

## TECHNOLOGY REPORT

Transgenic Mice That Express Cre Recombinase Under Control of a Skeletal Muscle-Specific Promoter From *mef2c*Analeah B. Heidt<sup>1</sup> and Brian L. Black<sup>1,2\*</sup><sup>1</sup>Cardiovascular Research Institute, University of California, San Francisco, California<sup>2</sup>Department of Biochemistry and Biophysics, University of California, San Francisco, California

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**Summary:** Genes expressed in skeletal muscle are often required in other tissues. This is particularly the case for cardiac and smooth muscle, both contractile tissues that share numerous characteristics with skeletal muscle, such that targeted inactivation can lead to embryonic lethality prior to a requirement for gene function in skeletal muscle. Thus, it is essential that conditional inactivation approaches are developed to disrupt genes specifically in skeletal muscle. In this report, we describe a transgenic mouse that expresses Cre recombinase under the control of a skeletal muscle-specific promoter from the *mef2c* gene. Cre expression in this transgenic line is completely restricted to skeletal muscle from early in development and is present in all skeletal muscles, including those of epaxial and hypaxial origins and in fast and slow fibers. This early skeletal muscle-specific Cre line will be a useful tool to define the function of genes specifically in skeletal muscle. *genesis* 42:28–32, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** skeletal muscle; Cre recombinase; transgenic mouse; MEF2C; ROSA26R

Many genes involved in skeletal muscle development, function, and regeneration are also necessary in other lineages. For example, cardiac, smooth, and skeletal muscle are similar in that each is a contractile tissue with similar methods of calcium handling, energy production, and force generation, yet these muscle types have specialized functions and distinct embryological origins. Because of the similar properties of these three muscle cell types, many genes are expressed in common in these lineages (Black and Olson, 1998). Therefore, in order to determine the function of genes in skeletal muscle independent of other cell types, conditional inactivation approaches are essential.

Several Cre mouse lines have been established previously using promoters from genes that are expressed in both cardiac and skeletal muscle, including *muscle creatine kinase* (MCK), human *smooth muscle  $\alpha$ -actin* and *cardiac  $\alpha$ -actin* (Miwa *et al.*, 2000; Wang *et al.*, 1999). These lines have been useful for inactivating skeletal muscle genes that do not have shared expression in the heart or that are not required for cardiac development

or in other tissue types. For example, MCK-Cre has been used to examine the role of the transcription factor PPAR $\gamma$  in skeletal muscle and its effects on insulin resistance distinct from the role of that factor in adipose tissue (Hevener *et al.*, 2003; Norris *et al.*, 2003). However, these Cre lines have limited use for disrupting genes with shared expression in cardiac and skeletal muscle, particularly if the gene is required early in cardiac development. An additional skeletal muscle-specific Cre line has been generated by homologous recombination of Cre into the *myosin light chain 1f* (*mlc*) locus, but Cre expression in this line is restricted to fast fibers (Bothe *et al.*, 2000) and is activated relatively late during myogenesis (Jiang *et al.*, 2002).

In order to circumvent the problem that arises due to the similarity between muscle types, we sought to establish an exclusively skeletal muscle-specific Cre transgenic mouse line. Our goal was to generate a line in which Cre expression was abundant throughout skeletal muscle, including fast and slow fibers, as well as muscles of hypaxial and epaxial origins and in which Cre was expressed from the earliest stages of skeletal muscle development. Previous studies have identified a highly conserved promoter from the mouse *mef2c* gene that lies 71 kb upstream of the first translated exon and is sufficient to direct expression exclusively to skeletal muscle (Dodou *et al.*, 2003; Wang *et al.*, 2001). The activity of this promoter fragment is first detected at 8.5 dpc (days post coitum), consistent with the earliest expression of *mef2c* in the developing myotome (Edmondson *et al.*, 1994). The early activation of this promoter from *mef2c*, combined with its highly restricted activity, suggested that it might serve as an ideal tool to direct Cre expression exclusively to skeletal muscle. Therefore, we cloned the Cre cDNA under the control of the *mef2c* skeletal

