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Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle

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SUMMARY

Early in embryogenesis, precursors of the limb musculature are generated in the somite, migrate to the limb buds and undergo terminal differentiation. Although myogenic differentiation in culture is affected by several growth factors including fibroblast growth factor (FGF), it remains uncertain whether migration and differentiation of myogenic cells in vivo are directly regulated by such growth factors. To investigate the roles of FGF signaling in the regulation of myogenesis both in the somite and the limb bud, mosaic chicken embryos were generated that consist of somitic cells carrying transgenes expressing one of the following: FGF1, FGF4, the FGF receptor type-1 (FGFR1) or its dominant negative mutant (ΔFGFR1). Cells infected with virus producing FGF ligand migrated into the somatopleure without differentiating into myotomal muscle, but differentiated into muscle fibers when they arrived in the limb bud. In contrast, cells overexpressing FGFR1 migrated into the limb muscle mass but remained as undifferentiated myoblasts. Cells infected with the ΔFGFR1-producing virus failed to migrate to the somatopleure but were capable of differentiating into myotomal muscle within the somites. These results suggest that the FGFR-mediated FGF signaling (1) blocks terminal differentiation of myogenic cells within the somite and (2) sustains myoblast migration to limb buds from the somite, and that (3) down-regulation of FGFRs or FGFR signaling is involved in mechanisms triggering terminal differentiation of the limb muscle mass during avian embryogenesis.

Key words: fibroblast growth factor receptor, retrovirus, myogenesis, cell migration, chick, limb muscle, differentiation

INTRODUCTION

All vertebrate skeletal muscle caudal to the branchial arches is derived from the myotome of the somite. One subset of myotomal cells begins to express muscle-specific genes within the somite, differentiating into epaxial trunk muscles, while another migrates to the somatopleure and becomes the progenitors of the appendicular musculature including the limb muscle mass (Christ et al., 1977; Chevallier et al., 1977; Solursh et al., 1987; Ordahl and LeDouarin, 1992). Thus, precursor cells of limb muscle remain undifferentiated during migration, enter the limb bud and then undergo terminal differentiation (Stockdale, 1992). Although all myogenic cell types become committed through the expression of one or more myogenic determination genes such as MyoD, myogenin, MRF4 or Myf-5 (Emerson, 1993; Olson and Klein, 1994), little is known about the molecular signals that cause some myotomal cells to suspend their differentiation, initiate migration into the somatopleure and, finally, trigger terminal differentiation within the limb muscle mass.

Terminal differentiation of myogenic cells is arrested in culture by growth factors (Konigsberg, 1971; Yaffe 1971), including fibroblast growth factor (FGF) (Kardami et al., 1985; Lathrop et al., 1985) and transforming growth factor-β (TGFβ) (Olson et al., 1986; Massagué et al., 1986; Florini et al., 1986). This has led to the hypothesis that myogenic precursor cells differentiate into postmitotic muscle fibers in response to low mitogen concentrations (Konigsberg, 1971; Yaffe 1971). However, this hypothesis, based on myogenesis in culture, stands in contrast to the fact that these growth factors are present in the differentiating muscle masses of the embryo as well as in adult skeletal muscle (Kardami et al., 1985; Seed and Hauschka, 1988; Joseph-Silverstein et al., 1989; de Lapeyriere et al., 1993). Furthermore, the picture is complicated by the presence of multiple isoforms within each growth factor family and their varied expression patterns during myogenesis in the embryo, particularly in the FGF gene family (Joseph-Silverstein et al., 1989; Haub and Goldfarb, 1991; Niswander and Martin, 1992; de Lapeyriere et al.,1993). In contrast to the variety and the complexity of expression patterns observed for FGF ligands, only one type of high affinity receptor, FGFR1, predominates in the myogenic cell lineage, exceeding levels of FGFR2, FGFR3 and FGFR4 (Peters et al., 1992; Patstone et al., 1992). Importantly, the levels of FGFR1 gradually decrease as muscle fibers develop both in vivo and in vitro (Olwin and Hauschka, 1988, 1990; Moore et al., 1991; Templeton and Hauschka, 1992). Therefore, the down-regulation of FGFRs may be involved in the mechanisms regulating the initiation of limb muscle differentiation in vivo, although both receptor-coupled and

uncoupled pathways have been proposed for FGF-signaling (reviewed in Logan, 1990).

The present study addresses (1) the role of FGF-signaling in the regulation of in vivo myogenic differentiation within the somite during migration to and after arrival in the limb bud; (2) whether receptor-mediated pathways are the main route of FGF-signaling in myogenic cells and (3) whether in vivo terminal differentiation is triggered by downregulation of the receptor or ligand. These questions can be examined if FGFs and FGFRs are targeted within the myogenic cell lineage in a restricted manner, both regionally and temporally, during embryogenesis. A set of replication-defective retroviruses (Mima et al., 1995a,b) has been engineered for constitutive expression of the following: FGF, FGFR1 or ΔFGFR1, a dominant negative mutant of FGFR1 which inhibits FGFR signaling by forming a kinase inactive heterodimer with endogenous FGFRs (Amaya et al., 1992; Ueno et al., 1992). In the present study, cells in newly formed somites of avian embryos were infected with these viruses, and assayed to determine whether the gain or loss of FGF-signaling in infected myogenic cells affects their migration and differentiation patterns during embryogenesis. Here we demonstrate that receptor-mediated FGF signaling is involved in the regulation of in vivo myogenesis and that down-regulation of FGFR function is directly coupled to mechanisms triggering terminal differentiation of the limb despite the continued expression of ligand.

