

Control of Myoblast Proliferation with a Synthetic Ligand*

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Skeletal myoblast grafts can form contractile tissue to replace scar and repair injured myocardium. Although potentially therapeutic, generating reproducible and sufficiently large grafts remains a challenge. To control myoblast proliferation *in situ*, we created a chimeric receptor composed of a modified FK506-binding protein (F36V) fused with the fibroblast growth factor receptor-1 cytoplasmic domain. Mouse MM14 myoblasts were transfected with this construct and treated with AP20187, a dimeric F36V ligand, to induce receptor dimerization. Transfected myoblasts proliferated in response to dimerizer (comparable with basic fibroblast growth factor (bFGF) treatment), whereas the dimerizer had no effect on non-transfected cells. Similar to bFGF treatment, dimerizer treatment blocked myotube formation and myosin heavy chain expression and stimulated mitogen-activated protein (MAP) kinase phosphorylation in transfected cells. Non-transfected cells differentiated normally and showed no MAP kinase phosphorylation with dimerizer treatment. Furthermore, myoblasts treated with dimerizer for 30 days in culture reduced MAP kinase phosphorylation, withdrawn from the cell cycle, and differentiated normally upon drug withdrawal, demonstrating reversibility of the effect. Thus, forced dimerization of the fibroblast growth factor receptor-1 cytoplasmic domain reproduces critical aspects of bFGF signaling in myoblasts. We hypothesize that *in vivo* administration of AP20187 following myoblast grafting may allow control over graft size and ultimately improve cardiac function.

Skeletal myoblast transplantation is a promising technique that may allow delivery of therapeutic genes (1) and can serve as a source of contractile muscle to repair damaged tissue, such as infarcted myocardium (2–5) and dystrophic skeletal muscle (6, 7). The success of such cell-based therapies is dependent on transplanting an appropriate number of myoblasts to elicit the desired therapeutic effect, which remains a significant challenge. One strategy for improving the outcome of myoblast grafting involves the implantation of a smaller number of cells initially, followed by *in vivo* expansion using mitogenic factors to increase graft size and improve integration with host tissue.

Basic fibroblast growth factor (bFGF)¹ induces proliferation of skeletal myoblasts and prevents differentiation and fusion into multinucleated myotubes (8). Thus its administration may be envisioned as a method for inducing grafted myoblast proliferation. However, bFGF can also stimulate proliferation of non-graft cells such as tissue fibroblasts, which may lead to fibrosis. A method for inducing proliferation selectively in the grafted cells would facilitate myoblast expansion without these undesired effects.

The myoblast proliferative response to bFGF is mediated through dimerization of fibroblast growth factor receptor-1 (FGFR-1, Refs. 9 and 10) and subsequent activation of intracellular signaling molecules. Although the exact mechanism by which this signal is transduced is not completely understood, it has been demonstrated that activation of the MAP kinase pathway is necessary for cell cycle progression (11) in MM14 cells. However, FGFR-1 dimerization leads to the activation of a number of other signaling molecules including phospholipase C γ (PLC γ) and Src, which may interact with proteins of the MAPK pathway to affect mitogenesis as well as chemotaxis and cell survival (12–14).

Recently, an alternative approach to activating receptor signaling has been developed in which the intracellular domain of a receptor is fused with a drug binding domain, followed by expression of the chimeric receptor in the target cell type (15). Treatment of the transfected cells with a bivalent drug results in forced dimerization of the receptor signaling domains in the absence of growth factor. This technique has been applied successfully to induce expansion of genetically modified hematopoietic stem cells expressing chimeric cytokine receptors *in vitro* (16–18) and *in vivo* (19) following bone marrow transplantation.

To generate a cell line in which FGFR-1 signaling occurs independently of bFGF binding, we transfected mouse MM14 skeletal myoblasts with a retroviral vector encoding a chimeric receptor consisting of a modified FK506-binding protein (FKBP) domain, F36V (20), fused with the cytoplasmic domain of FGFR-1. The F36V domain contains a binding site for a bivalent synthetic ligand, AP20187 (ARIAD Pharmaceuticals, Cambridge, MA). In the present study, we demonstrate that addition of AP20187 to MM14 cells stably expressing the fusion receptor stimulates myoblast proliferation, blocks differentiation into myotubes, and mimics bFGF signaling by activating the MAP kinase pathway.

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¹ The abbreviations used are: bFGF, basic fibroblast growth factor; FGFR-1, fibroblast growth factor receptor-1; MAP, mitogen-activated protein; FKBP, FK506-binding protein; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; BrdUrd, bromodeoxyuridine; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; PLC γ , phospholipase C γ ; PDGF, platelet-derived growth factor; MAPK, MAP kinase.

EXPERIMENTAL PROCEDURES

Materials—bFGF was kindly donated by Scios Inc. (Sunnyvale, CA). AP20187 was provided by ARIAD Pharmaceuticals Inc.