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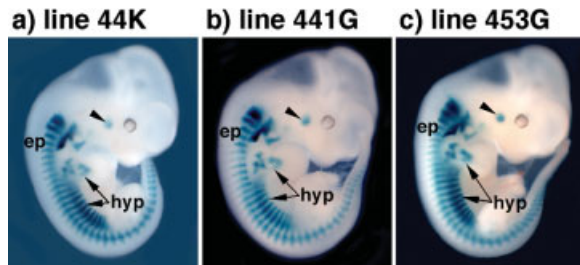
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**FIG. 1.** Schematic representation of the skeletal muscle-specific Cre transgene construct, *mef2c*-73k-Cre. A 954-bp highly conserved promoter from the mouse *mef2c* gene was cloned upstream of the Cre cDNA and a splice and polyadenylation sequence from SV40 and used to generate transgenic mice.



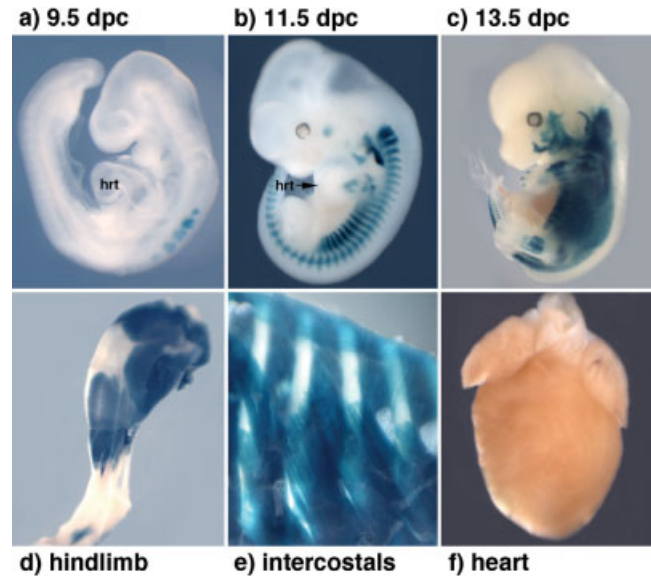
**FIG. 2.** The *mef2c*-73k-Cre transgene directs skeletal muscle-specific expression of Cre during embryogenesis. Three independent transgenic founder lines, 44K (a), 441G (b), and 453G (c) were crossed to ROSA26R Cre-dependent *lacZ* reporter mice and embryos were collected and X-gal-stained at 11.5 dpc. Cre induced recombination in the hypaxial (hyp) and epaxial (ep) lineages, and also in the developing muscle of the face (arrowhead).

muscle promoter and used this construct, designated *mef2c*-73k-Cre, to generate transgenic mice (Fig. 1).

Eight Cre transgene-positive founders were crossed to ROSA26R Cre-dependent *lacZ* reporter mice (Soriano, 1999) and screened for  $\beta$ -galactosidase activity at 11.5 dpc. Five of the eight *mef2c*-73k-Cre lines induced recombination at the ROSA26 locus exclusively in skeletal muscle. The remaining three transgene-positive lines displayed no detectable expression of  $\beta$ -galactosidase when crossed to ROSA26R reporter mice (data not shown). Among the five transgenic lines that demonstrated Cre activity at 11.5 dpc, three displayed robust excision at the ROSA26 locus (Fig. 2). The expression of Cre by all three of these independent transgenic lines was restricted exclusively to skeletal muscle, consistent with the previously described activity of this *mef2c* promoter element (Dodou *et al.*, 2003; Wang *et al.*, 2001).

