

Loss of FGF Receptor 1 Signaling Reduces Skeletal Muscle Mass and Disrupts Myofiber Organization in the Developing Limb

Heather Flanagan-Steet,^{*,1} Kevin Hannon,^{†,1} Michael J. McAvoy,^{*,2}
Ronald Hullinger,[†] and Bradley B. Olwin^{*,3}

^{*}Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309; and [†]Basic Medical Sciences, Purdue University, West Lafayette, Indiana 47907

The identities of extracellular growth factors that regulate skeletal muscle development *in vivo* are largely unknown. We asked if FGFs, which act as repressors of myogenesis in culture, play a similar role *in vivo* by ectopically expressing in the developing limb a truncated FGF receptor 1 (dnFGFR1) that acts as a dominant negative mutant. Hind limbs and the adjacent somites of Hamburger and Hamilton (HH) stage 17 chickens were infected with a replication-competent RCAS virus encoding dnFGFR1. By ED5, the virus had spread extensively within the limb and the adjacent somites with little rostral or caudal expansion of the infection along the axial midline. Viral infection and mutant receptor expression were coincident as revealed by the distribution of a viral coat protein and an HA epitope tag present on the carboxy terminus of dnFGFR1. Within 48 h following injection of dnFGFR1, we could detect no obvious changes in skeletal muscle precursor cell migration into the hind limb as compared to control limbs infected with an empty RCAN virus. However, by 3 days following infection of RCAS-dnFGFR1 virus, the level of skeletal muscle-specific myosin heavy chain was decreased and the expression pattern altered, suggesting disruption of skeletal muscle development. Two striking muscular phenotypes were observed in dnFGFR1-expressing limbs, including an average loss of 30% in skeletal muscle wet weight and a 50% decrease in myofiber density. At all ages examined the loss of skeletal muscle mass was accompanied by a loss of myoblasts and an unexpected concomitant loss of fibroblasts. Consistent with these observations, explants of infected cells revealed a reduction in the number of myonuclei in myotubes. Although the myofiber density per unit area was decreased over 50% compared to controls there were no detectable effects on myofiber diameter. The loss in myofiber density was, however, accompanied by an increase in the space surrounding individual myofibers and a generalized loss of myofiber integrity. It is noteworthy that long-bone development was unaffected by RCAS-dnFGFR1 infection, suggesting that FGFR2 and FGFR3 signaling was not disrupted. Our data provide conclusive evidence that FGFR1 signaling is necessary to maintain myoblast number and plays a role in myofiber organization. © 2000 Academic Press

Key Words: FGF; skeletal muscle; limb development.

INTRODUCTION

Formation of skeletal musculature in the vertebrate limb requires the migration of muscle precursor cells (mpcs) into

¹ These authors contributed equally to this work and are listed in alphabetical order.

² Current address: NIH, NCI, Metabolism Branch, Bethesda, MD 20892.

³ To whom correspondence should be addressed at Campus Box 347, Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309. Fax: (303) 492-1587. E-mail: Bradley.olwin@colorado.edu.

the developing limb, extensive expansion of the precursor population, and coordination of differentiation and patterning. While significant progress has been made in culture toward identifying the extracellular factors that may control these events, the mechanisms utilized *in vivo* are not well understood. Recent evidence from transgenic animals has, however, identified potential *in vivo* roles for hepatocyte growth factor/scatter factor (HGF/SF), sonic hedgehog (shh), IGF1, and fibroblast growth factor 6 (FGF6). HGF/SF has been shown to be a critical agent for the initiation of mpc migration into the limb (Bladt *et al.*, 1995; Maina *et al.*, 1996; Tatsumi *et al.*, 1998) and shh for proliferation of mpcs

(Duprez, 1998). IGF1 and FGF6 are implicated in the late-stage muscle processes of repair and regeneration (Floss *et al.*, 1997; Barton-Davis, 1998). In addition, ectopic overexpression of a retrovirus carrying a bicistronic truncated FGFR1/LacZ mRNA in muscle precursor cells eliminated their appearance in the limb, suggesting that FGFR1 signaling may also be required for migration of these skeletal muscle precursors (Itoh *et al.*, 1996).

In contrast to the few factors currently known to affect skeletal muscle development *in vivo*, 30 years of experimentation on cultured skeletal muscle cells has identified over two dozen extracellular factors that regulate myoblast proliferation and differentiation. Among these, members of the fibroblast growth factor (FGF) family are likely to play a role in skeletal muscle development *in vivo*. This is supported by several lines of evidence. First, primary cells cultured from human (unpublished data), rat (Allen *et al.*, 1984), mouse (Gospodarowicz *et al.*, 1975; Linkhart *et al.*, 1980), and chicken (Kardami *et al.*, 1985a; Seed and Hauschka, 1988) are responsive to exogenous FGF. Addition of purified FGFs has been shown to both delay the onset of myogenesis and stimulate proliferation of these cells. Further, the mammalian and avian expression patterns of several FGFs and their receptors are consistent with a role in myogenesis. Two of the four known FGF receptor tyrosine kinases (FGFRs), FGFR1 and FGFR4, are expressed in developing chicken skeletal muscle (Szebenyi *et al.*, 1995; unpublished data).

Although the current evidence strongly supports a role for FGF signaling in skeletal muscle development, actual demonstration of its function *in vivo* has been largely unsuccessful. First, mice null for FGFR1 do not gastrulate and thus its role in skeletal muscle development has not been demonstrated (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994). Second, FGFR4 null mice exhibit no obvious phenotypes, suggesting it is not essential for skeletal muscle development (Weinstein *et al.*, 1998). Third, ectopic expression of a truncated FGFR1/LacZ bicistronic message using a replication-defective retrovirus eliminated all infected skeletal muscle precursors from the developing limb. FGFR1 signaling was therefore proposed to be required for mpc migration. However, since no infected muscle precursors were present in the limb, these studies could not address a role for FGFs in the development and organization of skeletal muscle tissue. Further complicating *in vivo* analysis of FGFs and myogenesis is the fact that signaling from FGFR2 and FGFR3 contributes to both limb outgrowth and long-bone development (Colvin *et al.*, 1996; Deng *et al.*, 1996; Xu *et al.*, 1998). Given this, and the observation that FGFs are also important for anterior-posterior patterning and maintenance of the apical ectodermal ridge (Fallon *et al.*, 1994; Niswander *et al.*, 1993), it is necessary to experimentally separate limb outgrowth and patterning from myogenesis. We have previously demonstrated that ectopic overexpression of FGF2 leads to duplications in skeletal elements with no detectable effects on muscle (Riley *et al.*, 1993). Since expression of growth factors at concentrations

well above physiological levels can induce artifactual responses, we elected instead to inhibit FGF signaling to determine if it is required for skeletal muscle development *in vivo*.

To spatially and temporally control inhibition of FGFR1 signaling, we overexpressed in the developing chick limb a truncated FGFR1 that functions as a dominant negative mutant (dnFGFR1) (Amaya *et al.*, 1991; Hannon *et al.*, 1996; Kudla *et al.*, 1998). Ectopic overexpression of this mutant was accomplished by infection of chick somites and limb buds with a replication-competent retrovirus (Petropoulos and Hughes, 1991) carrying the altered coding sequence (RCAS-dnFGFR1). This approach enabled broad expression of the mutant receptor in the developing limb and allowed for assessment of the global effects of inhibiting FGFR1 signaling on skeletal muscle development. As expected, expression of the recombinant protein was coincident with the presence of a viral coat antigen, as confirmed by staining for the HA epitope tag encoded by the mutant protein. While inhibition of FGFR1 signaling did not detectably affect limb outgrowth or long-bone development, nearly all of the limbs expressing dnFGFR1 exhibited a loss of skeletal muscle mass that averaged 30%. In addition to the loss of muscle mass, we observed both a reduction in myofiber density and a decrease in the structural integrity of the developing muscle. We therefore propose that FGFR1 signaling plays important roles in the control of myoblast differentiation during skeletal muscle development and is critical for myofiber organization.

MATERIALS AND METHODS

Embryo culture and manipulation. Fertilized White Leghorn chick embryos were purchased from SPAFAS. Embryos were incubated at 99.75°F and 55% humidity. Embryos were windowed at (HH) stage 17 (Hamburger and Hamilton, 1951), and RCAS-dnFR1-HA virus was injected into somites 26–31 and the dorsal and ventral regions of hind limb buds where premuscle masses will form. Injections were performed using 6- to 8- μ m micropipettes and a Picospritzer (General Valve Corporation).

Viral constructs. A truncated chicken FGF receptor 1 sequence spanning amino acids 1 through 423 was generated by amplification of a chicken CEK1 plasmid (Pasquale and Singer, 1989) with the forward (gaattccatggggtttacctggagggtgcctc) and reverse (gacggatcctagaggctagcataatcaggaacatcatagttgaagtctg) primers. The reverse primer also contained the coding region for an influenza hemagglutinin (HA) epitope tag (Meloche *et al.*, 1992). DNA sequencing of the PCR product confirmed that no mutations were present. The HA-tagged chicken dnFGFR1 sequence was cloned into a shuttle vector (ClaNco) (Hughes *et al.*, 1987) and then subcloned into RCAS-BP(A) (provided by Connie Cepko). The resulting RCAS construct was named RCAS-dnFGFR1.