F36Vfgfr-1 Plasmid Construction—The FGFR-1 cytoplasmic domain was amplified by polymerase chain reaction from a rat FGFR-1 β cDNA template (B. J. Ballermann and co-workers, Ref. 21; S540048) using *Taq* polymerase (Promega) and primers flanking the cytoplasmic domain (sense: 5'-GGGGGGTTCGACAAGATGAAGAGCGGCACCAAGGAAGA-3' and antisense: 5'-GGGGGGTTCGACGCGCGTTTGTAGTCCACCATTTGCA-3') to which *Sac*I restriction sites were added (underlined). Following amplification, the fragment was digested with *Sac*I and ligated in-frame into the pBJF36V cloning vector (22) at the C-terminal end of the F36V domain. This created a fusion gene containing the c-Src myristylation domain, the modified FKBP domain (F36V), the FGFR-1 cytoplasmic domain, and a hemagglutinin epitope tag at the C terminus. The resulting F36Vfgfr-1 region was excised by a *Sac*II/*Bam*HI double digest and subsequently ligated into a bicistronic expression vector (23) containing the murine stem cell virus long terminal repeat with an enhanced green fluorescent protein (GFP) reporter upstream of an internal ribosomal entry sequence. The resulting F36Vfgfr-1 expression plasmid (Fig. 1) was sequenced in both directions and subsequently expressed in retroviral packaging cell lines.

Retrovirus Production—GP+E86 ecotropic packaging cells were transiently transfected with F36Vfgfr-1 plasmid DNA using Superfect (Qiagen) transfection reagent. After 24 h, supernatant was collected, supplemented with 10% fetal bovine serum (HyClone) and 8 μ g/ml polybrene (Sigma), and transferred to a cell culture dish containing PA317 amphotropic packaging cells. After 24 h, the retroviral supernatant was removed and replaced with Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (Life Technologies, Inc.). PA317 cells stably expressing the F36Vfgfr-1 construct were selected by fluorescence-activated cell sorting (FACS) using a FACStar Plus cell sorter (BD Biosciences) based on GFP expression. Stably transfected cells were expanded in culture, and supernatants were collected at 24-h intervals and stored at -80°C until subsequent transfections were performed.

Myoblast Transfection and Selection—Murine skeletal myoblasts (MM14, Ref. 24) were maintained at low density in growth medium (Ham's F-10 medium (Life Technologies, Inc.), 15% horse serum (ICN Flow), 6 ng/ml bFGF, 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (Life Technologies, Inc.)) on gelatin-coated tissue culture dishes. MM14 cells were transfected by incubation with PA317 F36Vfgfr-1 retroviral supernatant supplemented with 15% horse serum, 8 μ g/ml polybrene, and 6 ng/ml bFGF. Following a 24-h incubation, retroviral supernatant was removed and replaced with fresh growth medium. Stably transfected MM14 myoblasts were selected by FACS based on GFP expression as described above. The resulting non-clonal population of GFP-positive cells was expanded in growth medium.

MTT Conversion Assay—Transfected MM14 and wild type non-transfected MM14 cells were plated into gelatin-coated 24-well plates at a density of 5,000 cells/well. Cells were cultured for 48 h in the presence of 6 ng/ml bFGF or 0, 10, or 100 nM AP20187. The medium was then replaced with 400 μ l of fresh F10 growth medium, and 40 μ l of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) was added. Following a 4-h incubation, the cells were lysed with 550 μ l of lysis buffer (10% SDS, 0.1 M HCl). The relative level of MTT conversion in each sample was then measured by spectrophotometry ($\lambda = 570$ nm). The absorbance values were normalized to the mean absorbance of the bFGF-treated, non-transfected cells. A Student's *t* test was performed to determine statistical significance at $p \leq 0.05$.

Myoblast Differentiation and Immunocytochemistry—Transfected and non-transfected MM14 myoblasts were plated into gelatin-coated 6-well plates at a density of $2.5\text{--}6.0 \times 10^3$ cells/cm 2 . Cells were incubated in differentiation medium (Ham's F-10 medium, 10% horse serum, 0.9 mM CaCl $_2$ (final concentration 1.2 mM), 6 μ g/ml insulin (Sigma), 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (Life Technologies, Inc.)) with 6 ng/ml bFGF, 100 nM AP20187, or were left untreated. After 48 h, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and the extent of differentiation was assessed by myosin heavy chain immunocytochemistry. Fixed cultures were blocked with 1.5% normal rabbit serum (Vector Laboratories) in PBS, followed by incubation with MF-20 hybridoma supernatant (1:10; Developmental Studies Hybridoma Bank, University of Iowa) to detect sarcomeric myosin heavy chain. Plates were washed with PBS, followed by incubation with rhodamine-

