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Nfix Regulates Fetal-Specific Transcription in Developing Skeletal Muscle

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SUMMARY

Skeletal myogenesis, like hematopoiesis, occurs in successive developmental stages that involve different cell populations and expression of different genes. We show here that the transcription factor nuclear factor one X (Nfix), whose expression is activated by Pax7 in fetal muscle, in turn activates the transcription of fetal specific genes such as MCK and β-enolase while repressing embryonic genes such as slow myosin. In the case of the MCK promoter, Nfix forms a complex with PKC theta that binds, phosphorylates, and activates MEF2A. Premature expression of Nfix activates fetal and suppresses embryonic genes in embryonic muscle, whereas muscle-specific ablation of Nfix prevents fetal and maintains embryonic gene expression in the fetus. Therefore, Nfix acts as a transcriptional switch from embryonic to fetal myogenesis.

INTRODUCTION

During skeletal myogenesis, stage-specific transcriptional changes occur in muscle fibers derived from differentiation of different precursors: embryonic, fetal myoblasts and satellite cells (Biressi et al., 2007a; Stockdale, 1992). "Embryonic" or primary fibers appear at E11 in the mouse and establish the basic muscle pattern. A second wave of myogenesis, termed fetal or secondary, takes place between E14.5 and E17.5 and involves the fusion of fetal myoblasts either with each other to form secondary fibers (initially smaller and surrounding primary fibers), or with primary fibers. At E16, satellite cells appear as mononucleated cells underneath the newly formed basal lamina of each individual fiber: they are responsible for muscle postnatal growth and regeneration. Previous work identified specific features of embryonic, fetal myoblasts and satellite cells and of the myotubes they give rise to (Biressi et al., 2007a). In particular, primary fibers express both slow and embryonic fast MyHCs and ubiquitous isoforms of metabolic enzymes. Conversely, secondary fibers express fast but not slow MyHC and muscle-specific enzymes such as MCK, β-enolase and PKCθ (Biressi et al., 2007a).

A genome-wide expression analysis on purified embryonic and fetal myoblasts (Biressi et al., 2007b) revealed that the transcription factor nuclear factor I X (*Nfix*) is robustly expressed in the fetus but absent in the embryo. Nuclear factor one (Nfi) proteins act as transcriptional activators and/or repressors of cellular and viral genes. In amniotes, the *Nfi* gene family consists of four closely related genes, named *Nfia*, *Nfib*, *Nfic*, and *Nfix* (Gronostajski, 2000). They encode for proteins with a conserved N-terminal DNA-binding and dimerization domain and a C-terminal transactivation/repression domain, which exhibits a high variability due to extensive alternative splicing. Nfi family members act as homo- and heterodimers and bind with high affinity to the palindromic consensus sequence 5′-PyTGGCA-N3-TGCCAPu-3′. Nfi binding motifs were detected in promoters of genes expressed in different organs, including brain, lung, liver, intestine, muscle, connective tissue, and skeletal elements (Gronostajski, 2000); recently *Nfia* was shown to regulate fate choice between erythrocytes and granulocytes (Starnes et al., 2009).

Gene ablation studies revealed that *Nfi* genes have essential and distinct roles in different organ systems including brain (*Nfia*) (das Neves et al., 1999); lung and brain (*Nfib*) (Steele-Perkins

et al., 2005); tooth (*Nfic*) (Steele-Perkins et al., 2003); and brain, intestine, and skeleton (*Nfix*) (Driller et al., 2007). *Nf1*-binding sites are present on the *Myogenin* promoter (Johanson et al., 1999) and Nfi can form a complex with Myogenin, thus increasing its affinity for a number of muscle-specific genes (Funk and Wright, 1992). This suggested a role for Nfi as co-factor in muscle differentiation but its precise function was not subsequently studied. We investigated the function of Nfix during mammalian myogenesis and report here that Nfix represses the expression of embryonic genes while activating fetal genes in developing skeletal muscle; thus it acts as a main regulator of the embryonic to fetal transcriptional switch, a role that has evolutionary significance in amniotes.