Cell and virus culture. Chicken embryo fibroblasts (CEFs) were isolated from ED13 embryos, grown, and transfected with RCAS-dnFGFR1, and virus was isolated from CEFs as described (Fekete and Cepko, 1993). Viral titer of the concentrated stock used for infections, determined as described (Fekete and Cepko, 1993), was estimated to be 2×10^8 CFU/ml. For Western analysis of HA-tagged dnFGFR1 protein, CEFs were transfected with RCAS-

dnFGFR1, washed twice with cold phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mM KH_2PO_4), and solubilized in lysis buffer (1% (v/v) Triton X-100, 25 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM EDTA, 2 mM PMSF, 10 μM leupeptin) for 30 min at 4°C. Insoluble material was removed by centrifugation at 14,000g for 15 min at 4°C, and the protein content of cell lysate was determined by the bicinchoninic acid protein assay (Pierce Chemical Corp., Rockford, IL). Protein extracts (25 μg) were subjected to SDS-PAGE and electrophoretically transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) in 25 mM ethanolamine, 25 mM glycine, 20% methanol, pH 9.5. Nonspecific membrane-binding sites were blocked in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 100 mM NaCl) containing 3% nonfat dry milk and 0.05% (v/v) Tween 20. HA-tagged dnFGFR1 protein was detected using a polyclonal anti-HA primary antibody (as described, below). Bound anti-HA antibodies were detected with an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Promega Corp., Madison, WI).

Immunohistochemistry. AMV-3C2 primary antibody [Developmental Studies Hybridoma Bank (Potts *et al.*, 1987)], which recognizes avian myoblastosis virus gag protein p19, was used to visualize cells infected with RCAS virus in the chick embryo. A polyclonal anti-HA antibody (BabCo; HA-11) was used to detect expression of HA-tagged dnFGFR1 protein. A monoclonal anti-myosin antibody [MF20; Developmental Studies Hybridoma Bank (Bader *et al.*, 1982)] was used to stain for differentiated skeletal muscle tissue. Bound HA-11 antibody was visualized by staining with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Promega Corp., Madison, WI). Monoclonal AMV-3C2 and MF20 primary antibodies were visualized using horse anti-mouse subtype-specific secondary antibodies (South-Western Biotechnologies, Inc.) or an HRP-conjugated goat anti-mouse antibody (Promega Corp.). HRP-conjugated secondary antibodies were detected by incubating with the substrate DAB (diaminobenzidine).

Sections were incubated with blocking buffer (TBS (20 mM Tris base, pH 7.4, 100 mM NaCl) + 1% goat serum) containing 0.3% H_2O_2 for 1 h at 22°C, followed by incubation with primary antibody diluted in blocking buffer (HA-11, 1/1000; 3C2, 1/5; MF20, 1/10) for 1 h at 22°C. Following two rinses in TBS, sections were refixed in 4% paraformaldehyde for 5 min at 22°C. Sections were then rinsed twice in TBS and incubated with secondary antibody diluted 1/400 in blocking buffer for 1 h at 22°C. Antibody visualization was performed using the DAB substrate as per manufacturer's instructions (Vector Laboratories Inc). All section analysis involved at least three to five sections from three to five animals.

Following myosin staining with the MF20 antibody, fiber diameters in muscles of E16–E19 sections were measured using an ocular micrometer. The number of intrafascicular fibers/unit area was determined using an ocular grid. More than 100 fibers were analyzed under each experimental condition.

Whole mounts. Embryos were harvested at various times following infection, rinsed in cold PBS, and fixed overnight in 4% paraformaldehyde. They were bleached in H_2O_2 :paraformaldehyde (1:5) and blocked 8 h in PBS, 0.2% Triton X-100, 5% goat serum. They were rocked overnight at 4°C in primary antibody (1:10 3C2; 1:5 MF20). Following three 30-min rinses in blocking solution, embryos were incubated for 8 h at 4°C with an HRP-conjugated goat anti-mouse secondary antibody (Promega Corp.). They were rinsed several times in blocking solution and visualized using the DAB substrate per the manufacturer's instructions (Vector laboratories).

Sections. Embryos were harvested at various stages, sacrificed, rinsed in 4°C 1X PBS, and fixed for several hours in 4% paraformaldehyde. Embryos were rinsed for 1 h at 4°C in PBS and incubated overnight in 30% sucrose. Whole embryos and limbs were embedded in OCT (Tissue Tek) and sectioned at 10–20 μm on a Leica brand cryostat. Treatment of sections is as described under Immunohistochemistry.

Western blot analysis of dnFGFR1 expression in vivo. Embryos were harvested 5 days postinfection and rinsed in cold PBS and the skeletal muscle tissue was removed from infected and contralateral limbs. Muscle tissue was homogenized using a Teflon homogenizer in a homogenization buffer containing 20 mM Hepes at pH 7.4, 150 mM NaCl, and a protease inhibitor cocktail (10 mM EDTA, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 20 TIU aprotinin). The homogenate was centrifuged at 15,000g for 20 min at 4°C. The pellet was resuspended in homogenization buffer containing 1% Triton X-100 and incubated on ice for 10 min. The sample was centrifuged again and the soluble fraction removed for protein determination and Western blot analysis.

Samples containing 25 μg of protein were boiled in SDS-PAGE sample buffer and subjected to SDS-PAGE on 7.5% gels. The proteins were transferred to Immobilon-P membrane (Millipore Corp.) and the blot was stained with anti-HA antibodies (BabCo; HA-11), as described under Cell and Virus Culture, except the Renaissance detection system (NEN Life Science Products, Boston, MA) was used. The blots were then stripped and reprobed using an anti-FGFR1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Primary skeletal muscle culture. For analysis of clonal cultures, hind limb skeletal muscle tissue was isolated from chicken embryos at the indicated stages, minced with two number 10 scalpels, triturated in 0.1% collagenase, and incubated for 20 min at 37°C. Following incubation, dissociated tissue was passed through a 40- μm Falcon cell strainer and preplated for 20 min at 37°C to remove fibroblasts. Very little fibroblast contamination was apparent during later stage analysis. Of the cells plated >99% were myoblasts, as identified by myosin staining (MF20 antibody). Nonattached cells were harvested, counted by hemocytometer, and plated at clonal density onto either gelatin-coated glass coverslips or gelatin-coated 100- mm^2 plates. Those cells isolated from uninfected animals were subsequently infected in culture with RCAS-dnFGFR1, RCAN, or RCAS-AP virus. Cells were grown in F12 containing 15% horse serum, 2% chicken serum, and 2.5% chick embryo extract for 4–5 days with no disturbance or media change. Cells were then incubated for 12 h with 5-bromo-2'-deoxyuridine (BrdU) and fixed 10 min in 4% paraformaldehyde. Cultures were stained for myosin using MF20, BrdU using an anti-BrdU antibody, dnFGFR1 with an antibody to the HA epitope tag, and nuclei with DAPI. Following fixation in paraformaldehyde, cells were permeabilized with 0.2% Triton X-100. Coverslips were incubated in 2 N HCl for 30 min at 37°C, rinsed in TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA- Na_2) and PBS, and then incubated in PBS + 5% goat serum for 1 h at 22°C. Cells were incubated with various combinations of (1) anti-BrdU hybridoma supernatant (G3G4, Developmental Studies Hybridoma Bank) diluted 1:100 in PBS + 5% goat serum; (2) Texas red-conjugated goat anti-mouse IgG1 (Fisher) diluted 1:100 in PBS containing 1% goat serum; (3) anti-myosin antibody (MF20 see above) diluted 1:10 in PBS + 1% goat serum; (4) FITC-conjugated goat anti-mouse IgG2b (Fisher) diluted 1:100 in PBS containing 1% goat serum. All incubations were for 1 h at 22°C. Coverslips were mounted onto slides with Vectashield fluorescent mount media containing DAPI. For each result, a minimum of five coverslips was scored and photographed. Similar results were obtained from four independent experiments.

Analysis of reduction in mass and total cell number. Embryos were harvested at 8, 12, and 19 days and euthanized, and hind limbs were removed. Total limbs and isolated muscle were weighed individually on an analytical balance. Muscle was isolated with number five forceps following removal of skin. In some cases isolated muscle from three infected and three corresponding uninfected contralateral limbs was then combined. Primary chick embryo fibroblasts and myoblasts were isolated as described under Primary Skeletal Muscle Culture. Total cell numbers were counted on a hemocytometer before and after the preplating step, which specifically separates the fibroblasts from the myoblasts.

Bone staining and measurement. Embryos were harvested at ED16–ED19 and their hind limbs removed. They were fixed in 95% ethanol overnight followed by a 24-h acetone incubation. The acetone was replaced by stain solution (85 ml 70% EtOH, 5 ml glacial acetic acid, 5 ml 0.3% Alcian blue in 70% EtOH, 5 ml 0.1% alizarin red in 95% EtOH) and incubated at 37°C for 6 h. Limbs were left in the stain for 3–10 days. Skeletons were cleared in 1% KOH for several days and passed through a glycerol:KOH series (20%, 50%, 80%, 100%). Once completed animals were photographed, and the lengths and diameters of limb bones were measured.

RESULTS

Inhibition of FGFR1 Signaling Induces Terminal Differentiation of Primary Skeletal Muscle Cells but Does Not Affect Primary Fibroblasts

Several lines of evidence obtained from cultured skeletal muscle cells have long supported a role for FGF as a potent mitogen and repressor of differentiation. Although addition of FGFs to chick embryo myoblasts has been shown both to delay differentiation and be required for the onset of myogenesis (Kardami *et al.*, 1985a, b; Seed and Hauschka, 1988), the requirement for myogenic identity was observed only in early muscle cells derived from forelimb muscle of ED3.5–ED5 chicks. While addition of FGFs clearly delays the onset of myogenesis in Day 12–14 chick embryo myoblasts, we were unable to detect changes in FGF signaling upon addition of FGF1 or FGF2 to chicken skeletal muscle cells (data not shown). These experiments included assessment of FGF-stimulated FGFR1 autophosphorylation and FGF-mediated stimulation of intracellular MKK1 or ERK1 activities. It is possible that autocrine production of FGF masked a response to its exogenous application in these experiments. Consistent with this hypothesis, we can detect expression of FGF1 and FGFR1 in chick embryo myoblasts (unpublished data), and similar autocrine production of FGFs has been investigated and well described in mammalian skeletal muscle cells (Fox *et al.*, 1994; Groux-Muscattelli *et al.*, 1990; Hannon *et al.*, 1996).

