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Foxj3 transcriptionally activates Mef2c and regulates adult skeletal muscle fiber type identity

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ABSTRACT

The mechanisms that regulate skeletal muscle differentiation, fiber type diversity and muscle regeneration are incompletely defined. *Forkhead* transcription factors are critical regulators of cellular fate determination, proliferation, and differentiation. We identified a *forkhead*/winged helix transcription factor, Foxj3, which was expressed in embryonic and adult skeletal muscle. To define the functional role of Foxj3, we examined Foxj3 mutant mice. Foxj3 mutant mice are viable but have significantly fewer Type I slow-twitch myofibers and have impaired skeletal muscle contractile function compared to their wild type controls. In response to a severe injury, Foxj3 mutant mice have impaired muscle regeneration. Foxj3 mutant myogenic progenitor cells have perturbed cell cycle kinetics and decreased expression of Mef2c. Examination of the skeletal muscle 5′ upstream enhancer of the Mef2c gene revealed an evolutionary conserved *forkhead* binding site (FBS). Transcriptional assays in C2C12 myoblasts revealed that Foxj3 transcriptionally activates the Mef2c gene in a dose dependent fashion and binds to the conserved FBS. Together, these studies support the hypothesis that Foxj3 is an important regulator of myofiber identity and muscle regeneration through the transcriptional activation of the Mef2c gene.

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Introduction

The molecular networks that govern muscle differentiation, fiber diversity and muscle regeneration remain incompletely defined. Previous studies have uncovered an essential role for the basic helix loop helix (bHLH) MyoD family members (i.e., MyoD, myf5, MRF4 and myogenin) and myocyte enhancer factor 2 (Mef2) factors, but the repertoire of interacting factors and upstream regulators that coordinately regulate skeletal muscle differentiation and fiber type specification remain an area of intense interest. Studies have demonstrated that the bHLH MyoD factors interact with Mef2 factors to synergistically coactivate the myogenic program. Global elimination of Mef2c results in early embryonic lethality due to cardiovascular defects (Lin et al., 1997). Conditional elimination of Mef2c in skeletal muscle revealed a role in fiber type specification as there was a significant decrease in Type I oxidative slow myofibers (Potthoff et al., 2007). While efforts have focused on downstream targets of Mef2c, few direct upstream regulators of Mef2c have been identified in the skeletal muscle lineage.

Forkhead/winged helix transcription factors perform an array of functions including fate specification, pattern formation, cellular proliferation, cellular differentiation and organogenesis (Lehmann et al., 2003). Members of the *forkhead*/winged helix transcription factors function through a DNA-binding dependent mechanism or alternatively through protein–protein interactions to regulate gene expression (Wijchers et al., 2006). For example, the *forkhead*/winged helix factor, Foxk1, is expressed in myogenic progenitor cells and functions as a cell cycle regulator by regulating the cyclin-dependent kinase inhibitor, $p21^{KIP}$(Garry et al., 2000). Mice lacking Foxk1 have perturbed skeletal muscle regeneration due to impaired activation/proliferation of the myogenic progenitor cell population (Hawke et al., 2003a). In addition to Foxk1, other *forkhead* factors including FoxO factors modulate cell signaling pathways, growth and atrophy of adult skeletal muscle (Sandri et al., 2004). These studies suggest that *forkhead* factors have critical roles in the regulation of skeletal muscle development and regeneration.

Nonstandard abbreviations: Fox, Forkhead box; FBS, forkhead binding site; Mef2c, Myocyte enhancer factor 2c; luc, luciferase; β-gal, β-galactosidase; bHLH, basic Helix-Loop-Helix; H&E, hemotoxylin and eosin; NFR, nuclear fast red; WT, wild type; GFP, green fluorescent protein; DMEM, Dulbecco's modification of eagle media; ES, embryonic stem; ChIP, chromatin immunoprecipitation; EDL, extensor digitorum longus; TA, tibialis anterior; MCK, muscle creatine kinase.

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To further identify *forkhead* factors expressed in the myogenic lineages, we undertook a candidate based screen. Using this strategy, we identified Foxj3 as being significantly upregulated in differentiating myoblasts. To further explore the functional role of Foxj3 *in vivo*, we obtained a Foxj3 gene-targeted ES cell line and generated mutant mice that have a β-*geo* cassette flanked by two splice acceptors inserted into the Foxj3 locus. The resulting mutant Foxj3 allele produces a transcript that encodes exons 1–5 (1–176 amino acids) of the Foxj3 protein that lacks a transcriptional activation domain. Mice homozygous for both the mutant alleles (referred to as Foxj3m/m) are viable, but have impaired skeletal muscle contractility and decreased Type I oxidative myofibers compared to their wild type controls. In addition, Foxj3m/m mice have impaired skeletal muscle regeneration following injury, impaired cell cycle kinetics of the myogenic progenitor cell population and decreased expression of Mef2c. Examination of the 5′ upstream skeletal muscle enhancer of Mef2c revealed a highly conserved *forkhead* binding site (FBS). Transcriptional assays in C2C12 myoblasts demonstrated that Foxj3 activates a Mef2c-*luciferase* reporter and mutagenesis of the FBS in the Mef2c skeletal muscle enhancer ablates this transcriptional activation. Together these studies reveal that Foxj3 is a transcriptional activator of Mef2c, and is an important regulator for adult muscle fiber type identity and skeletal muscle regeneration.