RESULTS

Nfix2 Represses Embryonic and Activates Fetal Muscle Genes

Although the pattern of embryonic, fetal, and adult gene expression in skeletal muscle has been described (Gunning and Hardeman, 1991), the underlying molecular control remains elusive. A genome-wide screen in fetal and embryonic myoblasts purified from Myf5$^{GFP-P/+}$ mice (Biressi et al., 2007b) identified the transcription factor nuclear factor one among the differentially expressed genes; in particular *Nfix* appeared as the most differentially expressed (>8-fold change) being abundant in the fetus and virtually absent in the embryo (Figures S1A–S1C available online). Of the various splicing isoforms, we focused on the best characterized, Nfix2, which was shown to transactivate expression of a variety of promoters in fibroblasts, glial cells, and other cell types (Chaudhry et al., 1998) and is strongly expressed in fetal skeletal muscle (Figure S1D). To investigate the possible role of Nfix in fetal myogenesis, we initially performed loss-of-function experiments (dominant negative Nfi-engrailed) in fetal myoblasts and gain of function experiments (expression of Nfix2 isoform) in embryonic myoblasts. As a model of fetal myogenesis, we initially used the myogenic cell line C2C12, which allows more in-depth biochemical analysis than in purified fetal myoblasts, which are limited in amount. Although derived from postnatal muscle, C2C12 cells share many features with fetal myoblasts, including the expression of high levels of Nfix (Biressi et al., 2007b) (Figure S1A). C2C12 cells were transduced with a lentiviral vector expressing the dominant-negative Nfi-engrailed (NfiEngr), containing the DNA binding and dimerization domain of Nfia fused together with the *Drosophila* Engr transcriptional repression domain that inhibitis transactivation activity of all Nfi proteins (Bachurski et al., 2003). A lentivirus expressing only the engrailed domain was used as control (Engr). As shown in Figure S1E, Nfi inhibition negatively affects myogenesis, likely because of reduced Myogenin expression and consequent reduced expression of Myosin Heavy Chains and MCK (Figure S1F). These data are consistent with previous studies (Funk and Wright, 1992; Johanson et al., 1999). BrdU incorporation indicated that in C2C12 cells the NfiEngr block of terminal differentiation was not accompanied by enhanced proliferation rate (data not shown). To test whether a possible modulation of embryonic and fetal gene expression might exist independently of a generic inhibition of terminal differentiation in the whole culture, we analyzed embryonic/fetal-specific gene expression in the few differentiated C2C12NfiEngr myotubes. We used the short trypsinization method (Kitzmann et al., 1998), which causes detachment of myotubes from substrate at a low dose of trypsin. C2C12Engr and C2C12NFIEngr differentiated myotubes (DM mt) were collected and analyzed by western blot in comparison with the whole differentiated culture (DM). As shown in Figure S1G, the C2C12NFIEngr myotubes express total sarcomeric MyHC at levels comparable to Engr controls, but show a strongly reduced expression of fetal markers such as MCK and β-enolase. Conversely, slow MyHC, normally repressed during fetal myogenesis and almost absent in control C2C12 is expressed at high levels. We repeated these experiments on Myf5$^{GFP-P/+}$-purified fetal myoblasts, which were infected by LentiNfiEngr or Engr, as a control. NfiEngr causes a less dramatic inhibition of differentiation (e.g., MyHC expression) in fetal myoblasts than in the C2C12 cells (Figures S1E and S1H). Nevertheless, NfiEngr-expressing fetal myoblasts acquire the typical aspect of their embryonic counterparts, characterized by smaller myotubes and a lower number of nuclei/myotube (data not shown); in addition, western blot analysis confirmed that the two fetal markers analyzed, MCK and β-enolase, were strongly downregulated, whereas slow MyHC was robustly induced in fetal myotubes (Figure S1I). Since NfiEngr blocks the function of all the members of the Nfi family, we investigated whether the effect on fetal myogenesis was specific to Nfix. Fetal myoblasts were isolated by cell sorting from the *Nfix* null//Myf5$^{GFP-P/+}$ mouse embryos (described below). In addition, small interfering RNA (siRNA) silencing of Nfix and Nfia was also performed. Myf5$^{GFP-P/+}$-purified fetal myoblasts were transduced with lentivectors expressing siNfix, siNfia, or the nontargeting control siRNA (CTL). As Figure 1D shows, the inhibition of both Nfix and Nfia does not modulate the expression of the other members of the family, suggesting the absence of reciprocal regulation among the Nfi proteins. Most importantly, the selective inhibition of Nfix, both in the Nfix null myoblasts and in the Nfix silenced cells, dramatically impairs fetal myoblast differentiation and fusion (Figures 1A and 1E) and abolishes the expression of MCK and β-enolase (Figure 1C); despite reduced differentiation, slow MyHC was activated in the *Nfix* null and siNfix myotubes. Silencing of Nfia did not inhibit myogenesis and did not modify the profile of gene expression. As a reciprocal experiment, purified embryonic myoblasts were isolated and infected by a lentiviral vector expressing the Nfix2 and Nfia1.1 isoforms (or an empty vector, as a control). After 3 days in culture, the Nfix2-embryonic myoblasts (Nfix2) formed larger myotubes with an increased fusion index (Figure 1E) in comparison with control cells (CTL) (Figure 1B). In contrast, induction of Nfia1.1 reduced myoblast fusion (Figures 1B and 1E). RT-PCR analysis showed that Nfix2- but not Nfia1.1-differentiated myotubes expressed the fetal marker β-enolase and downregulated expression of the slow MyHC; unexpectedly, MCK, the other fetal marker analyzed, was not expressed (Figure 1C), suggesting the requirement of a cofactor(s) for Nfix2-mediated induction of MCK. In approaches of both loss and gain of function, myogenin was not dramatically affected (data not shown), suggesting that Nfix modulates fetal/embryonic gene expression independently from its role of