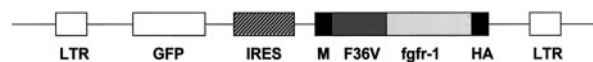


FIG. 1. The bicistronic F36Vfgfr-1 retroviral plasmid encodes an enhanced (GFP) marker upstream of an internal ribosomal entry sequence (IRES) and the F36Vfgfr-1 receptor. Expression is driven by the murine stem cell virus long terminal repeat (LTR). M, myristylation sequence; F36V, modified FK506-binding protein; fgfr-1, fibroblast growth factor receptor-1 cytoplasmic domain; HA, hemagglutinin epitope tag.

conjugated rabbit anti-mouse secondary antibody (1:20; Dako Corp.). Cells were counterstained with Hoechst 33342 dye (4 μ g/ml; Molecular Probes) to visualize myoblast nuclei. Images were acquired using a fluorescence microscope (Olympus) equipped with a SPOT digital camera (Diagnostic Instruments).

Western Blotting—Transfected and non-transfected MM14 myoblasts were plated into gelatin-coated 100-mm dishes at a density of $2\text{--}5 \times 10^5$ cells/cm 2 . Cells were incubated in growth medium with either 6 ng/ml bFGF, 100 nM AP20187, or were left untreated. After 48 h, cells were washed twice with ice-cold PBS, lysed in sample buffer (50 mM Tris-HCl, 1% SDS, 10% glycerol, 240 μ g/ml pefabloc, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin (Roche Molecular Biochemicals)), and scraped and transferred to tubes on ice. Cell lysate protein concentrations were assessed by BCA assay (Pierce) to ensure equivalent loading. Samples were loaded onto precast 4–15% gradient polyacrylamide gels (Bio-Rad), separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Membranes were blocked with 20 mM Tris-HCl, pH 7.8, 500 mM NaCl, and 5% nonfat milk (Carnation) and probed with a monoclonal antibody against phosphorylated ERK1/2 (1:1000). The membranes were then washed with TBS-T (20 mM Tris-HCl, pH 7.8, 300 mM NaCl, 0.1% Tween-20) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:20,000; Jackson ImmunoResearch). Blots were developed by incubation with chemiluminescent reagent (SuperSignal West Dura; Pierce) followed by exposure to ECL film (Hyperfilm ECL, Amersham Pharmacia Biotech).

Bromodeoxyuridine (BrdUrd) Incorporation Proliferation Assay—Transfected MM14 cells were cultured for 30 days in growth medium supplemented with 100 nM AP20187 with complete media exchange every 1–2 days. Following dimerizer treatment, naive and 30-day AP20187-treated cells were seeded into gelatin-coated 6-well dishes at $2\text{--}8 \times 10^3$ cells/cm 2 and cultured in differentiation medium supplemented with 6 ng/ml bFGF, 100 nM AP20187, or were left untreated. After 3 days in culture, 10 μ M BrdUrd (Roche Molecular Biochemicals) was added to the culture medium for 1 h, followed by fixation with a 9:1 MeOH/acetic acid solution for 10 min at room temperature. Samples were treated with 1.5 N HCl for 15 min at 37°C , neutralized by washing twice with 0.1 M borax, pH 8.5 and once with PBS, and then the samples were blocked with 1.5% normal rabbit serum in PBS and incubated overnight with a rat anti-BrdUrd primary antibody (1:100; Harlan Bioproducts for Science, Inc.). Cells were then incubated with a fluorescein-conjugated rabbit anti-rat secondary antibody (1:100; Vector Laboratories) and were counterstained with Hoechst 33342 dye. Total nuclei and BrdUrd-positive nuclei were visualized using a fluorescence microscope (Olympus) and were manually counted (~ 400 nuclei/well) from triplicate wells. A Student's *t* test was performed to determine statistical significance at $p \leq 0.05$.

RESULTS

Chimeric Receptor Construction and Expression—Mouse MM14 myoblasts were stably transfected with a retrovirus encoding a chimeric receptor composed of a modified FK506-binding protein domain (F36V) fused with the cytoplasmic domain of FGFR-1 (Fig. 1). The receptor construct also includes a hemagglutinin epitope tag as well as an N-terminal myristylation site to target the protein to the plasma membrane. Cells expressing this construct were selected by FACS based on expression of the reporter protein eGFP. Subsequent studies were performed using a polyclonal population of GFP-positive myoblasts with non-transfected wild type MM14 cells used as controls.

