Neogenin Regulates Skeletal Myofiber Size and Focal Adhesion Kinase and Extracellular Signal-regulated Kinase Activities In Vivo and In Vitro

Gyu-Un Bae,*‡‡ Youn-Joo Yang,*‡‡ Guoying Jiang,* Mingi Hong,* Hye-Jin Lee,† Marc Tessier-Lavigne,§ Jong-Sun Kang,*† and Robert S. Krauss* 

*Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029; ‡Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, South Korea; and §Division of Research, Genentech, Inc., South San Francisco, CA 94080

A variety of signaling pathways participate in the development of skeletal muscle, but the extracellular cues that regulate such pathways in myofiber formation are not well understood. Neogenin is a receptor for ligands of the netrin and repulsive guidance molecule (RGM) families involved in axon guidance. We reported previously that neogenin promoted myotube formation by C2C12 myoblasts in vitro and that the related protein Cdo (also Cdon) was a potential neogenin coreceptor in myoblasts. We report here that mice homozygous for a gene-trap mutation in the Neo1 locus (encoding neogenin) develop myotomes normally but have small myofibers at embryonic day 18.5 and at 3 wk of age. Similarly, cultured myoblasts derived from such animals form smaller myotubes with fewer nuclei than myoblasts from control animals. These in vivo and in vitro defects are associated with low levels of the activated forms of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK), both known to be involved in myotube formation, and inefficient expression of certain muscle-specific proteins. Recombinant netrin-2 activates FAK and ERK in cultured myoblasts in a neogenin- and Cdo-dependent manner, whereas recombinant RGMc displays lesser ability to activate these kinases. Together, netrin-neogenin signaling is an important extracellular cue in regulation of myogenic differentiation and myofiber size.

INTRODUCTION

Skeletal muscle is the most abundant tissue, by mass, in the vertebrate body. Muscles of the trunk and limbs arise from the somites, with myogenic progenitor cells derived from the dorsal region of the maturing somite, the dermomyotome (Tajbakhsh and Buckingham, 2000; Pownall et al., 2002; Charge and Rudnicki, 2004). In response to signals from the adjacent notochord, neural tube, and surface ectoderm, some dermomyotomal progenitors become committed to the muscle lineage and form the myotome, a set of differentiated muscle cells that underlies the dermomyotome. Subsequent embryonic, fetal, and postnatal stages of myogenesis are thought to involve additional muscle progenitors that migrate from the dermomyotome and ultimately establish the muscle cells that underlies the dermomyotome. Subsequent embryonic, fetal, and postnatal stages of myogenesis are thought to involve additional muscle progenitors that migrate from the dermomyotome and ultimately establish the muscle lineage and form the myotome, a set of differentiated muscle cells that underlies the dermomyotome. Subsequent embryonic, fetal, and postnatal stages of myogenesis are thought to involve additional muscle progenitors that migrate from the dermomyotome and ultimately establish the muscle lineage and form the myotome, a set of differentiated muscle cells that underlies the dermomyotome.

Somatic progenitor cells are specified to become muscle lineage-committed myoblasts through the action of the myogenic basic helix-loop-helix transcription factors Myf5, MRF4, and MyoD, whereas differentiation of myoblasts is regulated by myogenin, MyoD, and MRF4 (Tajbakhsh, 2005). These and other transcription factors coordinate the process of differentiation, including cell cycle withdrawal, expression of muscle-specific proteins, cellular elongation, and fusion into multinucleated myofibers. Although transcriptional regulation is at the core of myogenesis, the formation and growth of myofibers is also controlled by a variety of signaling ligands and their receptors, including insulin-like growth factor-1, fibroblast growth factors, Wnts, transforming growth factor-β superfamily members, and others (Tajbakhsh and Buckingham, 2000; Pownall et al., 2002; Charge and Rudnicki, 2004).

Neogenin is a vertebrate member of an evolutionarily conserved subfamily of immunoglobulin (Ig) superfamily receptors (Cole et al., 2007; Wilson and Key, 2007). Other members of this subfamily include, in vertebrates, deleted in colorectal cancer (DCC); in Drosophila, Frazzled; and in Caenorhabditis elegans, UNC40 (Huber et al., 2003; Round and Stein, 2007). All these receptors contain an ectodomain of four Ig repeats followed by six fibronectin type III repeats, a single pass transmembrane region, and a divergent cytoplasmic tail that lacks known catalytic activity but which harbors short, highly conserved sequences that bind signaling proteins (Huber et al., 2003; Round and Stein, 2007). The members of this family share a role as receptors for netrins, a group of secreted proteins related to laminins that associate with cell membranes and extracellular matrix; the best understood functions of netrins and their DCC/UNC40 family receptors are as regulators of axon guidance (Huber et al., 2003; Round and Stein, 2007).

This article was published online ahead of print in MBC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–06–0491) on October 7, 2009.

† These authors contributed equally to this work.

Address correspondence to: Robert S. Krauss (robert.krauss@mssm.edu) or Jong-Sun Kang (jskang@med.skku.ac.kr).
The intracellular regions of DCC and neogenin interact with FAK and the Src family kinase, Fyn, and stimulation of mammalian cortical neurons with netrin-1 in vitro results in tyrosine phosphorylation of both the kinases and the receptors (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Furthermore, FAK and the activity of Src family kinases (such as Fyn) are required for netrin-mediated turning of axonal growth cones (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Netrin-1 treatment of neurons also leads to DCC-dependent activation of ERK mitogen-activated protein kinase (MAPK) and inhibition of ERK signaling blocks neurite outgrowth and growth cone turning in response to a netrin gradient in vitro (Forcet et al., 2002; Campbell and Holt, 2003). Additional signaling pathways are also activated by netrin-1 signaling via DCC and neogenin (Xie et al., 2005; Zhu et al., 2007; Li et al., 2008).

