Transcriptional regulation of myotube fate specification and intrafusal muscle fiber morphogenesis

Y’vonne Albert,¹ Jennifer Whitehead,¹ Laurie Eldredge,¹ John Carter,¹ Xiaoguang Gao,¹ and Warren G. Tourtellotte¹,²,³

¹Department of Pathology, ²Neurology, and ³The Institute for Neuroscience, Northwestern University, Chicago, IL 60611

Vertebrate muscle spindle stretch receptors are important for limb position sensation (proprioception) and stretch reflexes. The structurally complex stretch receptor arises from a single myotube, which is transformed into multiple intrafusal muscle fibers by sensory axon–dependent signal transduction that alters gene expression in the contacted myotubes. The sensory-derived signal transduction pathways that specify the fate of myotubes are very poorly understood. The zinc finger transcription factor, early growth response gene 3 (Egr3), is selectively expressed in sensory axon–contacted myotubes, and it is required for normal intrafusal muscle fiber differentiation and spindle development. Here, we show that overexpression of Egr3 in primary myotubes in vitro leads to the expression of a particular repertoire of genes, some of which we demonstrate are also regulated by Egr3 in developing intrafusal muscle fibers within spindles. Thus, our results identify a network of genes that are regulated by Egr3 and are involved in intrafusal muscle fiber differentiation. Moreover, we show that Egr3 mediates myotube fate specification that is induced by sensory innervation because skeletal myotubes that express Egr3 independent of other sensory axon regulation are transformed into muscle fibers with structural and molecular similarities to intrafusal muscle fibers. Hence, Egr3 is a target gene that is regulated by sensory innervation and that mediates gene expression involved in myotube fate specification and intrafusal muscle fiber morphogenesis.

Introduction

Muscle spindles are stretch receptors that mediate myotatic stretch reflexes as well as limb and axial body position sensation (proprioception) in vertebrates. They consist of specialized, encapsulated muscle fibers (intrafusal muscle fibers) that are innervated by sensory (groups Ia and II) and motor (fusimotor) axons to form mechanoreceptors that relay skeletal muscle stretch sensation to the central nervous system. Late prenatal or early postnatal sensory denervation, but not motor denervation, of developing skeletal muscles leads to spindle degeneration, which indicates that normal spindle development is dependent on sensory, but not fusimotor, innervation (Zelená, 1957; Zelená and Soukup, 1973; Kucera and Walro, 1992). Spindle morphogenesis requires intact sensory innervation, and this has been convincingly demonstrated in mice that lack either neurotrophin-3 (NT-3) or its cognate, tyrosine kinase receptor/TrkC (Enfors et al., 1994; Klein et al., 1994; Tessarollo et al., 1994). Proprioceptive sensory neuron survival during development depends on NT-3–tyrosine kinase receptor C signaling, and when it is abrogated in mutant mice Ia-afferents do not form properly to induce spindle morphogenesis in the periphery. Thus, Ia-afferent–derived signaling has been recognized as an essential aspect of muscle spindle induction for several decades, but not until recently have some specific signaling mediators been identified. For example, neuregulin-1 (Nrg1) appears to be an essential Ia-afferent–derived mediator of spindle morphogenesis, and although Ia-afferents establish contact with myotubes, muscle spindle morphogenesis and some intrafusal muscle fiber–specific genes are not appropriately induced in mice when sensory neurons are deficient in Nrg1 (Hippemeyer et al., 2002). Similarly, muscle spindle morphogenesis is impaired in mice with a muscle-specific deficiency of erythroblastic leukemia viral oncogene homologue 2 (ErbB2), an essential component of the Nrg1 tyrosine kinase receptor signaling complex (Andrechek et al., 2002; Leu et al., 2003). Together, these results indicate that Ia-afferents induce spindle morphogenesis by releasing Nrg1 to engage ErbB2-dependent signal transduction in a subpopulation of myotubes that receive sensory myoneural contacts.
Comparatively little is known about the gene regulatory networks that are engaged by ErbB2 signaling in Ia-afferent–contacted myotubes in order to induce their transformation to intrafusal muscle fibers. Presumably, de novo gene expression is required after sensory myoneural contact is established to specify the fate of myotubes that will become morphologically and biochemically distinct intrafusal muscle fibers; to mediate terminal Schwann cell differentiation that will generate the fusiform spindle capsule; and to regulate growth factors that are induced to establish and/or maintain specialized, spindle-related sensory nerve terminals that coincides with Ia-afferent innervation, and it is not expressed in the adenovirus-infected myotubes was compared by using Affymetrix microarray analysis. The experiment was repeated twice, and a four-way analysis of the gene expression datasets identified 83 unique genes that were up-regulated and five unique genes that were down-regulated by Ergr3WT in primary myotubes. Ergr3 appears to be a transcriptional activator in the cellular contexts where it has been studied, which is consistent with the observation that many more genes were up-regulated by Ergr3WT (Table I, 83 genes, 2–244-fold) than were down-regulated (Table II, five genes, two- to threefold). Although most of the 83 genes identified have not been evaluated further, up-regulation was confirmed in 15/15 genes examined by using a real-time PCR analysis on cDNA samples that were prepared from independently infected primary myotubes (Fig. 1 D, results from 12 genes shown; Table I).