MATERIALS AND METHODS

Retrovirus production and in vivo injection

The retroviruses used for the present study are replication-defective variants of the spleen necrosis virus (Dougherty and Temin, 1986). Construction of the viral vectors and propagation of the recombinant viruses have been described elsewhere (Mima et al., 1995a,b; Mikawa 1995). In brief, after removal of polyadenylation signals, the cDNA of FGF1 converted to the secreted form (Forough et al., 1993), FGF4, FGFR1 or a truncation mutant of FGFR1 was inserted between the 5′LTR and a bacterial β-galactosidase (β-gal) gene of a vector plasmid, pSNZ (Mikawa et al., 1992). An internal ribosome entry sequence (IRES) derived from the 5′ noncoding region of the encephalomyocarditis virus genome (Ghattas et al., 1991) was inserted between the *FGFR1* and *lacZ* genes to equivalently express both genes from dicistronic messages. Procedures for propagating these recombinant retroviruses and testing of the helper virus-free stocks have been described (Mikawa et al., 1991; Mikawa and Fischman 1992). On average, we obtained ~10^6 virions/ml for control β-gal virus, FGF1 virus, and FGF4 virus, ~10^5 virions/ml for FGFR1 virus, and ~10^4 virions/ml for ΔFGFR1 virus in the supernatant of each clone of virus-producing cells. To increase production of ΔFGFR1 virus to ~10^5 virions/ml, the packaging cells were treated with 100 nM of phorbol-2-myristate-13-acetate (PMA, Sigma) for 10 minutes 1 day prior to viral harvest as described (Mima et al., 1995a). When viral concentrations of greater than 10^7 active virions/ml were required, culture supernatants of packaging cells were concentrated by ultracentrifugation as described (Mikawa et al., 1992). Co-translation of two genes from the IRES-containing dicistronic constructs in all daughter populations from the infected cells has been demonstrated both in culture and in the embryo (Ghattas et al., 1991; Mima et al., 1995a; Mikawa 1995). The biological activities of FGF virus, FGFR virus and ΔFGFR virus have been examined by infecting somatic mesoderm, mesenchyme in limb bud, cardiogenic mesoderm and coronary vascular cells, and assaying the effects on morphology, differentiation and cell proliferation as in Mima et al. (1995a,b) and Mikawa (1995).

Fertilized eggs were obtained from an outbred flock (Spafas) and incubated for 48-60 hours at 37.5°C in a humidified atmosphere, at which time they had developed to stages 13-17. A small window of ~5mm diameter was opened in the shell at the blunt end of the egg and the underlying shell membrane removed. Embryos were staged according to Hamburger and Hamilton (1951) after introduction of a biologically inert dye, India ink (10% in PBS), beneath the embryos with a 30-gauge needle. A microcapillary tube filled with virus solution was inserted into the lumen of newly formed somites or dermamyotome, and then 1-5 nl of virus solution containing 100 μg/ml polybrene (Sigma) was pressure-injected under the control of a Picospritzer II (General Valve Co., NJ). Somites at the wing and leg levels were identified according to Solursh et al. (1987) and Jacob et al. (1979), respectively. Other general procedures for microsurgery, injection of virus and incubation of infected embryos have been described previously (Mikawa et al., 1992). All embryos were fixed in freshly prepared 2% paraformaldehyde in PBS for 2-4 hours at 4°C and stained with X-gal for detection of β-gal in whole mount as described (Mikawa et al., 1991). Limb muscle masses exhibiting β-gal-positive cells were embedded in paraffin, serially sectioned at 10 μm thickness and examined by bright-field, phase or Hoffman modulation optics.

Primary culture of myogenic cells and in vitro infection

Both fibroblasts and myoblasts were isolated from leg or pectoralis muscle from either infected or uninfected embryos on embryonic day 9-11 (E9-11) according to Konigsberg (1971) with a slight modification as described (Nawrotzki et al., 1995). The isolated myoblasts were plated in a 1-3% gelatin-coated 60 mm dish with 5 ml of Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 2% chick embryonic extract (Gibco) and 10-20% fetal bovine serum (FBS). Effects of FGFs were examined by adding recombinant human FGF1 (Upstate Biotechnology, Inc., NY), FGF2 or FGF4 (gift from Dr L. Niswander, NY) at various concentrations up to 50 ng/ml with or without 50 nM heparin (Sigma) to media containing 2.5% FBS or 5% horse serum (HS). Recombinant FGFs were added every other day. Cells from uninfected E9-11 embryos were plated at a density of ~2×10^5 per 60 mm culture dish one day before infection. They were inoculated with virus solutions containing 10 μg/ml of polybrene for 2-4 hours, rinsed 3 times with PBS and then incubated in media with and without FGFs or FBS. Biological activities of FGF1 and FGF4 secreted from the infected cells was determined by transferring medium to PC12 cells and monitoring induction of neurite growth. The purified recombinant FGFs were used as a standard. In some cases, infected cells were incubated with 30 μg/ml the thymidine analogue, 5-bromo-2′-deoxyuridine (BrdU) overnight, and the incorporation of BrdU in the genomic DNA was monitored immunohistochemically as described (Sugi et al., 1993).

Antibodies

The following mouse monoclonal antibodies were used: an antibody (IgG1) to the extracellular domain of FGFR1 (gift from Dr P. Maher, The Whittier Institute, CA); MF20 (IgG2b) for sarcomeric myosin heavy chains (Bader et al., 1982); F310 for myosin light chain of fast skeletal muscle (Crow et al., 1983); an antibody for β-gal (IgG1; Sigma); an antibody for BrdU (IgG2a; Amersham). The secondary antibodies were: Texas Red-conjugated goat anti-mouse IgG2b (Hyclone); FITC-conjugated goat anti-mouse IgG1 and IgG2a (Hyclone); alkaline phosphatase-coupled goat anti-mouse IgG (Promega); horse radish peroxidase-coupled goat anti-mouse IgG (Sigma).

Immunoblotting

Prior to sample preparation, cells were briefly washed in PBS containing the protein inhibitor cocktail as described (Nawrotzki et al.,

1995). Cell extracts were collected in SDS sample buffer, boiled for 1 minute at 100°C, mechanically sheared through 30-gauge needle using a 1 ml syringe to reduce viscosity due to genomic DNAs in the sample and then microfuged for 15 minutes. 5-20 μg proteins of each sample were displayed by SDS-gel electrophoresis and transferred to nitrocellulose overnight at 100 mA at 4°C in the transfer buffer containing 0.1% SDS. Procedures for incubation with antibodies and NBT/BCIP color reaction (Promega) for alkaline phosphatase have been described elsewhere (Nawrotzki et al., 1995). Chemiluminescence reactions for horse radish peroxidase were carried out according to the protocol from E.I. du Pont Inc.

Immunohistochemistry

Cell were fixed with 2% paraformaldehyde in PBS for 10-15 minutes at room temperature, rinsed 3 times with PBS. Prior to the addition of primary antibodies, fixed cells were treated with 1% Triton in PBS for 10 minutes, rinsed with PBS and incubated for 15 minutes with 1% bovine serum albumin in PBS. After incubation for 1-2 hours at 37°C, excess primary antibodies were removed by washing 3 times in PBS and then cells were incubated with secondary antibodies. Stained cells were immersed with an anti-bleach mixture containing 10 mg/ml DABCO (Sigma), 10 mM Tris in 90% glycerol and examined with a Nikon epifluorescence microscope.