Although neogenin and DCC are similar in their signaling responses to netrin-1 (although not identical, see Xie et al., 2006; Ren et al., 2008), neogenin plays a broader role in development than DCC. First, neogenin, but not DCC, binds to members of a second family of ligands, the GPI-anchored RGMs (Rajagopalan et al., 2004; Zhang et al., 2005). Neogenin functions as a receptor for RGMs in axonal chemorepulsion and neuronal survival and differentiation (Matsunaga et al., 2004; Rajagopalan et al., 2004; Matsunaga et al., 2006; Conrad et al., 2007). Furthermore, RGM-neogenin signaling is required for neural tube closure in Xenopus embryos (Kee et al., 2008). RGMc (also known as hemojuvelin [Hjv] or Hfe2) is expressed mainly in liver and skeletal muscle and is involved in iron metabolism (Kuninger et al., 2004; Niederkofler et al., 2004; Oldekamp et al., 2004; Papanikolaou et al., 2004). RGMs also function as bone morphogenetic protein (BMP) coreceptors, and RGMc is required for BMP-mediated induction of hepcidin, a key regulator of iron homeostasis (Babitt et al., 2006; Xia et al., 2008). Neogenin mediates shedding of RGMc from the cell surface, and soluble RGMc inhibits BMP-induced expression of hepcidin (Babitt et al., 2007; Zhang et al., 2007).

Second, although DCC is expressed mainly in the central nervous system (CNS), neogenin is expressed widely during development (Gad et al., 1997). In the developing mammary gland, netrin-1 is expressed in preluminal cells and neogenin is expressed in adjacent cap cells of terminal end buds; mice lacking either factor display cell dissociation and tissue disorganization from loss of netrin-1/neogenin adhesive interactions (Srinivasan et al., 2003). Furthermore, vascular smooth muscle cells are attracted by and adhere to netrin-1 in a neogenin-dependent manner (Park et al., 2004). Neogenin morphant zebrafish display an aberrant neural tube and defects in somitogenesis, suggesting possible roles in convergent extension and/or cell polarity during formation of the rostrocaudal axis (Mawdsley et al., 2004).

Neogenin is expressed throughout the developing mouse embryo but is at high levels in developing skeletal muscle; netrin-1, netrin-3, and RGMc are also expressed in precardiac masses of the mouse embryo (Gad et al., 1997; Keeling et al., 1997; Wang et al., 1999; Kuninger et al., 2004; Niederkofler et al., 2004; Fitzgerald et al., 2006; Rodriguez et al., 2007). We recently showed that mouse myoblast cell lines and cultured primary myoblasts derived from satellite cells express netrin-3 and neogenin and that these factors promote formation of multinucleated myotubes (Kang et al., 2004). Furthermore, neogenin forms cis complexes with a second promyogenic Ig/fibronectin type III-repeat protein, Cdo (also called Cdon), and Cdo-null myoblasts fail to respond to soluble netrin, suggesting that neogenin-Cdo complexes might be required for some aspects of netrin/neogenin signaling (Kang et al., 2004). We report here that mice homozygous for a gene-trap mutation in the Neo1 gene (encoding neogenin) develop myotymes normally but have small myofibers at embryonic day (E)18.5 and at 3 wk of age. Similarly, myoblasts derived from such animals fail to form large myotubes in vitro. These defects are associated with low levels of the activated forms of FAK and ERK, and inefficient expression of certain muscle-specific proteins, both in vivo and in vitro. Finally, soluble netrin activates FAK and ERK in cultured myoblasts in a neogenin- and Cdo-dependent manner, whereas soluble RGMc displays lesser ability to activate these kinases. Together, neogenin signaling, probably activated via netrin ligands, is an important extracellular cue in regulation of myogenin differentiation and myofiber size.

MATERIALS AND METHODS

Mice

Mice carrying a secretory gene-trap vector insertion into intron 7 of the Neo1 gene were constructed previously (Leighton et al., 2001; Mitchell et al., 2001). The embryonic stem (ES) cell line that carried the secretory gene trap from which this mouse line was developed was designated KST265, and we therefore refer to the allele as Neo1secretory (abbreviated Neo1s). We mapped the insertion site to nucleotides 58,780,672 to 58,780,672 (mouse chromosome 11; NCBI Mouse Genome Reference Assembly, Build 37) through a combination of Southern blotting, polymerase chain reaction (PCR), and sequencing. The insertion was accompanied by a deletion of 922 base pairs and an inversion of a 923-base pair sequence immediately adjacent to the insertion site (encoded nucleotides are 58,780,672–58,779,784). This permitted design of a PCR genotyping strategy in which a single oligonucleotide (5′-CAACCTGGGT- CATAGCAAGTG-3′) served as the reverse primer for the wild-type allele and the forward primer for the mutant allele. The forward primer for the wild-type allele was 5′-GGTGGACAAAGAAGCAGAAAGGCA-3′ and the reverse primer for the mutant allele was 5′-TAAATGACACACGACGATGCC-3′. A three-primer PCR produces a 704-base pair fragment for the wild-type allele and a 917-base pair fragment for the mutant allele (Figure 1A). We also genotyped with a previously reported strategy (Burgess et al., 2006) and found the two methods concordant. The insertion is predicted to produce a mutant transcript that encodes a transmembrane protein consisting of the four Ig repeats of neogenin fused in-frame with a vector-encoded transmembrane domain and a cytoplasmic domain consisting of β-gal (β-galactosidase fused to neomycin phosphotransferase). Previous studies suggest that such fusion proteins are targeted to intracellular compartments (Leighton et al., 2001).

All mice and embryos were of a largely C57BL/6 background and were generated from intercrosses of Neo1s/+ animals. Noon of the plug date was designated E0.5.