If an autocrine FGF response were present in chick skeletal muscle cells, we would expect its signaling to be blocked by ectopic expression of a dominant negative FGFR mutant. Therefore, we constructed a truncated HA epitope-tagged FGF receptor 1 lacking the majority of the intracellular domain. This truncated receptor should function as a dominant negative mutant, as has been demonstrated in other systems (Amaya *et al.*, 1991; Kudla *et al.*, 1998).

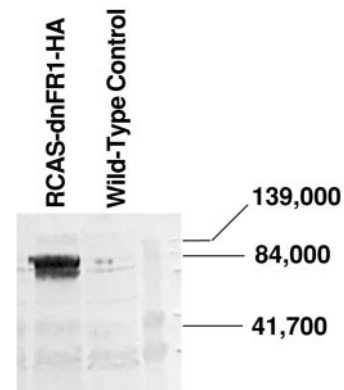


FIG. 1. An 80-kDa protein is recognized by anti-HA antibodies in RCAS-dnFGFR1-infected cells. RCAS-dnFGFR1 transfected chick embryo fibroblasts express high levels of an 80-kDa protein containing an HA epitope. Uninfected cells do not exhibit any detectable anti-HA antigens.

Transfection of chick embryo fibroblasts with the RCAS-dnFGFR1 construct resulted in the production of a protein product of approximately 80 kDa (Fig. 1). While analysis of the sequence encoding dnFGFR1 predicted a protein with a MW of 50 kDa, the observed increase in MW is likely due to glycosylation of the HA-dnFGFR1 extracellular domain (Fig. 1).

Myoblasts explanted from ED10 and ED14 chickens were plated at clonal density and infected with the RCAS-dnFGFR1 or RCAN viruses, or left uninfected. Following 5 days in culture, clones were examined independently for the extent of proliferation, myogenic differentiation, and expression of the HA-tagged dnFGFR1 (Fig. 2). While uninfected clones continued to proliferate and in some cases initiated differentiation (Figs. 2A, C, E), clones infected with RCAS-dnFGFR1 virus quickly withdrew from the cell cycle (Fig. 2D) and underwent terminal differentiation (Figs. 2D, E). Inhibition of FGF signaling resulted in small myotubes containing few nuclei. All of these cells stained positive for the HA epitope indicating that high level expression of dnFGFR1 was achieved following infection (Fig. 2F).

Induction of terminal differentiation by ectopic expression of dnFGFR1 is further supported by the following observations. First, clones infected with the control RCAN-A virus revealed no effect of virus alone on proliferation or differentiation (data not shown). Second, the dnFGFR1-expressing clones were 81% smaller than control-infected or uninfected clones, and the majority of these cells were differentiated as determined by myosin staining (Fig. 2 and Table 1). Third, only 7% of the total clones infected with RCAS-dnFGFR1 were actively proliferating (Fig. 2 and Table 1), while 92% of the control clones contained proliferating nuclei as indicated by BrdU incorporation (Fig. 2 and Table 1.). Further, viral spread occurs rapidly since staining with the 3C2 antibody 24 h following

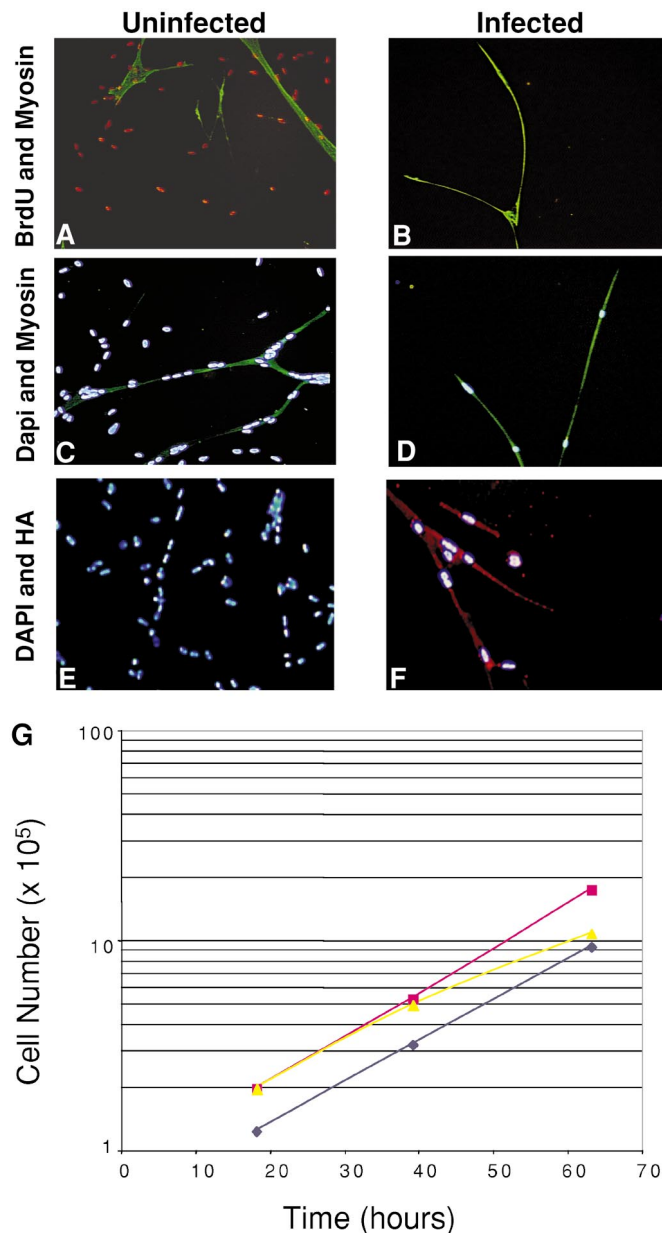


FIG. 2. Infection of chick primary cells with RCAS-dnFGFR1 induces terminal differentiation of myoblasts but does not effect fibroblasts. Primary chick myoblasts explanted from ED10 embryos were plated at clonal density on gelatin-coated coverslips and infected in culture with RCAS-dnFGFR1-HA virus (B, D, F) or left uninfected (A, C, E). Cells were treated with 10 μ M BrdU, fixed, and stained for myosin and BrdU (A, B), myosin and DAPI (C, D), or DAPI and HA epitope (E, F). Staining for BrdU (Texas red) and myosin (FITC) in panels A and B show reduced proliferation in infected versus uninfected clones, while myosin and DAPI-stained clones demonstrate a reduction in the size of individual myotubes and the entire clone. (G) Primary chick fibroblasts explanted from ED12–14 embryos were either left uninfected (◆), infected with RCAN virus (■), or infected with RCAS-dnFGFR1 virus (△). Cells were grown for several days prior to initiation of the growth

infection demonstrated that 100% of the RCAS-dnFGFR1 and RCAN-infected cells expressed the viral coat protein antigen (data not shown). Similar infection of chick embryo fibroblasts revealed no detectable effect of ectopic dnFGFR1 expression on cell doubling time (Fig. 2G).

Together these data demonstrate a marked effect of inhibition of FGFR1 signaling in cultured myoblasts. The requirement by chicken myoblasts for FGFs to repress differentiation was potentially masked by autocrine FGF production. Loss of FGF signaling as revealed by overexpression of a dominant negative FGFR1 mutant suggests that a large percentage of chicken embryo myoblasts utilize FGFs to repress myogenesis. It is also clear, however, that not all myoblasts are FGF dependent, since a small percentage (7%) of the RCAS-dnFGFR1-infected clones are indistinguishable from uninfected or RCAN-infected myoblasts (Table 1). Thus, the myoblast population in ED10 and ED14 chick embryos appears to be composed of both FGF-dependent and FGF-independent cell populations. This result is distinct from the conclusions that early muscle colony forming cells may require FGFs to confer a myogenic phenotype (Seed and Hauschka, 1988).

Injection of Somites and Limbs Efficiently Infects the Majority of the Resultant Limb and Somite Skeletal Muscle

Infection of myoblasts *in vivo* was effectively accomplished by injecting RCAS-dnFGFR1 virus into limb somites and buds prior to skeletal muscle cell migration. Previous work has demonstrated that the majority of the myogenic precursor cells contributing to skeletal muscle of the hind limb originate from the lateral dermomyotome of somites 26–31 (Jacob *et al.*, 1979). We therefore infected these five somites and the area of presumptive premuscle mass in the hind limbs of (HH) stage 16–17 embryos.

Embryos stained with 3C2 for the p19 viral coat protein demonstrated that skeletal muscle cells infected with RCAS-dnFGFR1 both migrate into the limb and accumulate as the premuscle masses (Fig. 3). Within 24 h injected somitic tissue was clearly infected and virus-expressing migratory cells were observed in the developing limb (Figs. 3A, C). At 48 h following infection, the majority of the limb level somites and the developing muscle masses expressed the p19 viral coat antigen (Figs. 3B, D). By 96 h the majority of the limb cells were infected, but virus had not extensively spread into the regions either rostral or caudal to the infected limb level somites (data not shown, see Fig. 5). These data suggest that infection of somitic tissue with the RCAS-dnFGFR1 virus does not elimi-

analysis to ensure complete infection of cells assayed. In addition, one plate of each condition was fixed and stained for viral coat protein 4 h following plating for the growth assay. Of the infected cell populations 100% were positive (not shown). Cells at each experimental condition doubled approximately every 15 h.

TABLE 1

	Muscle clones per plate	Nuclei per clone	% Nuclei in myosin + cells	% Clones with proliferating cells
Uninfected myoblasts	28.5	62.4	37	92
Infected myoblasts	19.5	12.1	85	7

Note. ED11 myoblasts were plated at clonal density, infected with RCAS-dnFGFR1 virus or left uninfected, and grown for 5 days with no media change prior to fixation. Following staining the cells were photographed and also scored for the parameters indicated. These include number of muscle clones as determined by myosin staining, number of nuclei per clone, the number of nuclei in differentiated and nondifferentiated cells, and the percentage of total clones containing proliferating nuclei (as determined by BrdU incorporation).

nate all migration of skeletal muscle cells into the limb, and that the majority of skeletal muscle tissue in the hind limb is infected 60–72 h following injection. This is substantiated by the observation that myoblasts explanted from infected limbs, which when stained for the viral coat protein 4–6 h postplating, demonstrate that >98% of the cells are infected (data not shown).