Results

Egr3-mediated gene regulation in primary murine myotubes

Egr3 expression is regulated in myotubes by sensory innervation and Nrg1 signaling in vivo (Tourtellotte et al., 2001; Hippiemeyer et al., 2002; Jacobson et al., 2004). Ia-afferents contact myotubes in Egr3-deficient mice; in the absence of Egr3-mediated gene regulation, they do not properly differentiate into spindles, which leaves the mice with profound sensory ataxia and proprioceptive deficits (Tourtellotte and Milbrandt, 1998). To identify target genes that are potentially regulated by Egr3 in Ia-afferent–contacted myotubes, primary myoblasts were isolated from newborn, wild-type mice and were differentiated into multinucleated myotubes. One population of myotubes was infected with an adenovirus that coexpressed enhanced GFP (EGFP) and transcriptionally active wild-type Egr3 (Egr3WT), whereas another population of myotubes was infected with an adenovirus that coexpressed EGFP and transcriptionally inactive truncated Egr3 (Egr3Tr; Fig. 1 A). Primary myotubes that were infected with 100% efficiency expressed high levels of transgenic protein and showed no evidence of toxicity (Fig. 1 B). Endogenous Egr3 was not detected in cultured myotubes, similar to myotubes that neither receive Ia-afferent innervation nor become intrafusal muscle fibers in vivo (Fig. 1 B, bottom right; Tourtellotte and Milbrandt, 1998). To identify genes regulated by Egr3 in this primary myoblast–cellular context, gene expression in the adenovirus-infected myotubes was compared using Affymetrix microarray analysis. The experiment was repeated twice, and a four-way analysis of the gene expression datasets identified 83 unique genes that were up-regulated and five unique genes that were down-regulated by Egr3WT in primary myotubes. Egr3 appears to be a transcriptional activator in the cellular contexts where it has been studied, which is consistent with the observation that many more genes were up-regulated by Egr3WT (Table I, 83 genes, 2–244-fold) than were down-regulated (Table II, five genes, two- to threefold). Although most of the 83 genes identified have not been evaluated further, up-regulation was confirmed in 15/15 genes examined by using a real-time PCR analysis on cDNA samples that were prepared from independently infected primary myotubes (Fig. 1 D, results from 12 genes shown; Table I).

Egr3-mediated gene regulation in developing muscle spindles

To examine whether Egr3 target genes identified in vitro may also be regulated by Egr3 in a similar cellular context in develop-
oping muscle spindles in vivo, we used in situ hybridization to characterize gene expression patterns in embryonic wild-type mouse muscles. Egr3 and Pea3 are transcription factors regulated by Ia-afferent–Nrg1 signaling in myotubes, and Ia-afferent contact myotubes in both Egr3-deficient and Pea3-deficient mice (Tourtellotte and Milbrandt, 1998; Livet et al., 2002). Pea3 is not necessary for spindle morphogenesis, but it is a useful marker for localizing Ia-afferent–contacted myotubes in the developing skeletal muscle (Hippenmeyer et al., 2002). We found that Pea3 was not regulated by Egr3 because Pea3 expression was identified in discretely labeled myotubes in Egr3-deficient mice (Hippenmeyer et al., 2002). However, Pea3-expressing myotubes were reduced by ~66% in Egr3-deficient muscles at the embryonic gestational day (E) 16.5 (Fig. 2 A) and were reduced by ~86% at E18.5 (Fig. 2 B). Because Pea3 is a marker of Ia-afferent–myotube contact, the decreased number of Pea3-expressing myotubes in Egr3-deficient mice is consistent with previous observations that demonstrate initial Ia-afferent contact of myotubes, progressive loss of afferents during prenatal development, and complete loss of afferents in adult Egr3-deficient mice (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001). Because some Ia-afferent–contacted, Pea3-expressing myotubes were present in prenatal Egr3-deficient muscles, it was possible to examine potential Egr3 target gene expression within them. Accordingly, Egr3-dependent gene expression in skeletal muscle should be restricted to Ia-afferent–contacted myotubes where Egr3 expression is restricted, and it should be abrogated in Pea3-expressing (Ia-afferent contacted), Egr3-deficient myotubes. We examined six target genes that were identified by Affymetrix microarray analysis (and were confirmed to be up-regulated in myotubes by real-time PCR analysis) using in situ hybridization on the muscle. Although three genes (rhophilin 2 [Rhpn2]; g-coupled protein receptor 50 [GPR50]; and activity-regulated cytoskeleton-associated protein [Arc]) showed no specific localization to muscle spindles (unpublished data), the three other genes, including two growth factor receptors (low affinity nerve growth factor receptor [NGFR (p75)]; and somatostatin receptor type 2 [SSTr2]) and the type III intermediate filament peripherin 1 (Prph1), appeared to be expressed selectively by spindles. In E18.5 wild-type skeletal muscle, NGFR (p75) (Fig. 3 A), SSTr2 (Fig. 3 B), and Prph1 (Fig. 3 C) expression was restricted to muscle spindles similar to the expression pattern of Egr3. In skeletal muscles from E18.5 Egr3-deficient mice, NGFR (p75), SSTr2, and Prph1 were not expressed in Pea3-expressing myotubes (Fig. 3, A–C, bottom). Hence, NGFR (p75), SSTr2, and Prph1 represent new
### Table I. Up-regulated genes (Egr3<sub>WT</sub> relative to Egr3<sub>S</sub>)

