Inhibition of p38 MAPK signaling promotes late stages of myogenesis

Andrea D. Weston[1,*], Arthur V. Sampaio[1], Alan G. Ridgeway[2,3] and T. Michael Underhill[1,‡]

[1]Department of Physiology and [2]Department of Biochemistry, Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ontario, N6A 5C1, Canada
[3]Department of Genetics, Harvard Medical School, Boston, MA 02115, USA
*Present address: Institute for Systems Biology, Seattle, WA, USA 98012
‡Author for correspondence (e-mail: tunderhi@uwo.ca)

Accepted 31 March 2003
Journal of Cell Science 116, 2885-2893 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00525

Summary

Signaling through the p38 mitogen-activated protein kinases (MAPKs) is essential for cartilage formation in primary cultures of limb mesenchyme. Here we show that, concurrent with a decrease in chondrogenesis, inhibition of p38 in limb bud cultures dramatically promotes muscle development. Specifically, treatment of primary limb bud cultures with p38 inhibitors increases the expression of myogenic markers and causes a striking increase in formation of myotubes, which were detected using antibodies specific for myosin heavy chain. These results are surprising in that they contrast with several previous reports describing a requirement for p38 during myogenesis. Nonetheless, the enhanced myogenesis leads to the formation of an extensive network of contractile myofibers, and this enhanced myogenesis can be conferred upon myogenic cells from clonal populations, such as G8 or C2C12 cells, if they are co-cultured with the limb mesenchymal cells. We provide evidence for the maintenance and rapid organization of existing, somitic-derived limb myoblasts in response to p38 inhibitors. These findings imply a novel and unexpected role for p38 MAPK inhibition in myogenesis and highlight the importance of the limb bud microenvironment in promoting the progression of limb myoblasts.

Key words: Myogenesis, p38 MAPK, Limb mesenchyme, Chondrogenesis

Introduction

Myogenesis is a multi-step process through which uncommitted mesodermal cells commit, differentiate and undergo profound phenotypic changes. These changes include elongation, polarization, aggregation and fusion, which ultimately give rise to functional muscle. The past two decades has seen major advances towards our current understanding of skeletal myogenesis (reviewed in Perry and Rudnicki, 2000). Of particular importance has been the identification of two families of transcription factors: the myogenic regulatory factors (MRFs) and the myocyte enhancer factors (MEFs). In mammals, the MRF group includes MyoD, myogenin, Myf5 and MRF4/herculin/Myf6 (Braun et al., 1990; Braun et al., 1989; Davis et al., 1987; Edmondson and Olson, 1989; Miner and Wold, 1990; Rhodes and Konieczny, 1989; Wright et al., 1989), which transactivate muscle-specific genes (Lassar et al., 1989). In addition, members of the MEF2 family (MEF2A, B, C and D) contribute to myogenesis by binding to regions within the promoters of muscle-specific genes (Gossett et al., 1989) and by cooperating with the MRFs to synergistically activate those genes (Kaushal et al., 1994; Molkentin et al., 1995).

Several extracellular factors modulate expression of the MRFs and/or MEF2 factors. Signal transducers such as the mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinases (ERK1 and 2), the Jun-N-terminal kinases (JNK1, 2, and 3) and the p38 isoforms (α, β, γ and δ) (Chang and Karin, 2001; Kyriakis and Avruch, 2001; Obata et al., 2000; Pearson et al., 2001), have all been widely studied with respect to their importance during myogenesis. Evidence from a number of studies strongly supports a requirement for p38 MAPK during myogenic progression. Transcripts for the upstream activator of p38, MKK6, are most abundant in skeletal muscle, and the p38 transcripts, particularly those encoding the γ isoform, are highly expressed in this tissue. Moreover, overexpression of p38 isoforms or upstream activators causes an upregulation of myogenic markers, enhances muscle reporter activity and accelerates myotube formation (Wu et al., 2000). In a similar manner, forced p38 induction can restore MyoD function and enhance MEF2 activity in rhabdomyosarcoma cells deficient for p38 MAPK activation, resulting in terminal differentiation (Puri et al., 2000). In addition to the effects of p38 activation, independent groups have demonstrated an inhibition of muscle differentiation in C2C12 and L6 cells (Cuenda and Cohen, 1999; Wu et al., 2000), human primary myocytes (Wu et al., 2000) and rhabdomyosarcoma cells (Puri et al., 2000) in response to specific p38 inhibitors. This apparent requirement for p38 in myogenesis is consistent with the demonstrated p38α- and β-specific induction of the transcription factor MEF2C (Wu et al., 2000; Yang et al., 1999).

