

# ***S. macrurus* myogenic regulatory factors (MRFs) induce mammalian skeletal muscle differentiation; evidence for functional conservation of MRFs**

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**ABSTRACT** The current-producing cells of the electric organ, i.e., electrocytes, in *Sternopygus macrurus* derive from skeletal muscle fibers. Mature electrocytes are not contractile, but they do retain some muscle proteins, are multinucleated, and receive cholinergic innervation. Electrocytes express the myogenic regulatory factors (MRFs) MyoD, myogenin, Myf5 and MRF4 despite their incomplete muscle phenotype. Although *S. macrurus* MRFs share functional domains which are highly conserved and their expression is confined to the myogenic lineage, their capability to induce the muscle phenotype has not been determined. To test the functional conservation of *S. macrurus* MRFs to transcriptionally activate skeletal muscle gene expression and induce the myogenic program, we transiently over-expressed *S. macrurus* MyoD (*SmMyoD*) and myogenin (*SmMyoG*) in mouse C3H/10T1/2 and NIH3T3 embryonic cells. RT-PCR and immunolabeling studies showed that *SmMyoD* and *SmMyoG* can efficiently convert these two cell lines into multinucleated myotubes which expressed differentiated muscle markers. The levels of myogenic induction by *SmMyoD* and *SmMyoG* were comparable to those obtained with mouse MRF homologs. Furthermore, *SmMyoD* and *SmMyoG* proteins were able to induce mouse MyoD and myogenin in C3H/10T1/2 cells. We conclude that *S. macrurus* MRFs are functionally conserved as they can transcriptionally activate skeletal muscle gene expression and induce the myogenic program in mammalian non-muscle cells. Hence, these data suggest that the partial muscle phenotype of electrocytes is not likely due to differences in the MRF-dependent transcriptional program between skeletal muscle and electric organ.

**KEY WORDS:** *MyoD*, *myogenin*, *differentiation*, *electric fish*, *skeletal muscle*

## **Introduction**

Skeletal muscle gene expression is controlled largely by the MyoD family of basic-helix-loop helix myogenic regulatory factors (MRFs), which includes MyoD, myogenin, Myf5, and MRF4 (Emerson, 1990; Weintraub, 1993). MRFs encode structurally related transcription factors that bind to E-box consensus promoter elements (CANNTG) (Emerson, 1990; Fujisawa-Sehara *et al.*, 1992; Weintraub, 1993) and are essential in the differentiation and commitment of cells to the muscle phenotype during development. Their role in the differentiation and commitment of cells to the skeletal muscle lineage is well established based on genetic studies demonstrating that myogenesis is severely deficient when

MRF genes are inactivated (Nabeshima *et al.*, 1993; Rudnicki *et al.*, 1993), and expression studies showing that MRFs can activate the myogenic program in non-muscle cells (Weintraub *et al.*, 1989; Choi *et al.*, 1990; Delfini and Duprez, 2004). Moreover, the activation of many muscle-specific genes including cytoskeletal, sarcomeric, metabolic, and cell signaling proteins is known to be under the transcriptional regulation of MRFs (Simon and Burden, 1993; Shield *et al.*, 1996; Lin *et al.*, 1991; Gramolini and Jasmin, 1999; Kraner *et al.*, 1999; Wheeler *et al.*, 1999; Angus *et al.*, 2001;

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*Abbreviations used in this paper:* EO, electric organ; MRF, myogenic regulatory factor; MyoG, myogenin; Sm, *Sternopygus macrurus*.

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Accepted: 23 September 2008. Published online: 12 June 2009.

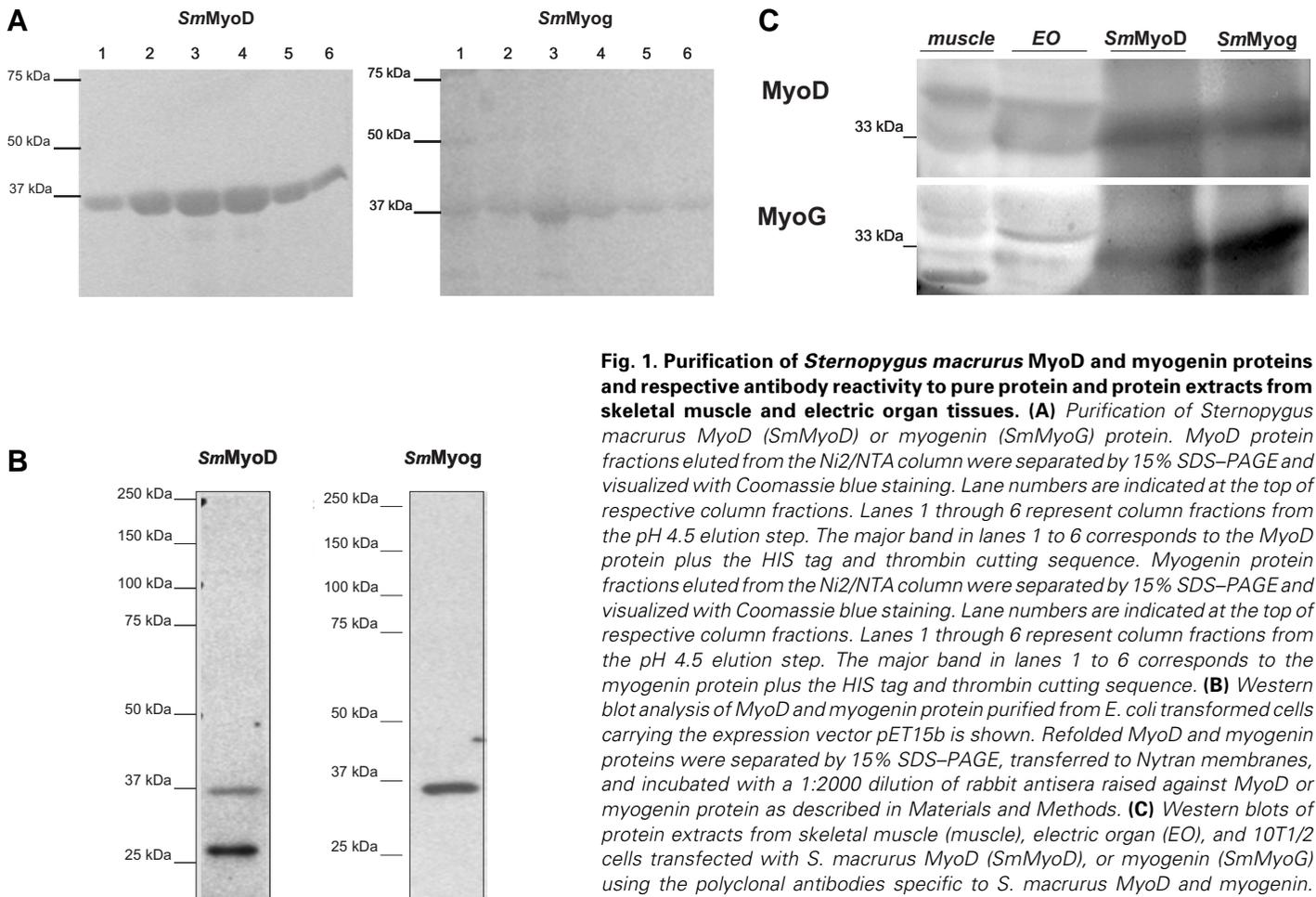
Li and Capetanaki, 1993; Marsh *et al.*, 1998).