Dimerizer Treatment Induces Proliferation in Transfected Myoblasts in the Absence of bFGF—In the absence of bFGF, wild type MM14 cells cease proliferating and differentiate into

multinucleated myotubes expressing structural proteins such as myosin heavy chain. Wild type and F36Vfgfr-1-transfected MM14 cells were cultured in 24-well plates for 48 h in the presence of 6 ng/ml bFGF or a bivalent F36V ligand, AP20187. Following the treatment period, the relative number of cells/well was assessed by MTT assay (Fig. 2). Cell number was normalized to the bFGF-treated control wells. In the absence of bFGF, wild type cells ceased proliferating, resulting in a reduced cell number in comparison to bFGF-treated cells (24.2 ± 1.6 versus $100 \pm 8.6\%$). Transfected MM14 samples also had significantly lower cell numbers in the absence of bFGF ($44.8 \pm$

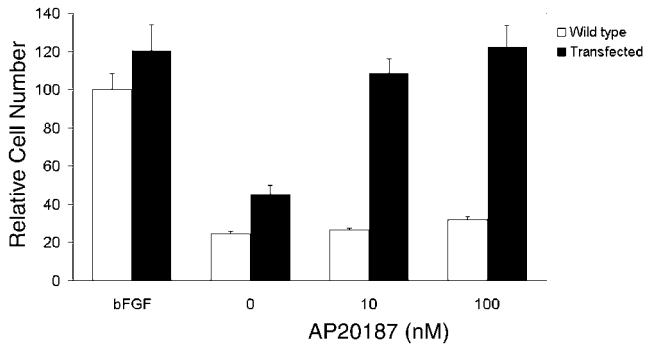


FIG. 2. **Stimulation of cell proliferation by AP20187.** MM14 myoblasts were treated with bFGF (6 ng/ml) or AP20187 for 48 h prior to incubation with MTT. MTT conversion was determined by spectrophotometry ($A^{570 \text{ nm}}$) as a measure of cell number and normalized to bFGF treatment. Myoblasts expressing the FGFR-1 chimeric receptor proliferated in response to dimerizer treatment, similar to the bFGF response. Wild type myoblasts did not proliferate in response to dimerizer. Data are the mean \pm S.D. from three replicate wells and are representative of four independent experiments.

5.1 versus $120.3 \pm 13.8\%$). Treatment of transfected cells with 10 or 100 nM AP20187, however, resulted in myoblast proliferation rates comparable with bFGF-treated controls ($108.6 \pm 7.5\%$ and $122.0 \pm 11.6\%$, respectively). The mean number of cells/well in AP20187-treated transfected cells was not significantly different from bFGF-treated transfected cells ($p > 0.05$) at either concentration. Treatment with 10 or 100 nM AP20187 did not stimulate proliferation in non-transfected control cells ($26.5 \pm 0.9\%$ and $31.9 \pm 1.6\%$, respectively). Thus, dimerization of the FGFR-1 cytoplasmic domain by AP20187 stimulated myoblast proliferation to the same extent as direct addition of bFGF. Interestingly, transfected cells appeared to proliferate at somewhat higher levels than non-transfected cells regardless of treatment (see "Discussion"). This difference was not significant between wild type and transfected cells treated with bFGF ($p > 0.05$); however, the number of cells in untreated transfected wells was significantly higher than in untreated wild type cells ($p < 0.005$).

Dimerizer Treatment Inhibits Differentiation of Transfected Myoblasts in the Absence of bFGF—Upon withdrawal or depletion of bFGF from the culture medium, wild type MM14 cells exit the cell cycle and begin to differentiate. Differentiation is characterized by expression of muscle-specific proteins (such as sarcomeric myosin heavy chains) followed by myoblast fusion to form multinucleated myotubes. To determine whether AP20187 treatment can prevent myoblast differentiation in the absence of bFGF, wild type and F36Vfgfr-1-transfected MM14 cells were cultured for 48 h in differentiation medium (low serum, high insulin, and high calcium compared with growth medium) containing either 6 ng/ml bFGF or 100 nM AP20187. Untreated samples were included as controls. Cells were sub-

