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 Mrf5, 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; Tapscoft, 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 Ponnall 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 (Klabar 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 EcoRI site of pBluescript (Stratagene, La Jolla, CA). A floxed neomycin cassette (Bi et al., 1999) was inserted into the first intron at a unique BamHI site at position +620 for positive selection. AloxP sequence was inserted into a unique BamHI site at position −303 in the promoter region of Myog. A second construct was generated with a modifiedloxP sequence inserted at position +40 in the 5′ untranslated region (UTR) of Myog. The modifiedloxP 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 Sall 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 cells (provided by Richard Behringer, The University of Texas M. D. Anderson Cancer Center) and positive colonies were selected using G418. 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.

**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 FSD (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 Myogflox/flox:CAGGC:notE3/4 or Myogflox/flox:CAGGC:notE3/4 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 Myogflox so that Myog primers corresponding to sequences in the first exon of Myog would amplify fragments specifically representing undeleted Myogflox. 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 Myogflox 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, DNA 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 Iglf1, Iglf2 and Iglfhp2, RNA was isolated from a 30 μm section of hindlimb with skin and fat removed. RNA was isolated using an RNaseasy 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-fifth 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’-CAAGTATTTTACCCACGAGGT-3’; il7 (reverse), 5’-TTATCCGCTCATAAAAAGCATGTTT-3’; ilmy5 (forward), 5’-TCAGTTGAGTGATCTGGGACTG-3’; ilmy5 (reverse), 5’-ACGTATAGTGATGTGGAGCAGTGC-3’; ilmy1d (forward), 5’-CTGCTCTCTCAGACGCG-3’; ilmy1d (reverse), 5’-GGCTGAGATGGTCCAAGGCT-3’. Myg1 (5’), 5’-CCCTGAAAAGAGGGGACCTGGG-3’; Myg1 (3’), 5’-TCATTCAGTTCTTGAGCAGAGACATCTTGC-3’; Mck (lower), 5’-AGGTTGAGTCAAAACAGGCTGCTTGG-3’. Mck (upper), 5’-CAATAGGCTTTCAAGTGGAGGAGG-3’. Igfl1a (forward), 5’-GGAGTTCATTTCTCATACCGAGTCTGCTC-3’; Igfl1a (reverse), 5’-CTGAGGACACAACTGTCCCTTCCT-3’. igm1a (forward), 5’-CTGGAAGGAAAGGGGACTGGG-3’; igm1a (reverse), 5’-TGAGAGGGATGCTTGAGGAGGAGG-3’. igm2a (forward), 5’-CTGGAAGGAAAGGGGACTGGG-3’; igm2a (reverse), 5’-TGGTACCGCTGCTAAGGCTGCTTGG-3’. igm2a (forward), 5’-ACATGGTTGAGGAGGAGGACCTGGG-3’. igm2a (reverse), 5’-TCATTCAGCTTGGACAGACATGCTGCTC-3’. Igfl2a (forward), 5’-TGGTACCGCTGCTAAGGCTGCTTGG-3’. Igfl2a (reverse), 5’-TGGTACCGCTGCTAAGGCTGCTTGG-3’. Igfl2a (forward), 5’-ACATGGTTGAGGAGGAGGACCTGGG-3’. Igfl2a (reverse), 5’-TCATTCAGCTTGGACAGACATGCTGCTC-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 Myoglox 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 Myoglox allele (Fig. 1A,B). Four Myoglox/lox heterozygous mouse lines were established for further investigation; mice containing the Myoglox allele are available upon request.

To determine whether functional myogenin protein was expressed from the Myoglox allele, Myoglox/lox mice were mated to mice heterozygous for a null allele of Myog (Myog+/-) to generate mice with a Myoglox 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 Myoglox 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 Myoglox behaves as a null allele following Cre recombine-mediated deletion. To do this, we mated Myoglox/lox mice to transgenic mice expressing CMV-Cre to generate a floxed allele, Myoglox, that lacked the first exon of Myog (Fig. 2A). CMV-Cre mice first express Cre recombine in the zygote, several days before the formation of skeletal muscle (Arango et al., 1999). As expected, Myoglox/lox,lox mice died as neonates and had skeletal muscle deficiencies resembling those of Myog-/- mice (Fig. 2B,C). Diaphragms from E18.5 Myoglox/lox,lox embryos were abnormally thin and lacked myofibers, closely resembling diaphragms from E18.5 Myog-/- embryos (Fig. 2B). Hindlimbs from E18.5 Myoglox/lox,lox,lox 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 Myoglox/lox,lox,lox embryos, corresponding hindlimbs from Myoglox/lox,lox,lox
embryos showed normal skeletal muscle in the hindlimbs and efficient expression of myogenin (Fig. 2C). These results demonstrate that the Myogflox allele can be effectively deleted using the Cre-loxP system.