Although MRFs can activate the myogenic program, the presence of MRFs does not ensure the full expression of the skeletal muscle program. In mammals, myoid cells of the thymus (Grounds *et al.*, 1992), myofibroblasts (Mayer and Leinwand, 1997), and the electrical conductive cells of the heart (Takebayashi-Suzuki *et al.*, 2001) express MyoD and/or myogenin yet these cells do not display a full muscle phenotype. A non-mammalian cell in which the expression of MRFs does not correlate with the level to which the myogenic program is manifested is the electrocyte, i.e., the current-producing cell of the electric organ in electric fishes. In all electric fish species known to date, mesodermal cells initially differentiate into myoblast-like cells that subsequently form myotubes. Conversion of these myotubes to mature electrocytes is characterized by the disassembly of sarcomeric structures, down-regulation of sarcomeric proteins, the replacement of myofibrils with amorphous material, relocation of many organelles to the periphery, and changes in cell size and shape (Bennett, 1970).

In the gymnotiform *Sternopygus macrurus*, mature electrocytes are similar to their muscle precursors in that they are multinucleated, express proteins common to muscle cells such as desmin, talin, acetylcholine receptors (AChRs),  $\alpha$ -actin,  $\alpha$ -actinin, dystrophin, and titin (Patterson and Zakon, 1993; Kim *et al.*, 2004; Cuellar *et al.*, 2006) and are innervated at a single endplate

(Bennett, 1970; Unguez and Zakon, 1998). However, mature electrocytes do not contain sarcomeres, sarcoplasmic reticulum, or T-tubules, nor do they contain tropomyosin, myosin heavy chains (MHCs), or troponin-T proteins (Patterson and Zakon, 1996; Cuellar *et al.*, 2006). How a differentiated electrocyte can down-regulate some, but not all components of the muscle program is an intriguing problem in our current understanding of the transcriptional regulation of skeletal muscle genes.

The partial muscle phenotype of electrocytes and the wide phylogenetic distribution of the MyoD family across animal taxa (Atchley *et al.*, 1994; Zhang *et al.*, 1999) prompted us to clone all four MRFs from *S. macrurus* and examine their expression patterns in skeletal muscle and electric organ to determine if the electrocyte phenotype is due to an MRF expression pattern that correlates to the level and number of the muscle proteins maintained after its differentiation. Our data showed that the *S. macrurus* genome contains MyoD, myogenin, Myf5, and MRF4 genes with predicted amino acid sequences that exhibit a high degree of sequence conservation in their functional domains (Kim *et al.*, 2004; 2008) suggesting that *S. macrurus* MRFs likely have myogenic regulatory functions similar to those of their mammalian counterparts. Previous studies also revealed MRFs to be expressed in the myogenic lineage exclusively, but that their quantitative patterns in muscle fibers and electrocytes could not be used to predict the level to which each cell manifests the muscle



**Fig. 1. Purification of *Sternopygus macrurus* MyoD and myogenin proteins and respective antibody reactivity to pure protein and protein extracts from skeletal muscle and electric organ tissues. (A)** Purification of *Sternopygus macrurus* MyoD (SmMyoD) or myogenin (SmMyoG) protein. MyoD protein fractions eluted from the Ni<sup>2+</sup>/NTA column were separated by 15% SDS-PAGE and visualized with Coomassie blue staining. Lane numbers are indicated at the top of respective column fractions. Lanes 1 through 6 represent column fractions from the pH 4.5 elution step. The major band in lanes 1 to 6 corresponds to the MyoD protein plus the HIS tag and thrombin cutting sequence. Myogenin protein fractions eluted from the Ni<sup>2+</sup>/NTA column were separated by 15% SDS-PAGE and visualized with Coomassie blue staining. Lane numbers are indicated at the top of respective column fractions. Lanes 1 through 6 represent column fractions from the pH 4.5 elution step. The major band in lanes 1 to 6 corresponds to the myogenin protein plus the HIS tag and thrombin cutting sequence. **(B)** Western blot analysis of MyoD and myogenin protein purified from *E. coli* transformed cells carrying the expression vector pET15b is shown. Refolded MyoD and myogenin proteins were separated by 15% SDS-PAGE, transferred to Nytran membranes, and incubated with a 1:2000 dilution of rabbit antisera raised against MyoD or myogenin protein as described in Materials and Methods. **(C)** Western blots of protein extracts from skeletal muscle (muscle), electric organ (EO), and 10T1/2 cells transfected with *S. macrurus* MyoD (SmMyoD), or myogenin (SmMyoG) using the polyclonal antibodies specific to *S. macrurus* MyoD and myogenin. Abbreviation: epi, epithelium.

program (Kim *et al.*, 2004; 2008). These analyses however did not allow us to draw firm conclusions about MRF protein activity in *S. macrurus* tissues.

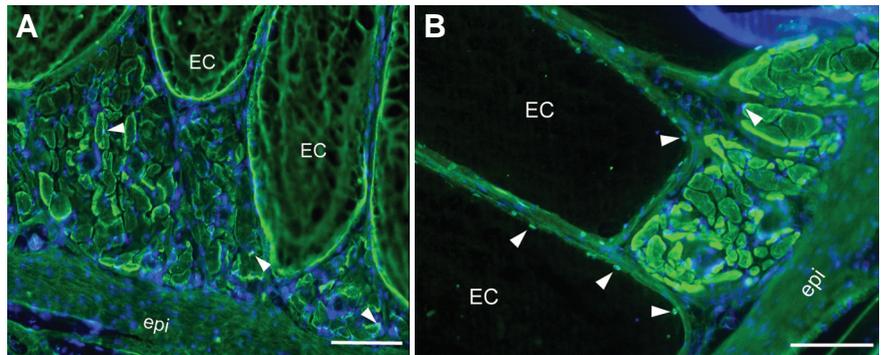
In the present study, we tested the hypothesis that *S. macrurus* MRFs can induce mammalian non-muscle cells to adopt a skeletal muscle phenotype. We show that forced expression of *S. macrurus* MyoD (*S<sub>m</sub>MyoD*) or myogenin (*S<sub>m</sub>MyoG*) in mouse embryonic stem cells (C3H/10T1/2 cells) and fibroblasts (NIH3T3 cells) results in the formation of multinucleated myotubes and up-regulation of sarcomeric proteins. The capacity of *S. macrurus* MRFs to activate the myogenic program in non-muscle cells was comparable to that obtained with mouse MRFs. *S<sub>m</sub>MyoD* and *S<sub>m</sub>MyoG* also induced the expression of mouse MRF transcripts and proteins in C3H/10T1/2 cells. Hence, these data are consistent with a functional conservation of an MRF-dependent transcriptional program of myogenesis in *S. macrurus*, and suggest that the partial muscle phenotype of electrocytes is not likely due to differences in the MRF-dependent transcriptional program between skeletal muscle and electric organ.