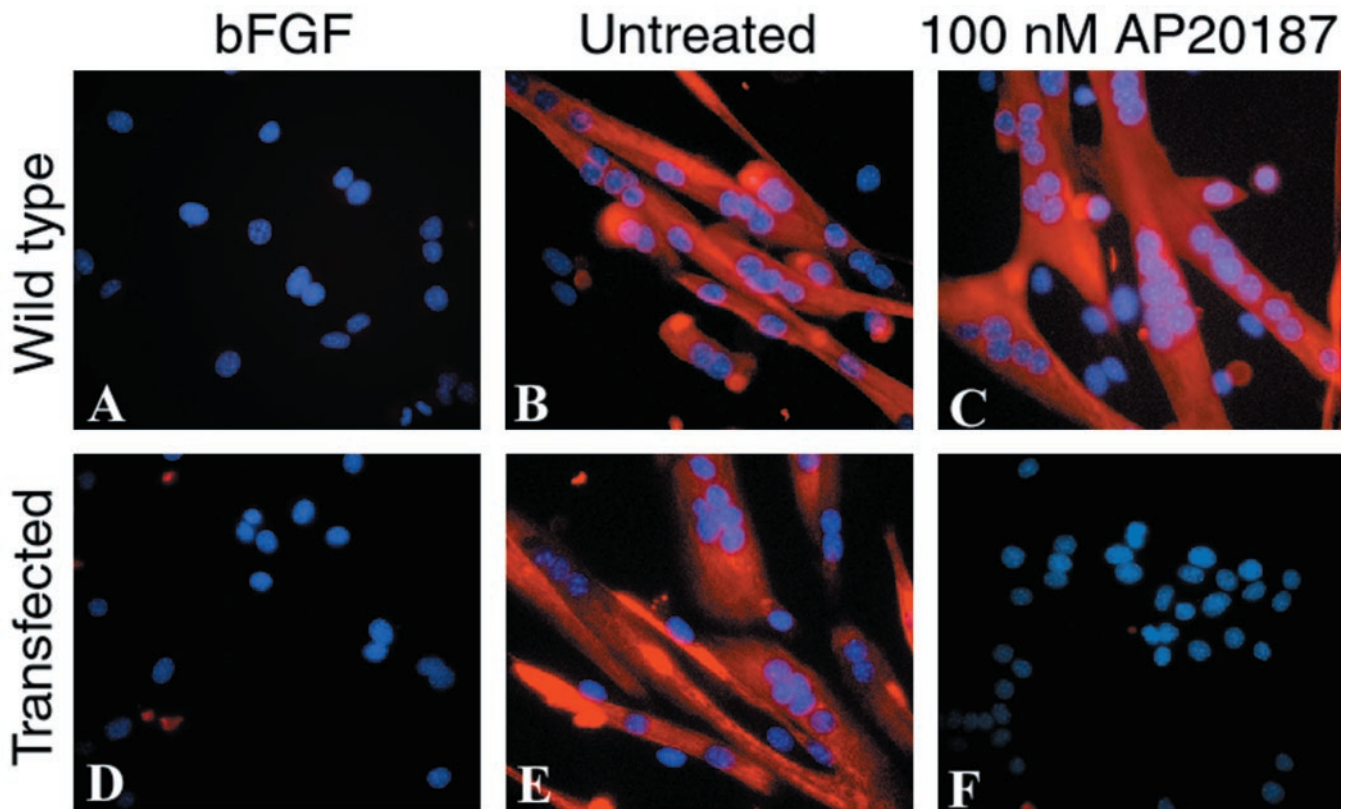


FIG. 3. **Inhibition of myoblast differentiation by AP20187.** Cells were treated for 48 h as indicated and fixed, and immunofluorescence was performed to evaluate expression of sarcomeric myosin heavy chain. In the presence of bFGF, both wild type (A) and transfected (D) myoblasts were proliferating mononuclear cells that did not express myosin heavy chain. When untreated, both wild type (B) and transfected (E) cells fused to form multinucleated myotubes that expressed myosin heavy chain. Treatment with the synthetic dimerizer AP20187 blocked formation of the myotubes and expression of myosin heavy chain in transfected cells (F), similar to treatment with bFGF. Dimerizer treatment had no effect on wild type cells (C). Red (rhodamine), myosin heavy chain immunofluorescence; blue, Hoechst 33342 nuclear staining.

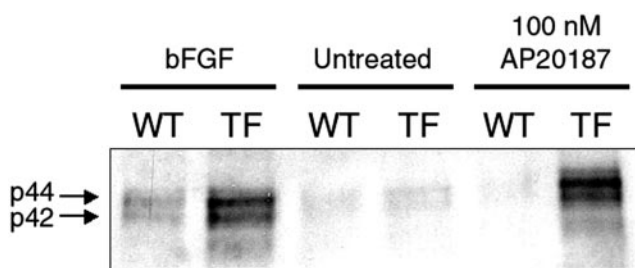


FIG. 4. Dimerizer-induced activation of the MAP kinase cascade. Wild type (WT) and F36Vfgfr-1-transfected (TF) cells were cultured in the presence of 6 ng/ml bFGF or 100 nM AP20187 or were left untreated. Western blotting of cell lysates was performed with an antibody against the phosphorylated form of MAP kinases ERK 1/2. ERK1 and ERK2 were phosphorylated in both wild type and transfected cells treated with bFGF. Note the enhanced phosphorylation in transfected cells. Phosphorylated ERK 1/2 was barely detectable in untreated wild type and transfected cells. AP20187 treatment did not induce ERK phosphorylation in wild type MM14 cells, whereas transfected cells treated with AP20187 showed intense phosphorylation of ERK 1 and 2.