| Affymetrix fold change (n = 4) | Up-regulated in myotubes >fivefold (n = 4) | Up-regulated in HS<sub>A</sub>-Egr3 mice >fivefold (n = 4) | Expression spindles | Gene symbol<sup>a</sup> | NCBI accession no.<sup>b</sup> |
|--------------------------------|--------------------------------------------|-------------------------------------------------|------------------|-----------------|-------------------------|
| 244.04                        | +                                          | +                                               |                  |                 | Rphpn2                 | BF228009               |
| 56.16                         | +                                          | +                                               |                  |                 | GRP50                   | BG032613               |
| 44.94                         | +                                          | +                                               |                  |                 | RIKEN_cDNA 2810417M05  | 2810417M05Rik          | AK014196.1             |
| 38.87                         | +                                          | +                                               |                  |                 | Plmaph                | NM_023938              |
| 29.07                         |                                             | +                                               |                  |                 | Snap25                | BC018249               |
| 27.94                         | +                                          | +                                               |                  |                 | Prph1                  | NM_013639               |
| 27.33                         | +                                          | +                                               |                  |                 | Ube3a                 | BB31387                |
| 17.63                         |                                             | +                                               |                  |                 | Keratinocyte diffrntiation assoc. protein | Krdnap | AV007706 |
| 14.37                         |                                             | +                                               |                  |                 | Sbns                  | AI507307               |
| 11.91                         |                                             | +                                               |                  |                 | Hey1                   | NM_010423.1            |
| 10.62                         |                                             | +                                               |                  |                 | Cdo1                   | NM_033037.1            |
| 10.26                         |                                             | +                                               |                  |                 | RIKEN_cDNA 1110065A22  | 1110065A22Rik          | BB768838              |
| 7.60                          |                                             | +                                               |                  |                 | Arc1                   | NM_018790.1            |
| 7.54                          |                                             | +                                               |                  |                 | Fbxo2                 | BB311718               |
| 7.48                          |                                             | +                                               |                  |                 | GABAA receptor, subunit a4 | GABRA4 | BB430205 |
| 7.46                          |                                             | +                                               |                  |                 | Cln2                   | NM_009900.1            |
| 6.77                          |                                             | +                                               |                  |                 | Lrnr6a                | BB078751               |
| 6.63                          |                                             | +                                               |                  |                 | Elnmifil I             | BB076147               |
| 6.61                          |                                             | +                                               |                  |                 | Rps6ka1                | NM_009097.1            |
| 6.48                          |                                             | +                                               |                  |                 | Dmkn                   | BA52905                |
| 6.33                          |                                             | +                                               |                  |                 | Krt18                  | NM_010664.1            |
| 6.23                          |                                             | +                                               |                  |                 | Snx2                   | NM_009217.1            |
| 5.97                          |                                             | +                                               |                  |                 | Neu4d                  | AW553317               |
| 5.94                          |                                             | +                                               |                  |                 | Atpase, Na<sup>+</sup>/K<sup>+</sup> transporting, a3 poly peptide | Atpa3 | BC027114.1 |
| 5.81                          |                                             | +                                               |                  |                 | Nkbie                  | BB820441               |
| 5.73                          |                                             | +                                               |                  |                 | Pras22                 | NM_133731.1            |
| 5.62                          |                                             | +                                               |                  |                 | LOC327991              | BB346082               |
| 5.59                          |                                             | +                                               |                  |                 | Ngfr                   | BB151515               |
| 5.23                          |                                             | +                                               |                  |                 | Eski                   | BB251859               |
| 4.99                          |                                             | +                                               |                  |                 | Riken_cDNA A030009H04  | A030009H04Rik          | NM_020591.1            |
| 4.82                          |                                             | +                                               |                  |                 | Inha                   | NM_010654.1            |
| 4.78                          |                                             | +                                               |                  |                 | Proc1                  | NM_011363.1            |
| 4.49                          |                                             | +                                               |                  |                 | Sdcsg3                 | AV291373               |
| 4.44                          |                                             | +                                               |                  |                 | Uclh1                  | NM_011670.1            |
| 4.41                          |                                             | +                                               |                  |                 | RIKEN_cDNA 8403427H17  | 8403427H17Rik          | BM120675              |
| 4.40                          |                                             | +                                               |                  |                 | Mus musculus, clone IMAGE:3368459, mRNA | Musm | MF68072 |
| 4.27                          |                                             | +                                               |                  |                 | Nacrl                  | AV010795               |
| 4.25                          |                                             | +                                               |                  |                 | Lapp                   | BM124366               |
| 4.24                          |                                             | +                                               |                  |                 | Riken_cDNA 2210010L05  | 2210010L05Rik          | BM126598.1            |
| 4.23                          |                                             | +                                               |                  |                 | Hey2                   | NM_013904.1            |
| 4.13                          |                                             | +                                               |                  |                 | Riken_hypothetical, 6330405H19 | 6330405H19Rik          | BB252256              |
| 4.04                          |                                             | +                                               |                  |                 | Car2                   | NM_009802.2            |
| 3.94                          |                                             | +                                               |                  |                 | Grasp                  | NM_019518.1            |
| 3.77                          |                                             | +                                               |                  |                 | Sept4                  | AW208509               |
| 3.75                          |                                             | +                                               |                  |                 | Riken_hypothetical, C130068N17 | C130068N17Rik          | AW548377              |
| 3.55                          |                                             | +                                               |                  |                 | Nse1                   | BC002154.1             |
| 3.53                          |                                             | +                                               |                  |                 | Tst                    | BC005644.1             |
| 3.51                          |                                             | +                                               |                  |                 | Cxcl3                  | AF071549.1             |
| 3.49                          |                                             | +                                               |                  |                 | Fos3                   | AK019022.1             |
| 3.44                          |                                             | +                                               |                  |                 | Prx                    | BG072867               |
| 3.41                          |                                             | +                                               |                  |                 | Preproepeptide 1       | M13227.1               |
| 3.41                          |                                             | +                                               |                  |                 | Ccnc1                  | C76734                 |
| 3.30                          |                                             | +                                               |                  |                 | Phospholipase C6      | P1c6i                 | NM_019676.1            |
| 3.21                          |                                             | +                                               |                  |                 | Riken_cDNA, A030009H04 | A030009H04Rik          | AK002962.1            |
| 3.19                          |                                             | +                                               |                  |                 | Riken_cDNA, 1810017F10 | 1810017F10Rik          | BC019563.1            |
| 3.12                          |                                             | +                                               |                  |                 | Ets transcription factor Sp1 | Sp1     | BM214016.1        |
| 3.09                          |                                             | +                                               |                  |                 | Riken_cDNA, B230331G10 | B230331G10Rik          | BB357157              |
| 3.07                          |                                             | +                                               |                  |                 | Ets1                   | BE948505               |
| 3.06                          |                                             | +                                               |                  |                 | Ephrin A3              | EA3a                  | AA388313              |
| 2.99                          |                                             | +                                               |                  |                 | Riken_cDNA, E030041H03 | E030041H03Rik          | BB353044              |
| 2.97                          |                                             | +                                               |                  |                 | Caspase recruitment domain, member 10 | Card10 | NM_130859.1 |
| 2.96                          |                                             | +                                               |                  |                 | Amn1                   | BB145065               |
| 2.94                          |                                             | +                                               |                  |                 | Phenylenediamine-N-methyltransferase | Phn | NM_023061.1 |
| 2.87                          |                                             | +                                               |                  |                 | Riken_cDNA, 2210010L05 | 2210010L05Rik          | BB225441              |
| 2.84                          |                                             | +                                               |                  |                 | Melanoma cell adhesion molecule | Mcam | NM_023061.1 |

<sup>a</sup> NCBI unified gene nomenclature according to Entrez Gene.