## Results

### Expression and purification of MyoD and myogenin proteins

BL21 (DE3) cells harboring the expression vector pET15b MyoD or pET15b myogenin were grown overnight and total protein was extracted under denaturing conditions. Solubilized protein was subsequently subjected to separation by nickel-affinity chromatography. Following removal of the majority of the contaminating proteins, MyoD or myogenin protein was eluted from the resin by lowering the buffer pH to 4.5. This released MyoD or myogenin protein into 6 fractions representing 6 column volumes (Fig. 1A). High yields of MyoD and modest levels of myogenin protein were extracted as shown in Fig. 1A. This one-step affinity purification yielded approximately 10–30 mg of MyoD or myogenin protein from 500 ml of cells.

Selected column fractions were pooled based on purity as visualized by Coomassie Blue staining and diluted to OD 0.2 at 595nm for refolding. MyoD or myogenin protein fractions were pooled and sent to Cell Essentials, Inc. as antigens for the production of monoclonal antibodies in rabbits. Purified refolded MyoD and myogenin proteins were examined for reactivity against sera from MyoD or myogenin protein-inoculated rabbits by Western blot (Fig. 1B). Western blot analysis of purified MyoD protein reacted against two bands. The band of approximately 28 kDa corresponds to the expected size of *S. macrurus* MyoD. The larger band most likely represents processing of the protein during refolding, since Coomassie Blue stained gels showed the presence of the lower dominant band during purification. The immunoanalysis with *S. macrurus* anti-myogenin showed one intense band corresponding to the expected size of approximately 33 kDa. These data suggest



**Fig. 2. Expression pattern of MyoD and myogenin proteins in adult *S. macrurus* skeletal muscle and electric organ.** Adult tail cryosections of skeletal muscle and electrocytes (EC) were immunolabeled with *S. macrurus* specific anti-MyoD (A) and anti-myogenin (B) antibodies and visualized with AlexaFluor-488 conjugated secondary anti-rabbit antibodies (green). Nuclei were counter-stained with Hoechst 33342 (blue). White arrows point to nuclei in electrocytes and muscle fibers that were double-labeled with antibody and Hoechst. Scale bar, 100  $\mu$ m.

that the expressed MyoD and myogenin proteins were stable and not affected by endogenous bacterial proteases, and the rabbit antisera labels proteins of the expected size.

Skeletal muscle and electric organ homogenates from adult *S. macrurus* were probed with the MyoD and myogenin antibodies. MyoD labeled bands of approximately 33 kDa and 37 kDa in size in both muscle and electric organ (EO) (Fig. 1C). These two bands are somewhat larger in size than the predicted product of ~28 kDa based on the inferred protein sequence. Myogenin labeled a band in both skeletal muscle and EO that corresponded to the expected size of ~33kDa (Fig. 1C). However, additional bands ranging between ~28 to 37 kDa were also labeled with anti-myogenin in both tissues (Fig. 1C). To further test the specificity of the MyoD and myogenin antibodies, we performed immunoblots on cell lysates from 10T1/2 cells transfected with *S<sub>m</sub>MyoD* and *S<sub>m</sub>MyoG*. MyoD and myogenin antibodies labeled a single band of approximately 33 kDa in each of the cell lysates (Fig. 1C). In sum, the cross-reactivity obtained with these antibodies in Western blots of purified proteins, muscle and EO tissue homogenates, and lysates of transfected cells with *S<sub>m</sub>MyoD* and *S<sub>m</sub>MyoG* suggests the detection of these two proteins and potentially their phosphorylated forms (Thayer *et al.*, 1989).

### MyoD and myogenin immunolabeling of *S. macrurus* muscle and EO tissue

In order to determine the expression pattern of MyoD and myogenin proteins in skeletal muscle fibers and electrocytes in *S. macrurus*, we examined the immunolabeling obtained with antibodies specific against MyoD and myogenin of *S. macrurus*. Tissue cryosections of adult fish tails immunoreacted with anti-MyoD and anti-myogenin are shown in Fig. 2A and 2B, respectively. Immunolabeling with the anti-MyoD antibody was detected in nuclei of muscle fibers as verified by Hoechst staining (Fig. 2A; arrows). Some background staining was found along the perimeter of electrocyte, and most electrocyte nuclei were not immunolabeled with the anti-MyoD antibody. Myogenin immunolabeling was detected in nuclei of both electrocytes and muscle fibers in adult tails of *S. macrurus* as shown in Fig. 2B.

These data suggest that MyoD and myogenin proteins are present in adult cells of the myogenic lineage in *S. macrurus*.

#### Induction of the myogenic program in mammalian non-muscle cells by *S. macrurus* MyoD and myogenin

To determine whether SmMyoD and SmMyoG exhibit functional conservation in mammalian skeletal muscle differentiation, we tested the ability of SmMyoD and SmMyoG to induce mammalian non-muscle cells to adopt a myogenic phenotype. Specifically, we transiently transfected mouse embryonic 10T1/2 cells with vectors expressing either SmMyoD or SmMyoG and then tested for the presence of sarcomeric MHC by immunofluorescence staining. Exogenously expressed SmMyoD and SmMyoG proteins were detected one day after transfection (Fig. 3A, top panel). At 3 days after transfection, we observed elongated and often multinucleated cells with myotube-like morphology that contained sarcomeric MHC in their cytoplasm (Fig. 3A, bottom panel). Detection of MHC staining was not restricted to multinucleated cells, as mononucleated cells were also immunolabeled by the anti-MHC antibody (Fig. 3A, bottom panel). In contrast, MHC immunolabel was not detected in cells transfected with empty vectors (data not shown).

In parallel with transfections with *S. macrurus* MRFs, we transfected 10T1/2 cells with mouse MyoD and myogenin cDNA to compare the transfection and conversion of non-muscle cells with MRFs from different species (Fig. 3A). Based on cell counts, we found that 10-20% of the cells expressed MRF proteins from plasmids containing either mouse or *S. macrurus* cDNAs. Transfection efficiencies obtained in this study that used mouse and fish MRF cDNAs fell within a range that is in agreement with that observed previously for mammalian MRFs (personal communication Stephen F. Konieczny). As evident by the number of cells with MHC immunolabel, the conversion of 10T1/2 cells to a myogenic phenotype after transfection with mouse MyoD or myogenin cDNA was also comparable to the conversion of 10T1/2 cells when they were transfected with the *S. macrurus* homologs (data not shown). To more thoroughly characterize the myogenic induction of 10T1/2 cells by *S. macrurus* MRFs, expression of different differentiation muscle markers was examined using RT-PCR to amplify mRNAs for fast MHC isoforms, tropomyosin and troponin-I (Table 1). Consistent with the immunofluorescence results, sarcomeric MHC transcripts were detected in MRF-

transfected cells (Fig. 3B). Bands corresponding to endogenous tropomyosin and troponin-I were also detected in 10T1/2 cells transfected with both murine and *S. macrurus* MRFs (Fig. 3B), although at apparently lower intensities than those of MHC mRNAs. These data demonstrate the induction of multiple differentiation muscle markers by murine and teleost MRFs in this non-muscle cell line.

The myogenic induction potential of *S. macrurus* MRFs on mammalian non-muscle cells was also tested on embryonic fibroblasts (NIH3T3 cell line). Fig. 3C shows a representative image of NIH3T3 fibroblasts that were transfected with *S. macrurus* MRF cDNA and subsequently immunolabeled with the anti-MHC antibody MF20 three days after transfection. Both SmMyoD and SmMyoG cDNAs induced some NIH3T3 cells to express MHC and fuse to give rise to multinucleated cells. Detection of MHC staining in NIH3T3 cells was not restricted to multinucleated cells after transfection with either murine or *S. macrurus* MRFs, as mononucleated cells were also immunolabeled by the anti-MHC antibody similarly to 10T1/2 cells. These data demonstrate that SmMyoD and SmMyoG can function in a heterologous system to induce the myogenic phenotype on different mammalian non-muscle cell lines (i.e., 10T1/2 cells, Fig. 3A; NIH3T3 cells, Fig. 3C), and are consistent with an MRF-dependent transcriptional program of myogenesis that is functionally conserved in *S. macrurus*.