In situ Hybridization, β-Galactosidase Staining, Histology, and Immunohistochemistry

For whole-mount RNA in situ hybridization, embryos were prepared essentially as described previously (Mulieri et al., 2002), except that they were treated with 10 μg/ml proteinase K (QIAGEN, Valencia, CA) in phosphate-buffered saline, 0.1% Tween-20 PBT according to stage (E10.5, 40 min; E11.5, 60 min). Embryos were rinsed, postfixed, and hybridized with digoxigenin-labeled probe in hybridization mix (50% formamide, 1× SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% SDS-3-[3-cholamidopropyl]dimethylammonio]propanesulfonate, and 100 μg/ml heparin) overnight at 65°C. After washing and blocking, embryos were incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000; Roche Diagnostics, Indianapolis, IN) in blocking buffer (2% blocking reagent [Roche Diagnostics]), 20% heat-inactivated sheep serum in 100 mM maleic acid, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 [MABT]). After washes in MABT and 500 mM NaCl, 150 mM Tris-HCl, pH 9.5, 30 mM MgCl2, and 1% Tween 20, signals were developed using BM Purple AP Substrate (Roche Diagnostics). Dissected embryos were stained for β-galactosidase activity essentially as described previously (Nait-Oumesmar et al., 2002) with the exception that fixation was dependent on the age of the embryo. Whole-mount staining was identical to the above peroxidase buffer, supplemented with 17.5 mM each K4Fe(CN)6 and K3Fe(CN)6, and 1 mg/ml 3-bromo-4-chloro-3-indolyl-β-d-galactoside (Sigma-Aldrich). Whole-mount embryos were dehydrated into 60% glycerol and PBS for photog-
Figure 1. The Neo1\textsuperscript{Gt} allele is variably hypomorphic. (A) Map of the gene-trap insertion site in the Neo1 locus. The arrow indicates the insertion site of the secretory gene-trap vector in intron 7. The positions of the primers used for genotyping are represented by red and black arrowheads (F, forward primer for wild-type allele; R, reverse primer for wild-type allele and forward primer for mutant allele). Lines above primers represent PCR products shown in B. The dotted line in the mutant allele represents a deleted sequence, and the white region in the wild-type and mutant alleles represents an inverted sequence. Within the gene-trap vector, the vertical white rectangle indicates a transmembrane (TM) domain, the blue segment is β-geo, the green segment an internal ribosome entry site (IRES), and the purple segment is an alkaline phosphatase reporter gene (PLAP). (B) Genotype analysis of Neo1\textsuperscript{+/+}, Neo1\textsuperscript{+/Gt}, and Neo1\textsuperscript{Gt/Gt} embryos. A three-primer PCR produces a 704-base pair fragment from the wild-type allele and a 917-base pair fragment from the mutant allele. (C) Western blot analysis of neogenin protein production in embryos and MEFs. Heads from E13.5 embryos of the indicated genotype were removed, and extracts were blotted with antibodies to the neogenin intracellular domain. Extracts from MEFs of the indicated genotype were also analyzed. The ~190-kDa band (arrow) represents full-length neogenin, and the ~60-kDa band (arrowhead) may correspond to a processed form of the neogenin intracellular region. The ~120-kDa band (asterisk) is a nonspecific band recognized in embryonic head, but not MEF, extracts. Extracts were also probed with a pan-Cadherin antibody as a loading control.

**Analysis of Dissected E15.5 Limbs by Immunoblotting**

Limbs from E15.5 embryos were removed, and muscle and associated connective tissues were dissected away from developing bones and frozen in liquid nitrogen. The tissues were ground and solubilized with lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) containing proteinase inhibitor (Roche Diagnostics), 50 mM NaF, and 1 mM sodium orthovanadate. Lysates were analyzed by immunoblotting as described previously (Kang et al., 1998). Polyvinylidene difluoride membranes were probed with antibodies against neogenin (Santa Cruz Biotechnology, Santa Cruz, CA), MyoD (Santa Cruz Biotechnology), Myogenin (Santa Cruz Biotechnology), Desmin (Dako North America), myosin heavy chain (MHC) (MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), pan-cadherin (Abcam, Cambridge, MA), ERK (Santa Cruz Biotechnology), ERK2 (Cell Signaling Technology, Goat anti-mouse, and streptavidin-peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections stained for myogenin were pretreated with target unmasking fluid (Zymed Laboratories, South San Francisco, CA) according to the manufacturer’s instructions.
Neogenin Regulates Skeletal Myogenesis

Table 1. Offspring of intercrosses between Neo1<sup>+/+</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>P10 (n = 453)</th>
<th>P21 (n = 432)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo1&lt;sup&gt;+&lt;/sup&gt;/Neo1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>147 (32)</td>
<td>145 (33)</td>
</tr>
<tr>
<td>Neo1&lt;sup&gt;+/Gt&lt;/sup&gt;</td>
<td>260 (57)</td>
<td>258 (60)</td>
</tr>
<tr>
<td>Neo1&lt;sup&gt;Gt/Gt&lt;/sup&gt;</td>
<td>46 (11)</td>
<td>29 (7)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

Defective Skeletal Muscle Development in Neo1<sup>Gt/Gt</sup> Mice

Neogenin mRNA and protein are expressed prominently in E14.5 fetal muscle mass and in adult muscle (Keeling et al., 1997; Fitzgerald et al., 2006; Rodriguez et al., 2007). However, little information exists on Neo1 expression during earlier stages of myogenesis. The gene trap insertion leads to expression of a β-galactosidase reporter from the Neo1 locus (Leighton et al., 2001; Mitchell et al., 2001) (Figure 1A), and β-galactosidase activity was examined in E9.5, E10.5, and E11.5 embryos. In each case, strong β-galactosidase activity was observed in the dorsal somite, the region of origin of muscle progenitor cells (Figure 2A). To assess the effects of loss of neogenin on early myogenesis, whole-mount RNA in situ hybridization was used to analyze expression of the critical differentiation gene myogenin in myotomes of E10.5 and E11.5 Neo1<sup>+/+</sup> and Neo1<sup>Gt/Gt</sup> mice (Figure 2B). No difference in myogenin expression was observed between the wild-type and homozygous mutant animals at either stage.

