

Differentially Expressed Fibroblast Growth Factors Regulate Skeletal Muscle Development through Autocrine and Paracrine Mechanisms

Kevin Hannon,* Arthur J. Kudla, Michael J. McAvoy, Kari L. Clase, and Bradley B. Olwin*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907; and *Walther Cancer Institute, Indianapolis, Indiana 46208

Abstract. Several FGF family members are expressed in skeletal muscle; however, the roles of these factors in skeletal muscle development are unclear. We examined the RNA expression, protein levels, and biological activities of the FGF family in the MM14 mouse skeletal muscle cell line. Proliferating skeletal muscle cells express FGF-1, FGF-2, FGF-6, and FGF-7 mRNA. Differentiated myofibers express FGF-5, FGF-7, and reduced levels of FGF-6 mRNA. FGF-3, FGF-4, and FGF-8 were not detectable by RT-PCR in either proliferating or differentiated skeletal muscle cells. FGF-1 and FGF-2 proteins were present in proliferating skeletal muscle cells, but undetectable after terminal differentiation. We show that transfection of expression constructs encoding FGF-1 or FGF-2 mimics the effects of exogenously applied FGFs, inhibiting skeletal muscle cell differentiation and stimulating DNA synthesis.

These effects require activation of an FGF tyrosine kinase receptor as they are blocked by transfection of a dominant negative mutant FGF receptor. Transient transfection of cells with FGF-1 or FGF-2 expression constructs exerted a global effect on myoblast DNA synthesis, as greater than 50% of the nontransfected cells responded by initiating DNA synthesis. The global effect of cultures transfected with FGF-2 expression vectors was blocked by an anti-FGF-2 monoclonal antibody, suggesting that FGF-2 was exported from the transfected cells. Despite the fact that both FGF-1 and FGF-2 lack secretory signal sequences, when expressed intracellularly, they regulate skeletal muscle development. Thus, production of FGF-1 and FGF-2 by skeletal muscle cells may act as a paracrine and autocrine regulator of skeletal muscle development *in vivo*.

P RIMARY skeletal muscle cells and many skeletal muscle cell lines are repressed from terminal differentiation by FGFs. Nine members of the FGF family have been identified (FGF-1 through FGF-9). The hallmarks of this family include (a) high affinity for heparin or heparan sulfate; (b) two invariant conserved cysteine residues; and (c) an overall homology of 30% (Baird and Klagsbrun, 1991). While exogenously added FGFs repress differentiation of cultured skeletal muscle cells (Gospodarowicz et al., 1975; Linkhart et al., 1981; Olwin and Hauschka, 1986), the role of endogenously expressed FGFs, particularly those lacking signal peptide sequences, are not clear. FGF-2, FGF-4, FGF-5, FGF-6, and FGF-8 mRNA appear to be expressed in skeletal muscle cells as they have been localized to the myotomal muscle region of the somites and in the developing limb muscle masses (for review see Olwin et al., 1994). As the majority of localization studies have not delineated whether FGFs are ex-

pressed in proliferating or in differentiated muscle cells, we examined FGF expression patterns in mouse myoblasts. Moreover, localization of FGF mRNAs cannot identify functional roles for these proteins in the regulation of skeletal muscle development.

Of the three members of the FGF family (FGF-1, FGF-2, and FGF-9) that lack a classical secretory signal sequence, FGF-1 and FGF-2 are implicated in regulating skeletal muscle development. Although both have been proposed to be released from cells only under conditions of cell lysis and cell death (McNiel et al., 1989; D'Amore, 1990; Muthukrishnan et al., 1991), we determined if intracellularly expressed FGFs that lack classical signal sequences could regulate skeletal muscle growth and differentiation. We found that a skeletal muscle satellite cell line (MM14) expresses a number of FGF-family members that are developmentally regulated. In addition, transfection of MM14 cells with FGF-1 or FGF-2 expression vectors mimics exogenously applied FGFs by repressing differentiation and stimulating DNA synthesis. To regulate MM14 cell differentiation, transfected FGFs require export from the cell and a functioning high affinity FGF-binding complex. These data suggest that FGF-mediated regulation of skeletal muscle development *in vivo* may be complex, involving

Address all correspondence to B.B. Olwin, Department of Biochemistry, Purdue University, 233C Hansen Life Sciences Research Building, West Lafayette, IN 47907. Tel.:(317) 494-1665. Fax: (317) 496-1739. e-mail: olwin@biochem.purdue.edu

K. Hannon and A.J. Kudla contributed equally to the work described in this manuscript.

both paracrine and autocrine action of intracellularly produced FGFs that lack classical signal sequences.

Materials and Methods

Cell Culture

Proliferating adult mouse MM14 skeletal muscle cells (Linkhart et al., 1980) and differentiation-defective cells (DD)¹ (Lim and Hauschka, 1984) were cultured on gelatin-coated plates in growth medium consisting of Ham's F-10 (GIBCO BRL, Gaithersburg, MD) supplemented with human FGF-2 (purified from a yeast strain expressing human FGF-2 [Rappreger et al., 1994]), 0.8 mM CaCl₂, 100 U of penicillin G per ml, 5 µg of streptomycin sulfate per ml, and 15% horse serum. The concentration of FGF-2 added to the cells was increased from 0.3 to 2.5 nM with increasing cell density. Differentiation medium consisted of Ham's F-10 supplemented with 1 µM insulin (Collaborative Biomedical Products, Waltham, MA), 0.8 mM CaCl₂, 100 U of penicillin G per ml, 5 µg of streptomycin sulfate per ml, and 2% horse serum. As determined by immunostaining for myosin, >95% of the nuclei in differentiated MM14 cell cultures were present in myosin-positive cells after 48 h of incubation (data not shown). DD cells were derived from MM14 cells and exhibit a mitogenic response to exogenously added FGF-2. However, in contrast to the parental MM14 cells, these cells do not differentiate after FGF withdrawal. Less than 5% of the nuclei in DD cells were myosin positive after growth in differentiation medium.

RT-PCR

RNA was isolated from MM14 and DD cells cultured in proliferation medium and for 48 h in differentiation medium (Chomczynski and Sacchi, 1987). 10 µg of total RNA was added to reverse transcriptase buffer (GIBCO BRL) containing 1 mM 2'-deoxynucleoside 5'-triphosphates, 200 pmol of random hexamer primer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), and 10 mM DTT in a total volume of 38 µl. From this mixture, 19 µl was removed and 200 U (1 µl) of Moloney murine leukemia virus reverse transcriptase (RT, GIBCO BRL) was added and incubated at 37°C for 1 h. The remaining 19 µl was used as a nonreverse-transcribed control (no-RT) in a PCR reaction. After the incubation, the cDNA and no-RT mixtures were diluted to 100 µl.