Clearly, evidence supports an essential role for p38 signaling during myogenesis. To date, however, biochemical dissection of the myogenic pathway has largely been done using homogeneous cell populations derived from adult muscle,

including the mouse C2C12 and rat L6 cells, both derivatives of satellite cells from adult muscle fibers (Cabane et al., 2003; Conejo and Lorenzo, 2001; Cuenda and Cohen, 1999; Gallea et al., 2001; Li et al., 2000; Puri et al., 2000; Zetser et al., 1999). Recently, however, p38 inhibitors were shown to reduce the expression of MyoD target genes in mouse embryonic fibroblasts. Importantly, this same study suggests distinct subprograms of myogenesis, which may differentially involve p38 (Bergstrom et al., 2002). To date, most of the studies that have implicated p38 in myogenesis focus on differentiation of precursors and acquisition of the myoblast phenotype. In the present study, we provide evidence to suggest that in primary limb mesenchymal cultures, p38 inhibition dramatically advances later stages of the myogenic program. This is revealed by the rapid alignment, aggregation and fusion of myocytes to form functional, twitching skeletal muscle. Given the extent of muscle seen in the limb mesenchymal cultures treated with p38 inhibitors, we believe that the role of p38 signaling in myogenesis is not as clear as originally thought.

Materials and Methods

Establishment of primary limb mesenchymal cultures

Limb mesenchymal cells were harvested from embryonic age (E) 11.25-E11.75 mouse embryos as previously described (Cash et al., 1997; Weston et al., 2000), with some modifications. To establish proximal or distal cultures, the proximal half of each limb bud was separated from the distal half, and each pool of limb fragments was processed separately. For these cultures, the cells were resuspended at a density of 1.5×10^7 cells/ml for seeding of 24-well culture plates (Corning, Corning, NY). Cell media was replaced daily or every other day. The p38 inhibitors SB202190 and SB203580 along with the inactive analog SB202474 were obtained from Calbiochem (San Diego, CA) and were dissolved in DMSO.

Generation of G8-βgeo and C2C12-βgeo cells

G8 embryonic myoblasts (American type-culture collection, ATCC) were maintained in Dulbecco's Modified Eagle's Media supplemented with 10% fetal bovine serum (FBS) and 10% horse serum (Christian et al., 1977). Cultures were subcultured prior to reaching ~80% confluence to minimize the loss of myoblasts. For generation of G8-βgeo cells and C2C12-βgeo cells, G8 and C2C12 cells were each infected with MSV-tk-βgeo. MSV-tk-βgeo retroviral particles were generated by co-transfection of MSV-tk-βgeo with pSVψ2 into COS cells. 48 hours after transfection, the supernatant from the cultures was collected and filtered. To infect G8 or C2C12 cells, the supernatant was added directly to each cell culture for 3 hours in the presence of 10 μg/ml polybrene. Within 24 hours of infection, two volumes of media were added to each cell culture. One day post-infection, the media was exchanged for fresh media containing 600 μg/ml active G418. Cells were subcultured three times during the next 10 days of selection in G418. At the end of the culture period >95% of the cells within each culture (G8 and C2C12 cells) stained positive for β-galactosidase.

Cell mixing experiments were performed by adding G8-βgeo or C2C12-βgeo cells to resuspended primary cells such that 5% of the entire cell suspension consisted of the tagged G8 or C2C12 cells. These mixtures were used to seed 24-well culture plates in 10 μl volumes.

Immunofluorescence, in situ β-galactosidase and alcian blue staining of cultures

The supernatant from a mouse myeloma cell line containing an anti-MyHC monoclonal antibody was used to detect the myogenic cells within the primary limb bud cultures (Bader et al., 1982). Detection of MyHc-positive cells was carried out as previously described (Ridgeway et al., 2000). To follow localization of LacZ-expressing cells in primary cultures, cells were briefly fixed and stained with Magenta Gal (BioShop Inc.) as previously described (Weston et al., 2000). Alcian blue staining was performed on fixed cultures also as described previously (Weston et al., 2000).