To further examine muscle development in Neo1<sup>Gt/Gt</sup> mice, sections from E15.5 embryos were analyzed for expression of muscle-specific markers by immunohistochemistry against desmin, myogenin and MHC. Transverse sections of developing intercostal muscles and of the premuscle masses of the trapezius and pectoralis revealed that although muscle patterning seemed largely normal in Neo1<sup>Gt/Gt</sup> embryos, the staining intensity for desmin and myogenin was clearly weaker than that seen in wild-type embryos. In contrast to 40% of the expected frequency (Table 1). By P21, additional animals had died, culminating in ~80% lethality by weaning age.

Because gene-trap mutations are sometimes hypomorphic alleles, extracts of heads removed from E13.5 embryos were Western blotted with antibodies directed against the neogenin intracellular region. Eleven Neo1<sup>Gt/Gt</sup> embryos from different litters were compared with a wild-type control, and all 11 expressed detectable full-length neogenin protein (~190 kDa), at low levels that were somewhat variable between animals (Figure 1C). Neogenin protein was not observed by Western blotting at this level of sensitivity in Neo1<sup>Gt/Gt</sup> mouse embryonic fibroblasts (MEFs) (Figure 1C), nor was it seen in dissected E15.5 Neo1<sup>Gt/Gt</sup> limbs or Neo1<sup>Gt/Gt</sup> myoblasts (vide infra). An immunoreactive band of ~60 kDa was also observed in wild-type heads and MEFs, but not in the mutants; this may correspond to a processed form of the neogenin intracellular region (Goldschneider et al., 2008). It is concluded that the Neo1<sup>+</sup> allele is hypomorphic in a stage- and/or tissue-dependent manner. All Neo1<sup>Gt/Gt</sup> mice were smaller than wild-type animals and had multiple abnormalities (Srinivasan et al., 2003; Burgess et al., 2006; Metzger et al., 2007). We focus here on skeletal muscle development.

RESULTS

The Neo1<sup>Gt</sup> Allele Is Hypomorphic and Results in Incompletely Penetrant Perinatal Lethality

Mice carrying a gene-trap insertion in the Neo1 gene have been constructed as described previously (Leighton et al., 2001; Mitchell et al., 2001), and homozygous mutants were reported to display perinatal lethality (Srinivasan et al., 2003), but this allele is not well characterized. We refer to the allele here as Neo1<sup>Gt/Kst6518mg</sup> (abbreviated Neo1<sup>Gt</sup>). The insertion site of the gene-trap vector was identified by PCR and sequence analysis, revealing that the vector integrated into intron 7 of the Neo1 locus (Figure 1A), and sequence analysis, revealing that the vector integrated into intron 7 of the Neo1 locus (Figure 1A), and sequence analysis, revealing that the vector integrated into intron 7 of the Neo1 locus (Figure 1A), and sequence analysis, revealing that the vector integrated into intron 7 of the Neo1 locus (Figure 1A), and sequence analysis, revealing that the vector integrated into intron 7 of the Neo1 locus (Figure 1A), and sequence analysis, revealing that the vector integrated into intron 7 of the Neo1 locus (Figure 1A). The insertion was accompanied by both a deletion of 922 base pairs and an inversion of a 923-base pair sequence immediately adjacent (3′) to the insertion site. This allowed design of a three-primer PCR genotyping protocol (Figure 1, A and B; see Materials and Methods). We intercrossed Neo1<sup>+/Gt</sup> mice and found that at postnatal day (P)10, Neo1<sup>Gt/Gt</sup> mice were present at only ~31% of the expected frequency (Table 1). By P21, additional animals had died, culminating in ~80% lethality by weaning age.

Because gene-trap mutations are sometimes hypomorphic alleles, extracts of heads removed from E13.5 embryos were Western blotted with antibodies directed against the neogenin intracellular region. Eleven Neo1<sup>Gt/Gt</sup> embryos from different litters were compared with a wild-type control, and all 11 expressed detectable full-length neogenin protein (~190 kDa), at low levels that were somewhat variable between animals (Figure 1C). Neogenin protein was not observed by Western blotting at this level of sensitivity in Neo1<sup>Gt/Gt</sup> mouse embryonic fibroblasts (MEFs) (Figure 1C), nor was it seen in dissected E15.5 Neo1<sup>Gt/Gt</sup> limbs or Neo1<sup>Gt/Gt</sup> myoblasts (vide infra). An immunoreactive band of ~60 kDa was also observed in wild-type heads and MEFs, but not in the mutants; this may correspond to a processed form of the neogenin intracellular region (Goldschneider et al., 2008). It is concluded that the Neo1<sup>+</sup> allele is hypomorphic in a stage- and/or tissue-dependent manner. All Neo1<sup>Gt/Gt</sup> mice were smaller than wild-type animals and had multiple abnormalities (Srinivasan et al., 2003; Burgess et al., 2006; Metzger et al., 2007). We focus here on skeletal muscle development.

Defective Skeletal Muscle Development in Neo1<sup>Gt/Gt</sup> Mice

Neogenin mRNA and protein are expressed prominently in E14.5 fetal muscle mass and in adult muscle (Keeling et al., 1997; Fitzgerald et al., 2006; Rodriguez et al., 2007). However, little information exists on Neo1 expression during earlier stages of myogenesis. The gene trap insertion leads to expression of a β-galactosidase reporter from the Neo1 locus (Leighton et al., 2001; Mitchell et al., 2001) (Figure 1A), and β-galactosidase activity was examined in E9.5, E10.5, and E11.5 embryos. In each case, strong β-galactosidase activity was observed in the dorsal somite, the region of origin of muscle progenitor cells (Figure 2A). To assess the effects of loss of neogenin on early myogenesis, whole-mount RNA in situ hybridization was used to analyze expression of the critical differentiation gene myogenin in myotomes of E10.5 and E11.5 Neo1<sup>+/+</sup> and Neo1<sup>Gt/Gt</sup> mice (Figure 2B). No difference in myogenin expression was observed between the wild-type and homozygous mutant animals at either stage.