RESULTS

Stable expression of retrovirally introduced genes during myogenesis

To control the region and developmental stage of exogenous FGF or FGFR expression, replication-defective retroviruses were generated to express recombinant FGF1, FGF4 and FGFR1 (Mima et al., 1995a,b; Mikawa, 1995). All viral constructs were designed to coexpress a reporter, bacterial β-galactosidase (β-gal), for detecting cells expressing the transgenes in whole mount (Fig. 1A). In addition, two control viruses were used: one to block FGFR function by overexpressing a competitive inhibitory (dominant negative) mutant (Amaya et al., 1992; Ueno et al., 1992) of FGFR1 (ΔFGFR1), the other to express only β-gal (Mikawa et al., 1992). Western blot analyses using an antibody specific for the extracellular region of FGFR1 revealed a 20-fold or more excess of virally produced FGFR1 or ΔFGFR1 over the endogenous receptors in myogenic cells infected in culture (Fig. 1B). On average 0.1-1 ng/ml of FGF1 and FGF4 were secreted from 10^6 infected cells per day.

To control for any effect of microsurgery or non-specific consequences of viral infection, the vector expressing only β-gal was first introduced into the cavity of newly formed somites or into the myocoele of more mature somites containing dermamyotomes in stage 13-17 chicken embryos. The progeny of infected cells were visualized with X-gal staining in whole mount at various stages after the infection. Some myogenic cells infected with the β-gal virus migrated into both the somatopleure and the prospective muscle forming regions of the limb bud, while other marked cells remained within somite regions (Fig. 2A). Later in development, the migratory cells were incorporated into well-differentiated muscle fibers in appendicular muscle masses such as the pectoralis and limb musculature, while those that remained in the somite differentiated into epaxial musculature (Fig. 2B-D, Table 1), in addition to connective tissue cells of the dermis (not shown). The distribution of β-gal-positive muscle fibers was consistent with previous lineage studies of somite-derived cells in chicken/quail chimeras (Chevallier et al, 1977; Christ et al., 1977; Solursh et al., 1987). In some cases, β-gal positive endothelial cells were present in blood vessels, probably due to viral leakage from the somite and diffusion into the somatopleure. Importantly, β-gal was stably expressed in normally differentiated muscle fibers even after hatching. These results indicate that virally infected myogenic cells expressing β-gal preserve their capacities for normal migration and differentiation during the course of embryogenesis.

Constitutive expression of FGF in myogenic cells

To test whether constitutive expression of FGF ligand in myogenic cells can block their terminal differentiation in the embryo, as observed in culture, viruses encoding FGF were introduced into somites. In contrast to the normal pattern of development seen in cells expressing β-gal alone, the majority of cells infected with the FGF virus were found in the somatopleure, with fewer infected cells seen within the somite (Fig.

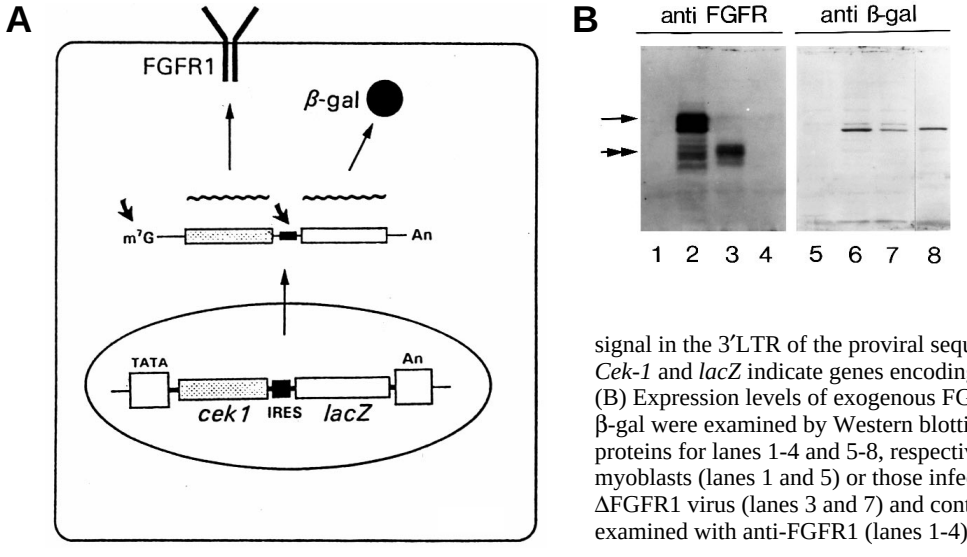

Fig. 1. (A) Schematic illustration of co-expression of FGFR1 and β-gal from the dicistronic proviral sequence in an infected cell. FGFR1 is translated by the 5′-cap (m^7G)-dependent manner, while β-gal translation depends upon ribosome entry from the IRES (filled box). Arrows mark these two ribosome entry sites. TATA and An represent the TATA-box in the 5′LTR and the polyadenylation signal in the 3′LTR of the proviral sequence in the host cell genome, respectively. *Cek-1* and *lacZ* indicate genes encoding FGFR1 and β-gal, respectively. (B) Expression levels of exogenous FGFR1 (arrow), ΔFGFR1 (double arrow) and β-gal were examined by Western blotting. Total extract (25 μg and 2.5 μg proteins for lanes 1-4 and 5-8, respectively) from chicken uninfected embryonic myoblasts (lanes 1 and 5) or those infected with FGFR1 virus (lanes 2 and 6), ΔFGFR1 virus (lanes 3 and 7) and control β-gal virus (lane 4 and 8) were examined with anti-FGFR1 (lanes 1-4) and anti-β-gal antibodies (lanes 5-8).