Transient transfections and reporter assays

For transfection purposes, cells were resuspended at 2.5×10^7 cells/ml and mixed with a DNA/FuGene6 mixture in a 2:1 ratio. FuGene6-DNA mixtures were prepared according to the manufacturer's instructions (Roche Biomolecular, Laval, Quebec, Canada). Briefly, 1 μg of reporter, 1 μg of expression vector and 0.05 μg of pRLSV40 (Promega) were mixed for a total of ~2 μg DNA in 100 μl of media and FuGene6. 50 ml of the DNA mixture was transferred into a sterile 1.5 ml eppendorf tube, followed by 100 μl of cells. Cells were gently triturated, and 10 μl was used to seed a single well of a 24-well culture dish. After 1.5 hours in a humidified CO_2 incubator, 1 ml of media was added to each well. 24 hours after transfection, the media was replaced and the appropriate supplements were added. G8 cells were transfected as described above except that monolayer cultures were transfected into 12-well plates, whereas C2C12 cells were transfected with jetPEI using conditions outlined by the manufacturer (Polyplus Transfection Inc., Illkirch, France). For experiments involving characterization of SB202190 activity, supplements were added immediately following transfection.

Analysis of reporter gene activity was carried out using the Dual Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WS). Briefly, approximately 48 hours post transfection, cells were washed once with PBS and lysed in 100 μl of passive lysis buffer for 20 minutes. Firefly and renilla luciferase activities were determined by using 40 μl of the cell lysate in a 96-well format Molecular Devices luminometer.

Western blot analysis

For western blot analysis, 10 individual limb mesenchymal cultures were established in each well of a 6-well culture dish and were treated with SB202190 (10 μM) daily, starting 24 hours after culture initiation. Lysates were collected immediately prior to SB202190 addition, and after 4 and 8 days of treatment, by adding 150 μl lysis buffer (Cell Signaling Technology, Beverly, MA) to each well. G8 and C2C12 cells were grown to confluence in 6-well culture dishes, lysed with 150 μl lysis buffer/well, and samples from 2 wells were pooled. Cleared lysates containing approximately 15 μg of protein were separated by SDS-PAGE gels and transferred to nitrocellulose. Antibodies for p38 (pan) and p38α (Cell Signaling Technology) were each used at a 1:1000 dilution, followed by a 1:3000 dilution of a secondary anti-rabbit IgG-HRP antibody (Santa Cruz, Santa Cruz, CA). The β-actin antibody (Sigma) was diluted 1:10,000 followed by incubation with an anti-mouse IgG-HRP antibody (Santa Cruz) at a 1:3000 dilution. HRP was detected using chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Expression plasmids and reporter constructs

To generate pGL3(4X48), a fragment containing the reiterated (4X48) Sox9 binding sequence upstream of the mouse *Col2a1* minimal promoter (–89 to +6) was liberated from the 4X48-p89 plasmid (Lefebvre et al., 1996) and subcloned into pGL3-basic as described previously (Weston et al., 2002). The cardiac actin reporter (pGL3-c-actin-Luc) was generated by subcloning a fragment of the cardiac actin promoter from –440 to +6 into pGL3-basic. The myogenin

promoter-luciferase construct was made by subcloning a 1.14 kb fragment of the myogenin promoter containing the region from pGZ1092, (Yee and Rigby, 1993) from plasmid pGBB into pGL3-basic. The pCMV-GAL4-MEF2A and pCMV-GAL4-MEF2C constructs were as described previously (Yang et al., 1999) and were co-transfected into cells with the pG5-Luc reporter containing five copies of a GAL4 DNA-binding element upstream of a TATA box and the luciferase gene (Stratagene, La Jolla, CA). MSV-tk-βgeo is a replication-defective retrovirus derived from the Mouse Sarcoma Virus containing a β-galactosidase-neomycin fusion gene. The p38 MAPK-responsive transactivation system (Stratagene) consists of an expression vector encoding a GAL4-CHOP10 fusion protein. This vector was co-transfected with pG5 into primary cells, G8 or C2C12 cells in the presence or absence of a constitutively active version of MKK6, termed MKK6E, both with and without expression vectors for p38α and p38β.

Northern blot analysis

Northern blots were carried out using total RNA from limb mesenchymal cultures as previously described (Weston et al., 2000). Briefly, total RNA was extracted from cells 1, 2, 3 or 4 days after cultures were initiated. Cells were treated with media alone or with SB202190-containing media. Blots were probed with radiolabelled DNA fragments derived from cDNAs for *Col2a1*, *Mef2c* or *myogenin*. Subsequently, blots were re-probed with a probe to the18S rRNA to normalize for loading.

Statistical analysis

All luciferase assays were performed a minimum of three times using separate preparations of cells each time. Each transfection or treatment was carried out in four separate wells for all experiments. All luciferase reporter data were analyzed by a one-way analysis of variance (ANOVA), followed by a Bonferroni post-test for multiple comparisons. Statistical analysis was carried out using GraphPad Prism, Version 2.0 (GraphPad Software Inc., San Diego, CA). One of at least two representative experiments is shown for all luciferase results.