To further examine muscle development in Neo1<sup>Gt/Gg</sup> mice, sections from E15.5 embryos were analyzed for expression of muscle-specific markers by immunohistochemistry against antibodies against desmin, myogenin and MHC. Transverse sections of developing intercostal muscles and of the premuscle masses of the trapezius and pectoralis revealed that although muscle patterning seemed largely normal in Neo1<sup>Gt/Gg</sup> embryos, the staining intensity for desmin and myogenin was clearly weaker than that seen in wild-type controls; staining intensity for MHC also seemed reduced, although less dramatically (Figure 3, A–E). To confirm and extend this observation at a biochemical level, extracts of dissected E15.5 hind limbs were analyzed by Western blotting with antibodies against muscle-specific proteins. Neogenin was easily detected in extracts from Neo1<sup>+/+</sup> limbs but, in contrast to E13.5 Neo1<sup>Gt/Gg</sup> heads, was not observed in extracts from E15.5 Neo1<sup>Gt/Gg</sup> limbs (Figure 3F). Similar to what was seen by immunohistochemistry, the levels of myogenin and desmin were strongly reduced in Neo1<sup>Gt/Gg</sup> limb extracts relative to control extracts, whereas...
the levels of MHC were lowered more modestly; MyoD levels were also modestly reduced (Figure 3F).

The production of phosphorylated forms of signaling proteins that lie downstream of neogenin was also analyzed. The levels of the phosphorylated forms of ERK and the ERK substrate p90RSK were both lower in Neo1Gt/Gt limbs than in control limbs, but total ERK and p90RSK levels were unchanged (Figure 3F). Similarly, there was a strong reduction in the concentration of FAK phosphorylated on Tyr576/577, Src family kinase sites required for maximal FAK kinase activity that are stimulated by netrin-1 in cultured cells (Calalb et al., 1995; Li et al., 2004; Liu et al., 2004; Ren et al., 2004; Mitra et al., 2005) (Figure 3F). Therefore, although muscle development was not grossly defective at E15.5 in Neo1Gt/Gt mice, these animals displayed diminished expression of both muscle-specific proteins and markers of activity of signaling pathways downstream of neogenin. Cell proliferation and apoptosis were also examined in sections of E15.5 embryos by immunostaining for phospho-histone H3 and TUNEL assay, respectively; significant differences between Neo1+/+ and Neo1Gt/Gt mice were not observed.

To assess muscle development at later stages, sections through E18.5 forelimbs of Neo1+/+ and Neo1Gt/Gt mice and through the quadriceps of P21 Neo1+/+ and surviving Neo1Gt/Gt mice were stained with hematoxylin and eosin and compared. Analysis of E18.5 sections revealed proper organization of muscle groups in Neo1Gt/Gt mice, although they were somewhat smaller than those of Neo1+/+ controls (Figure 4A). However, the average cross-sectional area of Neo1Gt/Gt forelimb myofibers was reduced by ~28% as compared with those of Neo1+/+ mice (Figure 4, B and C). In contrast, the diameter of the radius and ulna was not significantly different between E18.5 Neo1+/+ and Neo1Gt/Gt mice (Figure 4A). This phenotype was exacerbated in surviving P21 Neo1Gt/Gt animals, where average cross-sectional area of myofibers in the quadriceps was reduced by ~50% compared with controls (Figure 4, D and E).

**Defective Differentiation of Neo1Gt/Gt Myoblasts In Vitro**

Primary myoblasts were isolated from surviving P21 Neo1+/+ and Neo1Gt/Gt mice and analyzed for their ability to differentiate in culture, as assessed by the formation of multinucleated myotubes and expression of muscle-specific proteins. When differentiated cultures were stained with an antibody against MHC, ~70% of both Neo1+/+ and Neo1Gt/Gt cells were positive, but visual inspection revealed that Neo1Gt/Gt cells formed smaller myotubes than Neo1+/+ cells (Figure 5A). The smaller size of Neo1Gt/Gt myotubes was further assessed by quantifying both the average number of nuclei per myotube, and the distribution range of numbers of nuclei per myotube. Neo1+/+ myotubes had an average of 6.2 ± 1.1 nuclei, whereas Neo1Gt/Gt myotubes had an average of 2.6 ± 0.6 nuclei. In addition, Neo1Gt/Gt cultures produced ~60% more singly nucleated MHC+ cells and only ~25% the number of large myotubes (those with ≥6 nuclei) seen with Neo1+/+ cultures (Figure 5B). Several independent isolates of Neo1+/+ and Neo1Gt/Gt myoblasts displayed similar characteristics; there was some variability between different isolates of Neo1Gt/Gt cells in their ability to form myotubes but all were at least as strongly defective as those shown in Figure 5.

The possibility that the differences in the size of myotubes formed by Neo1+/+ and Neo1Gt/Gt myoblasts was due to alterations in cell proliferation or apoptosis was examined by measuring BrdU incorporation and by TUNEL assay, respectively. Neo1+/+ and Neo1Gt/Gt cells cultured in growth medium (15% FBS plus bFGF) displayed similar levels of BrdU incorporation (Figure 5, C and D). Apoptosis was negligible in cultures of either genotype in growth medium but 48 h after switching to differentiation medium, Neo1Gt/Gt cultures displayed more TUNEL-positive cells than did Neo1+/+ cultures (13.7 vs. 6.9%, respectively; Figure 5, E and F). Although this ~7% increase in apoptosis may contribute to the partially defective differentiation of Neo1Gt/Gt myoblasts, it is insufficient to explain the quantitatively much stronger effects on myotube formation as assessed by the percentage of nuclei in multinucleated cells or number of nuclei per myotube.