For PCR amplification, 2 µl of cDNA or no-RT mixture was added to 18 µl of PCR buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl₂, and 0.001% gelatin) containing 250 µM 2'-deoxynucleoside 5'-triphosphates, 0.4 µCi [³²P]dCTP (Amersham Corp., St. Louis, MO, 3,000 Ci/mmol), 0.25 µM of forward primer (Table I), 0.25 µM of reverse primer (Table I), and 0.03125 U/µl of TAAQ DNA polymerase (Roche). Cycling parameters were denaturation at 95°C for 45 s, annealing at various melting temperatures (Table I) for 30 s, and elongation at 72°C for 1 min. After amplification for various cycle numbers, 10 µl of each PCR mixture was electrophoresed through 6% polyacrylamide gels. The gels were dried and an image was obtained using a phosphorimager (Bio-Rad Labs, Hercules, CA). The amount of ³²P-product amplified was examined to determine if PCR amplification was exponential. If a particular product was amplified exponentially, then the amount of PCR product was quantitated (Hannon et al., 1992). All PCR products were visible after agarose gel electrophoresis when stained with ethidium bromide. Labeling PCR products with ³²P was performed solely for quantitation. In cases where both MM14 and DD cells did not express a detectable RT-PCR product, cDNAs made from various stage embryonic mouse RNAs were used as positive controls (Table I). RNA isolation and RT-PCR was replicated twice. Approximately equal amount of product was amplified using 18 s ribosomal primers, ensuring that similar amounts of RNA were reverse transcribed and PCR-amplified to similar extents in all samples. No product was amplified in any no-RT controls.

Western Analysis

Proliferating and differentiated MM14 cells were washed twice with cold

1. *Abbreviations used in this paper.* CFR, cyteine-rich FGF receptor; CMV, cytomegalovirus; DD, differentiation-defective cells; dpc, days post coitum; FGFR, FGF receptor; RT, Moloney murine leukemia virus reverse transcriptase; TM, annealing temperature.

Table I. Primers, TMs, and Positive Controls Used in PCR Amplifications

	Forward/reverse primers	TM	Positive control
FGF-1	atggctgaagggagatc ctagtcaagaagacaccgg	65	MM14 myoblast cDNA
FGF-2	agcggcatcacctcgcttcc tggagaacagatggcctctgtcc	65	MM14 myoblast cDNA
FGF-3	atgccctctgattcatt caacctctgtctctaca	60	11 dpc mouse embryo head cDNA
FGF-4	gaccgccgcaccaacgg tcattgtaggcgacactc	55	BC3H1 myoblast cDNA
FGF-5	ctgatccacagcgcttgg agtcacctcgttaatttgg	55	Differentiated MM14 myofiber cDNA
FGF-6	gaacacacagaggagaacc cagtgcaatgtaggtccc	60	MM14 myoblast cDNA
FGF-7	atgcgcaatggatactg ttaggtattgcatatagg	55	MM14 myoblast cDNA
FGF-8	ggcaaggactcgctattc ctatcgggctccggggc	65	11 dpc mouse embryo whole body cDNA
18 s	tacctggtgatcctgcc aggttatctagatcacc	55	MM14 myoblast cDNA

Primer sequences, annealing temperatures (TMs), and cDNA used for positive controls in PCR amplifications as described in Materials and Methods.

phosphate-buffered saline and solubilized in lysis buffer (1% [vol/vol] Triton X-100, 25 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM EDTA, 2 mM PMSF, 1 µg/ml leupeptin) for 30 min at 4°C. Insoluble material was removed by centrifugation at 14,000 g for 15 min at 4°C, and the protein content of each cell lysate was determined by the bicinchoninic acid protein assay (Pierce Chem. Co., Rockford, IL). Proteins (25 µg) from cell lysates were separated in 12% polyacrylamide gels by 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% non-fat dry milk and 0.05% (vol/vol) Tween-20. FGF-1 and FGF-2 were detected using a polyclonal antiserum and monoclonal antibody (Savage et al., 1993), respectively. Bound anti-FGF antibodies were detected with the appropriate horseradish peroxidase-conjugated secondary antibody (Promega Corp., Madison, WI). Horseradish peroxidase was visualized by chemiluminescence using the Amersham ECL system.

DNA Constructs

α-Cardiac actin-luciferase is a luciferase reporter gene driven by a skeletal muscle specific α-cardiac actin promoter (gift of Steve Konieczny, Purdue University) described previously (Kudla et al., 1995). Cytomegalovirus (CMV)-*lacZ* is a *lacZ* gene controlled by a CMV promoter (Centre Commercial de Gros). pcFGF-1 is a mouse FGF-1 gene (Hébert et al., 1990) cloned into the EcoRV site of pcDNA3 (Invitrogen, San Diego, CA). pcbFGF-2 is a bovine FGF-2 gene encoding the 18-kD form of FGF-2 (Rogelj et al., 1988), cloned into the EcoRV site of pcDNA3. pcDNFR1 is the extracellular and transmembrane domain of the mouse FGF receptor-1 (FGFR1) (Yayon et al., 1991) containing an Xba linker stop codon inserted at the Ball site of the intracellular domain, and cloned into the EcoRV site of pcDNA3.

Transient Transfections

Proliferating MM14 cultures (5 × 10⁴ cells/100-mm plate) were passaged and plated 6–8 h before transfection. A calcium phosphate-DNA precipitate containing 1.5 µg of α-cardiac actin-luciferase reporter gene, 1 µg of an expression construct containing CMV-*lacZ*, and either 25 µg of pcFGF-1, pcbFGF-2, or control (pcDNA3) was prepared in 0.55 ml of Hepes-buffered saline (25 mM Hepes [pH 7.05], 140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, 6 mM dextrose) containing 0.11 M CaCl₂. The cells were incubated with 0.5 ml of the precipitate for 20 min before the addition of growth medium and 0.5 nM FGF-2. After 4 h, the cells were osmotically shocked for 2.5 min with 15% glycerol in Hepes buffered saline. Growth medium with or without exogenous FGF-2 was then added back to the cells. A portion of the cells receiving the pcDNA3 control vector was sup-

plemented with either exogenous FGF-2 (0.3–2.5 nM with increasing cell density) or 0.3 nM human recombinant FGF-7 (Jeffery Rubin, National Cancer Institute, Bethesda, MD). Luciferase and β -galactosidase activity were analyzed 36 h after the osmotic shock in a Berthold Lumat luminometer using the luciferase (Promega) and Galacto-Light Plus (TROPIX) assay systems, respectively, with the exception that 2 mM PMSF and 1 μ g of leupeptin per ml were added to the solubilization buffers. Normalization of transfection efficiency was performed by correcting the luciferase activity for the levels of β -galactosidase present in each assay. Each transient transfection was replicated twice with treatments run in triplicate.