Results

Inhibition of p38 attenuates chondrogenesis and promotes myogenesis

Using primary cultures of mouse limb mesenchyme, we recently identified an essential role for activation of p38 in chondrogenesis (Weston et al., 2002). Cartilage formation in these cultures is reduced in the presence of a p38 MAPK inhibitor, SB202190 (Fig. 1). Surprisingly, the reduction in chondrogenesis is accompanied by a dramatic increase in the formation of cells that immunoreact with a myosin heavy chain (MyHC) antibody (Fig. 1). MyHC is a muscle-specific gene that is upregulated upon myoblast differentiation. If treated with the inhibitor

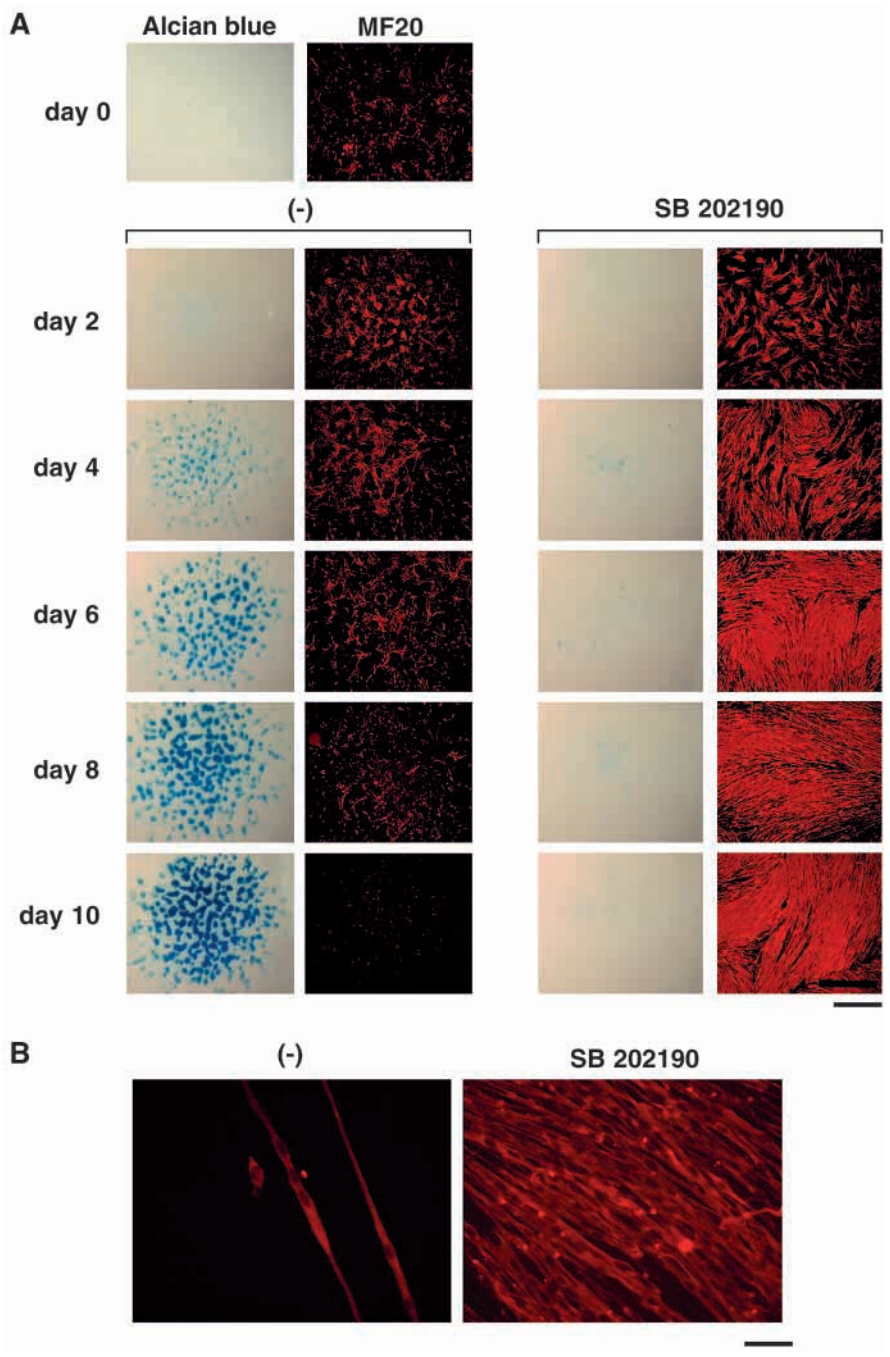

Fig. 1. Effects of p38 inhibition on chondrogenesis and myogenesis in PLM cultures. Primary limb mesenchymal cells were plated at high density, and cultures were fixed for detection of cartilage and muscle. Cartilage was detected by alcian blue staining, and muscle cells were identified by immunofluorescence with a mouse MyHC antibody. In untreated cultures, cartilage nodule formation increases over time, whereas the number of MyHC-positive cells declines to only a few by day 10 (A). Exposure of limb mesenchymal cultures to SB202190 (10 μm) (A) inhibits cartilage nodule formation, whereas there is a substantial increase in the presence of MyHC-positive cells in comparison to control cultures. At later stages the SB202190-treated cultures exhibit extensive myocyte fusion accompanied by the organization of the myocytes into parallel arrays (B). Bars, (A) bright field, 1.0 mm; fluorescence images, 500 μm; (B) 65 μm.

for ~2 or more weeks, the effect becomes increasingly pronounced, with the formation of myotubes (Fig. 1B) and the production of spontaneously contracting fibers.

Although most of our studies focused on the effects of 10 μM SB202190, concentrations as low as 1 μM elicited the same responses, albeit to a lesser extent. The effects of SB203580 on muscle formation are indistinguishable from those of SB202190, whereas the inactive analog of these inhibitors (SB202474) has no noticeable effect, even at concentrations as high as 20 μM (data not shown). At the concentrations used throughout this study (1-10 μM), SB202190 and SB203580 are believed to selectively inhibit the α and β isoforms of p38, leaving the other two isoforms (γ and δ) fully active (Davies et al., 2000). Thus, activation of p38α and β, either alone or in combination, appears to have an inhibitory effect on myogenesis.