We therefore examined expression of muscle-specific and signaling proteins by Western blot analyses of differentiating cultures of Neo1+/+ and Neo1Gt/Gt myoblasts. As was observed with E15.5 limbs, neogenin protein levels were...
Figure 3. Reduced expression of muscle-specific proteins and diminished concentration of phospho-FAK and phospho-ERK in premuscle masses of E15.5 Neo1<sup>Cre/Cre</sup> embryos. (A–E) Transverse sections through developing intercostal muscles (A and E), premuscle mass of the pectoralis (B and C) and premuscle mass of the trapezius (D) stained with antibodies against the indicated proteins. Within each panel, the lower micrographs are higher magnification views of the ones directly above and are delineated by the boxed areas in A and D. (F) Western blot analysis of production of muscle-specific and signaling proteins in extracts of dissected E15.5 hind limbs. Bars, 0.5 mm (A, top), 50 mm (A, bottom), and 50 mm (B–E).
high in Neo1/+ cells but not detected in Neo1Gt/Gt cells (Figure 5G). Levels of MyoD and Cdo, both of which are expressed in proliferating and differentiating myoblasts (Kang et al., 1998; Sabourin et al., 1999; Cole et al., 2004), were similar in wild-type and Neo1Gt/Gt cells. However, as seen with developing Neo1Gt/Gt muscle masses in vivo, Neo1Gt/Gt cells had lower levels of both myogenin and MHC compared with Neo1/+ cells. Also, similar to the in vivo situation, the amounts of the phosphorylated forms of ERK and of its substrate, p90RSK, were lower in Neo1Gt/Gt cells than Neo1/+ cells (Figure 5G). However, the inefficient activation of ERK signaling was specific, in that the levels of the phosphorylated forms of two other kinases involved in myogenesis, p38 MAPK and AKT, were not substantially different between Neo1/+ and Neo1Gt/Gt cells. The concentration of FAK phosphorylated on Tyr576/577 was also lower in Neo1Gt/Gt cells than Neo1/+ cells (Figure 5G). Lack of neogenin therefore resulted in similar myogenic phenotypes in vivo and in vitro: production of small myofibers/myotubes with reduced expression of muscle-specific proteins and diminished ERK and FAK activation.

**Defective Response to Netrin Signaling in Neo1Gt/Gt and Cdo−/− Myoblasts**

The low concentrations of the phosphorylated, active forms of FAK and ERK in developing Neo1Gt/Gt muscles in vivo and differentiating myoblasts in vitro suggest a loss of ligand-initiated, neogenin-dependent signaling. Specific netrin and RGM family members function as ligands for neogenin (Cole et al., 2007; Wilson and Key, 2007). Netrin-1 and netrin-3 are expressed in developing mouse skeletal muscle, and cultured murine myoblasts express netrin-3 but little or no netrin-1 (Wang et al., 1999; Kang et al., 2004). We have shown previously that recombinant chicken netrin-2 promotes myotube formation by C2C12 myoblasts in a neogenin-dependent manner (Kang et al., 2004) (note that mouse netrin-3 is related to chicken netrin-2 and that there is no netrin-2 in mice; netrin-2, unlike netrin-3, can be obtained in soluble form and is therefore used in our studies; Kang et al., 2004). RGMc, but not RGMa or RGMb, is expressed in developing muscle and is induced during myoblast differentiation in vitro (Kuninger et al., 2004, 2006; Niederkofler et al., 2004; Oldekamp et al., 2004). Although RGMc has been demonstrated to be a ligand for neogenin in the developing CNS (Matsunaga et al., 2004, 2006; Rajagopalan et al., 2004; Conrad et al., 2007; Kee et al., 2008), it is not clear that RGMc has a similar function in muscle. We therefore assessed whether soluble netrin-2 and/or RGMc were capable of inducing FAK and ERK phosphorylation in myoblasts and whether neogenin was required for such activity through analysis of Neo1Gt/Gt and Neo1Gt/Gt myoblasts. Cdo is an Ig superfamily member that binds in cis to neogenin and may function as a neogenin coreceptor (Kang et al., 2004); Cdo−/− and Cdo−/− myoblasts were therefore also examined.

**Figure 4.** Neo1Gt/Gt mice form small myofibers. (A) Cross-sections though the hind limbs of E18.5 Neo1+/+ and Neo1Gt/Gt mice stained with hematoxylin and eosin. Note that overall muscle patterning is not different between wild-type and mutant animals. R, radius; U, ulna. (B) Cross-sections though the hind limbs of E18.5 Neo1+/+ and Neo1Gt/Gt mice stained with hematoxylin and eosin. (C) Quantification of myofiber cross-sectional area (CSA) from B. Asterisk indicates different from control, p < 0.0001 by Student’s t test. (D) Cross-sections though the hind limbs of P21 Neo1+/+ and Neo1Gt/Gt mice stained with hematoxylin and eosin. (E) Quantification of myofiber CSA from D. Asterisk indicates different from control, p < 0.0001 by Student’s t test. Bars, 0.5 mm (A) and 50 mm (B and D).
and Neo1Gt/Gt myoblasts were treated with recombinant netrin-2 or RGMc, and cell lysates collected over a 3-h time course. Production of phospho(576/577)-FAK, total FAK, phospho-ERK, and total ERK2 was then analyzed by Western blotting. A low level of phospho(576/577)-FAK was present in both Neo1+/+ and Neo1Gt/Gt cells before any treatment (time 0). Treatment with netrin-2 elevated phospho(576/577)-FAK concentration in Neo1+/+ cells within 30 min, and this increase was sustained for at least 1 h (Figure 6A). In contrast, netrin-2 did not induce phospho(576/577)-FAK in Neo1Gt/Gt cells. Similar results were obtained with Cdo+/+ and Cdo−/− myoblasts: netrin-2 enhanced phos-