#### Induction of mammalian MRFs by *S. macrurus* MyoD and myogenin

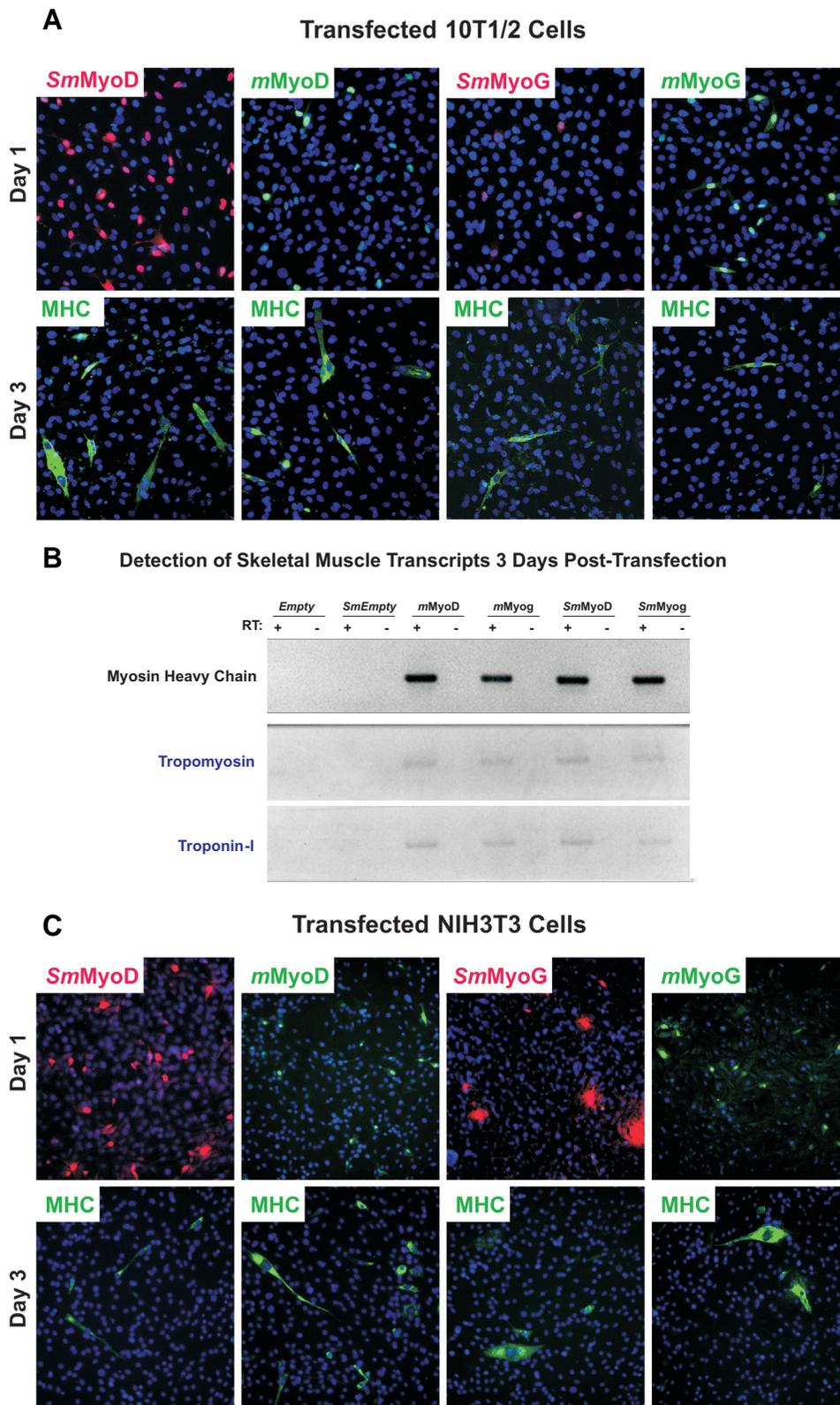
*In vitro* studies have shown that MyoD and myogenin can regulate their own expression via a positive feedback mechanism of transcriptional activation (Braun *et al.*, 1989; Weintraub *et al.*, 1989; Tapscott, 2005). In addition, endogenous MyoD and myogenin can be activated by exogenously expressed MRFs during skeletal differentiation in 10T1/2 cells (Auradé *et al.*, 1994). To test whether *S. macrurus* MRFs can also induce the expression of endogenous MRFs, we assayed for the expression of mouse MyoD and myogenin mRNAs and proteins in 10T1/2 cells 2 days after transfection with each *S. macrurus* MRF. RT-PCR analysis showed that transfection with either SmMyoD and SmMyoG resulted in the transcription of endogenous MyoD and myogenin (Fig. 4A). Western blots and immunofluorescence studies revealed that induction of the endogenous myogenin by each *S. macrurus* MRF and by mouse MyoD was also detected at the protein level (Fig. 4B and 4C).

Auto- and cross-regulation of MRFs have been shown to be cell line dependent. Unlike in 10T1/2 cells, forced expression of MyoD in NIH3T3 cells does not induce endogenous MyoD. NIH3T3 cells do respond to exogenous MyoD and myogenin by expressing myogenin and MyoD, respectively (Thayer *et al.*, 1989; Russo *et al.*, 1998). In the present study, we detected MyoD and myogenin transcripts in NIH3T3 cells transfected with both mouse MRFs (Fig. 4D). Detection of MyoD mRNA in NIH3T3 cells transfected with mouse MyoD (mMyoD in Fig. 4D) does not necessarily contradict published observations that exogenous MyoD does not induce its own expression in this cell line. Since our experimental approach could not differentiate between exogenous and endogenous MRFs, MRF transcripts detected 2 days after transfection with mMyoD and mMyoG, respectively, could likely reflect the levels of exogenous MRFs in the cells. However,