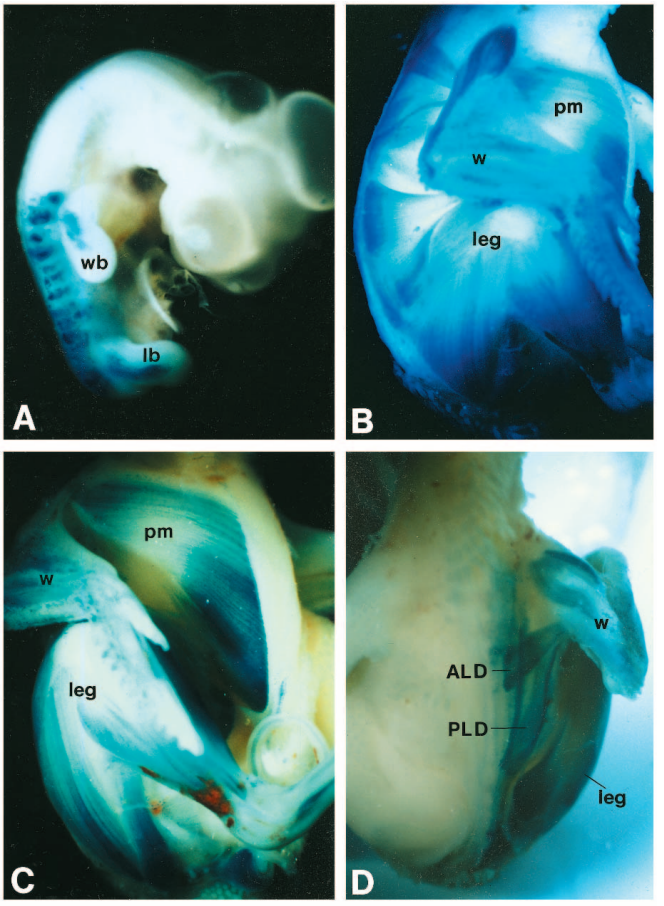

Fig. 2. Distribution of β-gal-positive cells in infected embryos. Control β-gal-virus was introduced into the lumen of multiple somites in only the right side of embryos at E2.5. The infected embryos were fixed on E5 (A) and E11 (B-D), and stained with X-gal in whole mount. Skin of the E11 embryo was removed to expose muscle masses. Note that the muscle-forming regions in both wing bud (wb) and leg bud (lb) of the E5 embryo (A) contain significant number of β-gal-positive cells. (B-D) Side, semi-front and dorsal view of the same embryo. Note that β-gal-positive cells are well differentiated into muscle fibers but restricted to the right side of the embryo. w, wing; pm, Pectoralis major; ALD, anterior latissimus dorsi; PLD, posterior latissimus dorsi.

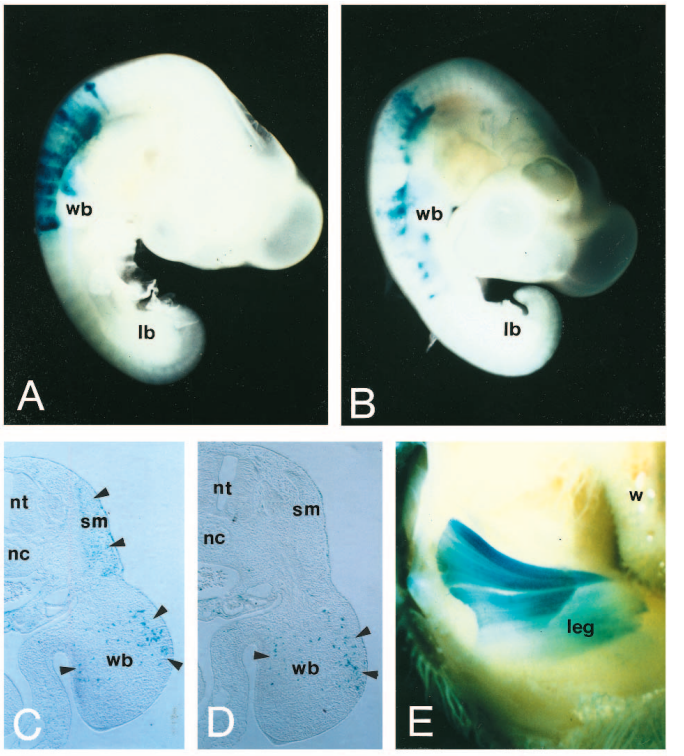

Fig. 3. Fate of somitic cells infected with FGF-producing virus. (A) Multiple somites at the wing level and those both anterior and posterior to the wing level were infected with control β-gal virus on E2, fixed on E4 and stained with X-gal in whole mount. (B) Same as in A but infected with FGF-producing virus. (C) Limb regions of the embryo infected with β-gal virus were processed for histological sections. (D) Same as in C but infected with FGF-producing virus. (E) Two consecutive somites at the hind-limb bud level were infected with FGF-producing virus on E3, fixed on E11 and stained with X-gal. The same results were obtained with FGF1 and FGF4 viruses. Skin of the embryo has been removed. wb, wing bud; lb, leg bud; nt, neural tube; nc, notochord; sm, somite; w, wing; arrowheads, β-gal-positive cells in the somite and those that migrated into the limb bud.

3A,B). As a consequence, there were few infected cells in the differentiated myotomal muscle mass (Fig. 3C,D; Table 1). It is currently unclear whether constitutive expression of FGFs promotes migration of the infected cells from the somite, or induces cell death within the somite. Limb level somitic cells infected with the FGF virus migrated into the limb bud and differentiated into muscle fibers in the correct position within the limb muscle mass (Fig. 3E; Table 1). Thus, cells infected with the FGF virus within limb level somites generate daughter populations that do not remain and/or survive within the somite but migrate to the limb buds to form limb muscle primordia which then undergo terminal differentiation.

In some myogenic cell lines, it has been demonstrated that depletion of FGFs from the medium downregulates its cognate receptor, suggesting that expression of FGFR is regulated by its ligand (Olwin and Hauschka, 1988; Moor et al., 1991; Templeton and Hauschka, 1992). To test whether down-regulation of FGFR occurs in cells infected with the FGF virus, we examined FGFR expression in infected cells before and after

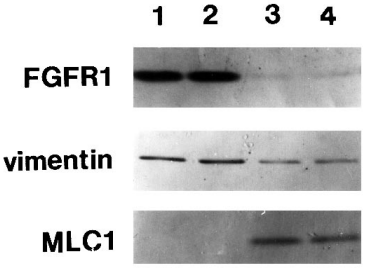

Fig. 4. FGFR-levels in cells infected with a control virus (lanes 1 and 3) and FGF4 virus (lanes 2 and 4) in culture were examined before (lanes 1,2) and after (lanes 3,4) differentiation. Whole cell extracts (25 μg proteins per lane) were separated by SDS-gel electrophoresis, transferred to nitrocellulose and reacted with antibodies to FGFR1, vimentin and myosin light chain 1 (MLC-1) specific to skeletal muscle. The FGFR1 band was visualized by chemiluminescence signal using an HRP-conjugated secondary antibody, while both the vimentin band and the myosin light chain 1 band were detected with conventional color reaction for the alkaline phosphatase-conjugated secondary antibody.

their differentiation in culture. Western blotting analysis of FGFR in these cells revealed a significant decline in FGFR levels when they formed muscle fibers (Fig. 4) despite the continued expression of FGF. These results strongly suggest a correlation between low FGFR levels and terminal differentiation, independent of exogenously expressed FGF.