Myogenic effects of p38 inhibition require factors present in limb mesenchymal cultures

The effects of SB202190 on myogenesis in primary cultures are reproduced in G8 myoblasts and in C2C12 cells that have been introduced into the primary limb bud cultures (Fig. 2). C2C12 cells are derived from adult muscle (Yaffe and Saxel, 1977), and G8 cells, while embryonic in origin, are from a clone isolated from a myogenic cell line that arose spontaneously in a culture of hind limb muscle cells from a fetal mouse (Christian et al., 1977). There is a major distinction between the primary cultures used here and G8 cells, as we dissect limb buds long before the emergence of functional muscle (E11.5), whereas G8 cells are from developed muscle of an older embryo. When cultured on their own, G8-βgeo cells show no noticeable response to SB202190 (Fig. 2A,B). However, when they are introduced into the primary limb mesenchymal cultures, initially comprising 5% of the total cells in the cultures, these tagged cells respond to SB202190 in a manner very similar to myocytes of the developing limb, becoming elongated and highly organized in parallel arrays of myocytes. In treated cultures, these tagged cells resemble the MyHc-positive cells from primary cultures that were treated with SB202190 (compare Fig. 2D with SB202190-treated cultures in Fig. 1).

C2C12-βgeo cells behave similarly to G8-βgeo cells in primary cultures. Normally, C2C12 cells require serum withdrawal to progress through the myogenic program, and this progression is blocked by p38 inhibition. In the primary cultures, however, the tagged C2C12 cells were induced to form myotubes upon SB202190 treatment, even in the presence of serum (Fig. 2G,H). When cultured on their own in the presence of serum, no obvious change is observed in response to SB202190 (Fig. 2E,F). Thus, combined with the effects on G8-βgeo cells, these results highlight the influence of the microenvironment on the progression of myogenic cells.

To extend our analysis of the cartilage and muscle phenotypes observed following treatment with SB202190, we followed the endogenous activity of cartilage- and muscle-specific genes by northern blot analysis, and monitored the activity of transiently transfected cartilage- and muscle-specific reporters (Fig. 3). As expected, the normal increase in *type II collagen (Col2a1)* expression over time in primary cultures is completely blocked in SB202190-treated cultures