**Generation of mice lacking myogenin in postnatal life**

Mice with the genotype Myogflox/flox;+/+ were mated to Myogflox/flox;CAGGCre-ERTM/+ 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 Myogflox/floxΔ mice were fully viable, a 1:1 ratio of Myogflox/floxΔ pups to Myogflox/+ pups would be expected. However, after either day of tamoxifen injection, we consistently found a ratio of 2:1 in the offspring from Myogflox/flox;+/+ matings (Fig. 3A,B). These results indicated that 50% of the Myogflox/floxΔ mice – those that inherited the CAGGCre-ERTM transgene and therefore had a Myogflox/floxΔ genotype – were dying before P10. It is not clear why these mice did not survive as the Myogflox/+ mice that did survive were fully viable, although by P10 they were noticeably smaller than their Myogflox/flox littermates (see below). Myogflox/flox and MyogfloxΔ/floxΔ embryos were found at 1:1 ratios at E18.5 (data not shown), suggesting that 50% of the MyogfloxΔ/floxΔ mice died immediately before birth or early in postnatal life. The reduced viability associated with MyogfloxΔ/floxΔ 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
129/C57BL/6 genetic background was inherited by 50% of the offspring and enhanced a lethal phenotype in $Myog^{floxed/flox}$ mice. We have not yet pursued the possibility of a modifier gene, and the basis for the lethality observed in some of the $Myog^{floxed/flox}$ 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 $Myog^{floxed/flox}+/+ \times Myog^{floxed/flox};CAGGCpe-ER^TM+/+$ crosses. We observed efficient cre-recombinase-mediated deletion of myogenin in the genomes of $Myog^{floxed/flox}$ mice that inherited the $CAGGCpe-ER^TM$ transgene. Genomic DNA from these pups contained only 3-20% of the floxed myogenin sequence, whereas the corresponding genomic DNA from $Myog^{floxed/flox}$ littermates that did not inherit the $CAGGCpe-ER^TM$ transgene contained 80-120% of the floxed myogenin sequence (Fig. 4A). The detectable residual myogenin DNA that was seen in $Myog^{floxed/flox}$ genomes was probably an overestimate because the high cycle threshold values for quantitative PCR with 605

**Fig. 3. Perinatal lethality associated with $Myog^{floxed/flox}$ mice.**
Mice with the genotype $Myog^{floxed/flox}/+$ were mated to $Myog^{floxed/flox};CAGGCpe-ER^TM+/+$ 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.

Fig. 4. Deletion of myogenin and attenuation of myogenin transcripts and protein in $Myog^{floxed/flox}$ mice.
(A) Quantitative PCR of tail DNA to determine the extent of deletion of floxed myogenin sequences in $Myog^{floxed/flox}$ and $Myog^{floxed/flox}$ mice at P10. The ratio of myogenin to Myf5 genomic DNA from one $Myog^{floxed/flox}$ mouse was arbitrarily set to 1 and all other ratios from both $Myog^{floxed/flox}$ and $Myog^{floxed/flox}$ genotypes were normalized to that value. (B) Southern genome hybridization with EcoRI-digested DNA extracted from the hindlimb skeletal muscle of 10-week-old mice from a $Myog^{floxed/flox};+/+ \times Myog^{floxed/flox};CAGGCpe-ER^TM+/+$ cross and probed with myogenin cDNA. The 6 kb and 3 kb bands represent the $Myog^{floxed}$ and $Myog^{floxed}$ alleles, respectively, as depicted in Fig. 1. The skeletal DNA in lanes 1 and 3 contained very little of the $Myog^{floxed}$ allele and therefore represents a $Myog^{floxed/flox}$ genotype, while the skeletal DNA in lanes 2, 4, 5 and 6 contained no $Myog^{floxed}$ allele and therefore represents a $Myog^{floxed/flox}$ genotype. (C) Quantitative RT-PCR to determine the levels of myogenin 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 myogenin to ribosomal protein L7 RNA for one RNA sample with a $Myog^{floxed/flox}$ genotype was arbitrarily set to 1, and all other ratios from both $Myog^{floxed/flox}$ and $Myog^{floxed/flox}$ 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 $Myog^{floxed/flox}$ (right panel) and $Myog^{floxed/flox}$ (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$^{\text{flox}/\text{flox}}$ mice tended to inflate the final calculation. However, even if 20% of the cells in Myog$^{\text{flox}/\text{flox}}$ 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$^{\text{flox}/\text{flox}}$ 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$^{\text{flox}/\text{flox}}$ 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$^{\text{flox}/\text{flox}}$ 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$^{\text{flox}/\text{flox}}$ pups (Fig. 4D, right panel), although control Myog$^{\text{flox/flox}}$ littermates showed robust expression in myofiber nuclei (Fig. 4D, left panel). In this case, genomic Myog in the Myog$^{\text{flox}/\text{flox}}$ 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$^{\text{flox}/\text{flox}}$ mice.