<sup>b</sup> NCBI GenBank accession number of sequence used to generate gene feature on Affymetrix chip.
It was not possible to generate transgenic founders that could be used to establish lines of transgenic mice because they were not viable. To facilitate the analysis of the transgenic founders, real-time PCR was used to examine the level of Egr3 expression from forelimb-derived RNA in every embryo that was generated. Animals were analyzed based upon their genotype and level of Egr3 expression by grouping them into those that did not carry the transgene (Tg−; wild type), those that carried the transgene but did not express transgenic Egr3 above endogenous levels (Tg+/ΔΦ), those that expressed low levels of transgenic Egr3 (Tg+/ΔL; 3–10-fold increased expression relative to Tg− mice), and those that expressed high levels of transgenic Egr3 (Tg+ΔH; >10-fold increased expression relative to Tg− mice; Fig. 4 B). Late gestation embryos that expressed the transgene (Tg+/ΔL and Tg+/ΔH) were consistently smaller than their transgene-negative (Tg−) or nontransgene-expressing (Tg−/ΔL and Tg−/ΔH) litter mates (Fig. 4 C). E18.5 transgene-expressing mice had beating hearts, flexion contractures of their extremities, and were not viable at birth (Fig. 4 D). The level of Egr3 expression in particular founder mice correlated with the relative number of muscle fibers that expressed Egr3. Egr3 expression in E18.5 wild-type skeletal muscle was restricted to developing spindles, which were relatively few in number (Fig. 4 E, arrows), whereas Egr3 was expressed by most of the muscle fibers in transgene-expressing mice (Fig. 4 F).

Transgene-expressing mice had poorly formed skeletal muscles that were generally more poorly formed in Tg+/ΔH muscles than in Tg+/ΔL muscles. As expected, muscles from mice that did not express the transgene (Tg+/ΔΦ) appeared normal. In E18.5 wild-type hindlimbs, discrete muscles were readily identified (Fig. 5 A), whereas distinct muscles were largely absent in transgene-expressing hindlimbs (Fig. 5 B). Wild-type muscle fibers contained skeletal myofilaments and subsarcolemmal nuclei that are typical of immature skeletal myofibers (Fig. 5, C and E). Occasional spindles were identified that consisted of intrafusal muscle fibers with scant myo-
fibrillary architecture and internal nuclei. Sections through the equatorial plane of the spindles showed prominent annulospiral axons (Ia-afferents) circumscribing the intrafusal muscle fibers within rudimentary spindle capsules (Fig. 5 E, arrowhead). In contrast, all muscle fibers from transgene-expressing mice contained scant myofilaments with centrally located nuclei. Although most were single multinucleated fibers that had myofibrillar structure and nuclear features indistinguishable from intrafusal muscle fibers, many were aggregated into clusters that were similar to intrafusal muscle fibers within spindles (Fig. 5, D and F [arrow]). Most of the intrafusal-like muscle fibers were not encapsulated or innervated as determined from an analysis of serially sectioned, resin-embedded hindlimbs. Occasional normal-appearing spindles were identified in muscles from transgene-expressing mice that had the typical configuration of three to four intrafusal muscle fibers, which were circumscribed by annulospiral sensory axons and rudimentary capsules (Fig. 5 F, arrowhead). Thus, Egr3 expression in skeletal myotubes independent of other Ia-afferent signaling has the remarkable capacity to transform them into muscle fibers that are structurally similar to intrafusal muscle fibers, presumably orchestrated by the network of genes it regulates.

Figure 2. Pea3 expression in wild-type and Egr3-deficient muscle is a marker of Ia-afferent–contacted myotubes in vivo. (A) At E16.5, shortly after Ia-afferent contact is made with myotubes, Pea3 is expressed in both wild-type and Egr3-deficient myotubes. In Egr3-deficient muscles, the number of myotubes that express Pea3 is reduced by 66% in E16.5 embryos and by 86% in E18.5 embryos, they are reduced by 66%. Ia-afferents are known to initially contact myotubes in Egr3-deficient mice and then withdraw during development, which is consistent with the progressive decrease in Pea3-expressing myotubes that are identified in Egr3-deficient muscles. Thus, Pea3 is not regulated by Egr3 in myotubes, and it is a marker for Ia-afferent contact. These results make it possible to use Pea3 to localize Ia-afferent–contacted myotubes and study potential Egr3 target gene expression within them (n = 3 animals for each genotype; mean and standard deviation shown). Insets are high power magnifications of labeled spindles (arrows). Bars: 50 µm; [inset] 10 µm.

Figure 3. Egr3 regulates specific target genes in developing spindles. Affymetrix microarray analysis demonstrated many potential target genes that are regulated by Egr3 in myotubes. Egr3 was necessary for the expression of (A) NGFR (p75), (B) SSTr2, and (C) Prph1 in muscle spindles. The muscle expression of all of these genes was restricted to the developing spindles (arrows), similar to the expression pattern of Egr3, whereas their expression was abrogated in Pea3-expressing (Ia-afferent contacted), Egr3-deficient myotubes. Bars: 50 µm; [inset] 10 µm.
Intrafusal-like muscle fibers in HSA–Egr3 transgenic muscle are not innervated