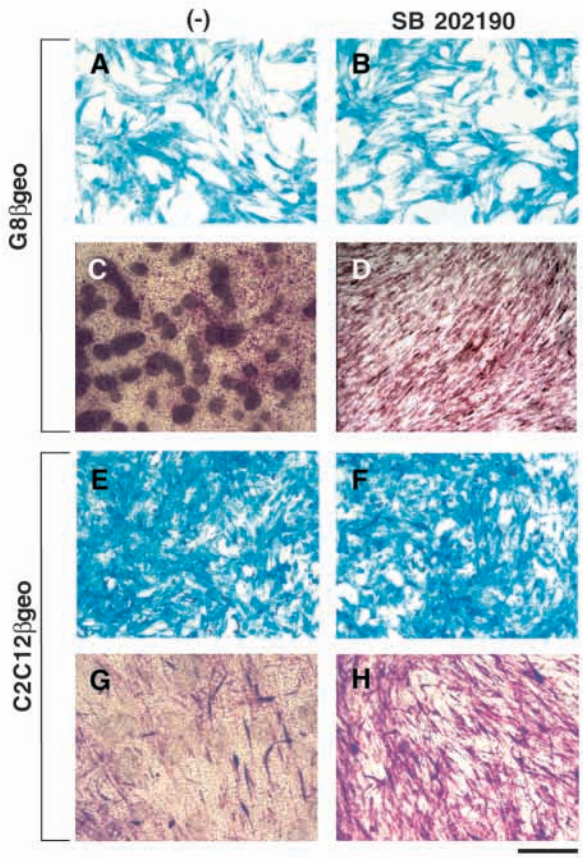

Fig. 2. Myogenic effects of p38 inhibition rely on factors present in primary limb mesenchymal cultures. G8 myoblasts (A-D) and C2C12 myocytes (E-H) were tagged using the pMSV-tk-βgeo retrovirus and were monitored by staining with X-gal or magenta-gal. When SB202190 is added for 4 days, there is no noticeable change in cell appearance, number or organization of either the G8 cells (compare A with B) or the C2C12 cells (compare E with F). To analyze the effects of SB202190 on the muscle cells in the primary limb mesenchymal cultures, cells were mixed at a ratio of 19:1, primary cells:G8 cells (C,D) or primary cells:C2C12 cells (G,H), and the tagged muscle cells were identified by magenta-gal staining after 12 days. When mixed into the primary cultures, both tagged cell populations become highly organized, resembling MyHC-positive cells from primary cultures that were treated with SB202190 (compare to Fig. 1). In contrast, in the untreated cultures, G8 and C2C12 cells appear to lose their muscle phenotype, becoming less bipolar and more round in appearance. Bars, (A,B) 125 μm; (C-D) 500 μm; (E-H) 250 μm.

(Fig. 3A), whereas expression of *myogenin* and *Mef2c* is increased in cultures treated with the inhibitor (Fig. 3A). Repression of cartilage-specific genes and activation of muscle-specific genes was observed using the pGL3(4X48) reporter, which is activated during chondrocyte differentiation (Lefebvre et al., 1996), and the muscle-specific reporters pGL3-E4-Luc (an E box reporter), pGL3-myogenin-Luc (a myogenin-promoter-based reporter) and pGL3-c-actin-Luc (a cardiac-actin-promoter-based reporter). SB202190 inhibited activity of pGL3(4X48) in a dose-dependent manner (Fig. 3B), but enhanced the activity of all of the muscle reporters (Fig. 3C). We also examined the activities of MEF2A and MEF2C by co-transfecting constructs containing the DNA-binding