Results

We have utilized an array of techniques to define the expression of *forkhead* family members in the skeletal muscle lineage during development and regeneration. To complement these studies, we utilized a C2C12 myoblast differentiation assay to analyze gene expression during discrete phases of myogenesis (Shi and Garry, 2006). We identified a novel member of the *forkhead*/winged helix family, Foxj3, which was dynamically expressed during C2C12 myogenic differentiation using semi-quantitative RT-PCR (Fig. 1 and Supplemental Figure S1). This differentiation assay revealed that Foxj3 and Mef2c preceded myoglobin expression during myogenesis. These studies support the hypothesis that Foxj3 is expressed and regulated during myogenesis.

Generation of Foxj3m/m mice

To define the functional role of Foxj3, we generated Foxj3 mutant mice (referred to as Foxj3m/m) from a BayGenomics© ES cell "trapped" line (Stryke et al., 2003). The Foxj3 trapped ES cell line contained an insertion of a β-galactosidase/neomyocin (β-*geo*) cassette with two separate splice acceptors (Fig. 2A). The resulting mRNA predicted transcript encodes exons 1 through 5 and contains all but the last six residues of the *forkhead* DNA-binding domain. No transcripts were detected following exon 6 of the native Foxj3 gene (Figs. 2A–C). Previous studies using Gal4 transcriptional assays demonstrated that the carboxy-terminus harbors the activation domain of Foxj proteins and is essential for Foxj family of proteins

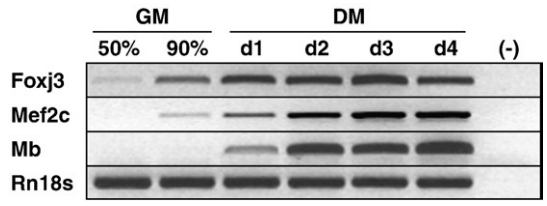

Fig. 1. Foxj3 is expressed in C2C12 myoblasts. Semiquantitative RT-PCR analysis of transcript expression during C2C12 differentiation from myoblasts (50% or 90% confluency, cultured in growth medium or GM) to myotubes (day 1 or d1 to day 4 or d4, cultured in differentiation medium or DM) demonstrates that Foxj3 is expressed in myoblasts prior to myoglobin (Mb) expression. Rn18s was used as a loading control. (−) represents a negative control (lacking reverse transcriptase).

functional activity (Pérez-Sánchez et al., 2000). Thus, we hypothesized that the fusion protein encoded from Foxj3m/m mice would result in a nonfunctional, transcriptionally inactive fusion protein. Additionally, we predicted that the Foxj3-β-gal fusion protein would recapitulate Foxj3 endogenous expression patterns.

Foxj3 mutant mice are viable

Mice homozygous for the Foxj3-β-*geo* targeted allele were generated from matings of heterozygote mice. Initially, our studies utilized the C57/B6J:129OlaP2Hsd mixed strain; however, we have backcrossed the original chimeric mice with inbred C57/B6J and 129OlaP2Hsd strains separately and over six generations and we observed no strain variances. Mice homozygous with the Foxj3 mutant alleles, generated from heterozygote matings, were viable and born at normal Mendelian ratios (Supplemental Figure S2). Semi-quantitative RT-PCR of gastrocnemius skeletal muscle from wildtype and homozygous Foxj3 mutant mice revealed that expression of the Foxj3 mRNA transcript was as predicted limited to exons 1–5, and that the carboxy-terminal activation domain in exons 8–12 were not transcribed (Fig. 2C).

Foxj3m/m mice have impaired skeletal muscle regeneration following injury

As previously described (Landgren and Carlsson, 2004), Foxj3 was expressed in the myogenic lineage during embyogenesis (Supplemental Figure S3) and in the in vitro myogenic differentiation assay (Fig. 1 and Supplemental Figure S1). We then examined the ability of Foxj3m/m mice to regenerate their skeletal muscle following a severe myonecrotic injury which destroys approximately 90% of the adult skeletal muscle. In response to this cardiotoxin-induced injury, wild type skeletal muscle regenerates within a 2-week period and has restoration of its architecture. In contrast, Foxj3 mutant mice had a severe regenerative impairment that was evident at 2 and 3 weeks following delivery of cardiotoxin. We observed persistent myonecrosis and widespread replacement of the myofibers with adipocytes (Fig. 2D). Using electron microscopy, we observed the presence of myogenic progenitor cells (i.e., satellite cells) in the unperturbed Foxj3m/m skeletal muscle and there were no significant differences in their number compared to the gender and age matched control mice (Supplemental Figure S4 and data not shown).

Foxj3 mutant myogenic progenitor cells have an increased proliferative capacity

To determine the proliferative capacity of the mutant and wildtype myogenic progenitor cell population, we performed cell cycle kinetics assay and an *in situ* proliferation assay. We isolated myogenic progenitor cells from wild type and mutant skeletal muscle and analyzed the cell cycle kinetics in asynchronized proliferating cells. The mutant myogenic progenitor cells had a significant decrease in the G0/G1 phase and a significant increase in the G2/M phase as assayed by flow cytometry ($p<0.05$; $n=3$ littermate pairs) (Figs. 3A and B). These cell cycle results were further supported using the proliferation marker Ki67 (which labels all proliferating cells) in regenerating Foxj3 mutant vs. control skeletal muscle performed 5 days following cardiotoxin induced injury. These results demonstrate an increased proliferative capacity, as measured by Ki67 expression (Fig. 3C) that represented a 65% increase compared to the wild type control ($p<0.05$; $n=3$) (Fig. 3D). Additionally, most of the Ki67-positive cells were also positive for MyoD expression, which supports the notion that the impaired regenerative defect may also be due in part to increased proliferation of the mutant myogenic progenitor cells (Fig. 3E). Importantly, we observed no significant differences in Ki67 expression in unperturbed Foxj3 mutant and wild type control