Single-Cell Fusion Studies

Proliferating MM14 cells were transfected as described above with 1 μ g CMV- β -gal and 25 μ g pcDNA3, 25 μ g pcFGF-1, or 25 μ g pcbFGF-2. After the osmotic shock, growth medium with or without exogenous FGF-2 was added back to the cells. 60 h postglycerol shock, cells were rinsed with PBS, fixed for 5 min with 0.5% glutaraldehyde in PBS, rinsed twice more with PBS and stained for β -galactosidase (1 mg per ml 5-bromo-4-chloro-3-indolyl- β -D-galacto pyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ in PBS at 37°C for 16 h). The number of β -galactosidase positive nuclei that fused into multinucleated (three or more nuclei) myotubes was then scored. A minimum of 300 cells/plate was scored for each assay point. Each single cell fusion assay experiment was repeated twice.

Analysis of DNA Synthesis

Proliferating MM14 cells were transfected as described above with 1 μ g CMV-*LacZ* and 25 μ g pcDNA3, 25 μ g pcFGF-1, or 25 μ g pcbFGF-2. After osmotic shock, growth medium with or without FGF-2 was added to the cells. One group of cells receiving pcDNA3 control vector was supplemented with exogenous FGF-2, while others received complete growth medium (15% horse serum) but no exogenously applied FGF. 24 h after the osmotic shock, methyl-³H]thymidine (New England Nuclear, Boston, MA; model NET027Z) was added to each plate to a final concentration of 2 μ Ci/ml. After 12 h of additional incubation, cells were fixed with 0.5% glutaraldehyde in PBS and stained for β -galactosidase as described above. After β -galactosidase staining, plates were coated with NTB-2 nuclear emulsion (KODAK), exposed for 1 wk, and developed according to instructions of the manufacturer. The number of β -galactosidase and [³H]thymidine-positive cells was then scored using bright-field microscopy. This assay was repeated twice.

In a second study, 6–8 h before transfection, proliferating MM14 cells were plated at 5×10^4 cells/100-mm plate in 0.7 nM FGF-1 (Olwin and Hauschka, 1986). Cells were then transfected as described above with 1 μ g CMV-*LacZ* and 25 μ g pcDNA3, or 25 μ g pcbFGF-2. After osmotic shock, growth medium with or without FGF-2 was added. In addition, 30 μ l of ascites fluid containing anti-FGF-2 antibodies (Savage et al., 1993) or anti-cysteine-rich fibroblast growth factor receptor (CFR) antibodies (Burrus et al., 1992) was added. Ascites fluid and/or exogenous FGF-2 were added to the cells every 12 h. Cells were supplemented with methyl-³H]thymidine, stained for β -galactosidase, coated with NTB-2 nuclear emulsion, and scored using bright-field microscopy as described above.

Results

To better understand the role(s) that FGFs play in skeletal muscle development, we analyzed the RNA expression, protein levels, and biological activity of FGF family members in MM14 cells. In these cells, FGF mRNAs are undetectable by Northern analysis (data not shown). Therefore, we examined the relative levels of FGF expression in MM14 cells by RT-PCR. Proliferating cells expressed FGF-1, FGF-2, FGF-6, and FGF-7 (Fig. 1). Differentiated cells express FGF-5 and FGF-7, while FGF-6 expression was reduced and expression of FGF-1 and FGF-2 was undetectable (Fig. 1). The expression of FGF-family members in the MM14 cells was compared to expression patterns in differentiation-defective (DD) cells. The latter cells are a variant of the MM14 cell line that fails to differ-

entiate (Lim and Hauschka, 1984). FGF-1, FGF-2, FGF-6, and FGF-7 expression was detected both in proliferating and differentiated DD cells (Fig. 1). Moreover, FGF-5 expression was not upregulated in DD cells cultured in differentiation medium (Fig. 1). Thus, regulation of FGF-1, FGF-2, FGF-5, and FGF-6 expression in MM14 cells is associated with skeletal muscle differentiation. The PCR products for FGF-1, FGF-2, and FGF-7 were first detectable at lower cycle numbers in DD cells than in MM14 cells, suggesting that more mRNA is present for these products in DD cells. FGF-1 was first detectable in DD cells after