sequently fixed, and immunocytochemical staining was performed to detect sarcomeric myosin heavy chain expression (Fig. 3). As expected, wild type cells treated with bFGF remained mononucleated and did not express myosin heavy chain (Fig. 3A), whereas bFGF withdrawal resulted in formation of myosin-positive multinucleated myotubes (Fig. 3B). Similarly, cells expressing the FGFR fusion protein remained mononucleated and myosin-negative after bFGF treatment (Fig. 3D) and differentiated normally after growth factor withdrawal (Fig. 3E). In contrast, treatment of the transfected cells with AP20187 completely prevented formation of myotubes and also blocked myosin heavy chain expression (Fig. 3F). AP20187 had no effect on control non-transfected cells (Fig. 3C). Thus, AP20187 appeared to block differentiation by inducing dimerization of the FGFR signaling domains in a manner similar to bFGF treatment.

Activation of the MAP Kinase Pathway in Transfected Myoblasts Treated with AP20187—Signaling studies were performed with whole cell lysates from MM14 cells to determine whether the observed bFGF-like effects of dimerizer treatment were caused by the activation of similar pathways. Wild type and F36Vfgfr-1-transfected cells were cultured in the presence of 6 ng/ml bFGF, 100 nM AP20187, or were left untreated. Western blots from cell lysates were probed with an antibody that specifically recognizes the phosphorylated forms of ERK 1/2, MAP kinases that are phosphorylated in response to FGFR-1 activation. Neither wild type nor transfected cells showed significant phosphorylation of ERK 1/2 in the absence of treatment (Fig. 4). After bFGF treatment both populations showed significant ERK 1/2 phosphorylation. The transfected cells actually showed an exaggerated ERK 1/2 phosphorylation after bFGF treatment (see “Discussion”). AP20187 treatment did not induce ERK phosphorylation in wild type MM14s. Importantly, transfected cells treated with AP20187 exhibited extensive phosphorylation of ERK 1/2, demonstrating that AP20187-induced activation of the chimeric receptor F36Vfgfr-1 can stimulate MAPK activation in the absence of bFGF.

Dimerizer-induced Proliferation Is Reversible—Although for grafting purposes controlled proliferation of transplanted myoblasts may be beneficial, uncontrolled proliferation may lead to undesirable effects such as cardiac tumor generation. To determine whether the mitogenic effect of AP20187 on transfected myoblasts is reversible, cells were cultured in growth medium supplemented with 100 nM AP20187 for 30 days. After the culture period, cells were transferred to gelatin-coated 6-well

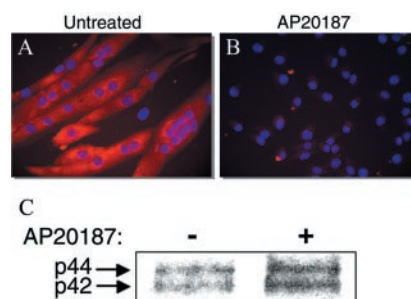


FIG. 5. Dimerizer-induced proliferation is reversible. Transfected MM14 cells were cultured in growth medium supplemented with 100 nM AP20187 (without bFGF) for 30 days and then switched to differentiation medium for 48 h in the presence (B) or absence (A) of AP20187. Following withdrawal of the dimerizer, myoblasts differentiated normally as evidenced by myotube formation and sarcomeric myosin heavy chain expression. Red (rhodamine), myosin heavy chain immunofluorescence; blue, Hoechst 33342 nuclear staining. To determine whether ERK activity is down-regulated in 30-day AP20187-treated cells, cell lysates were prepared and analyzed by Western blotting. In the absence of AP20187, ERK phosphorylation was reduced in comparison with cells that received continued dimerizer treatment (C).

plates and cultured in differentiation medium with or without AP20187 for an additional 2 days. As was observed previously, dimerizer-treated myoblasts fused to form multinucleated myotubes and expressed myosin heavy chain upon mitogen withdrawal (Fig. 5B), whereas cells treated with AP20187 continued to proliferate and failed to differentiate (Fig. 5A). To determine whether the observed differentiation was accompanied by a down-regulation in MAPK activity, cell lysates were collected from 30-day AP20187-treated cells cultured in differentiation medium in the presence or absence of dimerizer. Western blots probed with an antibody against the phosphorylated forms of ERK 1/2 demonstrated that ERK phosphorylation was reduced following dimerizer withdrawal in comparison with cells receiving continued AP20187 treatment (Fig. 5C). However, this reduced level of ERK phosphorylation was fairly high compared with that of untreated transfected or wild type cells (in which phosphorylated ERK was barely detectable), even though these cells differentiate normally.