TABLE 1

#### HOMOLOGOUS PRIMERS USED FOR AMPLIFICATION OF MUSCLE-SPECIFIC GENE TRANSCRIPTS FROM TRANSFECTED CELLS

| Gene                            | Orientation | Primer sequence                          | T <sub>m</sub> (°C) | PCR product size |
|---------------------------------|-------------|--|---------------------|------------------|
| MyoD (mouse)                    | Forward     | 5'- AGTGAATGAGGCCCTTCGAGA -3'            | 53                  | 259 bp           |
|                                 | Reverse     | 5'- CATCATGCCATCAGAGCAGT -3'             |                     |                  |
| Myogenin (mouse)                | Forward     | 5'- GGCATGCAAGGTGTGAAGA -3'              | 60                  | 411 bp           |
|                                 | Reverse     | 5'- ACGATGGACGTAAGGGAGTG -3'             |                     |                  |
| Fast MHC (mouse)                | Forward     | 5'- CATCGTCCAGGAAGCCC -3'                | 51                  | 574 bp           |
|                                 | Reverse     | 5'- TTCGCTTATGATTTTGGTGTGAACC -3'        |                     |                  |
| Tropomyosin (mouse)             | Forward     | 5'-TGAGTTTGCAGAGAGATCAGTAACC-3'          | 53                  | 189 bp           |
|                                 | Reverse     | 5'-GGGACATCCGGCTTGATGAAGGAGTCTTGGGAGG-3' |                     |                  |
| Troponin-I (mouse)              | Forward     | 5'- GAAGGAGAAGTACCTGTGCAGA -3'           | 51                  | 437 bp           |
|                                 | Reverse     | 5'- TGGCAGTTTAGGACTCAGACTC -3'           |                     |                  |
| MyoD ( <i>S. macrurus</i> )     | Forward     | 5'- GCACGTCTACGAATCCTAATCAG -3'          | 53                  | 394 bp           |
|                                 | Reverse     | 5'- CAAGGGCTGCCATCAGAG -3'               |                     |                  |
| Myogenin ( <i>S. macrurus</i> ) | Forward     | 5'- CGTACTTTTTCCCTGACCAGC -3'            | 55                  | 658 bp           |
|                                 | Reverse     | 5'- ATGTTAGCATGTTCCGGAGGATC -3'          |                     |                  |



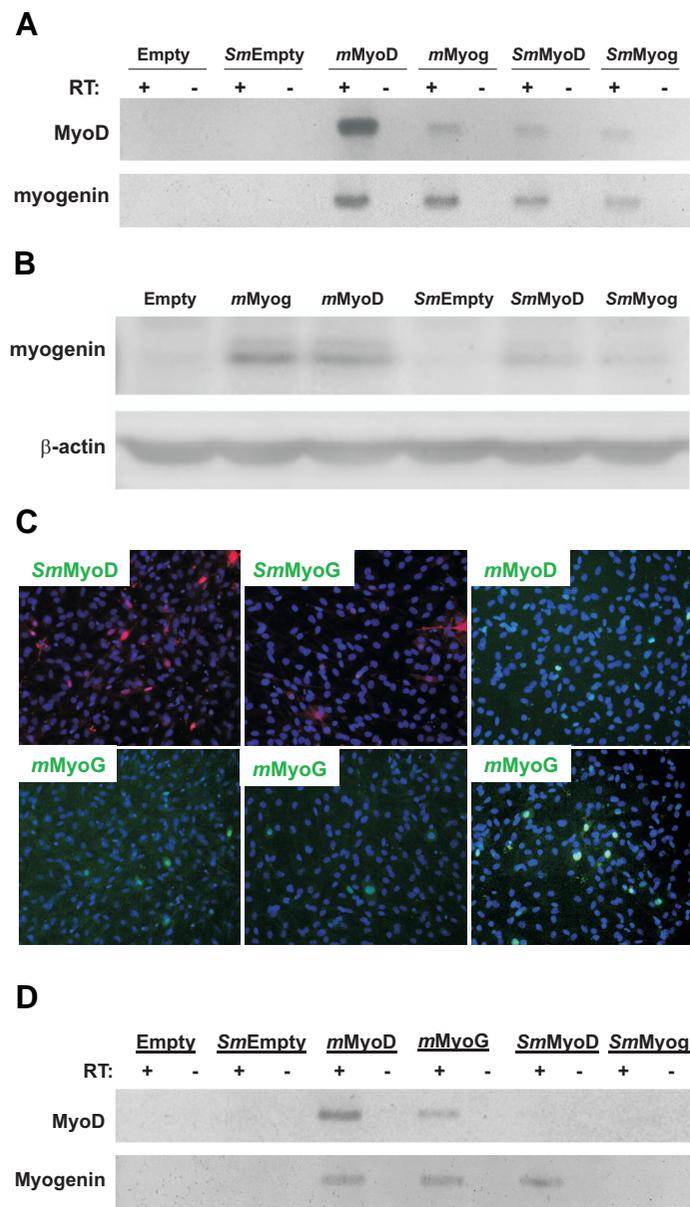
the detection of myogenin after mMyoD transfection and MyoD after mMyoG transfection is interpreted to indicate a clear cross-regulation between these mammalian MRFs. Only SmMyoD was found to induce endogenous myogenin (Fig. 4D). Together, these results demonstrate that both SmMyoD and SmMyoG are similar in function to their mammalian counterparts by demonstrating their capability of transcriptional activation of endogenous MRF genes in 10T1/2, but not completely in NIH3T3 cells.

## Discussion

Previous studies on the transcriptional regulation of the muscle program in electrocytes of *S. macrurus* revealed that all four MRFs are expressed in mature electrocytes despite the fact that these cells do not maintain a normal muscle phenotype (Kim *et al.*, 2004; 2008). Moreover, with the exception of MyoD, the endogenous levels of myogenin, Myf5, and MRF4 transcripts in electrocytes were greater than those detected in muscle fibers (Kim *et al.*, 2008). Although previous analyses did not allow us to draw firm conclusions about MRF protein activity in muscle and EO, the present study demonstrates that *S. macrurus* MRFs can induce the myogenic program in mammalian non-muscle cells. Our conclusion that SmMyoD and SmMyoG act as molecular switches for the myogenic

**Fig. 3. Myogenic conversion potential of *S. macrurus* MyoD and myogenin.** (A) 10T1/2 cells were transiently transfected with SmMyoD, SmMyoG, mouse MyoD (mMyoD) or mouse myogenin (mMyog) expressing vectors and assayed for expression of exogenous MRFs (top row) and sarcomeric myosin heavy chain (MHC; bottom row below corresponding MRFs) using immunofluorescence staining with antibodies specific to each protein at day 1 and day 3, respectively. AlexaFluor-546 (red) or AlexaFluor-488 (green) conjugated secondary antibodies were used. (B) 10T1/2 cells were transfected with mouse MyoD (mMyoD), mouse myogenin (mMyog), *S. macrurus* MyoD (SmMyoD), or myogenin (SmMyoG) and total RNA isolated from cells 3 days after transfection. Total RNA then was processed for RT-PCR as described in Materials and Methods by using the primers specific for mouse fast-MHC, tropomyosin, and troponin-I cDNAs indicated in Table 1. PCR products were resolved on 1% agarose gel and stained with ethidium bromide. The negative image of the gel is shown. Data shown is representative of results obtained from three different experiments. RT: reverse transcriptase. (C) NIH3T3 cells were transiently transfected with the same expression vectors as used for 10T1/2 cells and assayed for expression of each MRF and MHC as indicated in the figure. Nuclei were counter-stained with Hoechst 33342 (blue). Images were taken at 100x magnification by fluorescence microscopy (Zeiss Axiovert 200M).

Materials and Methods by using the primers specific for mouse fast-MHC, tropomyosin, and troponin-I cDNAs indicated in Table 1. PCR products were resolved on 1% agarose gel and stained with ethidium bromide. The negative image of the gel is shown. Data shown is representative of results obtained from three different experiments. RT: reverse transcriptase. (C) NIH3T3 cells were transiently transfected with the same expression vectors as used for 10T1/2 cells and assayed for expression of each MRF and MHC as indicated in the figure. Nuclei were counter-stained with Hoechst 33342 (blue). Images were taken at 100x magnification by fluorescence microscopy (Zeiss Axiovert 200M).