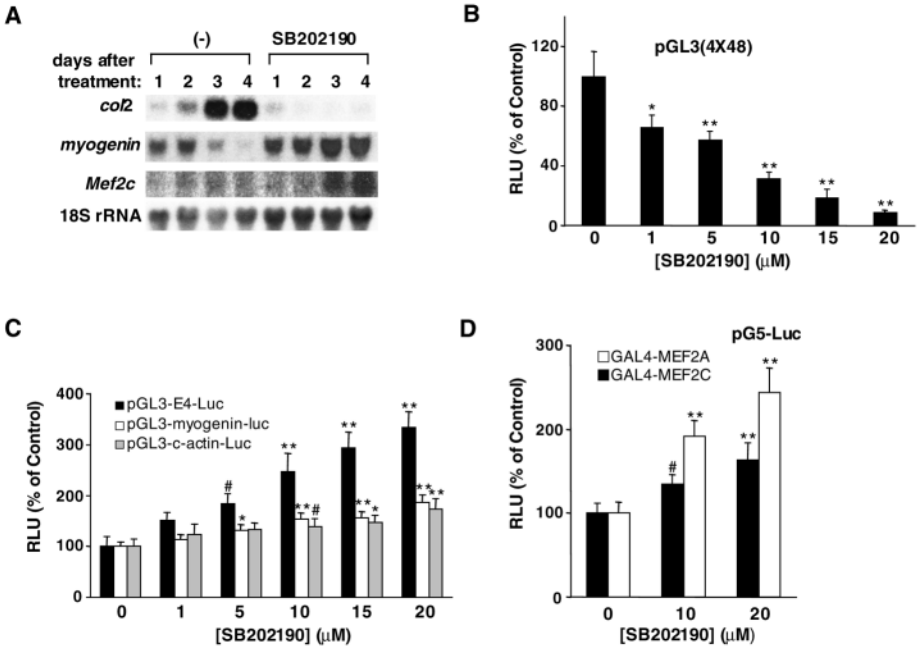

Fig. 3. Attenuation of p38 MAPK promotes expression of the myogenic phenotype. The normal increase in *Col2a1* expression over time in primary cultures is blocked by SB202190, whereas myogenin, present at low levels early on, decreases in expression in the untreated cultures while continuing to increase in response to SB202190 (A). *Mef2c* is also more abundant in SB202190-treated cultures by day 3 compared with the untreated control (A). SB202190 causes a concentration-dependent decrease in the activity of a pGL3(4X48) reporter gene (B), whereas the activity of a transfected E-box-luciferase (pGL3-E4-Luc) reporter was increased in response to increasing concentrations of SB202190 (C). A modest increase was also observed in the activities of reporters containing the myogenin promoter (pGL3-myogenin-Luc) and the cardiac actin promoter (pGL3-c-actin-Luc) (C). When co-transfected with GAL4-MEF2A or GAL4-MEF2C, the activity of a GAL4 reporter (pG5-Luc) was enhanced by SB202190 (D). [ANOVAs (C-E) $P<0.0001$; Bonferroni post-tests, (C-E): #$P<0.05$, *$P<.01$, **$P<0.001$)].

domain of GAL4 fused to each MEF2 (GAL4-MEF2A and GAL4-MEF2C) with the pG5-Luc reporter containing Gal4 response elements. SB202190 induced luciferase activity in cells co-transfected with GAL4-MEF2A or GAL4-MEF2C (Fig. 3D), implicating these two MEFs in the myogenic response to p38 inhibition and further demonstrating the myogenic response to SB202190.

To confirm the presence of p38 in all three cell types, western blots were carried out using p38- (pan) and p38α-specific antibodies. p38 is clearly present in limb mesenchymal cultures at comparable levels over 8 days, and levels are not noticeably affected by SB202190 (Fig. 4A). Similarly, G8 and C2C12 cells express p38 at levels detectable by western blot analysis (Fig. 4A). To assess the ability of SB202190 to block p38 activity, pFA-CHOP, an expression vector containing the GAL4 DNA-binding domain fused to the transactivation domain of CHOP10, was co-transfected with a Gal4-reporter gene, *pG5-Luc*. CHOP10, a transcription factor, is a known target of p38α, whose phosphorylation by p38 is blocked by the SB203580 inhibitor in other cell types (Wang and Ron, 1996). The activity of the Gal4 reporter is also attenuated in the limb mesenchymal cultures by SB202190 (10 μM). Specifically, the