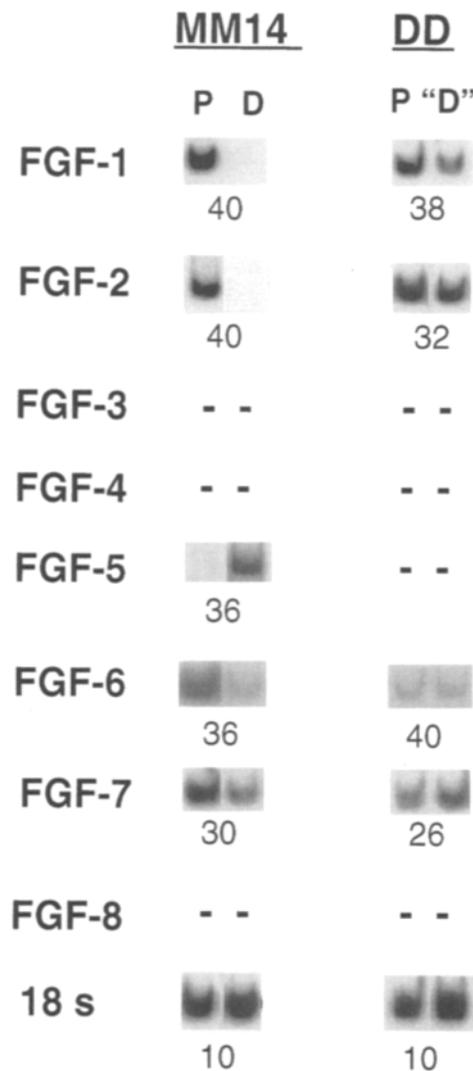


Figure 1. RT-PCR analysis of FGF-family and 18 s ribosomal RNA in MM14 and differentiation-defective (DD) cells. 5 μ g of total RNA from either proliferating MM14 (P), proliferating DD (P), differentiated MM14 (D), or mock-differentiated DD ("D") cells was reverse transcribed and 1/40 of this mixture PCR amplified with [³²P]dCTP. After amplification for various cycles (cycle titration) to ensure amplification was exponential, products were electrophoresed through 6% polyacrylamide gels, exposed to film and quantified on a BioRad phosphorimager. Representative results from each amplification are shown. The number of cycles of amplification for each product pair are indicated. (–) indicates RNA expression was not detected. RNA isolation and RT-PCR was replicated twice with similar results.

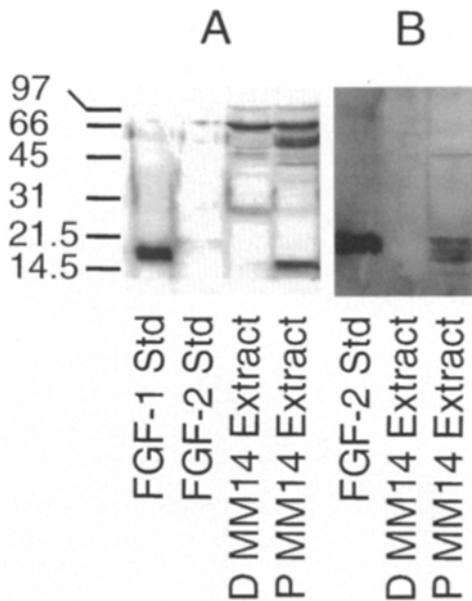


Figure 2. FGF-1 and FGF-2 protein are present in proliferating MM14 cells. Cell lysates (25 μ g total protein) from proliferating (*P MM14 Extract*) and differentiated (*D MM14 Extract*) MM14 cells were analyzed by Western blotting with (A) a polyclonal anti-FGF-1 antibody and (B) a monoclonal anti-FGF-2 antibody as described in Materials and Methods. FGF-1 and FGF-2 Std are purified bovine FGF-1 and human recombinant 18 kD FGF-2, respectively. Molecular mass standards (kD) are indicated.

only 32 cycles while it was not detected in MM14 cells until after 38 cycles, FGF-2 was first observed in DD cells after 34 cycles compared to detection in MM14 cells after 40 cycles, and FGF-7 was detectable after 26 cycles in DD cells and not until after 30 cycles in MM14 cells (data not shown). Expression of FGF-3, FGF-4, or FGF-8 was not detectable in either MM14 or DD cells (Fig. 1). Expression of FGF-9 was not examined as the mouse FGF-9 cDNA sequence was not available for PCR primer design.

Western blot analysis of FGF-1 and FGF-2 proteins in proliferating MM14 cell extracts identified a major FGF-1 protein migrating at 14 kD and three FGF-2 proteins migrating at 17.5, 19.5, and 21 kD (Fig. 2). The anti-FGF antibodies are specific for the FGF family member towards which they were generated. The anti-FGF-1 polyclonal antibodies were specific for FGF-1 as no cross reactivity was detected to FGF-2 (Fig. 2). The monoclonal anti-FGF-2 antibodies are FGF-2 specific (Savage et al., 1993). Higher molecular weight proteins recognized by both the anti-FGF-1 and anti-FGF-2 antibodies are either proteins recognized nonspecifically by the antibodies or proteins that share an epitope with FGFs. Neither FGF-1 nor FGF-2 was detected 4 d after terminal differentiation (Fig. 2). The three detected isoforms of FGF-2 have been described previously and most likely correspond to alternate upstream translational initiation of FGF-2 (Florkiewicz and Sommer, 1989; Prats et al., 1989; Renko et al., 1990). The biological role(s) of the multiple FGF-2 isoforms has not been established.

The presence of FGF-1 and FGF-2 protein in proliferating MM14 cells was unexpected as these cells are dependent on exogenously supplemented FGFs. As it seemed

unlikely that these proteins were biologically active, especially due to their lack of signal sequences, we designed an experiment to address the role(s) of these FGFs in regulation of myogenesis. Proliferating MM14 cells were transiently cotransfected with a series of expression vectors encoding a skeletal muscle differentiation-specific reporter (luciferase controlled by the α -cardiac actin promoter), FGF-1, the 18-kD form of FGF-2 and β -galactosidase. In cells transfected with the differentiation-specific reporter and cultured without FGFs, luciferase activity is enhanced severalfold, indicative of skeletal muscle cell differentiation (Fig. 3 A). Addition of exogenous FGF-2 or transfection of proliferating MM14 cells with expression vectors encoding FGF-1 or FGF-2 inhibits the differentiation-specific increase in luciferase activity (Fig. 3 A). Similar results were obtained using a luciferase reporter gene controlled by the troponin-I differentiation-specific *cis*-acting regulatory elements (data not shown). Addition of FGF-7 protein to the tissue culture medium had no effect on the differentiation-specific increase in luciferase activity, consistent with the observation that MM14 cells do not respond to FGF-7 (Olwin and Rapraeger, 1992; Patrie et al., 1995) nor express FGFR-2 (Templeton and Hauschka, 1992), a splice variant of which binds FGF-7.

Transfection of MM14 cells with expression vectors encoding either FGF-1 or FGF-2 mimics exogenously added FGF-2. Since exogenously added FGF-2 requires high affinity cell surface binding sites to repress terminal differentiation (Olwin and Rapraeger, 1992), we determined if intracellularly produced FGF-1 or FGF-2 requires a functioning high affinity cell surface-binding site to repress myogenesis. As one component of a high affinity-binding site is an FGF receptor tyrosine kinase, an expression vector encoding a truncated FGF receptor-1 that functions as a dominant negative mutant was cotransfected with expression vectors encoding FGF-1 or FGF-2. Cotransfection of the construct encoding the dominant negative mutant abrogated the activity of exogenously applied or intracellularly expressed FGFs (Fig. 3 B). In addition, when the dominant negative mutant construct and FGF-expression construct were cotransfected the differentiation-specific reporter gene and CMV-*LacZ* were activated to levels similar to that observed in cells transfected with a control vector alone. These results demonstrate that transfection of an FGF-1 or FGF-2 expression construct does not specifically promote cell death and that cell death or lysis is unlikely to be responsible for release of these FGFs.