Upregulation of FGFR level suspends limb muscle differentiation

In order to determine whether the loss of FGFR is necessary for myogenic differentiation within the limb muscle mass, a constitutive FGFR transgene was introduced into somitic cells. In most cases, myogenic cells infected with the FGFR-producing virus, like those infected with the FGF-producing virus, migrated into somatopleure, although in some cases a portion of infected cells were found in the myotome (Table 1). In striking contrast to myogenic cells expressing only β-gal (Fig. 2B-D) or the exogenous FGF-ligand (Fig. 3E), most of

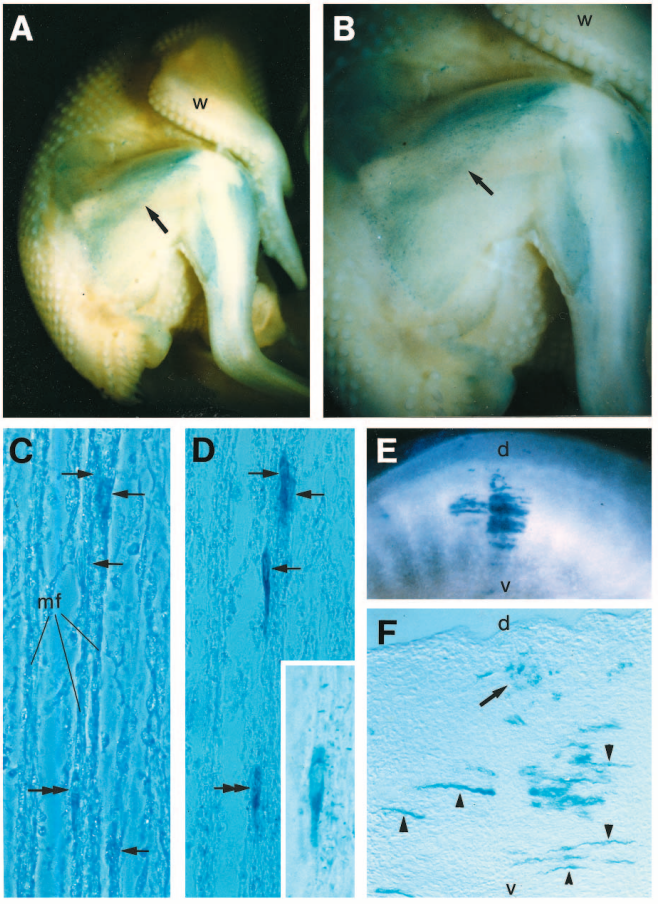

Fig. 5. Targeting of FGFR to cells forming limb muscle. (A) Somites at the hind-limb bud level were infected with FGFR1 virus on E3, fixed on E11 and stained with X-gal. Skin covering the leg of the embryo has been removed. (B) Higher magnification of the region indicated with arrow in A. Note that most β-gal-positive cells are distributed within the muscle mass (arrow) but remain unfused. (C) The muscle mass containing β-gal-positive cells was processed for paraffin sectioning and photographed under phase optics. (D) Same as in C viewed under bright field. The insert in D shows higher magnification of region containing a cell marked with a double-arrow in C and D. Note that uninfected cells have differentiated into muscle fibers (mf) while the β-gal-positive cells (arrows and double arrow) are unfused and mononucleated. (E) Virus encoding the truncation mutant ΔFGFR1 was introduced into two consecutive somites anterior to the forelimb bud on E2, fixed on E4 and stained with X-gal. (F) Histological sections of somitic regions containing β-gal-positive cells infected with ΔFGFR1-virus. d and v indicate dorsal and ventral sides of the embryo, respectively. Arrowheads and arrow mark typical myotomal muscle cells and those that migrated to dermis, respectively.