Expression of the Viral Coat Protein Antigen, 3C2, Is Coincident with HA Epitope Staining in RCAS-dnFGFR1-Infected Hind Limbs

It was critical to establish that cells infected with the RCAS-dnFGFR1 retrovirus were actually expressing the dominant negative mutant. We performed three different experiments to assess the expression level of the mutant receptor and the coincidence of viral infection and ectopic receptor expression. First, infected tissue was dissected and examined for expression of dnFGFR1 by Western blot

analysis using anti-HA antibodies. As expected, 5 days following infection we observed high level expression of an anti-HA epitope migrating at ~80 kDa (Fig. 4). Reprobing the blot with an anti-FGFR1 antibody that recognizes a C-terminal epitope failed to identify the endogenous FGFR1 present, most likely due to low levels of FGFR1 protein. These data demonstrate that infection with RCAS AdnFGFR1 results in high level ectopic overexpression of dnFGFR1 in the infected tissue. Moreover, expression levels between various animals are similar (Fig. 4, lanes 1–3).

Second, we asked what percentage of skeletal muscle cells was infected *in vivo*. Myoblasts from ED10 and ED 14 embryos infected at (HH) stage 16–17 were plated and fixed 4 h later. Staining these cells for the HA epitope tag revealed that >98% of the cells were positive for the HA epitope, suggesting that the vast majority of skeletal muscle cells are infected with the virus and overexpressing dnFGFR1. Muscle precursor cells isolated from animals prior to ED7–8 require the addition of conditioned media for growth in culture. In an effort to maintain continuity throughout our experiments, we did not perform explant analysis on cells from these early ages.

To establish that viral infection and ectopic dnFGFR1 expression were coincident, we compared the expression patterns of the p19 viral coat protein antigen with the HA epitope present on the intracellular domain of the dnFGFR1. A comparison of the 3C2 and HA staining patterns in sections of embryonic hind limbs revealed that all infected cells appear to express the HA epitope (Fig. 5). At 48 h following injection, prior to extensive skeletal muscle development, the infected limbs demonstrate that a large percentage of the cells stain positively for both the viral coat antigen and the HA epitope tag (Figs. 5A, B), while uninfected controls show little background (Figs. 5C, G). At 6 days after infection, both 3C2 and anti-HA staining pervade the entire limb with developing skeletal muscle and limb mesenchymal cells extensively infected (Figs. 5E, F). In contrast, the developing bone exhibits partial infection (Figs. 5E, F). A higher magnification illustrates that all discernable cells expressing the viral coat protein antigen also stain with the anti-HA antibody (Figs. 5D, H). These data demonstrate that the RCAS-dnFGFR1 virus infects the majority of the limb with extensive expression throughout developing skeletal muscle. Although bone tissue is clearly

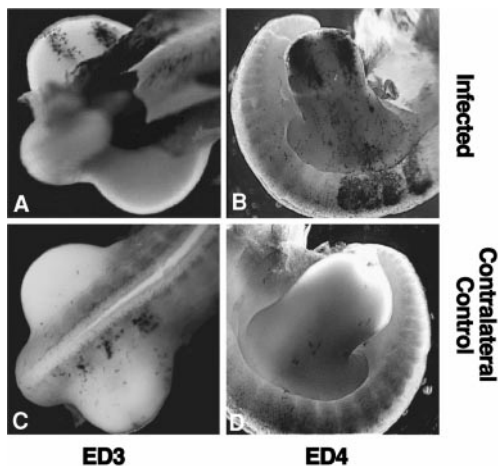


FIG. 3. Infection of somites and limbs with RCAS-dnFGFR1 virus infects the migratory skeletal muscle cells and, subsequently, the entire limb. Viral spread was assessed in infected (A upper limb, B) and uninfected (C upper limb, D) limbs at the indicated stages. Immunohistochemical whole-mount staining was performed with the 3C2 antibody.

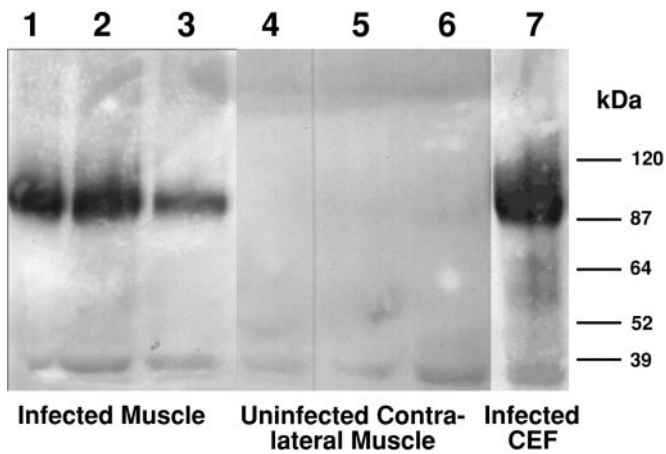


FIG. 4. The HA epitope is highly overexpressed in infected limbs. Total tissue was harvested and membrane proteins were isolated (as described under Materials and Methods) from three independently infected limbs (infected muscle, lanes 1–3) and their contralateral controls (uninfected muscle, lanes 4–6) of ED5 chicks. Western analysis was performed using the HA-11 antibody. Lane 7, which is protein from chicken embryo fibroblasts infected with the RCAS-dnFGFR1.HA, serves as a positive control for expression of the mutant receptor.

infected, the extent of viral spread appears less than that seen in skeletal muscle (Figs. 5D, H). Moreover, no discrepancies between 3C2 and HA epitope tag staining were observed, suggesting that all virally infected cells express high levels of the dnFGFR1 mutant protein. Therefore, we conclude that FGFR1 signaling should be inhibited in the majority of the developing skeletal muscle cells.

Expression of RCAS-dnFGFR1 Inhibits Skeletal Muscle Development *in Vivo*

The effects of retroviral infection with RCAS-dnFGFR1 on chick embryo myoblasts confirmed that FGFs are critical regulators of myogenesis in culture and likely to play important roles during myogenesis *in vivo*. Moreover, the lack of effect of exogenous FGFs we observed on chick skeletal muscle cells was likely due to autocrine responses from its endogenous production. In support of a role for FGF in myogenesis, myosin heavy chain (MHC) staining was consistently reduced in RCAS-dnFGFR1-infected limbs as compared to contralateral controls (Figs. 6A–D). Unlike the limb, the MHC staining of somitic muscle was visually

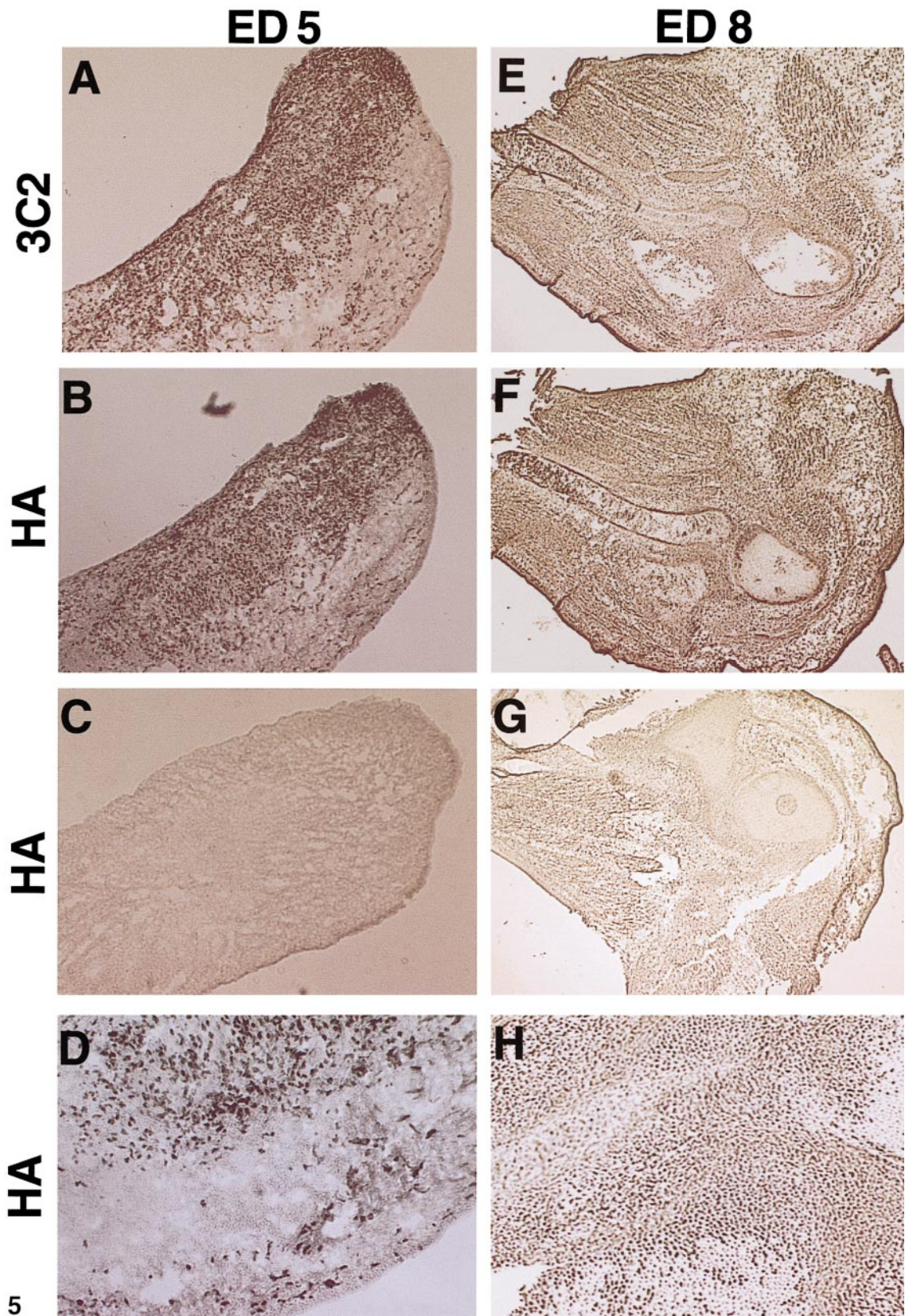
indistinguishable in infected versus uninfected hind limbs (Figs. 6A, B). As early as 96 h following infection, the muscle mass appeared reduced in RCAS-dnFGFR1-infected limbs when compared to contralateral controls (Figs. 6C, D). The reduction in MHC staining was even more pronounced 2 days later at ED8 (Figs. 6C, D). Embryos infected with the RCAN control virus demonstrated that this was not a result of the injection procedure or viral spread. We detected no changes in the MHC staining of these animals by 96 h following infection (Figs. 6E, F). These data demonstrate that inhibition of FGFR1 signaling may affect long-term skeletal muscle development.