To further assess whether long term dimerizer treatment affects the differentiation capacity of transfected myoblasts, naive and 30-day dimerizer-treated cells were cultured in differentiation medium with or without bFGF or AP20187 for 3 days followed by a 1-h BrdUrd incubation. Cells were fixed, and immunocytochemistry was performed to identify BrdUrd-positive nuclei. The percentage of nuclei that incorporated BrdUrd in each sample was determined by a manual count to measure the fraction of cells undergoing cell division. The percentage of naive and dimerizer-treated cells incorporating BrdUrd was comparable in response to bFGF ($27.3 \pm 4.0\%$ and $32.6 \pm 2.1\%$; $p > 0.05$) and AP20187 ($31.0 \pm 3.9\%$ and $34.1 \pm 4.2\%$; $p > 0.05$). Importantly, the percentage of untreated naive and dimerizer-treated cells incorporating BrdUrd was substantially lower than bFGF- and AP20187-treated cells ($6.2 \pm 2.9\%$ and $7.0 \pm 1.3\%$; $p > 0.05$) and was not significantly different from each other. In summary, these results suggest that although long term dimerizer-treated cells may have higher MAPK activity levels than wild type or naive cells following mitogen withdrawal, they still differentiate normally and cease proliferating in the absence of AP20187. Thus extended dimerizer treatment does not appear to induce uncontrolled cell proliferation, although it is possible that prolonged signaling through the chimeric receptor may affect activity levels of downstream effector molecules.

DISCUSSION

Recently, several studies have demonstrated that skeletal muscle stem cells, or satellite cells, can be isolated from adult tissue, expanded in culture, and reimplanted into the hearts of the donors to generate contractile muscle to replace scarring following myocardial infarction (2–5, 25). These successes have led to initiation of the first clinical trials of this technique (26). Despite its promise, myoblast grafting requires the harvesting and expansion of large numbers of cells, and graft size can vary enormously even when the injection procedure and cell number are kept consistent (27). These technical limitations may be overcome by developing a means of specifically and reversibly stimulating grafted skeletal muscle cell proliferation *in vivo*.

In the present study, we demonstrate that dimerization of the FGFR-1 cytoplasmic domain with a bivalent synthetic ligand, AP20187, can mimic critical aspects of bFGF-induced FGFR-1 signaling in myoblasts. Previous studies have demonstrated that bFGF is required for cell cycle progression and repression of differentiation in MM14 myoblasts (8). In the absence of bFGF, AP20187 stimulated proliferation and prevented differentiation in MM14 cells expressing the F36Vfgfr-1 chimera in a manner that was indistinguishable from bFGF treatment. Administration of AP20187 also activated the MAPK pathway in MM14 cells expressing the chimeric receptor, thereby reproducing key events involved in bFGF-mediated signal transduction. Importantly, cells treated with dimerizer for 30 days withdrew from the cell cycle and differentiated normally upon withdrawal of the dimerizer, demonstrating that the effect was reversible. Thus, the data presented offer proof of the concept that a genetically modified population of myoblasts can be expanded specifically and reversibly in response to a small synthetic molecule.

It is interesting to compare our results with those recently published by Kudla *et al.* (28). These investigators studied MM14 cells expressing a chimeric receptor composed of the PDGF- β R extracellular domain and the FGFR-1 intracellular domain. In response to PDGF-BB treatment, the MM14 cells (which express no intrinsic PDGF receptors) phosphorylated the chimeric cytoplasmic domains and activated the MAPK pathway. Similar to our results, activation of the chimeric receptor blocked myoblast differentiation. In contrast to our findings, however, activation of this chimeric receptor was insufficient to induce cell proliferation. It is not clear why we observed cell proliferation when they did not. Both systems employed forced dimerization of the FGFR-1 cytoplasmic domain in MM14 cells, and both resulted in MAPK activation, although our system used a small molecule to dimerize the receptors whereas the other system employed a relatively large dimeric protein ligand and included the extracellular domain of the PDGF receptor. It is possible that the extracellular domain of the PDGF receptor induced unfavorable allosteric changes in the FGFR-1 cytoplasmic domain. Alternatively, the spacing of the receptor dimers induced by PDGF binding may be different from that induced by AP20187 binding. Such differences may influence coupling of the receptor to additional downstream signaling pathways, *e.g.* PLC γ .

We observed that treating transfected cells with dimerizer resulted in greater levels of MAPK phosphorylation than was seen when wild type cells were bFGF-treated. This result may be explained by differences in the level of endogenous FGFR-1 expression, as only ~700 molecules are expressed on MM14 cells (29), compared with the chimeric receptor in which expression is driven by a retroviral promoter. We were surprised to note, however, that MM14 cells expressing the chimeric receptor demonstrated more robust MAPK activation than did wild type cells when both were treated with bFGF. Similarly,