Intrafusal-like muscle fibers in Egr3 transgene-expressing muscles do not have fusiform capsules, which may derive from terminal Schwann cells of innervating axons. To examine whether the transformed muscle fibers were innervated by either fusimotor or proprioceptive axons, parvalbumin (Pv) immunohistochemistry was used as a selective marker for proprioceptive neurons and Ia-afferents (Tourtellotte and Milbrandt, 1998), and ATP1α/β3 immunohistochemistry was used as a marker for both Ia-afferent and fusimotor axons in skeletal muscles (Dobretsov et al., 2003). There was no quantitative difference in the number of proprioceptive neurons in the Tg/H11002 or Tg/H11001/H11002 fifth lumbar dorsal root ganglion (DRG; Fig. 6 A), and there was no qualitative difference in the number of Pv/H11002 neurons in any first through fourth cervical or first through fifth lumbar DRG that were examined, suggesting that the number of innervated and structurally normal spindles (Fig. 5 F, arrowhead) was normal in Tg/H11001/H mice. However, whereas robust Pv/H11002 Ia-afferent axons terminated on spindles in Tg/H11002 mice (and occasional normal-appearing spindles in Tg/H11001/H mice), the intrafusal-like muscle fibers from Tg/H11001/H mice showed no evidence of Ia-afferent innervation (Fig. 6 B). Similarly, the tochemistry was used as a selective marker for proprioceptive neurons and Ia-afferents (Tourtellotte and Milbrandt, 1998), and ATP1α/β3 immunohistochemistry was used as a marker for both Ia-afferent and fusimotor axons in skeletal muscles (Dobretsov et al., 2003). There was no quantitative difference in the number of proprioceptive neurons in the Tg− or Tg+/H fifth lumbar dorsal root ganglion (DRG; Fig. 6 A), and there was no qualitative difference in the number of Pv+/H neurons in any first through fourth cervical or first through fifth lumbar DRG that were examined, suggesting that the number of innervated and structurally normal spindles (Fig. 5 F, arrowhead) was normal in Tg+/H mice. However, whereas robust Pv+/Ia-afferent axons terminated on spindles in Tg− mice (and occasional normal-appearing spindles in Tg+/H mice), the intrafusal-like muscle fibers from Tg+/H mice showed no evidence of Ia-afferent innervation (Fig. 6 B). Similarly, the
transformed muscle fibers were not able to sustain motor innervation as indicated by the near complete loss of motor neurons in the spinal cord and the absent ventral roots in Tg+/H mice (Fig. 6 C). In the periphery, ATP1α3 identified fusimotor and Ia-afferent axons that terminated selectively on normal spindles, whereas there was no evidence of such innervation in the transformed muscle fibers in Tg+/H muscles. ATP1α3 labeling the spindles innervated by Ia-afferents that would otherwise be expected to be labeled in Tg−/H mice (Fig. 6 C). In the periphery, ATP1α3 immunohistochemistry, whereas no ATP1α3-labeled axons innervated the transformed myotubes in Tg+/H muscles. Arrowheads indicate the spindles innervated by ATP1α3-containing axons. (E) Consistent with the observation that PV− neurons are not altered in Tg+/H mice, Ia-afferent–contacted, Pea3-expressing myotubes were observed in Tg+/H muscles, and Pea3 expression was not significantly different from wild-type (Tg−) or nontransgene-expressing (Tg+/H) muscles (n = 4 for each condition; mean and standard deviation shown). Inset shows a magnified image of the Pea3-expressing spindle (arrow). Bars: (A, C, and E) 50 μm; (B and D) 20 μm; (inset) 10 μm.

Muscle fibers from HSA-Egr3 transgenic mice express genes similar to those of intrafusal muscle fibers

Enforced expression of Egr3 in skeletal myotubes is sufficient to transform them into muscle fibers that structurally resemble intrafusal muscle fibers. To examine whether they also express genes that are characteristic of intrafusal muscle fibers, several well-established intrafusal muscle fiber markers and some newly identified markers were examined in HSA-Egr3 transgenic muscles. In wild-type muscle, Sd-MyHC is expressed by the Ia-afferent contact of myotubes as one of the earliest markers of intrafusal muscle fiber formation. Sd-MyHC is not expressed in Ia-afferent–contacted, Egr3-deficient myotubes, suggesting that they are impaired in their capacity to differentiate into intrafusal muscle fibers (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001). Sd-MyHC was localized to intrafusal muscle fibers in developing spindles in wild-type (Tg−) muscle as previously reported, whereas it was expressed by 15-fold more fibers in Tg+/H muscles, indicating that some myotubes had acquired at least one phenotype of intrafusal muscle fibers (Fig. 7 A). The neurotrophins NT-3 and glial-derived neurotrophic factor (GDNF) are preferentially expressed by intrafusal muscle fibers in E18.5 muscle and are required for their sensory and fusimotor innervation, respectively (Ernfors et al., 1994; Whitehead et al., 2005). Relative to Tg− muscles, neither GDNF nor NT-3 was up-regulated in Tg+/H muscles, but both were up-regulated 4- and 5.2-fold, respectively, in Tg+/H muscles (Fig. 7 B). The newly identified Egr3 target genes NGFR (p75), SST2, and Prph1 were not up-regulated in Tg+/H muscles relative to Tg− muscles, whereas they were all up-regulated in Tg+/H muscle 6.4-, 6.7-, and 4-fold, respectively (Fig. 7 C). Finally, several other genes identified as potential Egr3 target genes in primary myotubes (Fig. 1 D) were also up-regulated in Tg+/H muscles. ATP1α3, harry
enhance of split 1 (Hey1), and 2810417MoRik were not up-regulated in Tg+/ϕ muscles relative to Tg− muscles, whereas they were up-regulated 11.2-, 10.3-, and 7.3-fold, respectively. Together, these data demonstrate that enforced Egr3 expression in skeletal myotubes leads to their transformation into muscle fibers that are structurally similar to intrafusal muscle fibers and that express many genes characteristic of them.

Discussion

During skeletal muscle development, Ia-afferents specify the fate of myotubes by engaging novel gene transcription that transforms them into biochemically and functionally distinct intrafusal muscle fibers. Recent studies have refined our understanding of this classic nerve–muscle induction process by demonstrating that Nrg1 produced by Ia-afferents and signaling through the Nrg1 tyrosine kinase receptor ErbB2 in contacted myotubes is essential to engage the spindle morphogenesis program in vivo (Andrechek et al., 2002; Hippenmeyer et al., 2002; Lee et al., 2003) and in vitro (Jacobson et al., 2004). Moreover, Nrg1 signaling from Ia-afferents is necessary to induce several transcription factors (Egr3, Pea3, ER81, and ERM), which represent at least some proximal mediators of the gene regulatory networks that are engaged by Ia-afferent contact with myotubes. We focused on the role of Egr3 in mediating particular aspects of Ia-afferent (Nrg1–ErbB2) signal transduction because our earlier work indicated that Egr3 is required for normal spindle morphogenesis (Tourtellotte and Milbrandt, 1998). Its expression is restricted to Ia-afferent–contacted myotubes, and Egr3-deficient, Ia-afferent–contacted myotubes do not acquire characteristic structural features or phenotypic markers of intrafusal muscle fibers (Tourtellotte et al., 2001; Chen et al., 2002).