**Figure 5.** Defective differentiation of Neo1Gt/Gt myoblasts in vitro. (A) Photomicrographs of Neo1+/+ and Neo1Gt/Gt myoblasts cultured in differentiation medium, and fixed and stained with an antibody to MHC. (B) Quantification of myotube formation by cell lines shown in A. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results and with multiple, independent isolates of the cells. Asterisks indicate different from control, p < 0.01 by Student’s t test. (C) BrdU incorporation into proliferating Neo1+/+ and Neo1Gt/Gt myoblasts. Cultures were exposed to BrdU for 2 h, fixed, and stained with an antibody to BrdU. (D) Quantification of BrdU incorporation shown in C. (E) Photomicrographs of Neo1+/+ and Neo1Gt/Gt myoblasts cultured in growth medium (GM) or differentiation medium (DM) for 48 h and analyzed for apoptotic cells by TUNEL assay (green). Cultures were also stained with 4,6-diamidino-2-phenylindole to reveal nuclei (blue). (F) Quantification of myotube formation by cell lines shown in E. Values represent means of triplicate determinations ± 1 SD. Asterisk indicates different from Neo1+/+ control, p < 0.01 by Student’s t test. (G) Western blot analysis of production of muscle-specific and signaling proteins in extracts of differentiating Neo1+/+ and Neo1Gt/Gt myoblasts.
phospho(576/577)-FAK levels in the control cells but this induction was largely absent in Cdo−/− cells (Figure 6B). RGMc treatment also induced phospho(576/577)-FAK in the control Neo1+/+ and Neo1/Gt/Gt myoblasts treated with RGMc for the indicated times. Similar results to those obtained with FAK phosphorylation were seen with ERK phosphorylation. Phospho-ERK was induced in control myoblasts 30 min to 1 h after treatment with netrin-2 and was maintained for at least 3 h, and both Neo1/Gt/Gt and Cdo−/− myoblasts treated with RGMc for the indicated times. In contrast, RGMc failed to enhance phospho-ERK levels above background in control or mutant myoblasts, though the levels of phospho-ERK in the presence of RGMc were generally higher in control than in mutant cells (Figure 6, G and H). Based on these results, it seems most likely that netrin-3 is the major neogenin ligand of relevance in skeletal myogenesis, although an auxiliary role for RGMc cannot be excluded.

**DISCUSSION**

A variety of signal transduction pathways participate in the development of skeletal muscle, but the extracellular cues that regulate such pathways in myofiber formation are not well understood (Pownall et al., 2002; Charge and Rudnicki, 2004). We reported previously that netrin-3 and neogenin promoted myotube formation by C2C12 myoblasts (Kang et al., 2004). In this report, it is demonstrated that mice homozygous for a gene-trap mutation (Neo1Gt/Gt) in the Neo1 locus develop small myofibers. Furthermore, myoblasts derived from such mice display a similar phenotype in vitro, forming smaller myotubes with fewer nuclei than myoblasts from control animals. In both cases, the phenotype is associated with inefficient expression of certain muscle-specific proteins, including myogenin, which is required for myoblast differentiation (Pownall et al., 2002), and MHC, a marker of terminal differentiation. The diminished expression of these muscle-specific proteins indicates that the differentiation process occurs inefficiently in the absence of...
Neogenin, and it is likely that myofibers fail to attain a normal size as a consequence of this defect. This phenotype is similar but not quite as strong as the one observed with the C2C12 cell line, where RNA interference (RNAi)-mediated knockdown of neogenin resulted in a sharply reduced ability to form myotubes (Kang et al., 2004). It is possible that during the process of immortalization, C2C12 cells acquired changes that rendered them more dependent on neogenin for differentiation than primary cells or developing muscles in vivo. Neo1\textsuperscript{Gt/Gt} mice and myoblasts also had reduced levels of the active, phosphorylated forms of FAK and ERK, both shown previously to be activated by neogenin signaling in cultured neurons (Li et al., 2004; Liu et al., 2004; Ren et al., 2004); this is the first study to demonstrate that these kinases are downstream of neogenin in vivo.

Neo1 is expressed in fetal premuscle masses and adult muscle (Keeling et al., 1997; Fitzgerald et al., 2006; Rodriguez et al., 2007) and, as shown here, developing somites. However, although Neo1\textsuperscript{Gt/Gt} mice displayed an obvious defect in the formation of fully sized myofibers, they did not show a defect in the formation of myotomes. Neogenin’s role may therefore be restricted to later stages of myogenesis. It should be noted, however, that the Neo1\textsuperscript{Gt} allele used in these studies is clearly hypomorphic, as demonstrated by the detection of full-length neogenin protein in extracts of E13.5 Neo1\textsuperscript{Gt/Gt} heads. In contrast, extracts of dissected E15.5 Neo1\textsuperscript{Gt/Gt} limb muscle and cultured Neo1\textsuperscript{Gt/Gt} myoblasts and MEFs did not reveal detectable neogenin protein at a similar level of sensitivity, indicating that the splicing events that allow bypass of the intronic gene-trap vector may occur in a tissue- and/or embryonic stage-dependent manner. Thus, although the phenotype observed in Neo1\textsuperscript{Gt/Gt} mice may be similar to that achieved with a true null allele, a final conclusion on the full role of neogenin in myogenesis must await the generation of a targeted, null mutation. Morpholino-mediated knockdown of neogenin in zebrafish and Xenopus embryos resulted in defective neural tube development, including failure of cavitation or closure, respectively (Mawdsley et al., 2004; Kee et al., 2008). These phenotypes were not seen in Neo1\textsuperscript{Gt/Gt} mouse embryos (Hong and Krauss, unpublished data). The lack of early neural tube phenotypes in Neo1\textsuperscript{Gt/Gt} mice may also be due to the hypomorphic nature of this allele, as the morpholino knockdown may be more effective at depleting neogenin. Alternatively, neogenin may play a less important role in these processes in mice than in fish and amphibians.