Infection of Hind Limbs with RCAS-dnFGFR1 Leads to Loss of Skeletal Muscle Mass

Although the reduction in MHC staining suggested that limbs expressing the dnFGFR1 may also exhibit losses of muscle mass, it was unclear whether FGF signaling would globally affect long-term muscle development. We therefore chose to analyze skeletal muscle wet weight at ED8, ED12, and ED19 (Fig. 7A). These ages correspond with the presence of distinct myoblast subtypes, as previously identified in culture by variations in media requirements and MHC staining patterns (Stockdale, 1990). Prior to ED7–8, the majority of myoblasts are considered embryonic and are thought to form the primary myofibers. Secondary myogenesis, which begins between ED10 and ED13, corresponds with the appearance of the fetal myoblasts. It is this population of muscle cells that is thought to be responsible for formation of the secondary myofibers. Finally, the adult myoblasts which appear late in development (ED16) are thought to contribute primarily to myofiber hypertrophy and generation of the satellite cell population (Stockdale, 1990).

Examination of wet muscle weight at ED8 revealed a 22% decrease in mass between dnFGFR1-infected limbs and their contralateral controls. This decrease in mass continued throughout development with 28 and 30% decreases occurring at ED12 and ED19, respectively. This indicates that loss of FGFR1 signaling may affect skeletal muscle during early events such as extensive embryonic myoblast proliferation (ED8) and during late events including skeletal muscle splitting, patterning, and hypertrophy. Of 112 embryos infected with the RCAS-dnFGFR1 virus 107 displayed a loss in skeletal muscle mass, demonstrating that 96% of the embryos infected with dnFGFR1 virus were detectably affected by inhibition of FGFR1 signaling.

FIG. 5. Expression of the viral coat antigen, 3C2, and dnFGFR1 are 100% coincident. Viral spread and expression of dnFGFR1 were assessed in sections of infected and uninfected limbs. Sections were probed with antibodies that recognize the viral specific coat protein (A, E) and the HA epitope tag (B, C, D, F, G, H) present on dnFGFR1. Hind limbs were harvested at ED5 (A, B, C, D) or ED8 (E, F, G, H) from RCAS-dnFGFR1-infected limbs (A, B, D, E, F, H) or uninfected limbs (C, G). Higher magnification of panels B and F are shown in D and H, respectively.



dnFGFR1 INFECTED

UNINFECTED

ED6



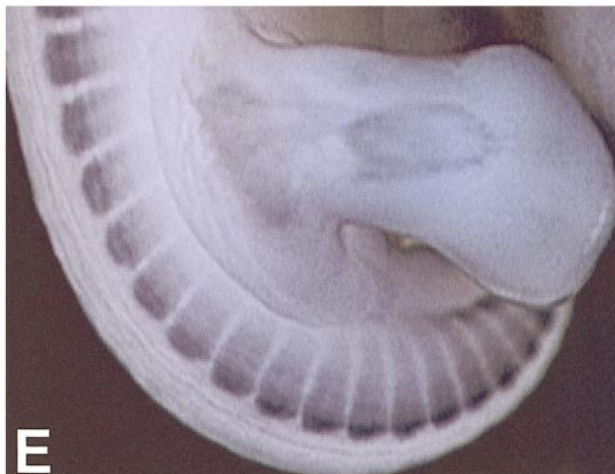
ED8



RCAN INFECTED

UNINFECTED

ED4



6

To determine whether similar losses in muscle mass would occur if the somites or limbs alone were infected, the somites, hind limbs, or both were infected with RCAS-dnFGFR1. Not surprisingly, the greatest weight differential between infected and uninfected controls occurred when both the limb bud and the somites were infected, with infection of the limb or somites individually having a lesser effect (Fig. 7B). Similar to uninfected limbs, infection of somites and the hind limb with a control RCAS-AP virus did not significantly alter the skeletal muscle mass (Fig. 7B), suggesting that the observed decrease in mass is specific for ectopic expression of dnFGFR-1.

Expression of dnFGFR1 Does Not Affect Long-Bone Development or Patterning

Loss of skeletal muscle tissue could be an indirect effect due to consequences of major alterations in bone development. Much existing evidence supports an essential role for FGF signaling in both bone development and limb patterning (Fallon *et al.*, 1994; Niswander *et al.*, 1993; Colvin *et al.*, 1996; De Moerlooze and Dickson, 1997; Deng *et al.*, 1996). Limbs stained for cartilage and bone showed no detectable effects of ectopic dnFGFR1 expression on long-bone length or diameter (Fig. 8). These data demonstrate that loss of FGFR1 signaling does not replicate the phenotypes that are observed in FGFR3 null mice (Colvin *et al.*, 1996; De Moerlooze and Dickson, 1997; Deng *et al.*, 1996) or in FGFR2 chimeric mice (Xu *et al.*, 1998). Particularly interesting are the observations that defects do occur in the distal bones of the foot and phalanges. These include shortening of the metatarsal and phalangeal bones, defects in cartilage ossification, and frequent fusions of the distal-most elements in infected limbs (in preparation). This is in accordance with recently published phenotypes of mice carrying hypomorphic alleles of FGFR1 (Partanen *et al.*, 1998). Together, our data suggest that ectopic overexpression of dnFGFR1 does not significantly alter signaling from FGFR2 or FGFR3, and further demonstrates that loss of skeletal muscle tissue is not due to morphological defects in long-bone development.

Myoblasts Infected *In Vivo* with RCAS-dnFGFR1 Differentiate into Myotubes That Possess Fewer Nuclei

Since our data reveal no gross alterations in skeletal development, a likely cause for loss of skeletal muscle

tissue is the decrease of skeletal muscle myoblasts. When cultured myoblasts were infected with RCAS-dnFGFR1, we observed rapid induction of myogenic differentiation and the formation of myotubes with low numbers of nuclei (see Fig. 2). If loss of FGFR1 signaling affects cells similarly *in vivo*, we would expect that explanted, infected myoblasts would also differentiate into small myotubes with few nuclei. In accordance with these data, myoblasts explanted from limbs infected *in vivo* formed significantly smaller myotubes than those from the contralateral uninfected limb (Fig. 9). The effect on explanted cells expressing dnFGFR1 was not, however, as striking as that observed when cultured myoblasts were infected with RCAS-dnFGFR1 *in vitro*. In contrast, myoblasts infected *in vivo* formed significantly fewer (43.8% less) myoblast clones containing more than 25 nuclei (Fig. 9), which corresponded with a concomitant increase (32.5% more) in the number of clones containing 1–10 nuclei (Fig. 9). In addition, the overall morphology of the myotubes in RCAS-dnFGFR1-infected limbs was different than the contralateral controls. In control cultures, the myonuclei were highly organized and aligned with the direction of the myotube (unpublished data). Normally, the myonuclei also appear longer and thinner than nuclei present in proliferating myoblasts. When infected myoblasts fuse, however, the myonuclei often appear disorganized and exhibit an irregular shape (unpublished data).

RCAS-dnFGFR1-Induced Loss of Skeletal Muscle Mass Corresponds with a Loss of Embryonic, Fetal, and Adult Myoblast Subtypes

As discussed previously, the formation of organized groups of complex skeletal muscle is thought to occur via waves of fiber fusion, each wave being associated with the emergence of distinct lineages of myoblasts. We have demonstrated that a loss of skeletal muscle mass is detectable as early as ED8 and continues throughout development. The loss of skeletal muscle mass could arise from decreased numbers of embryonic myoblasts, which would only be expected to affect the number of primary fibers formed but still cause a reduction in the overall tissue mass. Alternatively, loss of FGFR1 signaling could affect multiple myoblast populations, causing reductions in mass throughout development. To distinguish between these possibilities total muscle was isolated from infected and contralateral uninfected limbs. Individual cells were isolated, and the fibroblasts and myoblasts obtained were separated and

FIG. 6. Infection with RCAS-dnFGFR1 leads to a reduction in muscle-specific myosin heavy chain. Hind limbs were infected with either RCAN or RCAS-dnFGFR1 retrovirus at (HH) stage 16–17 and the myosin heavy chain content was analyzed by whole-mount immunohistochemistry using the MF20 antibody. Infection with RCAS-dnFGFR1 significantly delayed expansion and patterning of skeletal muscle at ED6 (A) and ED8 (C). The uninfected controls demonstrate that differentiation of the premuscle mass normally occurs at ED4 (F), expansion of muscle mass at ED6 (B), and splitting and patterning of skeletal muscle by ED8 (D). Infection with RCAN virus did not affect accumulation or expansion of the premuscle masses at ED 4 (B).