Egr3 gene regulation in Ia-afferent–contacted myotubes

Egr3 regulates a relatively large network of genes within myotubes, and although not all of the genes identified were characterized further in this study, some of them were regulated by Egr3 in developing intrafusal muscle fibers. We found that NGFR (p75), SSTr2, and Prph1 are regulated by Egr3 in intrafusal muscle fibers. Of the 83 genes identified by expression analysis in myotubes, we verified that 15/15 genes tested were up-regulated, suggesting that the list of Egr3 target genes has a high degree of specificity. Moreover, the Egr3-mediated gene regulatory network is evidently complex because the target genes have tremendous functional diversity, including processes such as transcriptional regulation, intracellular signal transduction, protein processing, and cytoskeletal organization. Interestingly, however, not all genes that were up-regulated by Egr3 in myotubes in vitro were Egr3 targets in the developing intrafusal muscle fibers in vivo because neither GPR50, Rphn2, nor Arc was expressed by them. Thus, enforced expression of Egr3 in myotubes leads to target gene expression that is not necessarily restricted to the myotube–cellular context. In support of this concept, studies in progress indicate that Arc is directly regulated by Egr3 in neurons, despite the fact that it is not expressed by intrafusal muscle fibers where Egr3 is expressed at high levels (unpublished data). These results indicate that the cellular context appears to be important in providing a permissive environment for expression of a particular repertoire of Egr3 target genes that are relevant to intrafusal muscle fiber morphogenesis. Whether the target genes identified are directly regulated by Egr3 or whether they are expressed by the activation of downstream mediators of Egr3 is currently not known. Moreover, it is not known whether any of these newly identified Egr3 target genes are essential for spindle morphogenesis.
An analysis of additional Egr3 target genes by in situ hybridization will be necessary to further define the Nrg1–ErbB2–Egr3 regulatory axis that mediates intrafusal muscle fiber morphogenesis.

Muscle-specific and skeletal myotubes were transformed into muscle fibers that had structural and molecular similarities to intrafusal muscle fibers when Egr3 expression was enforced independently of Ia-afferent signaling. Moreover, several established intrafusal muscle fiber markers, including Sd-MyHC, NT-3, and GDNF were up-regulated in HSA–Egr3 transgene-expressing muscles. Similarly, three new Egr3 target genes that were selectively expressed by intrafusal muscle fibers (NGFR [p75], SSTr2, and Prph1) and three additional Egr3 target genes (ATP1a3, Hey1, and 2810417MoRik) were all up-regulated in transgene-expressing muscles. However, the transformed muscle fibers did not acquire the motor or sensory innervation that is characteristic of normal intrafusal muscle fibers, suggesting that Egr3 has a role that is distinct from other Ia-afferent–mediated pathways that may be required to establish and/or maintain these interactions. Moreover, the transformed myotubes did not acquire fusiform capsules that may normally be derived from terminal Schwann cells of the innervating sensory axons. Finally, somatic motor neurons were almost completely absent in HSA–Egr3 transgenic mice, indicating that the transformed muscle fibers were not capable of sustaining skeletomotor innervation that would have been present had they not expressed Egr3 and differentiated into intrafusal muscle fibers. It will be necessary to conditionally express Egr3 in skeletal myotubes in order to study the fate of motor and sensory innervation during development because the transgenic founders died and could be generated only in small numbers.

Sensory control over the morphogenesis of many mechanoreceptors is recognized as a common theme in their ontogeny. A detailed understanding of the gene regulatory mechanisms that are governed by sensory axon innervation of muscle spindles may be applicable to mechanisms used during the ontogeny of other sensory mechanoreceptors. These studies define a set of Egr3-regulated target genes, some of which appear to be relevant to intrafusal muscle fiber morphogenesis. Egr3 is regulated by Nrg1–ErbB2 signaling, and it appears to mediate gene expression that is necessary and sufficient to specify the fate of Ia-afferent–contacted myotubes to become intrafusal muscle fibers and to mediate their morphogenesis.

Materials and methods

Animals and preparation of tissues

Egr3-deficient mice were mated on a C57BL/6J background and were generated and genotyped as previously described (Tourtellotte and Milbrandt, 1998; Whitehead et al., 2005).

The HSA–Egr3 transgenic mice were generated by using the previously characterized HSA promoter to express Egr3 in skeletal myotubes (Muscat et al., 1992). The transgenic construct was derived from pBSX-HSAvpA (Crawford et al., 2000; provided by J. Chamberlain, University of Washington, Seattle, WA) by cloning a full-length, HA-tagged Egr3 cDNA into the NotI site of pBSX-HSAvpA. The construct was injected into B6SJL embryo pronuclei and transplanted into recipient females using standard procedures (provided by L. Doglio and R. Alvarez, Northwestern University, Chicago, IL). The transgene-expressing founder mice were not postnatally viable, and, therefore, recipient females were killed to analyze founder embryos at various gestational ages. Each embryo was genotyped using primers 5'-AAACCTCGCGGAAGCTGCGTG-3' (sense) and 5'-TGTGCTACACCGCTGCTG-3' (antisense) to amplify a 230-bp fragment of the endogenous Egr3 genomic locus. A third primer, 5'-TCAGGGAGACGTGGAGGCCATGTA-3', was used to amplify a 413-bp fragment of the Egr3 cDNA transcript (nontron containing) when paired with the sense probe in a multiplex PCR reaction.

Myoblast isolation and myotube culture

Hindlimb skeletal muscle tissues were isolated from wild-type, postnatal (3–5) mice. Myoblasts were isolated by enzymatically dissociating (disperse, grade II, 2.4 U/ml, Boehringer), and the muscle tissues were triturated according to previously published procedures (Rando and Blau, 1994). Myoblasts were purified by multiple rounds of preplating on collagen-coated tissue culture plates (0.01% type I, Sigma-Aldrich) and expanded in growth medium (Ham’s F10; Mediatech), 20% FBS, and 2.5 ng/ml basic FGF (Promega). Purified primary myoblasts were plated onto collagen-coated plates and were differentiated into multinucleated myotubes for 10 d using differentiation medium (Ham’s F10 and 5% horse serum).
Recombinant adenovirus preparation, characterization, and myotube infection

Recombinant adenoviruses were generated using homologous recombination in Escherichia coli as previously described (He et al., 1998). To generate an adenovirus that expressed transcriptionally active Eg3, the full-length rat Eg3 cDNA (GenBank/EMBL/DDB accession no. NP058782, aa 1–187) was cloned into the BglII site of pAdTrackCMV. Similarly, a transcriptionally inactive, COOH-terminally truncated of Eg3 that lacked the entire three zinc finger DNA-binding and COOH-terminal domains (GenBank/EMBL/DDB accession no. NP058782, aa 1–245) was cloned into the BglII site of pAdTrackCMV. Each recombinant pAdTrack shuttle vector, including pAdTrackCMV without a cDNA insert, was recombined by homologous recombination into the adenoviral genomic plasmid (pAdTrackCMV). Non-intron-spanning primers amplified the cDNA encoding E3. coli to generate a recombinant, replication-deficient adenoviral genome plasmid. The three replication-deficient viruses (Eg3ΔVR, Eg3ΔV, and Eg3ΔE) were packaged and amplified in transfected HEK-293 cells, purified, concentrated on cesium chloride gradients, and titered using EGFP fluorescence and TCID50.