**Fig. 4.** Auto- and cross-regulation of MRFs by *S. macrurus* MRFs. Mouse 10T1/2 cells or NIH3T3 cells were transfected with *S. macrurus* MRF (SmEmpty, SmMyoD and SmMyoG) and mouse MRFs (Empty, mMyoD and mMyog) and assayed for expression of mouse MyoD or myogenin 2 days after transfection. **(A)** Detection of transcripts of mouse MyoD and myogenin in 10T1/2 cells by RT-PCR. PCR products were resolved on 1% agarose gels and stained with ethidium bromide (negative images shown). **(B)** Detection of mouse myogenin protein in 10T1/2 cells by Western blot analysis using monoclonal anti-myogenin antibody.  $\beta$ -actin was used as equal loading control. **(C)** Detection of endogenous myogenin (mMyoG) protein in 10T1/2 cells by immunofluorescence staining (bottom row) was assessed following transient transfection with *S. macrurus* MyoD (SmMyoD) and myogenin (SmMyoG) and mouse MyoD (mMyoD). Expression of exogenous MRFs was detected with respective antibodies indicated in the figure (top row). Hoechst 33342 was used to stain nuclei (blue) **(D)** Mouse NIH3T3 cells were transfected with the expression plasmids as described above and assayed for mouse MyoD and myogenin transcript by RT-PCR.

program is based on their conversion of non-muscle cells into multinucleated cells that express endogenous MRFs and muscle differentiation markers. To our knowledge, the present data represent the most complete test of the functional conservation of vertebrate non-mammalian MRFs using two murine non-muscle cell lines. Our cell conversion assays at the transcript and protein levels of MRF auto- and cross-regulation and induction of different terminal myogenic markers provide compelling evidence in support of an MRF-dependent transcriptional program of myogenesis in *S. macrurus*. This is also the first report on the expression pattern of MyoD and myogenin in mature skeletal muscle fibers and electrocytes of *S. macrurus* using species-specific antibodies against these MRFs.

#### ***S. macrurus* MRFs can substitute for mammalian MyoD and myogenin to induce the myogenic program in non-mammalian cells**

Our immunofluorescence assays showed the presence of MHC expression in 10T1/2 cells and NIH3T3 cells after SmMyoD or SmMyoG transfections. MHC expression was not exclusive to multinucleated myotubes as MHC label was also detected in mononucleated cells, an observation consistent with previous studies using mammalian MRFs (Weintraub, 1989). These data revealed that sarcomeric MHC expression might be independent of cell fusion in culture conditions. Although SmMyoG also induced 10T1/2 and NIH3T3 cells to adopt a myogenic phenotype, it did so with an apparent lower frequency of conversion than SmMyoD. However, our data also show that the frequency of conversion by mouse myogenin was lower than that by mouse MyoD (compare Fig. 3A and 3C).

We should note that in the present study comparisons between the ability of MRFs to induce muscle differentiation were not correlated to the transfection efficiency for each MRF overexpression experiment. Cell counts of MHC-positive cells in our preliminary experiments did not yield conclusive relationships between the transfection levels by either murine or *S. macrurus* MRFs and the levels of myogenic cell conversion observed. Given that the MRF cDNAs are not integrated with a transient transfection approach, and there is a high variability in the number of MRF molecules expressed per cell so as to not allow an accurate assessment of cell conversion, the muscle gene detection assays performed in the present study, i.e., RT-PCR, Western blots, and immunolabeling profiles, were deemed more appropriate in our assessment of the functional conservation of *S. macrurus* MRFs.

The phenotypic conversion of mammalian non-muscle cells by a non-mammalian MRF is not unique to SmMyoD or SmMyoG. Transfection of 10T1/2 cells with the MyoD homolog from the sea urchin (Venuti *et al.*, 1991) and the nematode *C. elegans* also converts 10T1/2 cells into multinucleated cells that express sarcomeric proteins (Venuti *et al.*, 1991; Krause, 1992; Zhang *et al.*, 1999). We are not aware of similar expression studies that have thoroughly tested the functional conservation of non-mammalian vertebrate MRFs.

#### **Regulation of endogenous MRFs by *S. macrurus* MyoD and myogenin**

The presence of mouse MyoD and myogenin transcripts in 10T1/2 cells transfected with SmMyoD or SmMyoG infers that *S. macrurus* MRFs can functionally bind to the promoters of their

endogenous mammalian counterparts. Given that methylation of the MyoD promoter renders it to be transcriptionally repressed in 10T1/2 cells, (Jones *et al.*, 1990), forced expression of *S. macrurus* MRFs almost certainly brings about a reduction of this inhibition. To date, it is not clear how exogenous MyoD can activate the endogenous MyoD promoter in 10T1/2 cells. Moreover, auto-regulation by exogenous *S. macrurus* MRFs is cell-line specific (e.g., 10T1/2 vs NIH3T3, Fig. 4). For example, exogenous MyoD converts NIH3T3 cells to a myogenic phenotype in the absence of induction of the endogenous MyoD (Thayer *et al.*, 1989; Russo *et al.*, 1998). Consistent with the literature, SmMyoD induced the expression of MHC in the absence of endogenous MyoD in NIH3T3 cells. In contrast, we could not detect endogenous MRFs in SmMyoG transfected NIH3T3 cells despite their expression of MHC – a finding unlike that obtained with murine MyoG (Fig. 4D). Nonetheless, these results demonstrate that both SmMyoD and SmMyoG are similar in function to the mammalian counterpart by demonstrating their capability of transcriptional activation of endogenous MRF genes in 10T1/2, but not completely in NIH3T3 cells.

Our analysis of the muscle program activation by *S. macrurus* MRFs does not substantiate any conclusions about the mechanisms by which they influence 10T1/2 and NIH3T3 to differentiate into muscle cells. For instance, we do not know whether SmMyoD directly activates the expression of MHC, tropomyosin, or troponin-I genes by binding to their promoters, or indirectly through the induction of endogenous MyoD or myogenin. Inclusion of highly conserved functional domains in SmMyoD and SmMyoG (Kim *et al.*, 2004) together with an efficiency of muscle gene activation similar to that observed with mouse homologs (present study), intimate that *S. macrurus* and mammalian MRFs are involved in similar mechanisms of MRF-dependent myogenesis. Studies designed to characterize the transcriptional activity of SmMyoD and SmMyoG conferred through homodimerization or heterodimerization with mammalian MRFs or E-proteins, respectively, will be necessary to more thoroughly evaluate their functional conservation.