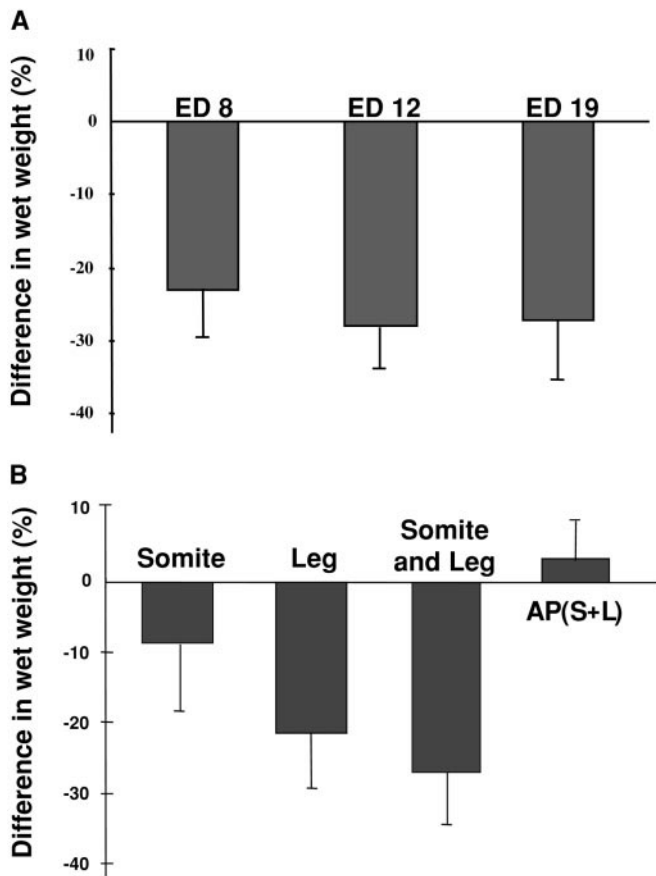


FIG. 7. Infection of primary chick limb myoblasts *in ovo* leads to a 30% overall reduction in muscle mass. (A) Hind limbs and somites 26–31 were infected at (HH) stage 16–17 with RCAS-dnFGFR1. Skeletal muscle tissue was dissected from the limb and the mass determined for infected limbs and contralateral controls at ED8, ED12, and ED19. The difference in wet weight was determined as described under Materials and Methods. In all cases, the mass of infected limbs was reduced as compared to the contralateral control limb. This is reflected in the negative value of percentage difference between mass of infected and uninfected limbs. (B) Hind limbs, somites 26–31, or both were infected with RCAS-dnFGFR1 and the wet weight was determined at ED19. In addition, hind limbs and somites 26–31 were infected with RCAS-AP and skeletal muscle wet weight was determined at ED19.

counted. Consistent with the decrease in muscle mass that occurred at each of the ages examined, there was a corresponding decrease in the number of myoblasts at ED8, 12, and 19 (Fig. 10). By ED8 21% fewer myoblasts were present in the dnFGFR1-infected limbs. This loss increased to 33% by ED12 and remained at this level (31%) through ED19 (Fig. 10).

Unexpectedly, a concomitant loss in the number of fibroblasts occurred with the myoblasts (Fig. 10). The effect on fibroblasts was not predicted since infection of fibroblasts in culture with RCAS-dnFGFR1 did not appreciably alter proliferation rates or the size of fibroblast clones (Fig. 2G). Moreover, explants of infected fibro-

blasts were indistinguishable from the contralateral controls or RCAN-infected hind limbs (data not shown). The reduction of fibroblasts that occurs following *in vivo* infection of hind limbs with the RCAS-dnFGFR1 does not occur in RCAN-infected or uninfected limbs. Since ectopic expression of dnFGFR1 in culture had no detectable effect on fibroblast growth (see Fig. 2G), these data suggest that communication critical for maintaining fibroblast number is likely to occur between myoblasts and fibroblasts *in vivo*.

RCAS-dnFGFR1-Infected Limbs Display Altered Patterns of Skeletal Muscle Organization

The loss of both myoblasts and fibroblasts is consistent with additional alterations we observed in the organization of skeletal muscle in hind limbs infected with the RCAS-dnFGFR1 virus. Whole-mount immunohistochemistry

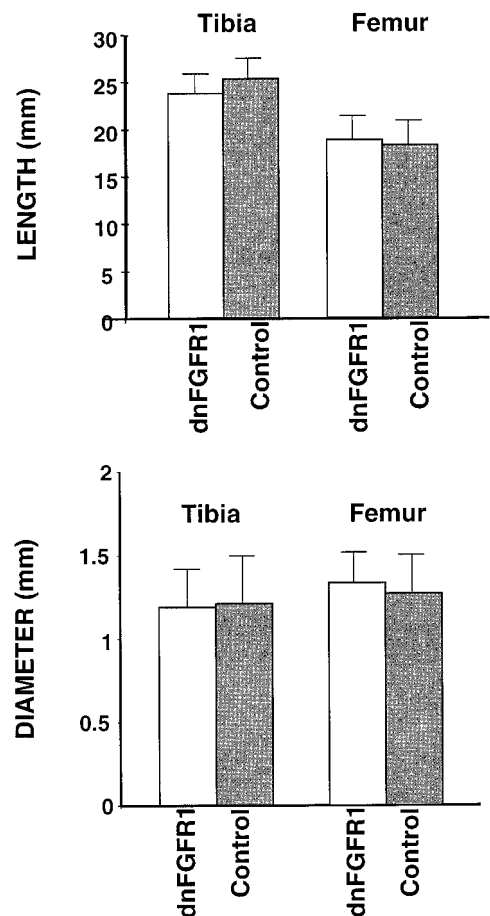


FIG. 8. Ectopic expression of dnFGFR1 does not affect long-bone length or diameter. Tibias and femurs of infected and contralateral control limbs were stained for cartilage and bone and their lengths and diameters measured. As indicated, no significant differences in the lengths or diameters were detected between the tibias and the femurs of dnFGFR1-infected and contralateral uninfected limbs.

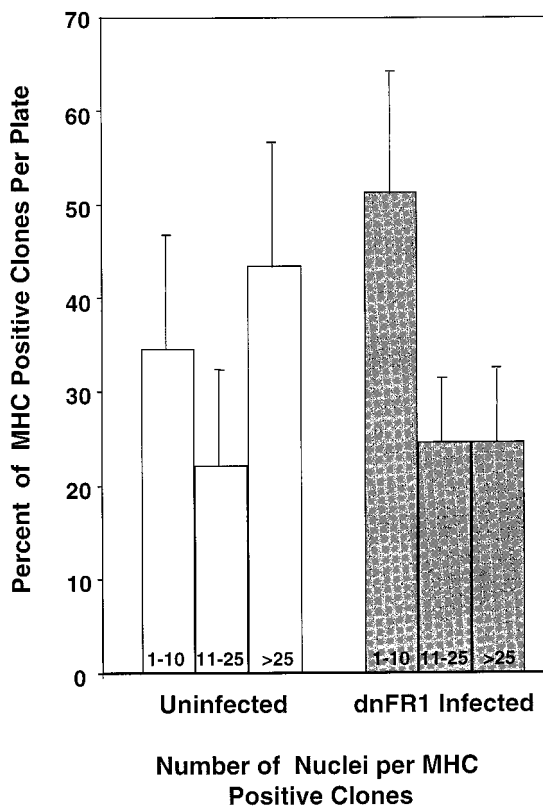


FIG. 9. Explanted myoblasts from dnFGFR1 infected hind limbs differentiate into myotubes with fewer nuclei than contralateral controls. Myoblasts were explanted at clonal density from dnFGFR1-infected and contralateral uninfected hind limbs of ED10 embryos and cultured for 5 days. The cells were then fixed and stained with MF20 antibody to myosin heavy chain (MHC) and the number of nuclei per clone was determined in clones exhibiting differentiation as determined by positive MHC staining. Five hundred cells were plated resulting in approximately 60 total clones on the uninfected plates, 25 of which were myosin positive, and 80 total clones on uninfected plates, 30 of which were myosin positive. The graph represents the percentage of uninfected (unshaded) and dnFGFR1-infected (shaded) MHC-positive clones (out of 100% total MHC-positive clones) per plate that contain 1-10, 11-25, or >25 nuclei.

with the MF20 antibody demonstrated defects in both the muscle pattern and the fiber organization of infected ED14 limbs. When infected and contralateral control limbs are compared, reductions in size between individual muscle groups are clearly evident (Figs. 11A, D). Further, cross sections through infected limbs demonstrate a reduction in fiber packing and overall organization (Figs. 11B, E). Defects in fiber organization are additionally supported by the observation that skin removal leads to torn and damaged muscle tissue in RCAS-dnFGFR1-infected limbs but not in uninfected or RCAN control-infected limbs (Figs. 11C, F). These morphological changes identify a phenotype distinct from the loss of muscle mass that also results from ectopic overexpression of the dnFGFR1 mutant.

Further examination of skeletal muscle sections revealed a 50% reduction in fiber packing density in the infected versus contralateral control limbs (Fig. 12A). This corresponds to the increase in space seen between skeletal muscle fibers in the myosin-stained whole mounts (Figs. 11B, C). Given the loss of muscle mass and myoblasts observed at ED19, we further expected to see reductions of skeletal muscle fiber diameter since the adult myoblasts are primarily responsible for late-stage muscle hypertrophy. The fiber diameters in dnFGFR1-infected limbs and contralateral controls were not, however, significantly different (Fig. 12B). Thus, we conclude that the observed loss of myoblasts and the reduction of myonuclei in clones cultured from dnFGFR1-infected limbs led primarily to altered fiber length and packing density but not fiber diameter. Although inhibition of FGF signaling led to a large reduction in the number of myofibers/unit area, no gross alterations in global muscle patterning were evident. All individual muscles were present and topographically located as

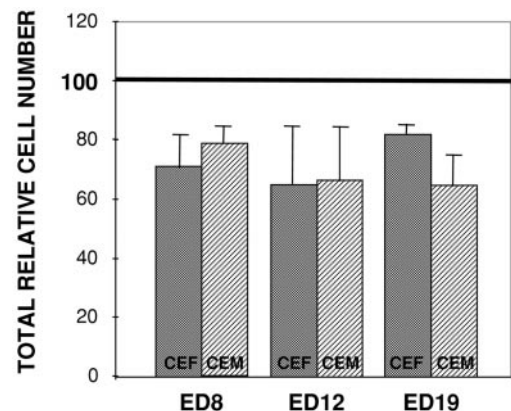


FIG. 10. Hind limbs infected with RCAS-dnFGFR1 have fewer myoblasts and fibroblasts than contralateral or RCAN-infected controls. Hind limbs were infected at (HH) stage 16-17 with RCAS-dnFGFR1 or RCAN retroviruses. At the indicated ED, the infected and contralateral control hind limbs were removed, the skeletal muscle was dissected, and the total number of cells and myoblasts was determined. The total number of cells was determined by counting in a hemocytometer prior to preplating. Myoblasts were quantitated by counting the number of cells that do not preplate on uncoated plastic. The number of fibroblasts was determined by subtracting the number of myoblasts from the total number of cells counted. The graph represents total relative numbers of chicken embryo fibroblasts (CEFs) and chicken embryo myoblasts (CEMs) in dnFGFR1-infected limbs (shaded and hatched bars, respectively) as compared to contralateral control limbs, which are normalized to 100% (represented by black line). Each determination is an average of three independent experiments composed of cells pooled from three infected limbs or three contralateral uninfected limbs. As an example, in one ED12 experiment total cell numbers from uninfected and infected limbs were 3.63×10^7 and 2.30×10^7 , respectively, while myoblast numbers for uninfected and infected (those that do not preplate) were 2.23×10^7 and 1.20×10^7 .