NIH-3T3 fibroblasts were infected with the recombinant adenoviruses at a multiplicity of infection (MOI) of 10–20 to obtain 70–80% infection efficiency. Total cell lysates were obtained 24 h after infection, and a Western blot using an NH2-terminal Eg3, rabbit polyclonal antibody (O’Donovan et al., 1998) demonstrated a high expression of full-length and truncated Eg3 protein as expected. Myoblasts were cotransfected with an early growth response element (ERE) containing a firefly luciferase reporter plasmid (pRlCTK) and a Renilla luciferase reporter plasmid (pRL; Promega) for transfection efficiency control. 16 h after plasmid cotransfection, the cells were infected with either control EGFP, Eg3ΔVR, or Eg3ΔE adenoviruses at an MOI of 10 to obtain 80–90% infection efficiency. 8 h after infection, a dual-reporter luciferase assay was performed to quantify ERE activation by the virally produced proteins according to the manufacturer’s specifications (Promega). Primary myotubes that differentiated for 10 d in vitro were infected with either Eg3ΔVR or Eg3ΔE adenovirus at an MOI of 100 to obtain 100% infection efficiency. Although myotubes were relatively resistant to infection and required a relatively high MOI for optimal infection, no evidence of toxicity was noted 24 h after infection at the time that total RNA was extracted from the infected myotubes.

Gene expression profiling

5 μg of total RNA was converted to cDNA using the superscript reverse transcriptase (Invitrogen) and the T7-Oligo (dT) promoter primer kit (Affymetrix, Inc.). The cDNA was purified using the GeneChip sample preparation kit (Affymetrix, Inc.) and was used for the in vitro synthesis of biotin-labeled cRNA using the GeneChip expression 3′amplification reagents for IVT labeling (Affymetrix, Inc.) at 37°C for 16 h. cRNA was fragmented into 35–200-bp fragments using a magnesium acetate buffer (Affymetrix, Inc.). 10 μg of labeled cRNA were hybridized to GeneChip mouse expression arrays (430A and 430B; Affymetrix, Inc.) for 16 h at 45°C. The chips were washed and stained according to the manufacturer’s recommendations (Affymetrix, Inc.) using the GeneChips fluidsic Next-Station天使 (model 450; Affymetrix, Inc.). This procedure included washing the chips with phycoerythrin-streptavidin, performing signal amplification by a second staining with biotinylated antistreptavidin, and performing a third amplification with phycoerythrin-streptavidin, performing signal amplification by a second staining with biotinylated antistreptavidin, and performing a third amplification with phycoerythrin-streptavidin, performing signal amplification by a second staining with biotinylated antistreptavidin, and performing a third amplification. Primary myotubes that differentiated for 10 d in vitro were infected with either Eg3ΔVR or Eg3ΔE adenovirus at an MOI of 100 to obtain 100% infection efficiency. Although myotubes were relatively resistant to infection and required a relatively high MOI for optimal infection, no evidence of toxicity was noted 24 h after infection at the time that total RNA was extracted from the infected myotubes.

5 μg of total RNA was converted to cDNA using the superscript reverse transcriptase (Invitrogen) and the T7-Oligo (dT) promoter primer kit (Affymetrix, Inc.). The cDNA was purified using the GeneChip sample preparation kit (Affymetrix, Inc.) and was used for the in vitro synthesis of biotin-labeled cRNA using the GeneChip expression 3′amplification reagents for IVT labeling (Affymetrix, Inc.) at 37°C for 16 h. cRNA was fragmented into 35–200-bp fragments using a magnesium acetate buffer (Affymetrix, Inc.). 10 μg of labeled cRNA were hybridized to GeneChip mouse expression arrays (430A and 430B; Affymetrix, Inc.) for 16 h at 45°C. The chips were washed and stained according to the manufacturer’s recommendations (Affymetrix, Inc.) using the GeneChips fluidsic Next-Station天使 (model 450; Affymetrix, Inc.). This procedure included washing the chips with phycoerythrin-streptavidin, performing signal amplification by a second staining with biotinylated antistreptavidin, and performing a third staining with phycoerythrin-streptavidin. Each chip was scanned using the GeneChips scanner (model 3000; Affymetrix, Inc.). Signal intensity and detection calls were generated using the GeneChip operating software (Affymetrix, Inc.). The absolute intensity values of each chip were scaled to the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons. Four chip comparisons were generated using the same target intensity value of 150 in order to normalize the data for interarray comparisons.
and the counting frame boundaries were 80 × 60 × 6 μm in height. Only immunopositive neurons with sharply focused nuclei (containing at least one nucleolus) within the optical dissector counting frame boundaries were tallied. The total number of Pv+ neurons per DRG was estimated by the StereoInvestigator software.

**Statistical measures**

The Kolmogorov-Smirnov test was used to detect departures of the data from normality. F-tests were used to check for equal variances, and, in the case of unequal variances, the nonparametric Mann-Whitney U test was used. When variances were equal, the t-test was used. The acceptance of significance was set to P < 0.05.

We thank the National Institute of Neurological Disorders and Stroke/National Institute of Mental Health Microarray Consortium (K. Ramsey) for performing the Affymetrix microarray analyses and the Northwestern University Transgenic and Targeted Mutagenesis laboratory (I. Doglio and A. Alvarez) for pronuclease clear injections. The HSA promoter construct was provided by J. Chamberlain. The Egr3 N-terminus, Pv, and S46 antibodies were provided by J. Baraban, K. Baimbridge, and D. Fischman, respectively. K. Felici and M. McKnight provided technical assistance.