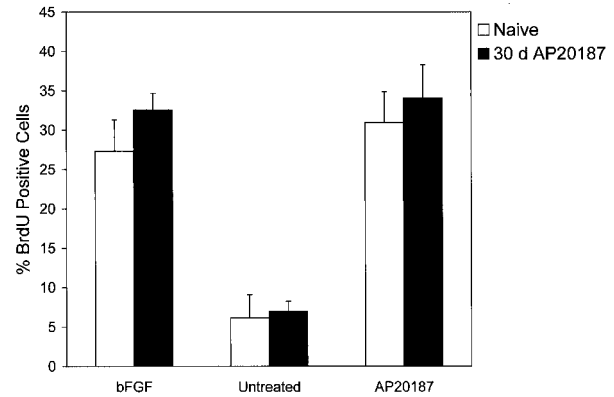


FIG. 6. Dimerizer-induced proliferation is reversible upon drug withdrawal after long term AP20187 treatment. The proliferation rate of transfected MM14 cells cultured for 30 days in AP20187 was compared with that of naive transfected cells to determine whether long term dimerizer treatment leads to uncontrolled cell proliferation. Cells were cultured in differentiation medium in the presence of 6 ng/ml bFGF, 100 nM AP20187, or left untreated. After 3 days, cells were pulsed with BrdUrd, and immunocytochemistry was performed to identify proliferating nuclei, followed by Hoechst counterstaining to identify total nuclei. Approximately 400 cells/well were counted manually in triplicate wells. The data are expressed as the mean percentage of BrdUrd-positive nuclei per treatment \pm S.D.

we observed that transfected myoblasts proliferated at a higher rate than non-transfected cells either basally or after bFGF treatment. Because the chimeric receptor cannot respond to bFGF, these data suggest that the modified receptor may have some constitutive activity. This hypothesis is in accordance with the work of Kudla *et al.* (30), who showed that overexpressing FGFR-1 in the absence of ligand could activate MAPK in MM14 cells. The level of activity in our cells was not sufficient, however, to interfere with myoblast differentiation, as the transfected cells differentiated normally upon withdrawal of AP20187 or bFGF, and MAPK phosphorylation was down-regulated in the absence of bFGF or AP20187 in both cell types. Another important distinction between bFGF and dimerizer treatment is that the dimerizer can only activate FGFR-1-mediated signal transduction, whereas bFGF can also bind to and activate isoforms of FGFR-2, -3, and -4 (31), thereby stimulating multiple receptor-induced signaling pathways within the same cell. Although FGFR-1 is the predominant FGFR subtype expressed in MM14 cells, this is an important consideration in comparing the effects of bFGF with dimerizer treatment (9, 10) in cells that express multiple FGFR isoforms.

Long term (30 days) treatment with dimerizer did not interfere with the ability of myoblasts to withdraw from the cell cycle or differentiate into myotubes. There were a few notable differences, however, between naive transfected cells and the long term-treated cells. As shown in Fig. 6, comparable fractions of naive and long term-treated cells were in S phase after bFGF or dimerizer treatment. On the other hand, when cell number was used to measure proliferation, the long term-treated cells showed increased cell accumulation compared with naive cells (data not shown). This suggests that the long term dimerizer treatment may select for a population of cells with shorter cell cycle times or greater survival rates. Additionally, we were surprised to note that bFGF treatment of the long term-treated cells actually resulted in reduced phosphorylation of ERK ($n = 3$ experiments; data not shown), despite the fact that the cells proliferated well in response to bFGF. Incubation with the MAPK/ERK kinase (MEK) inhibitor U0126 significantly reduced proliferation of these cells when either bFGF or dimerizer was used to drive proliferation. Thus, it appears that the long term-treated cells require the MAP

kinase pathway for proliferation, but steady-state levels of ERK phosphorylation do not reflect the pathway's activity following bFGF treatment. Taken together, the results presented support the hypothesis that dimerizer-mediated proliferation acts through induction of MAPK signaling, similar to bFGF treatment. The results also strongly suggest, however, that subtle differences in signaling dynamics exist between the two receptor pathways, which may be amplified following long term signaling through the chimeric receptor in the absence of bFGF. Importantly, transfected MM14 cells appeared phenotypically normal with respect to differentiation, and uncontrolled proliferation following dimerizer treatment was not observed.

Using a small molecule to expand genetically modified myoblasts offers several advantages over conventional grafting approaches, including the possibility of using fewer cells for injection, better control over the graft size, and the potential for increasing cell-cell communication with host myocardium by reducing the intervening scar area. Use of a promoter active in myoblasts but not fibroblasts, *e.g.* the desmin promoter, may permit selective expansion of myoblasts from impure primary muscle cultures. Furthermore, the drug has proven non-toxic for *in vivo* applications (19). The F36V modification of FKBP (20), coupled with the synthesis of novel bivalent F36V ligands (20, 32), has led to an improved dimerization system without immunosuppressive or other potentially harmful effects. Future studies will focus on determining whether AP20187 can induce proliferation specifically and reversibly in transplanted genetically modified myoblasts *in vivo*.

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