Since the transient transfection assays measure only reporter gene activity, we performed single cell analyses to determine the effects of transient transfection of FGF-1 and FGF-2 on cell fusion. Transfected cells were identified by expression of β -galactosidase from a cotransfected plasmid encoding *lacZ*. The nuclei from cells transfected with *lacZ* were blue and easily discernible from nontransfected cell nuclei in myotubes, allowing the analysis of an individual cell's developmental fate (Fig. 4). MM14 myoblasts cotransfected with control DNA and cultured in the absence of FGFs were used to determine the maximal myoblast fusion index (\sim 35% of total β -galactosidase positive nuclei were in multinucleated myotubes after 60 h). Cotransfection of *lacZ* with FGF-1 (data not shown) or FGF-2 expression vectors inhibited myoblast fusion (Fig. 4).

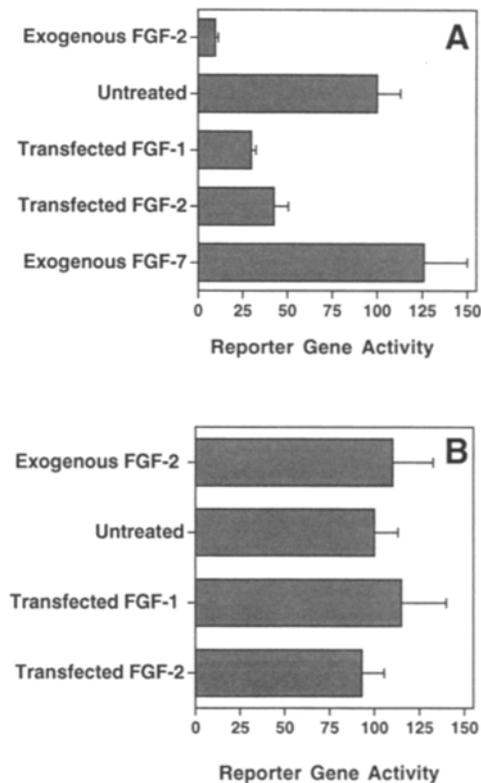


Figure 3. Intracellularly expressed FGF-1 and FGF-2 require a functioning high affinity tyrosine kinase receptor to inhibit skeletal muscle-specific gene expression in MM14 cells. (A) Proliferating MM14 cells were transiently cotransfected with an α -cardiac actin-luciferase reporter construct, a *LacZ* expression construct, and either a control vector, an FGF-1 expression vector, or an FGF-2 expression vector. After transfection with the control vector, cells were cultured in the presence of exogenously supplemented FGF-2 (Exogenous FGF-2), exogenously supplemented FGF-7 (Exogenous FGF-7), or no additions (Untreated). Cells transfected with expression vectors encoding FGF-1 (Transfected FGF-1) or FGF-2 (Transfected FGF-2) were incubated for 36 h in the absence of exogenous FGF. After a 36-h incubation, luciferase activity was determined and normalized to β -galactosidase activity as described in Materials and Methods. (B) Transfections and growth conditions were identical to A except that all cells were cotransfected with an expression vector encoding a truncated FGFR1. Error bars represent standard deviation of triplicate points. All experiments were replicated twice with similar results.

To completely reproduce the effects of exogenously applied FGF, cells transfected with FGF expression constructs should be proliferated as well as repressed from terminal differentiation. Therefore, a single cell analysis of DNA synthesis was performed. Transfected cells were identified by β -galactosidase expression and DNA synthesis was analyzed by scoring the number of [3 H]thymidine-positive cells. We examined cultures transiently transfected with expression constructs encoding FGF-1 or FGF-2, or cells cultured in the presence of exogenously applied FGF-2. Control cultures not supplemented with FGF exhibit only a low level of [3 H]thymidine incorporation indicating the majority of cells withdrew from the cell cycle (Fig. 5, A and E). Cells cultured in the presence of exogenous FGF-2

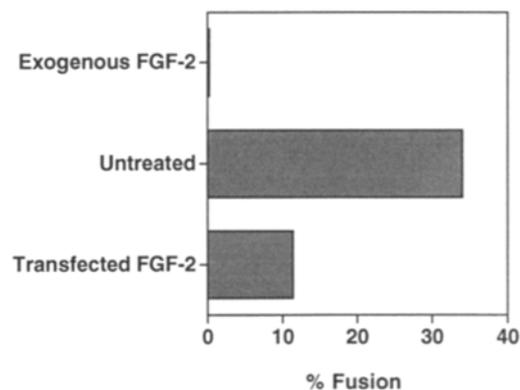


Figure 4. Transfection of an FGF-2 expression construct inhibits fusion of MM14 myoblasts. Proliferating MM14 cells were cotransfected with expression vectors containing the *LacZ* gene and an FGF-2 cDNA or a vector control. Cells cotransfected with a *LacZ* expression vector and a control vector were cultured in the presence of exogenously supplemented FGF-2 (Exogenous FGF-2) or with no additions (Untreated) for 60 h. Cells cotransfected with a *LacZ* expression vector and the FGF-2 cDNA expression vector (Transfected FGF-2) were incubated for 60 h in the absence of exogenous FGF. After the 60-h incubation cells were stained for β -galactosidase and the number of stained cells that fused into multinucleated myofibers (three or more nuclei) was counted. A minimum of 300 cells/plate were scored. The experiment was replicated twice with similar results.

exhibited a maximal level of [3 H]thymidine incorporation (Fig. 5, B and E). Cells transfected with FGF-1 or FGF-2 expression vectors exhibited high levels of [3 H]thymidine incorporation (Fig. 5, C–E).