To determine the expression pattern of Cre directed by the *mef2c* skeletal muscle promoter in more detail, we examined  $\beta$ -galactosidase activity in embryos and offspring from crosses of 73k-*mef2c*-Cre with ROSA26R reporter mice (Fig. 3).  $\beta$ -Galactosidase expression was evident by 9.0 dpc and X-gal staining could easily be detected in the somites by 9.5 dpc (Fig. 3a).  $\beta$ -Galactosidase activity intensified until 11.5 dpc (Fig. 3b) and by 13.5 dpc, X-gal staining was obvious in every skeletal muscle in the embryo (Fig. 3c).  $\beta$ -Galactosidase expression in neonatal mice could be observed robustly in every skeletal muscle in the animal and was not detected in any other tissue, even those of sclerotomal origin, including the bones of the limbs and ribs (Fig. 3d,e). Notably,  $\beta$ -galactosidase expression was never observed in the heart at any stage (Fig. 3f).

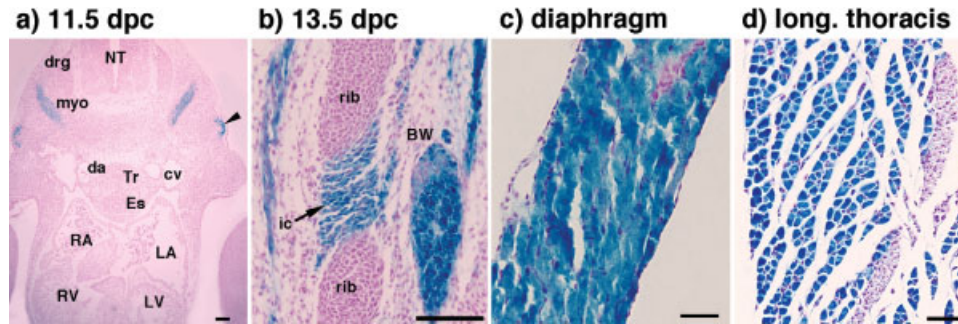
To define skeletal muscle promoter-directed Cre expression more precisely, transverse sections from Cre-



**FIG. 3.** The *mef2c*-73k-Cre transgene directs Cre expression exclusively in skeletal muscle and is active from early in muscle development. Whole-mount X-gal-stained embryos resulting from crossing Cre-positive transgenic mice from line 441G to ROSA26R Cre-dependent *lacZ* reporter mice are shown at 9.5 dpc (a), 11.5 dpc (b), 13.5 dpc (c), exhibiting a strictly skeletal muscle-specific pattern with no staining ever witnessed in the heart (hrt), or any other tissue. Tissues collected from neonatal mice also exhibit Cre-dependent recombination exclusively in skeletal muscle, including the hindlimb (d) and intercostal muscles (e). No recombination was observed in the neonatal heart (f).

positive, ROSA26R transgenic embryos and neonatal muscles were examined by X-gal staining. Sections from transgenic embryos collected at 11.5 dpc showed expression in the myotomal component of the somites and in muscle cells in the limb buds (Fig. 4a). Expression was completely absent from all four chambers of the heart and from all of the smooth muscle in the embryo, including the smooth muscle cells in the trachea, esophagus, and the vascular system. These results support the observation that the activity of this *mef2c* Cre transgene is completely restricted to skeletal muscle. By 13.5 dpc, *lacZ* expression marked all of the skeletal muscle fibers in the embryo, while nonmuscle tissues all remained unstained by X-gal (Fig. 4b). Similarly, sections of X-gal stained neonatal muscles showed that every muscle fiber in the animal was positive for  $\beta$ -galactosidase activity, including muscles of hypaxial (diaphragm) and epaxial (longissimus thoracis) origins (Fig. 4c,d).

To test directly the overlap of  $\beta$ -galactosidase activity in neonatal *mef2c*-73k-Cre Tg/0; ROSA26R Tg/+ mice, we compared the staining generated with an antibody against  $\beta$ -galactosidase to the staining of muscle cells by anti-skeletal muscle MyHC (MY-32) in transverse sections of neonatal limb muscles (Fig. 5). Anti-myosin stained every muscle fiber in the neonatal limb, as expected (Fig. 5a), and anti- $\beta$ -galactosidase also stained every muscle fiber in the animal, including those in the limb

