et al., 1997). It is therefore unlikely that the few *Myog*-positive cells that were present in *Myog*$^{flox\Delta/flox\Delta}$ mice would be capable of suppressing any potential postnatal skeletal muscle defects that might be associated with *Myog*-deleted cells.

It is also possible that, in some cells, only one of the floxed *Myog* alleles was deleted, thereby resulting in some cells that were heterozygous for functional *Myog*, some that were *Myog* null and some that were wild type. However, the overall levels of *Myog* transcript and protein in *Myog*$^{flox\Delta/flox\Delta}$ mice were still well below those required for embryonic and postnatal skeletal muscle development, as we showed previously using a hypomorphic, low-expressing allele of *Myog* (Vivian et al., 1999).

Satellite cells are crucial for postnatal muscle growth, and if these cells were compromised by the loss of myogenin, severe consequences for skeletal muscle growth would result. Clearly,

this was not what we observed. Although we have not determined directly that floxed *Myog* sequences were deleted from the satellite cells of $Myog^{floxΔ/floxΔ}$ mice, we never observed myogenin-positive cells in regions where active satellite cells reside. In addition, we showed that genomic DNA from postnatal skeletal muscle, which contains both multinucleated myofibers and satellite cells, was deficient in floxed *Myog* sequences. Because tamoxifen-induced Cre recombinase-mediated deletion of *Myog* was effective in both tail and hindlimb DNA, it seems unlikely that recombination at the *Myog* locus would be selectively inhibited in satellite cells.

The most likely interpretation of our results is that mice lacking functional levels of myogenin are fully capable of generating skeletal muscle in postnatal life. Except for their proportionally smaller size, $Myog^{floxΔ/floxΔ}$ mice behaved identically to their control littermates. Moreover, $Myog^{floxΔ/floxΔ}$ mice up to 2 years of age do not appear to be noticeably different from wild-type mice, except for their small size.

Embryonic lethality and small body size associated with $Myog^{floxΔ/floxΔ}$ mice

Two unexpected features of $Myog^{floxΔ/floxΔ}$ mice emerged from our analysis. First, ~50% of the $Myog^{floxΔ/floxΔ}$ mice died between E18.5 and P10; and second, the $Myog^{floxΔ/floxΔ}$ mice that did survive were approximately two-thirds the size of wild-type mice of the same age. Although the basis of the perinatal lethality associated with $Myog^{floxΔ/floxΔ}$ mice is uncertain, it is possible that some $Myog^{floxΔ/floxΔ}$ mice inherited a modifier gene from either the C57BL/6 or 129 genome that uncovered a cryptic $Myog^{floxΔ/floxΔ}$ phenotype associated with an essential process in perinatal skeletal muscle growth. Whatever the explanation for the perinatal lethality, our results show clearly that the $Myog^{floxΔ/floxΔ}$ mice that do survive beyond P10 have skeletal muscle that is indistinguishable from that of wild-type mice.

The proportionally smaller body size and mass of $Myog^{floxΔ/floxΔ}$ mice when compared with wild-type mice may reflect a subtle role for myogenin in energy homeostasis (Carbo et al., 2001). Although a great deal is known about the role of adipose and nerve tissue in energy homeostasis, little is known about the role that skeletal muscle might have in this process (Argiles et al., 2005). Skeletal muscle accounts for greater than 30% of the energy expenditure in mice (Smith and Muscat, 2005). Myogenin has been implicated in influencing energy metabolism in skeletal muscle by inducing a shift of enzyme activity from glycolytic to oxidative metabolism (Hughes et al., 1999; Ekmark et al., 2003). This could indicate that myogenin has broader roles in regulating genes involved in body-wide homeostasis. However, we found no substantial alterations in the expression of *Igf1*, *Igf2* and *Igfbp2* in *Myog*-depleted hindlimbs, suggesting that their effects on long bone growth are not regulated by myogenin. Nonetheless, skeletal muscle is a source of many other growth factors and cytokines and these secreted factors may have functions in communicating globally for regulating growth (Zoico and Roubenoff, 2002). Myogenin could directly or indirectly control the expression of genes encoding secreted factors. Myostatin, a member of the TFGβ superfamily, is a potent negative regulator of muscle differentiation but also appears to modulate adipose metabolism, indicating that myostatin acts systemically in adult mice to regulate both skeletal muscle and adipose growth (Zimmers et al., 2002; Argiles et al., 2005). In addition to myostatin, interleukin 15, interleukin 16 and TNFα are all expressed in skeletal muscle, and have been implicated in regulating skeletal muscle and adipose metabolism (Argiles et al., 2005).

Myogenin-independent mechanisms for postnatal skeletal muscle growth

The role of myogenin as an important regulator of skeletal muscle gene expression and its relationship with Myod1, Myf5 and Mrf4 are well defined (Penn et al., 2004; Blais et al., 2005; Tapscott, 2005). It was therefore surprising to find that removing myogenin in early postnatal life did not interfere with skeletal muscle growth. Our results, however, do not formally exclude an earlier function for myogenin in establishing the appropriate genetic program in Pax3/Pax7-positive cells, which are the precursors of postnatal satellite cells (Relaix et al., 2005; Kassar-Duchossoy et al., 2005). Myogenin might have had a crucial function in satellite cell development before it was depleted from postnatal $Myg^{floxΔ/floxΔ}$ mice.

Although an earlier function in satellite cell development cannot be ruled out, we favor the simpler hypothesis that mechanisms entirely independent of myogenin are required for postnatal skeletal muscle growth. In fact, the massive growth in skeletal muscle observed in mice in perinatal and postnatal life may have only minor dependency on the myogenic bHLH regulators. Pax3 and Pax7 are likely to be the transcription factors responsible for activating the downstream differentiation events in postnatal satellite cells (Relaix et al., 2005; Kassar-Duchossoy et al., 2005). Downstream regulators may include SRF and MRTF-A, which have been shown to be crucial for perinatal muscle growth (Li et al., 2005). However, it is unlikely that skeletal muscle growth relies entirely on SRF/MRTF-A-mediated mechanisms as these factors appear to be involved in controlling protein accretion within myofibers rather than in regulating satellite cell proliferation and differentiation.

Mef2 proteins may be major regulators of skeletal muscle growth, based on comparisons between vertebrate and invertebrate species. Vertebrates depend much more on myogenic bHLH factors than do invertebrates, which depend mainly on Mef2 proteins for embryonic muscle development (Olson and Klein, 1998). The dependence of vertebrate embryonic skeletal muscle development on the myogenic bHLH regulators suggests that in the vertebrate lineage, these proteins evolved specialized functions at multiple steps in the myogenic pathway. Mef2 factors may have retained their importance in perinatal and postnatal skeletal muscle growth in vertebrates, while the myogenic bHLH factors have evolved novel regulatory functions in embryonic muscle development that have supplanted Mef2 factors. It is known that Mef2 proteins act as major transducers of Ca^{+2} signaling events and that these events have a central role in the hypertrophic growth and remodeling of adult skeletal muscle in response to mechanical load (Olson and Williams, 2000). It is therefore possible that postnatal skeletal muscle growth depends more on Ca^{2+} signaling and Mef2 proteins than on the myogenic bHLH factors.

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