Unexpectedly, transfection of FGF-1 or FGF-2 exerted a global effect on neighboring cells. Although 15% of the cell population was transfected as determined by β -galactosidase staining, DNA synthesis was observed in >50% of the nontransfected cells (Fig. 5). The maximum number of cells transfected was 15% after transfection of either 0.5, 1.0, 5.0, 10, or 30 μ g of CMV-*LacZ* (data not shown). These data demonstrate that the number of FGF-transfected cells does not exceed 15% and cannot account for the global increase in DNA synthesis. These data indicate that protein products of the FGF expression vectors are released from the transfected cells and stimulate DNA synthesis in surrounding cells. We confirmed this hypothesis by demonstrating that the increase in DNA synthesis was blocked when monoclonal anti-FGF-2 antibodies were added to the medium of cells transfected with an FGF-2 expression vector. Maximal levels of [3 H]thymidine were observed in cells transfected with a control vector and not treated with anti-FGF-2 antibody, while cells transfected with an FGF-2 expression vector exhibited high levels of [3 H]thymidine incorporation (Fig. 6). However, in the presence of a monoclonal anti-FGF-2 antibody, neither cells treated with exogenous FGF-2 nor those transfected with FGF-2 incorporated [3 H]thymidine (Fig. 6). This effect was specific as an avian-specific anti-CFR monoclonal antibody had no effect (Fig. 6). These results demonstrate that intracellularly produced FGFs exported from the cell mimic the biological activities of exogenously applied FGFs.

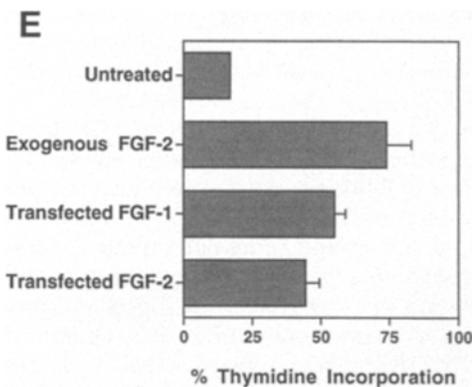
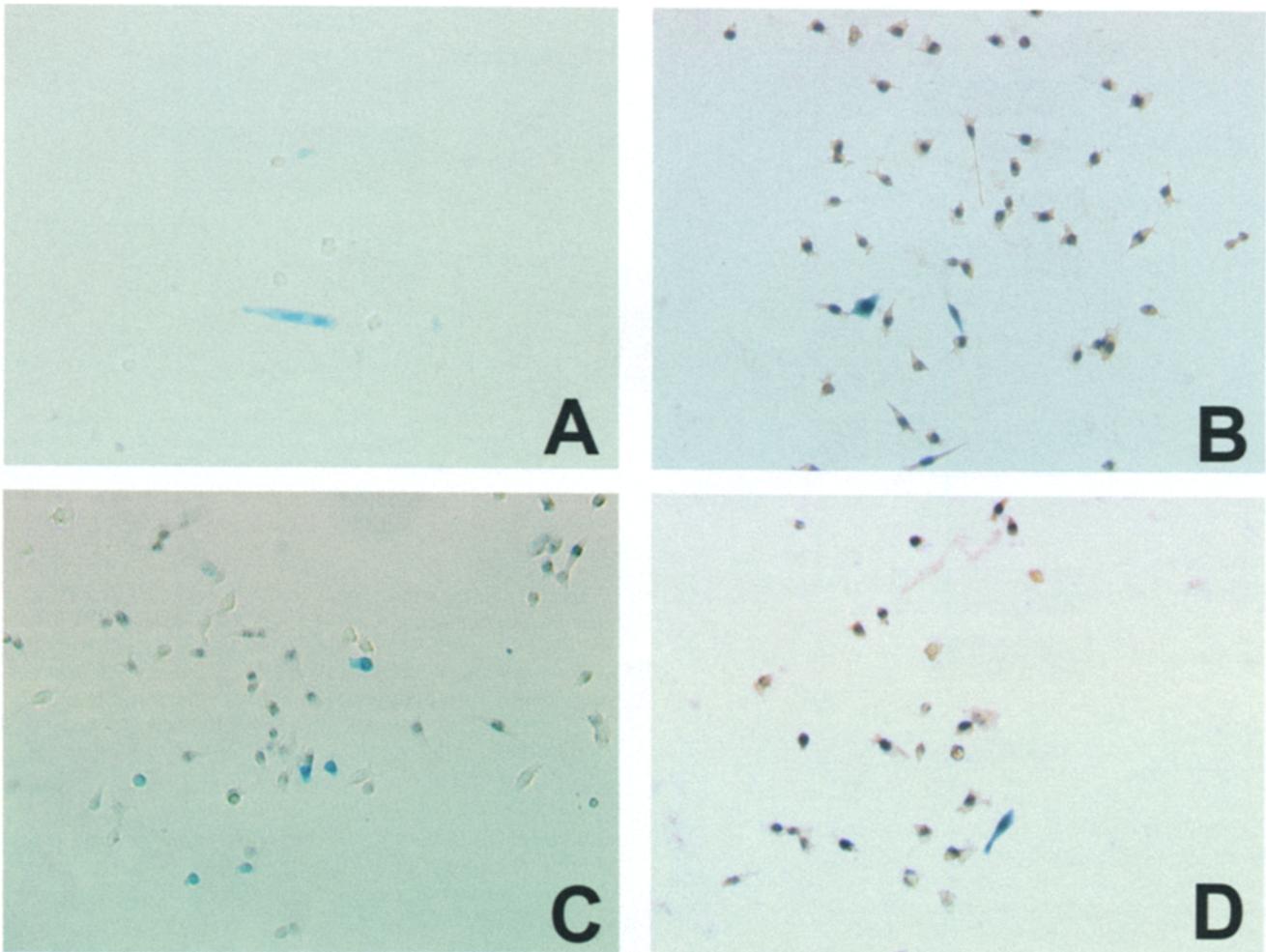


Figure 5. Intracellular production of FGF-1 and FGF-2 stimulate DNA synthesis in MM14 myoblasts. Cells were cotransfected with expression constructs encoding β -galactosidase and FGF-1, FGF-2, or a control and then cultured in the presence or absence of exogenously added FGF-2. 24 h after transfection, [^3H]thymidine was added and the cells were incubated for an additional 12 h. Cells were then fixed, stained for β -galactosidase, and exposed to an autoradiographic emulsion. The number of [^3H]thymidine-positive MM14 myoblasts was scored. Control cells not supplemented with FGFs (*A*) withdrew from the cell cycle and exhibited low levels of [^3H]thymidine incorporation. Cells transfected with a control vector and treated with exogenous FGF-2 (*B*) stimulated maximal [^3H]thymidine incorporation. The number of cells transfected with either FGF-1 (*C*) or FGF-2 (*D*) that incorporated [^3H]thymidine was similar to that observed for cells treated with exogenous FGF-2. A quantitative summary of two separate experiments is shown in *E*. Approximately 15% of the cells were transfected as determined by the blue β -galactosidase stain. A minimum of 500 cells/plate was scored. The experiment was replicated twice with similar results.