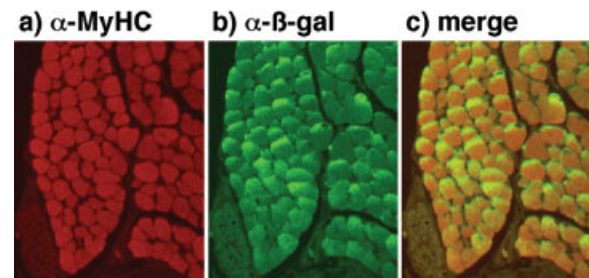


**FIG. 4.** Cre expression from the *mef2c*-73k-Cre transgene is completely restricted to skeletal muscle and every muscle fiber exhibits Cre dependent recombination. Transverse sections through the developing embryo at 11.5 dpc (a) and 13.5 dpc (b) show that  $\beta$ -galactosidase activity due to Cre-dependent recombination in ROSA26R reporter mice occurs in the myotome (myo), intercostals (ic), and body wall muscles (BW), but not in the smooth muscle of the trachea (Tr), esophagus (Es), or the vasculature. Expression was not observed in cardiac muscle in the four chambers of the heart. Additionally, staining was not observed in the neural tube (NT), dorsal root ganglia (DRG), or sclerotome-derived tissue of the ribs (rib). Sections through neonatal muscles of hypaxial (c, diaphragm) and epaxial (d, longissimus thoracis) origins show Cre-induced recombination in every skeletal muscle fiber, indicating that the *mef2c*-73k-Cre transgene is expressed in every skeletal muscle progenitor population. Arrowhead marks the limb bud. Scale bar = 100  $\mu$ m. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; da, dorsal aorta; cv, cardinal vein.

(Fig. 5b). Importantly, no nonmuscle cells were stained by the anti- $\beta$ -galactosidase antibody, which was consistent with the X-gal staining results and indicated that Cre had not been expressed outside the skeletal muscle lineage (Fig. 5c). These results further demonstrate that *mef2c*-73k-Cre directs Cre expression throughout skeletal muscle during development and that expression is completely restricted to the skeletal muscle lineage.

The expression of Cre directed by the *mef2c* promoter is observed in both hypaxial and epaxial muscles and is not restricted to any specific fiber type. This is an important distinction from a previously described Cre transgenic line in which Cre expression directed from the *mhc* locus is restricted during development to fast fibers (Bothe *et al.*, 2000). In addition, the Cre transgene described here is active from very early in skeletal muscle development (Fig. 3). This is also in contrast to other previously described muscle Cre lines, which are activated later in skeletal muscle development than the *mef2c* promoter. Indeed, the *mck* and *mhc* promoters are direct transcriptional targets of MEF2 in skeletal muscle, indicating that they are activated after significant accumulation of MEF2 proteins in the myotome (Amacher *et al.*, 1993; Ferrari *et al.*, 1997; Gossett *et al.*, 1989; McGrew *et al.*, 1996; Rao *et al.*, 1996). By contrast, we have shown previously that the *mef2c* skeletal muscle promoter is activated directly by MyoD and other myogenic bHLH family members and is not subject to autoregulation by MEF2 factors (Dodou *et al.*, 2003).

Several transcription factors have been implicated in skeletal muscle development, but play essential roles elsewhere in the embryo at early developmental times, making it difficult to determine the function of these genes in skeletal muscle using conventional targeting approaches. For example, the transcription factor GATA2, a member of the GATA family of zinc finger transcription factors, has been implicated strongly in postnatal skeletal muscle function (Musaro *et al.*, 1999), but



**FIG. 5.** The *mef2c*-73k-Cre transgene expresses Cre in every skeletal muscle fiber. (a) The expression of myosin heavy chain (MyHC) in neonatal limb skeletal muscle was compared to the expression of  $\beta$ -galactosidase (b). The merged image (c) shows that every MyHC-positive fiber was also positive for  $\beta$ -galactosidase protein produced from Cre-dependent recombination at the ROSA26R locus.