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

Skeletal muscle of Myog$^{\text{flox}/\text{flox}}$ mice and their Myog$^{\text{flox/flox}}$ 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$^{\text{flox}/\text{flox}}$ littermates were notably thinner than their Myog$^{\text{flox/flox}}$ 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$^{\text{flox}/\text{flox}}$ mice examined ($n=20$) had normal musculature in their diaphragms (Fig. 5J).
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$^{+/+}$ and Myog$^{+/\Delta} \Delta$ mice were indistinguishable from each other (Fig. 5K). Moreover, the Myog$^{+/\Delta} \Delta$ mice were as active as Myog$^{+/+}$ 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$^{+/\Delta} \Delta$ mice

The lack of noticeable skeletal muscle defects in adult Myog$^{+/\Delta} \Delta$ 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 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$^{+/+}$ and Myog$^{+/\Delta} \Delta$ 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$^{+/+}$ and Myog$^{+/\Delta} \Delta$ 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 downregulated in Myog$^{-/-}$ mice (Hasty et al., 1993), but we observed less than twofold downregulation of Mrf4 expression in Myog$^{+/\Delta} \Delta$ mice compared with Myf4 expression in Myog$^{+/+}$ 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$^{+/\Delta} \Delta$ mice at 3 days and 2 weeks after birth, respectively, compared with Myog$^{+/+}$ control mice (Fig. 6). Although the differences in Myod1 expression between Myog$^{+/\Delta} \Delta$ and Myog$^{+/+}$ 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 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).

Small size of Myog$^{+/\Delta} \Delta$ mice

Despite the normal histological appearance of their skeletal muscle, Myog$^{+/\Delta} \Delta$ mice were uniformly smaller than Myog$^{+/+}$ mice. Fig. 7A shows a representative image of Myog$^{+/\Delta} \Delta$ and Myog$^{+/+}$ littermates at 12 weeks after birth. The Myog$^{+/\Delta} \Delta$ mouse weighed 30% less than its Myog$^{+/+}$ littermate, although it was not thinner or wasted, but rather proportionally smaller. To show that the smaller size of Myog$^{+/\Delta} \Delta$ 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$^{+/\Delta} \Delta$ mice weighed less than their Myog$^{+/+}$ littermates (Fig. 7B). Moreover, the smaller size and decreased weight was not specific to this litter, as the weights of 6-week-old Myog$^{+/\Delta} \Delta$ and Myog$^{+/+}$ mice from multiple Myog$^{+/+}$ mice.
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 concomitantly 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 genotypes, implying that while most skeletal muscle cells in Myog<sup>−/−</sup> mice were Myog-null, a few were wild type. However, the Myog<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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,

\[ \text{Expression levels of Igf1, Igf2 and Igfbp2 are not significantly altered in the absence of myogenin} \]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change of Myog&lt;sup&gt;−/−&lt;/sup&gt; vs Myog&lt;sup&gt;+/+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igf1</td>
<td>1.06±0.16</td>
</tr>
<tr>
<td>Igf2</td>
<td>0.84±0.12</td>
</tr>
<tr>
<td>Igfbp2</td>
<td>0.86±0.13</td>
</tr>
</tbody>
</table>

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

Myog<sup>flox/flox</sup>-CAGGCre-ERT<sup>TM/+</sup> matings were significantly different (Fig. 7C). The mean weight for Myog<sup>flox/flox</sup> mice was 20.0 g, and for Myog<sup>flox/flox</sup> mice, 17.5 g.

From birth until adulthood, Myog<sup>flox/flox</sup> mice ate and drank with the same regularity as their Myog<sup>flox/flox</sup> littermates. We have not established whether Myog<sup>flox/flox</sup> 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 Myog<sup>flox/flox</sup> 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).
this was not what we observed. Although we have not determined directly that floxed Myog sequences were deleted from the satellite cells of Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} 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\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} mice behaved identically to their control littermates. Moreover, Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} 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\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} mice**

Two unexpected features of Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} mice emerged from our analysis. First, ~50% of the Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} mice died between E18.5 and P10; and second, the Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} 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\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} mice is uncertain, it is possible that some Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} mice inherited a modifier gene from either the C57BL/6 or 129 genome that uncovered a cryptic Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} phenotype associated with an essential process in perinatal skeletal muscle growth. Whatever the explanation for the perinatal lethality, our results clearly show that the Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} 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\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} 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 TGF\beta 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 TNF\alpha 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 Myog\textsuperscript{flox}\textsuperscript{\Deltalox}\textsuperscript{\Deltalox} 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\textsuperscript{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\textsuperscript{2+} signaling and MeF2 proteins than on the myogenic bHLH factors.

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**References**