Discussion

A number of factors that regulate terminal differentiation of skeletal muscle cells *in vitro* have been identified (for review see Olwin et al., 1994). The role(s) of these factors in the development or regeneration of skeletal muscle *in vivo* is unknown, as a direct involvement of any of these factors in induction, growth, maintenance, or regeneration of skeletal muscle *in vivo* has not been shown. Members of the FGF family are likely to be critical regulators of skele-

tal muscle development *in vivo* as a number of FGF family members and FGF receptors are (*a*) localized to skeletal muscle (Joseph-Silverstein et al., 1989; Orr-Urtreger et al., 1991; Niswander and Martin, 1992; Peters et al., 1992; de Lapeyrière et al., 1993; Han and Martin, 1993; Savage et al., 1993; Savage and Fallon, 1995); (*b*) present in high levels in diseased and regenerating skeletal muscle (DiMario and Strohman, 1988; DiMario et al., 1989; Garrett and Anderson, 1995); and (*c*) required for the maintenance of primary mouse and chick skeletal muscle cultures (Linkhart

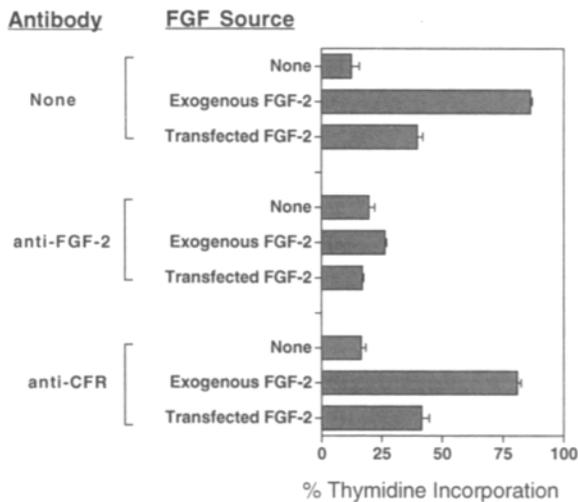


Figure 6. Intracellularly produced FGF-2 requires cell export in order to stimulate DNA synthesis in MM14 myoblasts. Cells were cotransfected with expression constructs encoding β -galactosidase and FGF-2 or a control vector and then cultured in the presence or absence of exogenously added FGF-2. After transfection, cells were treated every 12 h with either a monoclonal anti-FGF-2 antibody, a monoclonal anti-CFR antibody, or no antibody. 24 h after transfection, cells were incubated with [3 H]thymidine for 12 h. Cells were then fixed, stained for β -galactosidase, and exposed to an autoradiographic emulsion. The number of [3 H]thymidine-positive MM14 myoblasts was scored. Cells supplemented with either no antibody or with anti-CFR antibody incorporated [3 H]thymidine when they were supplied with exogenous FGF or were transfected with an FGF-2 expression vector. [3 H]Thymidine incorporation was reduced in the presence of an anti-FGF-2 antibody when cells were supplied with exogenous FGF-2 or were transfected with an FGF-2 expression vector. Approximately 15% of the cells were transfected as determined by the blue β -galactosidase stain. A minimum of 500 cells/plate were scored. The experiment was replicated twice with similar results.

et al., 1980, 1981; Kardami et al., 1985a,b; Seed and Hauschka, 1988; Rando and Blau, 1994). The roles of FGF-1 and FGF-2 as *in vivo* regulators of skeletal muscle development have been questioned as both factors lack signal secretory sequences that normally allow secretion via the classical secretory pathway. A number of investigators have postulated that these FGFs are released only during cell lysis and are stored in the extracellular matrix until they are to be used (Burgess and Maciag, 1989; Klagsbrun and Edelman, 1989; D'Amore, 1990; Baird and Klagsbrun, 1991). Recent data suggest that FGFs may be released from living cells although the biological role(s) of the growth factors in the cell lines examined is not clear (Mignatti and Rifkin, 1991; Jackson et al., 1992; Mignatti et al., 1992; Maciag et al., 1994; Bikfalvi et al., 1995; Florkiewicz et al., 1995; Jackson et al., 1995).

We have examined the expression and biological activities of FGF-1 and FGF-2 in an FGF-dependent skeletal muscle cell line derived from adult mouse satellite cells. The MM14 cell line expresses a variety of different FGFs in developmentally regulated patterns. These changes in FGF expression are not simply due to removal of exogenous FGF-2 and reduction of serum to 2% (the conditions

used for differentiation), as the levels of FGF-1, FGF-2, and FGF-6 do not decline in DD cells cultured in differentiation medium as they do in differentiated MM14 cells. In addition, FGF-5 is not upregulated in DD cells as it is in differentiated MM14 cells. If 5% of the DD cells did differentiate, we would have expected a low level of FGF-5 expression in the DD cells grown in differentiation conditions. Typically DD cell differentiation was <5%, indicating that the expression of FGF-5 was below detectable limits. Although possible, it is unlikely that the DD cells that express skeletal muscle myosin heavy chain are distinct from differentiated MM14 cells in their FGF expression patterns. The higher level of FGF-1, FGF-2, and FGF-7 expression in differentiation-defective cells is consistent with a direct role for the involvement of these FGFs in regulating skeletal muscle differentiation.

Although the levels of FGF-1 and FGF-2 mRNA were extremely low in MM14 cells, FGF-1 and FGF-2 protein were detectable by Western analysis. FGF-2 protein detected by Western analysis is unlikely to be due to contaminating human recombinant FGF-2 used for maintenance of cell growth as (a) FGF-1 protein was also detected by Western analysis in proliferating cells fed with FGF-2; (b) FGF-2 protein was present in cells fed with exogenously applied FGF-1 (data not shown); (c) three forms of FGF-2 were observed and two of them migrated at a relative molecular weight distinct from exogenously supplied recombinant FGF-2; (d) loss of mRNAs for FGF-1 and FGF-2 correlates with loss of protein; and (e) the extraction procedure used to isolate proteins for Western analysis would not release exogenously applied FGFs bound to heparan sulfate. Despite the presence of detectable FGF-1 and FGF-2 protein, MM14 cells remain absolutely dependent on exogenously supplied FGFs. These data suggest that FGF supplied by both paracrine and autocrine mechanisms may be critical for maintenance of myoblast growth.