mice lacking *Gata2* exhibit embryonic lethality prior to 11.5 dpc due to hematopoietic defects (Tsai *et al.*, 1994). Similarly, members of the MEF2 family of transcription factors play key roles in the differentiation of multiple cell types (Black and Olson, 1998). Conventional targeted inactivation of the *mef2c* gene results in early embryonic lethality due to severe cardiovascular defects, making it impossible to address the genetic function of *mef2c* in skeletal muscle (Bi *et al.*, 1999; Lin *et al.*, 1997, 1998). The *mef2c*-73k-Cre line described here should be useful for early skeletal muscle-specific inactivation of these and other genes that are important in the heart or elsewhere during development.

## MATERIALS AND METHODS

### Cloning and Generation of the Skeletal Muscle-Specific Cre Mice

A 954-bp fragment of the *mef2c* gene containing the skeletal muscle promoter and enhancer was isolated as

a SacI/Clal fragment from 73k-HSP68-*lacZ* (Dodou *et al.*, 2003). This enhancer fragment was then cloned into a Cre expression plasmid containing the Cre cDNA and the SV40 splice and polyA signal sequence to create plasmid *mef2c*-73k-Cre. The ~2.5 kb *mef2c*-73K-Cre transgene fragment was purified as a NotI fragment and injected into the male pronuclei of fertilized oocytes as described previously (Dodou *et al.*, 2003). Cre-positive founder mice were identified by Southern blot using a Cre-specific radiolabeled probe on genomic DNA isolated from tail biopsies. All experiments using animals complied with federal and institutional guidelines and were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

### Analysis of Cre Expression and Recombination

Transgenic male founders or transgenic male offspring of female founders were crossed to female ROSA26R reporter mice (Soriano, 1999). Embryos were collected at 11.5 dpc and stained with 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-gal) to detect  $\beta$ -galactosidase activity, as previously described (Anderson *et al.*, 2004). After establishing three transgenic lines that exhibited robust skeletal muscle-specific expression, embryos at various time points and neonatal tissues were collected for X-gal staining. For analysis of sections, representative X-gal-stained embryos or neonatal tissues were prepared and stained as described previously (Anderson *et al.*, 2004) and counterstained with Neutral Fast Red for better visualization of histology. Immunohistochemistry (IHC) was performed on paraffin sections of neonatal limbs, as described previously (Anderson *et al.*, 2004). Tissues were collected, fixed, sectioned at a thickness of 5  $\mu$ m, and sections were blocked for 20 min in 3% normal goat serum diluted in phosphate-buffered saline (PBS). Mouse monoclonal anti-skeletal muscle myosin (MY-32; Sigma, St. Louis, MO) and rabbit anti- $\beta$ -galactosidase (ICN, Irvine, CA) were diluted 1:300 in 3% normal goat serum. Incubation in both primary antibodies was performed concurrently for 1 h at room temperature in a humid chamber. Following incubation with the primary antibodies, the sections were washed three times for 10 min each with PBS. The secondary antibodies, biotin-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) and tetramethyl rhodamine isocyanate (TRITC)-conjugated antimouse (Sigma), were diluted 1:300 into 3% normal goat serum and slides were incubated in both for 1 h at room temperature in a humid chamber in the dark. Following incubation, the secondary antibody mix was removed and replaced by Streptavidin-Alexa Fluor (SA-488, Molecular Probes) diluted 1:500 in 3% normal goat serum in PBS for 1 h. After washing three times for 10 min in PBS, slides were mounted using a SlowFade Light antifade kit (Molecular Probes) and photographed on a fluorescence microscope.