#### **Implications on the partial muscle phenotype of electrocytes in *S. macrurus***

The presence of some cytoskeletal, sarcomeric and cell signaling proteins in mature electrocytes, such as desmin,  $\alpha$ -actin, dystrophin, titin, and acetylcholine receptors reflects their differentiation from the myogenic lineage (Kim *et al.*, 2004; Cuellar *et al.*, 2006). Because MRFs are essential for the transcriptional activation of these muscle genes in mammals (Li and Capetanaki, 1993; Marsh *et al.*, 1998; Simon and Burden, 1993; Shield *et al.*, 1996; Lin *et al.*, 1991; Gramolini and Jasmin, 1999; Wheeler *et al.*, 1999; Angus *et al.*, 2001), evaluation of the functional conservation of *S. macrurus* MRFs is warranted to determine whether a unique transcriptional mechanism of muscle gene regulation is present in electrocytes. Our present data demonstrate that SmMyoD and SmMyoG are capable of programming muscle differentiation in non-muscle cells similarly to their mammalian counterparts by inducing the expression of known MRF targets MHC, troponin-T, tropomyosin, and troponin-I (Wheeler *et al.*, 1999; Hirao *et al.*, 2004; Toutant *et al.*, 1993; Banerjee-Basu and Buonanno, 1993). Interestingly, previous expression analyses revealed that mature electrocytes transcribe MHC, tropomyosin,

and troponin genes but the corresponding protein products could not be detected (Cuellar *et al.*, 2006, unpublished data). Further studies of the differential expression of select muscle genes in electrocytes are likely to provide insight into the possible roles that additional transcriptional and post-transcriptional mechanisms might play in the homeostasis of muscle-specific properties of the EO after differentiation.

## **Materials and Methods**

### **Sub-cloning of full length cDNA sequences of *S. macrurus* MyoD and myogenin**

Full-length coding cDNA sequences of *S. macrurus* MyoD and myogenin (Kim *et al.*, 2004; GenBank accession sequences AY396566 and AY396565, respectively) were first cloned into the pDrive vector (Qiagen, Valencia, CA), and subsequently subcloned into the pET15b vector (Novagen, San Diego, CA). For subcloning, primers were designed following the BD In-Fusion™ Dry-Down PCR cloning Kit user manual (BD Bioscience, San Jose, CA). Briefly, the 5'-end of both forward and reverse primers (sense MyoD: 5'-CCG CTC GAG ATG GAA CTG TCC GAT ATC CC-3', anti-sense MyoD: 5'-CGG GAT CCT TAC AGA ACC TGG TAG ATC GGA T-3', sense myogenin: 5'-CCG CTC GAG ATG GAA CTC TTC GAG ACT AA -3', anti-sense myogenin: 5'-CGG GAT CCT TAT TTA ATA TCC ACG GAG TAG G-3') shared 15 bases of sequence homology with the sequences on either side of the point of insertion and one extra base that included an XhoI and BamHI site, which flanked the gene-specific sequence of MyoD or myogenin. PCR was performed with HiFi Taq DNA polymerase (Invitrogen, Carlsbad, CA) for 25 cycles, and the fragments were purified using the Qiaquick PCR Purification Kit (Qiagen). The pET15b vector was digested with XhoI and BamHI restriction enzymes and gel-purified using the Qiaquick Gel Extraction Kit (Qiagen). Recombination between the pET15b and each PCR product was carried out using the BD In-Fusion™ Cloning Procedure in the BD In-Fusion™ Dry-Down PCR Cloning Kit per the manufacturer's instructions. Bacterial colonies were screened for plasmids with the MyoD or myogenin gene insertion by colony PCR, and confirmed by DNA sequencing using the ABI 3130xl Genetic Analyzer.

### **Protein purification and antibody production**

MyoD and myogenin plasmids were expressed in *Escherichia coli* BL21 (DE3) as N-terminal hexahistidine-tagged fusion protein using Studier's auto-induction procedure (Studier, 2005) and purified from the insoluble fraction as described previously (Jonsson *et al.*, 1993). Briefly, cells were grown in 500 ml of ZYM-5052 and supplemented with 50  $\mu$ g/ml ampicillin following the auto-induction procedure (Studier, 2005). Cells were then centrifuged, resuspended in 40 ml Buffer A (20 mM Tris pH 8.0, 6 M guanidine HCl, 1 M NaCl, 0.1 mM EDTA and 1 mM DTT), shaken at room temperature (RT) for 1 hr at 150 RPMS, and centrifuged for 30 min at 30,000  $\times$  g to harvest the supernatant. The material was purified through a 1 ml nickel agarose resin (Qiagen) equilibrated with 1X Buffer A and total protein was eluted with a stepwise decrease in pH (6.3, 5.9 and 4.5). Fractions containing protein were dialyzed and refolded in 3 M urea/Buffer B (40 mM HEPES pH 7.4, 1 M NaCl, 10 mM  $\beta$ -mercaptoethanol, 0.4% NP40, 10% glycerol) and the urea concentration dropped by 1 M. A final dialysis was made in Buffer C (40 mM HEPES pH 7.4, 0.2 M NaCl, 1 mM DTT, 0.4% NP40, 10% glycerol). Total protein concentration was measured by the Bradford method (BioRad, Hercules, CA) and stored in aliquots at -80°C.

### **MyoD and myogenin polyclonal antibody generation and Western blot analyses**

Rabbits were immunized with the purified proteins for antibody production (Cell Essentials, Inc. Boston, MA). After initial immunization, rabbits were boosted twice, and test bleeds were performed. After the final boost, rabbits were bled and sera from production bleeds were used

in the experiments in this study. For immunoblot analysis, purified MyoD (10  $\mu$ l; 0.3  $\mu$ g/ $\mu$ l) and myogenin (10  $\mu$ l; 0.7  $\mu$ g/ $\mu$ l) proteins were electrophoresed on 4-12% Bis-Tris SDS-polyacrylamide gel electrophoresis (PAGE) gels with running buffer (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes (Biorad, Hercules, CA) according to the manufacturer's recommendations. Membranes were incubated in blocking buffer (5% nonfat dried milk in TBS-Tween: 50 mM Tris, pH 7.6, 150 mM NaCl) for 1 hr at RT. Antibodies were diluted 1:2000 in blocking buffer and incubated with the membrane for 1 hr at RT. Goat anti-rabbit IgG (Fc) alkaline phosphatase-conjugated (Promega, Madison, WI) was diluted 1:2,000 in blocking buffer and incubated with the membrane for 1 hr at RT. Antibody label was detected by phosphatase activity with NBT/BCIP (Sigma, St. Louis, MI) as per manufacturer's instructions.

#### Cell culture and transfection with mouse and *S. macrurus* MRFs

The C3H/10T1/2 (referred to as 10T1/2 hereafter) mouse embryonic stem cell line was purchased from American Type Cell Culture (Manassas, VA) and maintained in growth medium [Basal Medium Eagle (BME) supplemented with 10% FBS and 2 mM L-glutamine]. NIH3T3 mouse embryonic fibroblasts were generously provided by Dr. Stephen Tapscott (Fred Hutchinson Cancer Research Center, Seattle, WA) and maintained in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum and 100 units/ml penicillin G and 100  $\mu$ g/ml streptomycin. Cells were transfected with plasmids expressing mouse MyoD or myogenin (cloned into pEMSV $\beta$ 2; a gift from Dr. Konieczny, Purdue University, West Lafayette, IN) or *S. macrurus* MyoD or myogenin (cloned into pcDNA4T/O, Invitrogen) to test for myogenic conversion. pEMSV $\beta$ 2 and pcDNA4T/O vectors without insert were used as controls for mouse and *S. macrurus* MRFs, respectively. Transfections were carried out using Fugene-6 (Roche, Indianapolis, IN) on 10T1/2 cells and lipofectamine (Invitrogen) on NIH3T3 cells following manufacturer's instructions. Transfection efficiency was determined after 24 hrs by MRF-positive nuclei to total cell number in 7 randomly selected microscopic fields per dish. Cells were switched to differentiation medium (DMEM supplemented with 2% horse serum) 24 hrs after transfection, and further incubated up to 2 days with daily medium change. Cell culture reagents (culture media, serum, antibiotics etc) were purchased from Invitrogen (Carlsbad, CA) unless otherwise indicated.

#### Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from each population of transfected cells at different time points using the Qiagen RNeasy Mini kit per the manufacturer's protocol. Residual DNA was removed by treatment with DNase I, Amplification Grade (Invitrogen), and total RNA concentrations were determined by spectrophotometry ( $A_{260}/A_{280}$ ). cDNAs were synthesized from 0.50  $\mu$ g total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). To monitor DNA contamination in all experiments, control reactions were performed without reverse transcriptase. Gene-specific homologous primers for mouse MyoD and myogenin, mouse fast myosin heavy chain (MHC) isoforms, and *S. macrurus* MyoD and myogenin, mouse tropomyosin and troponin-I (Table 1) were used to characterize the transcription profile of these cells. PCR amplification of the cDNAs (~1  $\mu$ g) was performed using either Platinum Taq polymerase (Invitrogen) or Paq polymerase (Stratagene, La Jolla, CA). For amplification of mouse MHC and MRF cDNAs, 27-35 cycles were performed with denaturation at 94°C for 30 seconds, primer-annealing at 51-60°C (Table 1) for 30 seconds, and elongation at 72°C for 30 seconds following an initial denaturation at 94°C for 2 min. For amplification of mouse tropomyosin and troponin-I cDNAs, 35 cycles were performed with denaturation at 95°C for 20 seconds, primer-annealing at 51-53°C (Table 1) for 20 seconds, and elongation at 72°C for 30 seconds following an initial denaturation at 95°C for 2 minutes. In all PCR reactions, a final elongation step was performed for 5 min at 72°C. Amplification was maximized by optimizing annealing temperature for

each PCR primer pair. PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide to verify product size. Amplicons were purified using Qiagen's QIAquick PCR Purification kit and their nucleotide sequences were verified by Elim Biopharmaceuticals (Hayward, CA). Sequence information was downloaded to align forward and reverse sequences in order to find the consensus sequence using VectorNTI software from Invitrogen. Consensus sequences were then entered into the NCBI database to identify mammalian homologs.

#### Immunofluorescence: cultured cells and tissue sections

Cultured cells transfected with MRF-expressing plasmids were fixed in cold methanol for 15min at -20°C and processed for staining. Briefly, cells were washed with phosphate buffered saline (PBS), permeabilized in PBS containing 0.1% Triton X-100 (PBS-T) for 5min and blocked in blocking buffer (3% bovine serum albumin in PBS-T). Cells were then incubated with primary antibody diluted in blocking buffer containing Hoechst 33342 for 1 hr at 37°C. Antibodies used were: monoclonal mouse anti-MyoD (1:100, BD Pharmingen), monoclonal mouse anti-myogenin (F5D, 1:5, Hybridoma Bank), monoclonal mouse anti-MHC (MF20, 1:5, Hybridoma Bank), polyclonal *S. macrurus* MyoD (1:20, Cell Essentials, Boston, MA), and polyclonal *S. macrurus* myogenin (1:20, Cell Essentials). Antibody-antigen complexes were visualized using AlexaFluor-488 or AlexaFluor-546 secondary antibodies (anti-mouse or anti-rabbit, 1:200; Molecular Probes, Eugene, OR). Images of immunolabeled cells were captured on a Zeiss Axiovert 200M microscope equipped with Axiovision software version 4.5 and interfaced with a CCD fluorescent camera (AxioCam MRm, Carl Zeiss, Thornwood, NY).

Serial transverse and longitudinal cryosections (20-30  $\mu$ m thick) of control adult tails were mounted on Superfrost Plus glass slides (Fisher, Pittsburgh, PA) and air-dried at RT. Sections were fixed by immersion in 4% buffered paraformaldehyde (pH 7.4, 20 min, 37°C) and rinsed twice in 0.1 M PBS (pH 7.4). Slides were immersed in permeabilization solution (0.5% Triton X-100 in PBS) for 30 min and subsequently incubated in 1% blocking reagent (TSA kit, Invitrogen) for 1 hr at RT. Rabbit polyclonal antibodies against *S. macrurus*-specific MyoD (1:20) and myogenin (1:20) (Cell Essentials) were diluted in 1% blocking solution and applied to separate slides for 15-20 hr at RT. Tissue sections were incubated in 1% blocking solution without primary antibodies to assess non-specific labeling by secondary antibodies. Sections were rinsed in PBS and incubated in horseradish peroxidase-conjugated goat anti-rabbit Ig G (TSA kit, diluted 1:100 in 1% blocking solution) for 1hr. Tissue sections were rinsed in PBS, and incubated in AlexaFluor-488 tyramide working solution (TSA kit, 10 min., diluted 1:1000 in 0.0015% H<sub>2</sub>O<sub>2</sub>). Sections were rinsed in PBS, counterstained with Hoechst 33342, rinsed in water, and cover-slipped with Gelmount (Biomedex, Foster City, CA).

#### SDS-PAGE and Western blot analysis of cultured cell lysates and tissue homogenates

Total cell lysates from MRF-transfected cells were prepared using lysis buffer [20mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin and 1 mM PMSF and protease inhibitor cocktail (Roche, Indianapolis, IN)] on ice for 5 min. The cell lysates were scraped off and homogenized with a 21-gauge needle. Lysates were then centrifuged at 13,000 x g for 15 min at 4°C, and supernatants were collected. Protein concentration was measured using the Bradford assay (Biorad, Hercules, CA). Total proteins (50  $\mu$ g per sample) were separated by SDS-PAGE (4-15%), and transferred onto PVDF membranes. Membranes were washed with TBS-T (0.1 % Tween-20 in Tris buffered saline) and incubated in blocking buffer (3% non-fat dry milk in TBS-T) for 60 min at RT. Membranes were then incubated with primary antibody diluted in 5% BSA in TBS-T at 4°C overnight. Primary antibodies used were the same as those used for immunofluorescence staining (see above). Membranes were washed with PBS-T and incubated with anti-mouse or anti-rabbit HRP-conjugated secondary antibody.

ies (1:5000 dilution, BioRad) for 1 hr at RT. Colorimetric analysis was used to visualize the specific protein bands using the Opti-4CN substrate (BioRad).

Similar procedures were followed for the immunoblot analysis of protein homogenates from skeletal muscle and electric organ with antibodies against *S. macrurus*-specific MyoD and myogenin proteins, except that total protein was isolated from skeletal muscle and electric organ following the protocol of Talmadge *et al.*, 1993). All subsequent steps including: measurements of protein concentration, protein separation through gel electrophoresis, protein transfer onto PVDF membranes, and immunoblotting with anti-MyoD (1:1,00) and anti-Myogenin (1:1,000) antibodies, were as described above for Western blots with cell lysates.

#### Acknowledgements

This research was supported by National Institute of Health Grants S06-GMO8136-27, RR16480-01, and U56-CA96286 (G.A.U.). We are grateful to Stephen Konieczny for mouse MyoD and myogenin clones; Tan Wang and Xialin Xu for purification of *S. macrurus* MyoD and myogenin proteins; Jung A. Kim and two anonymous reviewers for providing critical suggestions in earlier manuscript drafts, and the Cell and Organismal Culture Facility at NMSU for its culture services.

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