An important question is which ligand or ligands work with neogenin in myogenesis. Mice homozygous for strong hypomorphic Ntn1 (encoding netrin-1) alleles are not reported to have defects in muscle development (Serafini et al., 1996; Salminen et al., 2000), and Hfe2 (encoding RGMc)-null mice are reported to have normal muscle development (Huang et al., 2005; Niederkofler et al., 2005). Mice lacking netrin-3 have not been generated. Netrin-2 (the chick family member most closely related to mammalian netrin-3) promoted C2C12 myotube formation in a neogenin-dependent manner, whereas stable overexpression of RGMc in C2C12 cells was reported to have no effect on myotube formation (Huang et al., 2005; Niederkofler et al., 2005; Kuninger et al., 2006). Furthermore, netrin-2 stimulated FAK and ERK activation in Neo1\textsuperscript{Gt/Gt}, but not Neo1\textsuperscript{Gt/GG}, myoblasts in vitro; in contrast, RGMc displayed lesser ability to do so, though the levels of phospho-FAK and phospho-ERK were generally a bit higher in wild-type than mutant cells in the presence of RGMc. Because developing muscles in Neo1\textsuperscript{Gt/Gt} embryos and Neo1\textsuperscript{Gt/GG} myoblasts had low concentrations of the activated forms of FAK and ERK, and Ntn1 and Hfe2 mice lack gross muscle phenotypes, we speculate that netrin-3 is the major ligand for neogenin in the context of myogenesis, perhaps redundantly with netrin-1. Obviously, Ntn3 mutant mice will be required to prove this point. It also is possible that unidentified neogenin ligands exist and play a role in myogenesis. Finally, it should be mentioned that we have used soluble forms of netrin-2 and RGMc, and these factors may be presented in vivo in a membrane- and/or matrix-bound manner, and this may also affect activity.

We reported previously that Cdo might function as a coreceptor for neogenin in myoblasts (Kang et al., 2004). Consistent with this possibility, netrin-2 treatment did not stimulate FAK or ERK phosphorylation in Cdo\textsuperscript{−/−} myoblasts, similar to the lack of response seen with Neo1\textsuperscript{Gt/GG} myoblasts. We have not observed obvious binding of netrins to Cdo (unpublished data), so Cdo may be a nonligand-binding coreceptor for neogenin within specific contexts. A similar situation exists with neogenin and the cell surface receptor UNC5b. Unc5b associates with neogenin as a coreceptor for RGMa in signaling growth cone collapse but does not bind RGMa directly (Hata et al., 2009). It is possible that neogenin associates in a cis-manner with a variety of coreceptors in a context-dependent manner to provide appropriate signaling capabilities to receptor complexes. Interestingly, Unc5b is a netrin-1 receptor, both independently and as part of a complex with DCC, that modulates short- and long-distance axon repulsion (Barallobre et al., 2005; Round and Stein, 2007). It is likely, therefore, that the specific component composition of axon guidance receptor complexes regulates responses to multiple ligands in a spatiotemporal manner; this may serve as a paradigm for many types of receptor complexes during development in general, including neogenin:Cdo complexes. It should be noted, however, that Neo1\textsuperscript{Gt/GG} and Cdo\textsuperscript{−/−} myoblasts do not have identical phenotypes (Cole et al., 2004; Takaesu et al., 2006; this study). Cdo\textsuperscript{−/−} mice display delayed myogenesis and form somewhat smaller muscles than controls, whereas myofiber size in Neo1\textsuperscript{Gt/GG} animals is more severely affected. Conversely, Cdo\textsuperscript{−/−} myoblasts have a more strongly disrupted differentiation program in vitro than Neo1\textsuperscript{Gt/GG} myoblasts. It may be that some but not all of these two proteins’ functions during myogenesis are interdependent.

Several signaling pathways are activated by netrin signaling through DCC/UNC40 family members, although neogenin is understudied compared with its mammalian paralogue DCC (Forcet et al., 2002; Campbell and Holt, 2003; Li et al., 2004, 2008; Liu et al., 2004; Ren et al., 2004; Xie et al., 2005; Zhu et al., 2007). Both FAK and ERK are activated by, and required for, netrin-1/DCC signaling in axon guidance (Forcet et al., 2002; Campbell and Holt, 2003; Li et al., 2004; Liu et al., 2004; Ren et al., 2004). FAK and ERK play complex roles in myogenesis. FAK activity is required for efficient myotube formation during muscle regeneration in response to injury, in fusion and costamere development of primary mouse myoblasts, and in survival of proliferating C2C12 myoblasts and their differentiation into myotubes (Clemente et al., 2005; Quach and Rando, 2006; Quach et al., 2009). ERK activity has both positive and negative roles in C2C12 cell differentiation (Wu et al., 2000; Li and Johnson, 2006; Cho et al., 2007; Yokoyama et al., 2007); it is also implicated in fiber type identity in vivo (Murgia et al., 2000; Shi et al., 2008). The ERK substrate p90RSK has also been implicated in C2C12 cell differentiation (Cho et al., 2007). Given the lower than normal levels of the phosphorylated forms of these kinases in Neo1\textsuperscript{Gt/GG} embryos and myoblasts, it is logical to suggest that they are involved in neogenin’s promyogenic function. However, FAK, ERK, and p90RSK activities are regulated by...
several different stimuli, including various soluble factors and adhesion proteins (Mitra et al., 2005; Ramos, 2008), and the approaches taken to explore their various functions in muscle cell differentiation generally affect their overall activity without regard to initiating stimulus (e.g., overexpression of wild-type or mutant proteins, RNAi-mediated depletion, pharmacological agents). Neogenin-dependent FAK, ERK, and p90RSK activities would presumably be regulated in concert with these kinases’ activities initiated by other promyogenic stimuli, such as integrins. Furthermore, they would also probably occur as part of a broader network of signaling events initiated by neogenin, as occurs in DCC-mediated turning of growth cones (Huber et al., 2003; Round and Stein, 2007). Further studies will be aimed at deciphering such networks.

ACKNOWLEDGMENTS

We thank Karen Schachter for critical review of the manuscript. This work was supported by National Institutes of Health grant AR46207 and the T. J. Martell Foundation (to R.S.K.) and a Korea Research Foundation grant funded by the Korean Government (KRF-2008-313-C00260) (to J.S.K.).

REFERENCES


Neogenin Regulates Skeletal Myogenesis