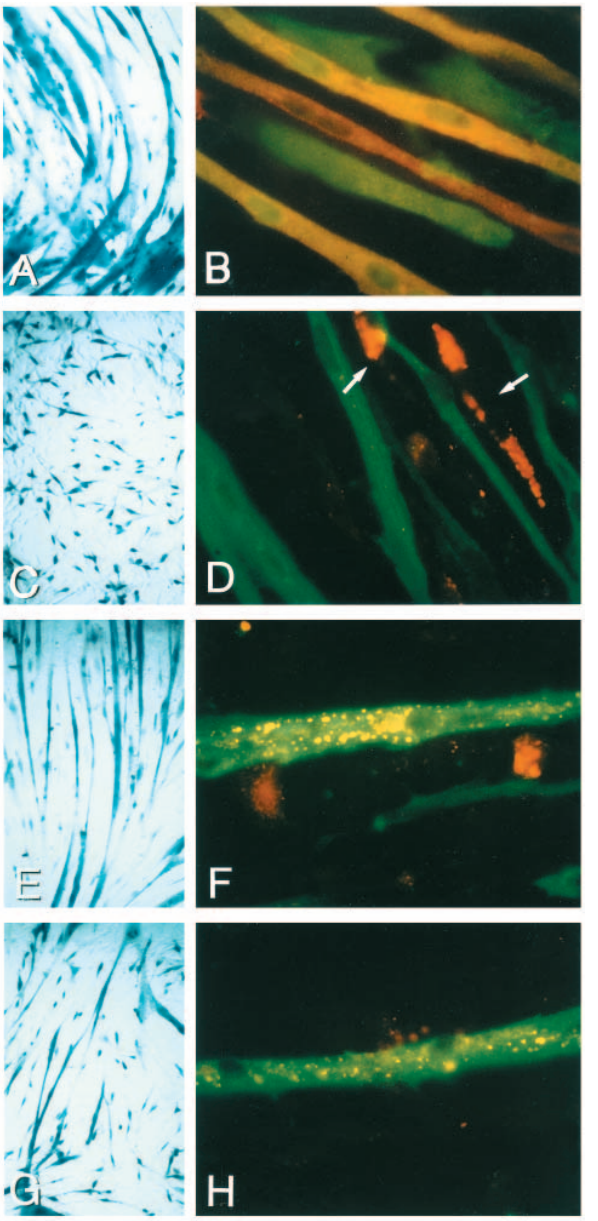

Fig. 6. In vitro response of myogenic cells to FGF ligands. Myoblasts infected with control β-gal-virus (A,B), FGFR1 virus (C-F) and ΔFGFR1 virus (G,H) were cultured in the presence (A-D, G,H) and absence (E,F) of FGFs, fixed 5 days after plating and stained either with X-gal (A,C,E,G) or with the MF20 antibody recognizing sarcomeric myosin heavy chain (red in B and green in D,F,H), with antibody to β-gal (green in A) and with antibody to FGFR (red in D,F,H). Note punctate appearance of FGFR staining in both MF20-negative myoblasts (arrows) and MF20-positive myotubes (F,H).

Table 1. Distribution of myogenic cells infected with viruses producing FGF, FGFR and ΔFGFR

Positions (somite level) and stages of infection		*% of embryos bearing β-gal+ cells in myotome and limb muscle mass			
		Virus types			
		β-gal	FGF	FGFR	ΔFGFR
Wing (somite 15-20)					
stages 13-15	M	0	0	0	100
	M + L	100	4	23	0
	L	0	96	77	0
		(*n*=53)	(*n*=48)	(*n*=48)	(*n*=38)
stages 16-17	M	0	0	0	100
	M + L	100	19	54	0
	L	0	81	46	0
		(*n*=34)	(*n*=46)	(*n*=39)	(*n*=41)
Leg (somite 26-32)					
stages 16-17	M	0	0	0	100
	M + L	100	8	34	0
	L	0	92	66	0
		(*n*=45)	(*n*=37)	(*n*=35)	(*n*=33)

*β-gal+ muscle cells were examined in myotome and limb muscles of infected embryos after whole mount X-gal staining at E7-E9. M, embryos bearing β-gal+ muscle fibers only in the myotome but not in the limb muscle; M + L, embryos containing β-gal+ fibers both in myotome and limb muscles; L, embryos exhibiting β-gal+ muscles only in the limb but not in the myotome; *n*, total number of embryos examined.

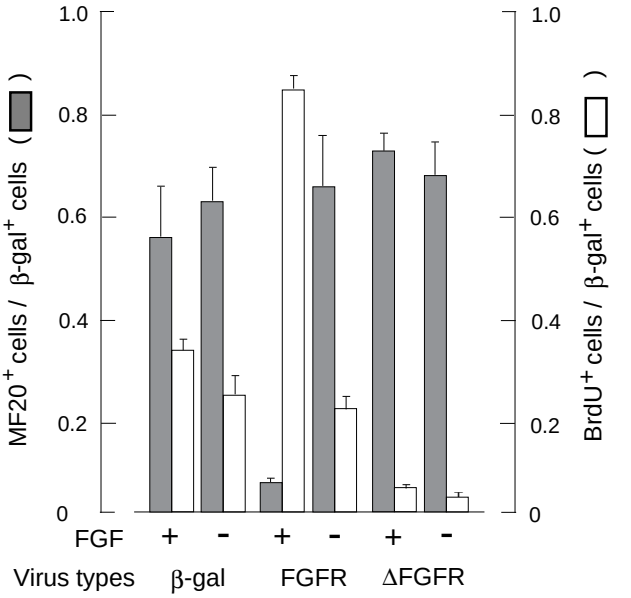

Fig. 7. Myoblasts infected with β-gal virus, FGFR1 virus and ΔFGFR virus were cultured, incubated with BrdU on day 3 of culture and fixed the next day. Six replica plates for each virus group were made. The cells were doubly stained with an anti-β-gal antibody and MF20 or with an anti-β-gal antibody and an anti-BrdU antibody. 200 β-gal-positive cells were randomly counted in each plate. Bar indicates standard deviation.

the cells constitutively expressing FGFR were not incorporated into muscle fibers even later in development but remained predominantly as unfused myoblasts (Fig. 5A-D). These results suggest that cells infected with the FGFR1-virus followed correct migration patterns but did not enter terminal differentiation at the appropriate stage of development.

Although these results demonstrate that muscle fiber formation, or the fusion of myoblasts, is inhibited by overexpression of FGFR, it was not clear whether other events of differentiation, including the expression of myofibrillar proteins, are also affected by expression of the FGFR transgene. To determine if altered FGFR levels regulate the induction of muscle-specific myosin expression and muscle differentiation, we cultured FGFR1-infected myoblasts with or without FGF in the media, and assayed expression of muscle-specific myosin. In parallel experiments, undifferentiated and mitotic cell populations were identified by incorporation of the thymidine analogue, BrdU, during DNA synthesis. As expected, myoblasts infected with control virus encoding only β-gal initiated myotube formation (Fig. 6A) within a few days in culture, yielding a smaller population of BrdU-positive (BrdU+) cells (Fig. 7). Expression of muscle-specific myosin heavy chain, as detected by the MF20 antibody, was evident soon after myotubes formed (Fig. 6B). Addition of FGFs to the culture medium delayed, but did not completely block, terminal differentiation of these cells.

In contrast, the majority of cultured myoblasts overexpressing FGFR1 in the presence of FGFs remained mononucleated (Fig. 6C), as observed in the embryo (Fig. 5A-D), and exhibited no significant myosin heavy chain expression (Fig. 6D). Under these conditions, the number of mononucleated, β-gal-positive cells expressing exogenous FGFR1 increased significantly, indicating their active proliferation. Indeed, these mononucleated cells were BrdU+ (Fig. 7). Importantly, cells expressing the FGFR1 transgene transferred to medium containing low levels of growth factors became mitotically inactive and successfully formed myosin-positive myotubes (Figs 6E,F, 7). Thus, the arrest of myogenesis in cells overexpressing FGFR1 is dependent upon extracellular FGF.