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### LITERATURE CITED

- Amacher SL, Buskin JN, Hauschka SD. 1993. Multiple regulatory elements contribute differentially to *muscle creatine kinase* enhancer activity in skeletal and cardiac muscle. *Mol Cell Biol* 13:2753-2764.
- Anderson JP, Dodou E, Heidt AB, De Val SJ, Jaehnig EJ, Greene SB, Olson EN, Black BL. 2004. *HRC* is a direct transcriptional target of MEF2 during cardiac, skeletal, and arterial smooth muscle development in vivo. *Mol Cell Biol* 24:3757-3768.
- Bi W, Drake CJ, Schwarz JJ. 1999. The transcription factor MEF2C-null mouse exhibits complex vascular malformations and reduced cardiac expression of angiotensin 1 and VEGF. *Dev Biol* 211:255-267.
- Black BL, Olson EN. 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol* 14:167-196.
- Bothe GW, Haspel JA, Smith CL, Wiener HH, Burden SJ. 2000. Selective expression of Cre recombinase in skeletal muscle fibers. *genesis* 26:165-166.
- Dodou E, Xu SM, Black BL. 2003. *mef2c* is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. *Mech Dev* 120:1021-1032.
- Edmondson DG, Lyons GE, Martin JF, Olson EN. 1994. *Mef2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* 120:1251-1263.
- Ferrari S, Molinari S, Melchionna R, Cusella-De Angelis MG, Battini R, De Angelis L, Kelly R, Cossu G. 1997. Absence of MEF2 binding to the A/T-rich element in the *muscle creatine kinase* (MCK) enhancer correlates with lack of early expression of the *MCK* gene in embryonic mammalian muscle. *Cell Growth Differ* 8: 23-34.
- Gossett LA, Kelvin DJ, Sternberg EA, Olson EN. 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol Cell Biol* 9:5022-5033.
- Hevener AL, He W, Barak Y, Le J, Bandyopadhyay G, Olson P, Wilkes J, Evans RM, Olefsky J. 2003. Muscle-specific *Ppar $\gamma$*  deletion causes insulin resistance. *Nat Med* 9:1491-1497.
- Jiang P, Song J, Gu G, Slonimsky E, Li E, Rosenthal N. 2002. Targeted deletion of the *MLC1f/3f* downstream enhancer results in precocious MLC expression and mesoderm ablation. *Dev Biol* 243:281-293.
- Lin Q, Schwarz J, Bucana C, Olson EN. 1997. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* 276:1404-1407.
- Lin Q, Lu J, Yanagisawa H, Webb R, Lyons GE, Richardson JA, Olson EN. 1998. Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development* 125: 4565-4574.
- McGrew MJ, Bogdanova N, Hasegawa K, Hughes SH, Kitsis RN, Rosenthal N. 1996. Distinct gene expression patterns in skeletal and cardiac muscle are dependent on common regulatory sequences in the *MLC1/3 locus*. *Mol Cell Biol* 16:4524-4534.
- Miwa T, Koyama T, Shirai M. 2000. Muscle specific expression of Cre recombinase under two actin promoters in transgenic mice. *genesis* 26:136-138.
- Musaro A, McCullagh KJ, Naya FJ, Olson EN, Rosenthal N. 1999. IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* 400: 581-585.

- Norris AW, Chen L, Fisher SJ, Szanto I, Ristow M, Jozsi AC, Hirshman MF, Rosen ED, Goodyear LJ, Gonzalez FJ, Spiegelman BM, Kahn CR. 2003. Muscle-specific PPARgamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. *J Clin Invest* 112:608-618.
- Rao MV, Donoghue MJ, Merlie JP, Sanes JR. 1996. Distinct regulatory elements control muscle-specific, fiber-type-selective, and axially graded expression of a myosin light-chain gene in transgenic mice. *Mol Cell Biol* 16:3909-3922.
- Soriano P. 1999. Generalized *lacZ* expression with the ROSA26 Cre reporter strain. *Nat Genet* 21:70-71.
- Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, Alt FW, Orkin SH. 1994. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 371:221-226.
- Wang J, Wilhelmsson H, Graff C, Li H, Oldfors A, Rustin P, Bruning JC, Kahn CR, Clayton DA, Barsh GS, Thoren P, Larsson NG. 1999. Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. *Nat Genet* 21:133-137.
- Wang DZ, Valdez MR, McAnally J, Richardson J, Olson EN. 2001. The *Mef2c* gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. *Development* 128:4623-4633.