This work was supported by the NIH (NS046468) and a Howard Hughes Faculty Scholar Award to W.G. Tourtellotte. L. Eldredge was provided by a predoctoral fellowship from the NIH (CA009560) and the NIH Medical Scientist Training Program (GM008152). J. Carter was supported by a predoctoral fellowship from the NIH (GM008061) and the NIH Medical Scientist Training Program (GM008152). J. Carter was supported by a predoctoral fellowship from the NIH (CA009560) and the NIH Medical Scientist Training Program (GM008152).

Submitted: 31 January 2005
Accepted: 10 March 2005

**References**

Andrechek, E.R., W.R. Hardy, A.A. Girgis-Gabardo, R.L. Perry, R. Butler, F.L. Graham, R.C. Kahn, M.A. Rudnicki, and W.J. Muller. 2002. Erbb2 is required for muscle spindle and myoblast cell survival. *Mol. Cell. Biol.* 22:4714–4722.

Arber, S., D.R. Ladle, J.H. Lin, E. Frank, and T.M. Jessell. 2000. ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell.* 101:485–498.

Chen, H.H., W.G. Tourtellotte, and E. Frank. 2002. Muscle spindle-derived neurotrophin 3 regulates synaptic connectivity between muscle sensory and motor neurons. *J. Neurosci.* 22:3512–3519.

Chen, Y.W., P. Zhao, R. Borup, and E.P. Hoffman. 2000. Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. *J. Cell Biol.* 151:1321–1336.

Crawford, G.E., J.A. Faulkner, R.H. Crosbie, K.P. Campbell, S.C. Froehner, and J.S. Chamberlain. 2000. Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. *J. Cell Biol.* 150:1399–1410.

Darby, I.A. 2000. Ed. In situ hybridization protocols. Methods in Molecular Biology, vol. 123. Totowa, NJ: Humana Press.

Dobretsov, M., S.L. Hastings, T.J. Sims, J.R. Stimers, and D. Romanovsky. 2000. Stretch receptor-associated expression of alpha 3 isofrom of the Na+ K+ ATPase in rat peripheral nervous system. *Neuroscience.* 116:1069–1080.

Ermfors, P., K.F. Lee, J. Kucera, and R. Jaenisch. 1994. Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell.* 77:503–512.

Haase, G., E. Dessaud, A. Garces, B. de Bovis, M. Birling, P. Filippi, H. Schmalbruch, S. Arber, and O. de la Peiryrie. 2002. GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. *Neuron.* 35:893–905.

He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adeno-viruses. *Proc. Natl. Acad. Sci. USA.* 95:2509–2514.

Hippenmeyer, S., N.A. Shneider, C. Birchmeier, S.J. Burden, T.M. Jessell, and S. Arber. 2002. A role for neuregulin1 signaling in muscle spindle differentiation. *Neuron.* 36:1035–1049.

Jacobson, C., D. Duggan, and G. Fischbach. 2004. Neuregulin induces the expression of transcription factors and myosin heavy chains typical of muscle spindles in cultured human muscle. *Proc. Natl. Acad. Sci. USA.* 101:12218–12223.

Klein, R., I. Silos-Santiago, R.J. Smeyne, S.A. Lira, R. Brambilla, S. Bryant, L. Zhang, W.D. Snider, and M. Barbacid. 1994. Disruption of the neurotrophin-3 receptor gene trkC eliminates the la muscle afferents and results in abnormal movements. *Nature.* 368:249–251.

Kucera, J., and J.M. Walro. 1992. Formation of muscle spindles in the absence of motor innervation. *Neurosci. Lett.* 145:47–50.

Kucera, J., W. Cooney, A. Que, V. Szedier, H. Stancz-Szedier, and J. Walro. 2002. Formation of supernumerary muscle spindles at the expense of Golgi tendon organs in Er81-deficient mice. *Dev. Dyn.* 223:389–401.

Leu, M., E. Bellmunt, M. Schwander, I. Farinas, H.R. Brenner, and U. Muller. 2003. Erbb2 regulates neuromuscular synapse formation and is essential for muscle spindle development. *Development.* 130:2291–2301.

Livet, J., M. Sigrist, S. Stroebel, V. De Paola, S.R. Price, C.E. Henderson, T.M. Jessell, and S. Arber. 2002. ETS gene PEA3 controls the central position and terminal arborization of specific motor neuron pools. *Neuron.* 35:877–892.

Muscat, G.E., S. Perry, H. Prentice, and L. Kedes. 1992. The human skeletal al-phaa-actin gene is regulated by a muscle-specific enhancer that binds three nuclear factors. *Gene Expr.* 2:111–126.

O’Donovan, K.J., E.P. Wilkens, and J.M. Baraban. 1998. Sequential expression of Egr-1 and Egr-3 in hippocampal granule cells following electroconvulsive stimulation. *J. Neurochem.* 70:1241–1248.

Rando, T.A., and H.M. Blau. 1994. Primary mouse myoblast proliferation, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125:1275–1287.

Swinoff, A.H., and J. Milbrandt. 1995. DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol. Cell. Biol.* 15:2275–2287.

Tessarollo, L., K.S. Vogel, M.E. Palko, S.W. Reid, and L.F. Paradis. 1994. Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. *Proc. Natl. Acad. Sci. USA.* 91:11844–11848.

Tourtellotte, W.G., and J. Milbrandt. 1998. Sensory axatia and muscle spindle agenesis in mice lacking the transcription factor Egr3. *Nat. Genet.* 20:87–91.

Tourtellotte, W.G., C. Keller-Peck, J. Milbrandt, and J. Kucera. 2001. The transcription factor Egr3 modulates sensory axon-myo-tube interactions during muscle spindle morphogenesis. *Dev. Biol.* 232:388–399.

Whitehead, J., C. Keller-Peck, J. Kucera, and W.G. Tourtellotte. 2005. Glial cell-line derived neurotrophic factor-dependent fusimotor neuron survival during development. *Mech. Dev.* 122:27–41.

Zelená, J. 1957. The morphogenic influences of innervation on the ontogenic development of muscle spindles. *J. Embryol. Exp. Morphol.* 5:283–292.

Zelená, J., and T. Soukup. 1973. Development of muscle spindles deprived of fusimotor innervation. *Z. Zellforsch. Mikrosk. Anat.* 14:435–452.