A dominant negative mutant of FGFR1 induces myogenic terminal differentiation

The responses of myogenic cells to overexpression of functional FGFR1 both in vivo and in vitro (Figs 1, 5, 6) suggest that upregulation of FGFR1 can suspend myogenic differentiation, but it does not prove that FGFR-mediated signaling is involved in the endogenous mechanisms regulating muscle differentiation. If FGFRs play an important role in terminal myogenic differentiation, blockage of FGFR-signaling should affect FGF-dependent responses of the myogenic cells. To test this idea, a virus encoding a truncation mutant, ΔFGFR1, which lacks the C-terminal kinase domain, was introduced into somitic myotomes. It has been demonstrated previously that the ΔFGFR1 inhibits receptor-mediated signaling by forming an inactive heterodimer with all isoforms of full-length FGFR (Ueno et al., 1992). In marked contrast to somitic cells infected with the FGFR virus, those cells infected with the ΔFGFR virus assumed a spindle-shape characteristic of myotomal muscle cells (Fig. 5G,H). Furthermore, these cells failed to migrate into the somatopleure (Table 1). These results suggest that introduction of ΔFGFR1 into somitic cells, inhibits their proliferation and migration, and triggers terminal muscle differentiation within the myotome.

Myosin expression and BrdU incorporation in cells infected with ΔFGFR virus were then examined. The majority of myoblasts infected with the ΔFGFR virus were detected as

myosin-positive and BrdU-negative cells and some of them formed myotubes (Figs 6G,H, 7). Neither myoblast proliferation nor BrdU incorporation was stimulated by adding FGF to the culture medium (Fig. 7). These data suggest that cells infected with the ΔFGFR virus readily entered terminal differentiation without active proliferation: i.e., disruption of FGFR function with the dominant negative mutant ΔFGFR1 suppresses mitosis of the myogenic cells and/or induces their terminal differentiation.

DISCUSSION

It is known that the commitment of skeletal muscle precursor within the somite requires the expression of one or more members of the *MyoD* gene family, which act as transcription factors upregulating muscle-specific gene expression (reviewed in Emerson, 1993; Olson and Klein, 1994). It remains uncertain how these committed cells in vivo temporarily keep from differentiating until reaching their final destination in the developing limb. Our data provide a molecular basis for this suspension of terminal differentiation in a subset of somitic cells as they migrate from the somite into the limb bud. Multiple subtypes of both FGF and FGFR are broadly distributed in embryos and blockage of FGF signaling in whole embryos inhibits either the induction or patterning of mesoderm, including precursors of the myogenic lineage (Amaya et al., 1992; Yamaguchi et al., 1994). To analyze the altered FGF and FGFR signalings in the myogenic lineage, we utilized retroviral-mediated gene transfer. Our results demonstrate that the functions of FGF signaling within the myogenic lineage can be analyzed in mosaic embryos that consist of myogenic cells constitutively expressing transgenes of FGF, FGFR or ΔFGFR.

In this mosaic analysis, it is critical to identify the cells expressing the transgenes. One method for tracing the infected cells would be to introduce an epitope tag on FGF or FGFR. Cells expressing the tagged sequence could be detected with appropriate immunohistochemical analyses of serially sectioned embryos. This procedure assumes that the tagged protein remains confined to the cell expressing it. However, FGFs encoding a secretion signal were used in the present study. The recombinant FGFs were secreted continuously (~0.1-1 ng/10^6 cells/day) from host cells infected with FGF virus in culture, although the exact levels of transgene expression in vivo are uncertain. Furthermore, somitic cells infected with the FGF virus exhibited active migration to somatopleure, probably leaving a trail of secreted protein. Thus, it is unlikely that the epitope would be localized exclusively to the cells encoding the provirus. In addition, epitope tagging has the potential for modifying the physiological properties of FGF or its cognate receptor. To minimize the complexity of epitope tagging, we utilized an IRES-containing dicistronic construct to coexpress FGF or FGFR with β-gal. This marker enzyme remains within the host cell as a biologically inert reporter protein. Thus, migration, proliferation and differentiation patterns of cells expressing the transgene can be followed with X-gal staining of whole embryos, without histological sectioning. Equivalent co-expression of both transgenes has been demonstrated in all daughter populations of the infected cells during chicken embryogenesis, by immunohistochemistry and PCR analyses (Mima et al., 1995a).

In our earlier studies, the same set of viruses were used to examine FGF signaling in other mesoderm-derived lineages: cardiogenic and somatic mesoderm (Mima et al., 1995a,b; Mikawa, 1995). Unlike somitic cells, somatic mesoderm infected with FGF virus generates limb-bud-like outgrowths, which differentiate into supernumerary limbs later in development (Mima et al., 1995b; Ohuchi et al., 1995). Mesoderm lateral to Hensen's node (Rosenquest and DeHaan, 1966) differentiates as cardiac myocytes, which form beating tubular hearts. Unlike skeletal muscle, the differentiated cardiac myocytes continue to proliferate. By infecting cells in the cardiomyocyte lineage with the same set of recombinant retroviruses used in the present study, we have shown that receptor-mediated FGF signaling regulates the proliferation of cardiac myocytes but not their migration or terminal differentiation (Mima et al., 1995a). Thus, a tight coupling of mitogenic suppression with terminal differentiation is unlikely in the cardiogenic lineage. In contrast, the differentiation of skeletal muscle cells is clearly linked with withdrawal from the cell cycle both in vivo and in vitro (Konigsberg, 1971; Clegg et al., 1987; Olson, 1992). Furthermore, expression of the MyoD gene family is susceptible to FGF signaling in the skeletal muscle lineage (Brunetti and Goldfine, 1990; Hannon et al, 1992; Li et al., 1992; Wolf et al., 1992). At present, there is no evidence for involvement of FGF-sensitive MyoD-like protein(s) in the regulation of cardiac muscle differentiation (reviewed in Litvin et al., 1992). Taken together, we suggest that, in both skeletal and cardiac muscle lineages, FGF signaling regulates cell proliferation, but that different mechanisms must trigger terminal differentiation in these two contractile lineages.

The FGF ligand gene family consists of at least nine members, which have been classified into two subgroups: one contains an N-terminal, signal sequence for secretion while the other lacks this sequence (Rogeli et al., 1988; Forough et al., 1993). Although the former type of FGF is secreted more effectively than the latter (Rogeli et al., 1988; Forough et al., 1993), FGFs lacking a secretion signal are also secreted by a currently unknown mechanism. In the embryo, several FGF isotypes are expressed during myogenesis. FGF1 and FGF2, lacking the secretion signal, are located within developing muscle during the entire course of myogenesis even in differentiated muscle fibers (Joseph-Silverstein et al., 1989). FGF4, FGF5 and FGF6, which contain a secretion signal, are expressed in somitic, migratory and the embryonic limb muscle masses but not in differentiated muscle (Haub and Goldfarb, 1991; Niswander and Martin, 1992; de Lapeyriere et al., 1993). Unlike the expression patterns of FGF family members, FGFR1 is the predominant receptor expressed in the myogenic lineage, exceeding other members of FGFR gene family (Peters et al., 1992; Patstone et al., 1993; de Lapeyriere et al., 1993).