To test if FGF supplied by an autocrine loop could support MM14 growth, cells were transiently transfected with expression vectors encoding FGF-1 or FGF-2. Expression of either factor blocked terminal differentiation and induced DNA synthesis in transfected cells. Unexpectedly, a global effect was observed as the majority of untransfected cells were also inhibited from terminal differentiation and stimulated to synthesize DNA. This activity required a functional high affinity FGF-binding complex as a dominant negative FGF receptor mutant inhibited the activity. Moreover, an anti-FGF-2 antibody blocked the ability of transfected FGF-2 to stimulate DNA synthesis. Therefore, the factor released from the FGF-2 transfected cells that acts on surrounding cells to repress myogenesis and to stimulate DNA synthesis is likely to be FGF-2. Other recent data have also implicated an autocrine acting FGF-1 as an important regulator of the differentiation of the Sol 8 skeletal muscle cell line. However, it was not determined if the FGF-1 was acting intracellularly or extracellularly or if FGF-1 activity was dependent on FGF receptor tyrosine kinases (Fox et al., 1994). In our studies, the export of intracellular FGF-1 or FGF-2 is not likely to be by cell death or lysis as several experimental results indicate the cells transfected with FGF expression vectors are not dying. First, the level of β -galactosidase activity is similar in cells transfected with a control expression vector or an FGF ex-

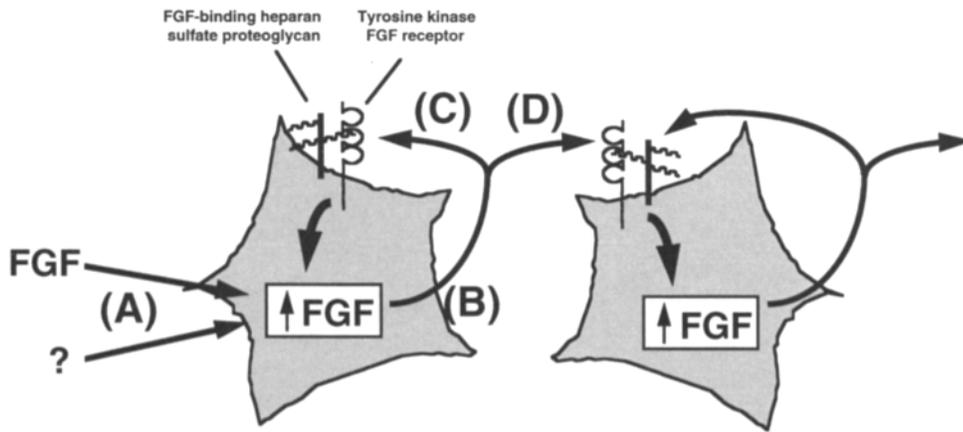


Figure 7. A model for regulation of myogenesis involving autocrine and paracrine action of FGFs. (A) Paracrine-mediated stimulation of intracellular FGF production by FGFs or unidentified factors. (B) Intracellular FGF is exported from the muscle cell and binds an FGF signaling complex on the cell surface acting as an autocrine (C) or paracrine (D) factor stimulating FGF production. An FGF positive-feedback loop is initiated by an exogenous factor and maintains myoblast proliferation.

Disruption of the autostimulatory loop and consequent terminal differentiation could be accomplished by inactivation of the FGF signaling complex, by inhibition of FGF synthesis or by inhibition of FGF export.

pression vector. Second, cells cotransfected with expression vectors encoding FGF and the dominant negative mutant receptor differentiated as well as the control cells. Third, cells transfected with an FGF expression vector differentiated when cultured with an anti-FGF-2 antibody. Recent data have demonstrated that FGF-2 can be exported from cells via an uncharacterized pathway independent of the Golgi (Bikfalvi et al., 1995; Florkiewicz et al., 1995). It is likely that this pathway operates in skeletal muscle cells.

We favor a model whereby intracellularly produced FGFs, particularly those FGFs lacking classical signal peptide sequences, are exported and act via an autocrine loop (Fig. 7). Exported FGFs would also function as paracrine regulators of skeletal muscle cells and would stimulate a positive-feedback loop for FGF production and release (Fig. 7 D). A similar positive-feedback loop may be intact in the Sol 8 skeletal muscle cell line. These cells synthesize sufficient endogenous FGF to support growth and thus do not require supplemental FGF. After transfection with an antisense FGF-1 construct, this FGF positive-feedback loop is disrupted and the Sol 8 cell line acquires an absolute dependency for exogenously applied FGF that is indistinguishable from the MM14 cell FGF requirement (Fox et al., 1994). There are four possibilities that may account for why this FGF positive-feedback loop is dysfunctional in some skeletal muscle cells such as MM14 cells. (1) Cells are unable to produce sufficient intracellular FGF to support growth; (2) cells differ in their efficiency of FGF-1 or FGF-2 export; (3) loss of receptor signaling complexes; and (4) FGFs may be posttranslationally modified so they are inactive. Consistent with the third hypothesis are the observations that FGF receptors are undetectable in differentiated skeletal muscle cell cultures (Olwin and Hauschka, 1988; Moore et al., 1991; Templeton and Hauschka, 1992) and in differentiated skeletal muscle tissue (Orr-Urtreger et al., 1991; Peters et al., 1992). However, we favor one or both of the first two hypotheses, as transfection of an FGF-1 or FGF-2 expression construct increases intracellular FGF, bypasses the requirement for exogenously supplied FGF, and initiates a functioning FGF positive-feedback loop. Many primary skeletal muscle cell cultures from neonates or adults also exhibit a dependency on ex-

ogenously supplied FGFs (Linkhart et al., 1980; Allen et al., 1984; Rando and Blau, 1994) while embryonic cultures seldom display an absolute dependency on supplemented FGF (Seed and Hauschka, 1988). If both responding cell types were present in developing skeletal muscle, our model provides a mechanism for how asynchronous differentiation might occur during expansion of the premuscle masses in the developing limb. A similar model proposes that TGF- β regulates growth of primary and secondary fibers in vivo (Cusella-DeAngelis et al., 1994). In summary, our data demonstrate that complex mechanisms involving both autocrine and paracrine regulation by FGFs are likely to affect the ultimate fate of a myoblast: to divide or not to divide.

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