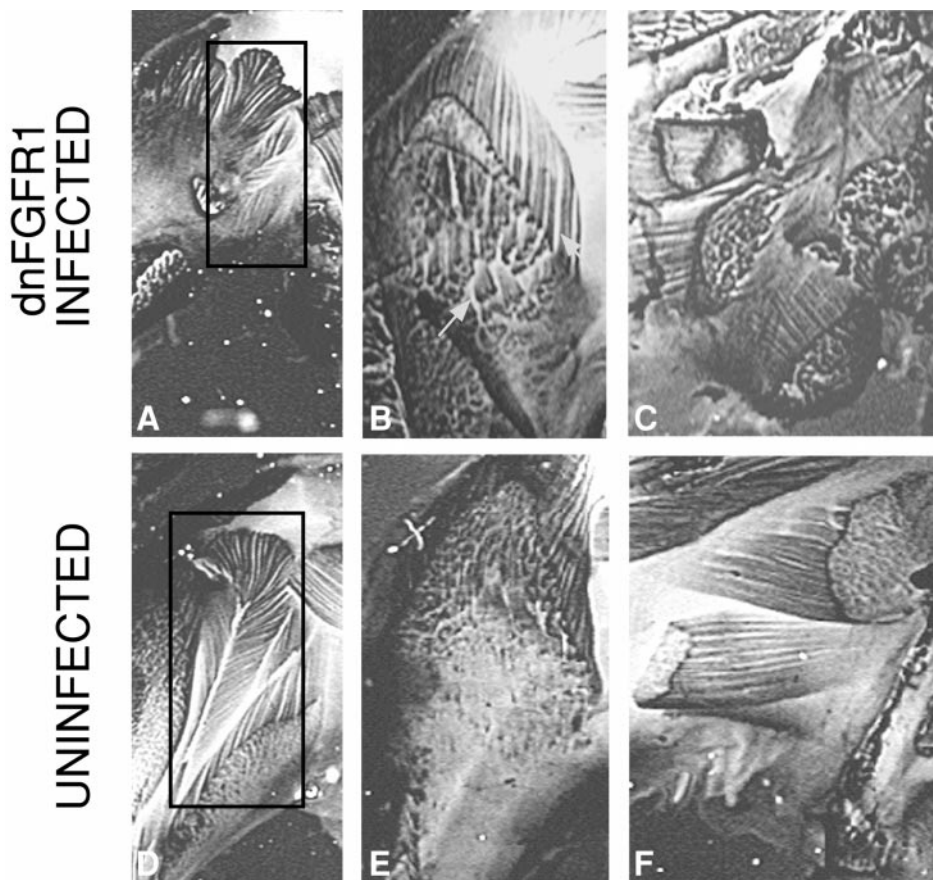


FIG. 11. RCAS-dnFGFR1-infected hind limbs have smaller muscles and a reduced density of myofiber packing per unit area. RCAS-dnFGFR1-infected hind limbs (A, B, C) and contralateral controls (D, E, F) from ED14 embryos were fixed and stained for muscle-specific myosin. In limbs ectopically expressing dnFGFR1, muscle groups appear smaller (compare A and D), fibers are less densely packed (compare F with C), and the muscle tissue is easily damaged when the skin is removed (compare C and E). Boxed areas (A and D) outline an affected muscle group. Yellow arrows (B) mark characteristic spaces between fibers of infected limbs.

observed in the contralateral limb. Identical results were observed in sections through the lower portion of the hind limb (data not shown).

DISCUSSION

Skeletal muscle precursor cells migrate into the forelimb and hind limb from the adjacent somites. Once in the limb these muscle precursor cells acquire skeletal muscle characteristics and expand to form the premuscle masses. As the premuscle masses develop neighboring cells decide to either proliferate or commit to terminal differentiation. Although the mechanisms involved are not understood, data from cultured primary cells and skeletal muscle cell lines suggest that growth factors play a critical role in this decision (Florini *et al.*, 1991). Members of the FGF and TGF β families (Cussella-DeAngelis *et al.*, 1996) as well as shh (Duprez *et al.*, 1998) and IGF-1 (Barton-Davis, 1998) have been shown to affect skeletal muscle growth or regeneration. In particular, FGFs have been implicated both

in migration of muscle precursor cells from the somites into the forelimb (Itoh *et al.*, 1996) and in regeneration of adult skeletal muscle (Floss *et al.*, 1997). FGFs are also excellent candidates for regulating skeletal muscle growth in the developing limb since: (i) the FGFs and their receptors are present in developing skeletal muscle (Szebenyi *et al.*, 1995); (ii) primary cells from avian and mammalian species are responsive to FGFs in culture (Allen *et al.*, 1984; Kardami *et al.*, 1985b; Seed and Hauschka, 1988; Zappelli *et al.*, 1996); and (iii) skeletal muscle cells themselves produce significant levels of FGFs (Fox *et al.*, 1994; Groux-Muscattelli *et al.*, 1990; Hannon *et al.*, 1996). We therefore asked if avian skeletal muscle myoblasts, which unlike mouse primary myoblasts do not require addition of exogenous FGF to maintain growth, are in fact dependent on FGF. Ectopic expression of dnFGFR1 in primary avian myoblast cultures resulted in rapid and complete differentiation. These data establish that FGFs are critical for maintaining cultured avian myoblasts and suggest that autocrine production is likely to mask or reduce responses of avian myoblasts to exogenously applied FGFs. To further

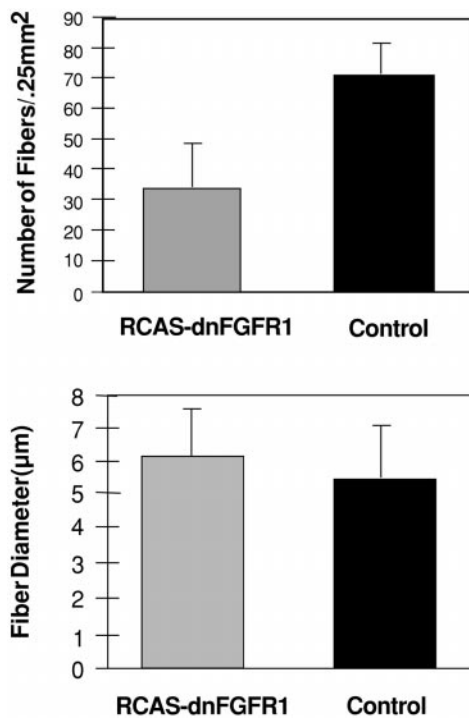


FIG. 12. Analysis of fibers in cross section demonstrates a reduction in fiber number but not fiber diameter in dnFGFR1-infected limbs. The fiber diameter and number of fibers per unit area (mm^2) were quantitated by examination of cross sections from RCAS-dnFGFR1-infected and contralateral controls.

determine if FGFs are involved in regulating myogenesis *in vivo*, we infected developing hind limbs with a modified avian leukosis virus encoding a dnFGFR1 mutant. It is estimated that a 10- to 50-fold molar excess of truncated tyrosine kinase receptor is required for it to efficiently function as a dominant negative mutant (Ueno *et al.*, 1991, 1992; Amaya *et al.*, 1991). We therefore confirmed (i) that expression levels of the ectopic dnFGFR1 were sufficient to inhibit signaling *in vivo*, (ii) that $\geq 98\%$ of all myoblasts are infected in ED8 to ED10 limbs, and (iii) that widespread expression of a viral coat protein was concomitant with expression of the HA epitope-tagged dnFGFR1. Although it was not possible to directly demonstrate that all cells expressing dnFGFR1 had lost the capacity to transduce FGFR1 signals, the level of epitope tag expression and the low level of endogenous FGFR1 protein suggest that the majority of the FGFR1 signal was blocked. Moreover, infection of explanted myoblast cultures induced the majority of the cells to differentiate ($\sim 85\%$), suggesting that the level of ectopic dnFGFR1 is sufficient to block FGF responses. We were unable to overcome this effect by addition of very high levels of FGF2 to the culture medium (unpublished data). These data are consistent with the hypothesis that a large percentage of myoblasts require FGFR1 signaling to maintain an undifferentiated phenotype.