Our results summarized in Fig. 8 are consistent with a model in which the level of FGFR is linked to the regulation of FGF-dependent myogenic responses. Under normal conditions, precursors of the limb musculature co-express FGFR and both secreted and non-secreted forms of FGF. These cells suspend terminal differentiation during migration from the somite to the limb bud probably because of high FGF ligand concentrations. After arrival at the muscle-forming region of the limb, FGFR levels are downregulated, thus uncoupling downstream signaling from FGF ligands. This loss of FGFR signaling

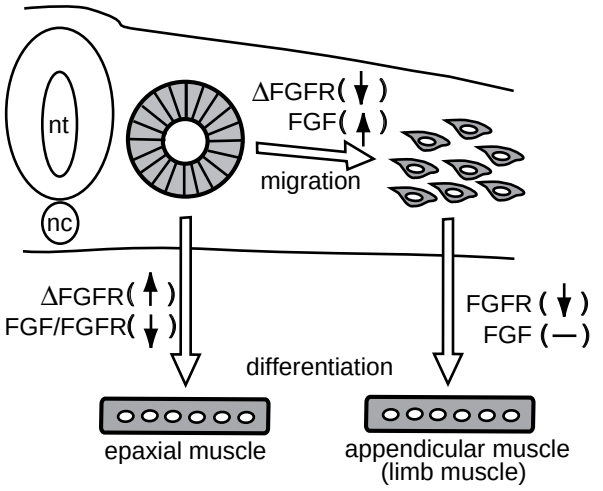

Fig. 8. A diagram illustrating the effects of exogenous FGF, FGFR and ΔFGFR in the regulation of myogenesis in early chicken embryos. (↑), activation; (↓), inhibition; (–), insignificant; nt, neural tube; nc, notochord; so, somite. One subset of myogenic precursor cells within the somite differentiates into epaxial musculature without migration, while the other suspends terminal differentiation, migrates into somatoplure (including limb buds) and then differentiates into appendicular musculature such as limb muscles. Exogenous FGF blocks the terminal differentiation in the somite but has no effect on either the migration into somatopleure or the subsequent differentiation in the limb. In striking contrast, constitutive expression of FGFR1, mediating the signals of endogenous FGFs present in the limb muscle mass, can block myogenic differentiation. Blocking the FGFR with a dominant negative mutant ΔFGFR1 induces premature myotomal muscle differentiation within the somite and blocks migration to limb buds.

triggers terminal muscle differentiation, including the formation of myotubes and the expression of muscle-specific genes. Constitutive expression of exogenous FGFs does block terminal differentiation in the somite but does not inhibit limb muscle differentiation since FGFR is already downregulated. In contrast, upregulated FGFR levels suspend terminal differentiation in the presence of FGF ligand in the muscle primordia of the limb. Inhibition of the FGFR with the truncation mutant ΔFGFR inhibits FGF signaling, leading to premature muscle differentiation within the somite as well as loss of the migratory subpopulations. The potential role of heparan sulphate proteoglycan, the low affinity FGF receptor (Olwin and Rapraeger, 1992), is not included in this summary. In addition to this receptor-coupled pathway, direct binding of ligand to the nucleus has been proposed as a part of the FGF signal cascade (Logan, 1990; Woodward et al., 1992). In support of our model, it has recently been shown that nuclear localization of FGFs is mediated by an internalization of the FGF-FGFR complex formed on the surface membrane (Amalric et al., 1994; Reliland and Rapraeger, 1993), thus levels of functional FGFR could regulate second message pathways at both the plasma membrane and at the nucleus, consistent with our observations. Roles for an FGFR-independent pathway of FGF signaling in myogenic cells remain to be established.

The results of the present study suggest that there must be a signal(s) that induces the downregulation of FGFR in the myogenic lineage in a temporally and regionally specific manner. A decline in FGFR synthesis is associated with in vitro myogenic differentiation of murine myogenic cell lines, as induced by FGF depletion in murine myogenic cell lines (Olwin and Rapraeger, 1992; Templeton and Hauschka, 1992). In contrast to the results obtained with murine cell lines, our data demonstrate rather weak coupling between FGFR expression and the level of ligand in avian myogenic cells. Furthermore, in developing limbs, FGF mRNAs and proteins are detected even after muscle fiber formation begins (Seed et al., 1988; Niswander and Martin, 1992; deLaperyriere et al., 1993; Fallon et al., 1994). Our data indicate that the levels of FGFs present in muscle-forming regions of the limb are high enough to activate FGFR. Taken together, these results strongly suggest the involvement of factor(s) other than the FGF ligand itself in the downregulation of FGFR in limb muscle. Recently, an interplay of FGF signaling with other signal cascades, such as insulin-like growth factor (IGF) and TGFβ family members, has been documented (Rosenthal et al, 1991; LaBonne and Whitman, 1994; Cornell and Kimelman, 1994). The IGF family, including IGFI and IGFII, stimulates spontaneous differentiation of myotubes in culture (Florini et al., 1991). Also, TGFβ induces myoblast differentiation in the presence of mitogen (Zentella and Massaqué, 1992), and the disruption of the TGFβ-signaling pathway in myogenic cells, with a dominant negative mutant of the type II TGFβ receptor, inhibits myoblast differentiation in culture by down-regulating MyoD expression (Filvaroff et al., 1994). It is known that interactions between early somites and axial structures such as the neural tube and notochord are important for induction of paraxial muscle formation in vertebrate embryos (Lipton and Jacobson, 1974; Emerson, 1993; Bober et al., 1994). Conceivably, an analogous factor(s) exists in the developing limb to induce differentiation of the limb muscle mass. These may include members of the TGFβ and/or IGF families but not the FGF ligand itself. The physiologic roles of those FGFs containing secretion signals and those lacking a secretion signal during terminal differentiation remain to be established. This study is the first to provide in vivo evidence that FGFR-mediated FGF signaling is a potential regulator of migration, proliferation and differentiation of myogenic cells during avian embryogenesis.

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