Consistent with its effect on primary cultures, infection of the hind limb and adjacent somites with RCAS-dnFGFR1 virus resulted in a loss of MHC that was easily discernible 3 days following infection. By ED8, 5.5 days following infection, an even greater reduction in MHC staining was seen when comparing infected limbs with contralateral controls. The reduction in MHC staining was accompanied by a reduction in skeletal muscle mass at all ages examined. This loss in skeletal muscle mass upon inhibition of FGFR1 signaling could have occurred either as a direct effect on the myoblast population or as an indirect effect, such as a reduction in bone outgrowth. An effect due to bone loss is a likely possibility since FGFs are known regulators of long-bone development. As demonstrated in chimeric mice, FGFR2 signaling is essential for limb bud formation and limb outgrowth (Xu *et al.*, 1998). Therefore, loss of FGFR2 signaling would be expected to alter both limb patterning and limb outgrowth, and might also alter bone development in the chick limb. Further, FGFR3 $^{-/-}$ mice display abnormal bone overgrowth defects (Colvin *et al.*, 1996; Deng *et al.*, 1996). Thus, a similar loss of FGFR3 signaling in the chick limbs would be expected to dramatically increase long-bone length and induce abnormal curvatures (Colvin *et al.*, 1996; De Moerloose and Dickson, 1997; Deng *et al.*, 1996). Surprisingly, we observed no changes in long-bone length or diameter when comparing dnFGFR1-infected limbs with control-infected or the contralateral uninfected limbs. Thus, we favor the hypothesis that a loss of FGFR1 signaling directly affects proliferation, differentiation, or survival of the myoblast population in the developing limb. This hypothesis is further substantiated by the observations that dnFGFR1-infected limbs display defects in distal digit development (manuscript in preparation; Flanagan *et al.*) that are strikingly similar to defects reported for hypomorphic FGFR1 mutant alleles in the mouse (Partanen *et al.*, 1998). Together, these data suggest that we have inhibited the majority of FGFR1 signaling in the developing limb. In addition, we conclude that ectopic overexpression of the dnFGFR1 mutant has little or no detectable effect on signaling from FGFR2, FGFR3, and FGFR4.

Inhibition of FGFR1 signaling resulted in a 30% reduction of total muscle mass, which conclusively establishes a role for FGFR1 signaling in hind limb skeletal muscle development. Based on the phenotypes observed from infection of primary chick myoblast cultures with dnFGFR1, we expected *in vivo* infection to result in loss of the majority of the skeletal muscle in developing hind limbs. Unexpectedly, we found that explanted myoblasts exhibit an increased dependency on FGF signaling as compared to myoblasts present in the developing limb. Removal of myoblasts from the surrounding tissue most likely disrupts cell-cell communication and cell-cell interactions that are normally present *in vivo*. This may in turn lead to an increased dependency by cultured myoblasts for exogenous application of growth factors such as FGF. Moreover, myoblasts lacking FGFR1 signaling *in vivo* may adapt and utilize other factors to overcome this loss. Since it was

possible that loss of FGFR1 signaling *in vivo* would affect skeletal muscle cells differently than that observed when FGFR1 signaling was blocked in cultured myoblasts, we explanted myoblasts from RCAS-dnFGFR1-infected limbs, RCAN-infected limbs (not shown), and the uninfected contralateral controls. Myoblasts from limbs infected with dnFGFR1 yielded clones with a reduced number of nuclei. In addition, the nuclei from these infected cells were misshapen and did not organize properly prior to differentiation. Although myoblasts explanted from infected limbs exhibit quantitative differences from myoblasts infected in culture, qualitatively the results are quite similar. Both explanted dnFGFR1-expressing cells and cells infected with the mutant in culture precociously differentiated, resulting in reduced numbers of myonuclei per fiber. We believe it is possible that myoblasts *in vivo* partially compensate for the loss of FGFR1 signaling and thus, the hind limbs developed an intact musculature that was reduced in mass. This could reflect a "community effect" that has been previously described for skeletal muscle development in *Xenopus laevis* (Kato and Gurdon, 1993).

Development of the limb musculature is thought to occur via multiple "waves" of muscle cell precursor emigration from the somite (Stockdale, 1990; Van Swearingen and Lance-Jones, 1995; Seed and Hauschka, 1988). The earliest muscle cells to appear in the limb bud are the embryonic myoblasts. These cells are both responsive to exogenous FGF addition (Kardami *et al.*, 1985a) and may even require exposure to FGFs in order to acquire a skeletal muscle phenotype (Seed and Hauschka, 1988). *In vivo* these embryonic myoblasts fuse to form small, sparse primary myotubes (Rutz and Hauschka, 1982). Secondary myotubes are thought to arise from the fetal myoblasts, which appear between ED10 and ED13 (Stockdale, 1992). Finally, adult myoblasts appear at approximately ED16–ED19 (Feldman and Stockdale, 1992). These cells are thought to primarily contribute to fiber hypertrophy and the satellite cell population (Stockdale, 1992). Since a loss in skeletal muscle mass was observed as early as ED8 it is possible that loss of FGFR1 signaling affected migrating precursor cells or the embryonic myoblasts. The continued loss in mass could have resulted if this early lineage of skeletal muscle precursor cells was required for subsequent development. However, if loss of FGFR1 signaling truly affected only one myoblast population we would expect that subsequent inhibition of FGFR1 signaling might not affect fetal or adult-type myoblasts. In support of this, cultured fetal myoblasts in the mouse appear unresponsive to exogenous FGF addition and require TGF β to repress myogenesis (Cusella-DeAngelis *et al.*, 1994). However, we found that infection of ED10–ED14 primary myoblasts with the RCAS-dnFGFR1 virus rapidly induced terminal differentiation in $\geq 85\%$ of the cells present. Thus, it is possible that FGFR1 signaling is critical for more than one myoblast population. These data are consistent with the 20 to 30% loss of myoblasts that occurred in the dnFGFR1-infected limbs between ED8 and ED19. Therefore, we believe that

the increase in cell loss that occurred throughout development may not result solely from effects of losing FGFR1 signaling in a single population of early myoblasts. Our data suggest that FGFR1 signaling may provide important information for myoblasts throughout embryogenesis.

Our results differ significantly from a previous report, which suggests that FGFR1 signaling is necessary for myoblast migration into the forelimb bud (Itoh *et al.*, 1996). In these experiments, a replication-defective virus was used to deliver a truncated FGFR1 to the somites adjacent to the developing forelimb. The expression of β -galactosidase from a bicistronic message was used to follow the course of infection and expression of the truncated FGFR1. Infection of (HH) stage 14–17 forelimbs with the dnFGFR1/LacZ virus eliminated the appearance of infected cells in the developing limb musculature. From these studies, it was concluded that FGFR1 signaling was required for migration of skeletal muscle precursors into the limbs. Although our results suggest that FGFR1 signaling is not essential for migration of all skeletal muscle precursor cells, it may be necessary for the migration of a subset of cells. The loss in muscle mass and skeletal muscle tissue that we observe in ED8 embryos could be attributed to a loss of a subpopulation of migratory precursors. Our culture data suggest that the loss of migratory precursors, if it occurs, is likely to be due to premature differentiation of these cells rather than a direct effect on migration itself. It is also possible that the discrepancies observed in the two studies are due to infection of single cells as opposed to mass infection of the entire limb. If the muscle of the developing limb functions as a community, it is possible that the response elicited by singly infected cells would be different than that observed when the whole population is globally altered (community effect reviewed by Gurdon *et al.*, 1993).

In light of these possibilities, our results build substantially on those previously reported. We have established that inhibition of FGFR1 signaling using our dominant negative construct is unlikely to affect signaling from other FGFRs. We suggest that FGFR1 signaling is required throughout skeletal muscle development. It is possible that FGFR1 signaling provides a general survival signal and/or participates with other factors to regulate terminal differentiation *in vivo*. Despite the observation that primary cells exhibit an FGF dependency in culture, our data suggest that all myoblasts in the developing limb are not absolutely dependent on FGFR1 signaling to maintain a proliferative state. In agreement with a previous report (Cusella-DeAngelis *et al.*, 1994), we propose the existence of distinct myoblast subpopulations that are responsive to different growth factors or growth factor combinations. Loss of signaling from one growth factor receptor may reduce skeletal muscle mass but will likely not eliminate the majority of muscle tissue.

Completely unexpected was a striking loss of fibroblasts that accompanied the loss of myoblasts at every developmental age examined. Since we observed no effects on fibroblast growth when cells were explanted from

dnFGFR1-infected limbs or when fibroblasts were infected in culture with the RCAS-dnFGFR1 virus, we believe that loss of FGFR1 signaling in myoblasts may also disrupt essential communication with surrounding cells. The observed decrease in the number of fibroblasts may have contributed to additional phenotypes we observed when late-stage embryos were stained for MHC. In these limbs, the organization and packing density of the myofibers were disrupted. Although the diameter of the individual fibers did not seem to be affected, there was a severe ($\geq 50\%$) reduction in the number of fibers per unit area. Large spaces, which appeared to vary in size, could be seen between individual fibers, suggesting that the organization of the fibers was also affected. In addition, removal of the skin layer from dnFGFR1-infected limbs caused a great deal of damage and disruption of the underlying muscle tissue. Myofiber disorganization could have arisen from defects in the connective tissue that resulted when fibroblasts were lost. We propose that either a critical number of myoblasts is required to properly organize and orchestrate myofiber organization or that the loss of fibroblasts reduced the production of extracellular matrix and adversely affected myofiber organization. Inhibition of FGFR1 signaling revealed this novel phenotype and suggests that the numbers of myoblasts and fibroblasts are coordinately regulated to properly organize patterning of muscle tissue.

These studies are the first to use a replication-competent retrovirus to inhibit signaling of a tyrosine kinase pathway. Our data illustrate that inhibition of growth factor signaling using the appropriate dominant negative receptor mutants is likely to prove a useful tool for elucidating the activity of specific signaling pathways in the developing embryo.

ACKNOWLEDGMENTS

We thank members of the Bradley B. Olwin and Jennifer Martin labs for helpful scientific discussions and critical comments with regard to the manuscript. This work was supported by Grants from the Muscular Dystrophy Association and the National Institutes of Health to B.B.O. H.F.S. was supported by a National Institutes of Health Predoctoral Training Grant GM07135. A portion of this work was also supported by a USDA grant to K.H.

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Received July 21, 1999

Revised September 28, 1999

Accepted September 28, 1999