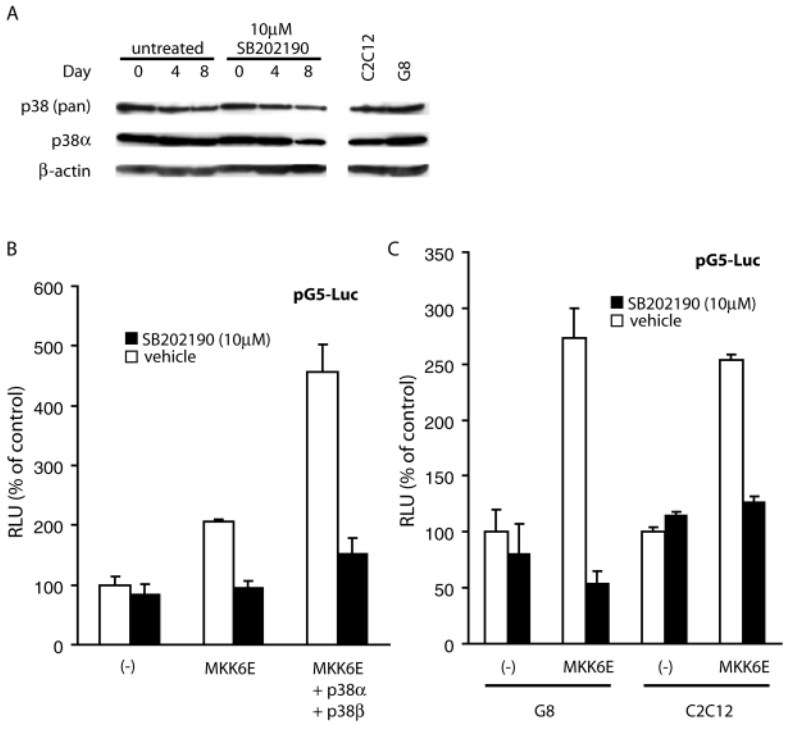

Fig. 4. p38 is expressed and effectively inhibited by SB202190 in primary limb mesenchymal, G8 and C2C12 cells. Limb mesenchymal cultures, G8 and C2C12 cells express at least p38α, as determined by western analysis using a pan-p38 and p38α-specific antibodies (A). A band corresponding to a molecular weight of ~42 kDa, consistent with the reported size of p38 isoforms, was observed with both antibodies. The expression of p38 in limb mesenchymal cultures does not noticeably change upon treatment with 10 μM SB202190. To control for loading all blots were subsequently incubated with an antibody against β-actin. SB202190 effectively inhibits p38 activity in limb mesenchymal cultures (B) and G8 and C2C12 cells (C). Activity of a GAL4-CHOP fusion protein, measured by pG5-luc activity, is induced by co-transfection with MKK6E and p38α and β in primary cultures and in G8 and C2C12 cultures (data shown only for MKK6E). This induction is attenuated by 10 μM SB202190 in all cell types.

increase in luciferase activity caused by co-transfection with MKK6E (a constitutively active version of MKK6) is completely blocked by SB202190. As expected, the ability of MKK6E to increase reporter gene activity is further enhanced by co-transfection of expression vectors encoding p38α and p38β, and this activity is also substantially attenuated by the addition of 10 μM SB202190 (Fig. 4B). These results provide convincing evidence that this inhibitor can effectively attenuate p38 activity in the limb mesenchymal cultures. Similar results are observed in G8 and C2C12 cells, where addition of 10 μM SB2020190 was able to reduce MKK6E-induced reporter gene activity to control levels (Fig. 4C).

Inhibition of p38 enhances myogenesis of somitic mesoderm-derived cells

During limb development, myogenic cells originate from the somites, whereas cartilage progenitors arise from the progress zone, a region at the distal tip of the limb bud. The increase in muscle formation caused by SB202190 could be due to enhanced myogenesis of somite-derived cells or the redirection of cells originally fated to become chondrocytes. Both possibilities could account for an increase in muscle that appears to be at the expense of cartilage. To identify which cells contribute to the increased muscle, cells of the proximal portion of the limb bud were cultured separately from those of the distal portion (Fig. 5). The distal region of the limb bud at this stage (Fig. 5A) contains fewer somite-derived myogenic cells compared with the proximal portion (Fig. 5G), although there are more prechondrogenic cells in the distal cultures (compare Fig. 5E with K). SB202190 attenuates chondrogenesis in both cultures (Fig. 5F,L). If the effects of p38 inhibition were the result of prechondrogenic cells being redirected to the myogenic lineage, the dramatic myogenic effect of SB202190 would be observed within the distal cultures. In contrast, however, the magnitude of the myogenic response is directly proportional to the number of somitic cells present in the culture at the time of initiation. Specifically, more muscle was seen in the proximal cultures after 6 days of SB202190 treatment (Fig. 5I) compared with the distal cultures (Fig. 5C). This suggests that p38 inhibition promotes myogenesis of somite-derived cells as opposed to redirecting the chondrogenic cells derived from the distal tip of the limb bud. Further support for this comes from the almost complete lack of MyHC-positive cells both in the presence and absence of SB202190 in primary cultures derived from E10 embryos (data not shown). At E10, very few somite-derived cells have migrated into the developing limb (Martin, 1990).

Inhibition of p38 induces the rapid re-organization of myocytes.

To further examine the effects of SB202190 on myogenesis, cells were fixed and analyzed for MyHC expression at earlier time points following treatment (Fig. 6). The increased muscle formation appears to be due to the rapid advancement of pre-existing myocytes. Even 2 hours after treatment with SB202190, MyHC-positive cells aggregate together and polarize, forming distinct foci of myocytes within 6 hours. The appearance of these discrete aggregations of bipolar cells is striking by 24 hours. These dramatic changes do not appear to be accompanied by changes in the number of MyHC-positive cells. Muscle cells expressing MyHC were counted 12 hours after treatment, at which time the effects of SB202190 became visibly apparent, but not to the extent to which aggregation and fusion of myocytes makes the counting of individual cells impossible. There was no significant difference in the number of MyHC-positive cells between SB202190-treated and untreated cells (data not shown). Thus, the enhanced muscle formation is probably not due to increased proliferation of myogenic precursors or of terminally differentiated myocyte, but rather the advanced progression of pre-existing myocytes. p38 inhibition in these cultures may therefore advance the post-differentiation stages

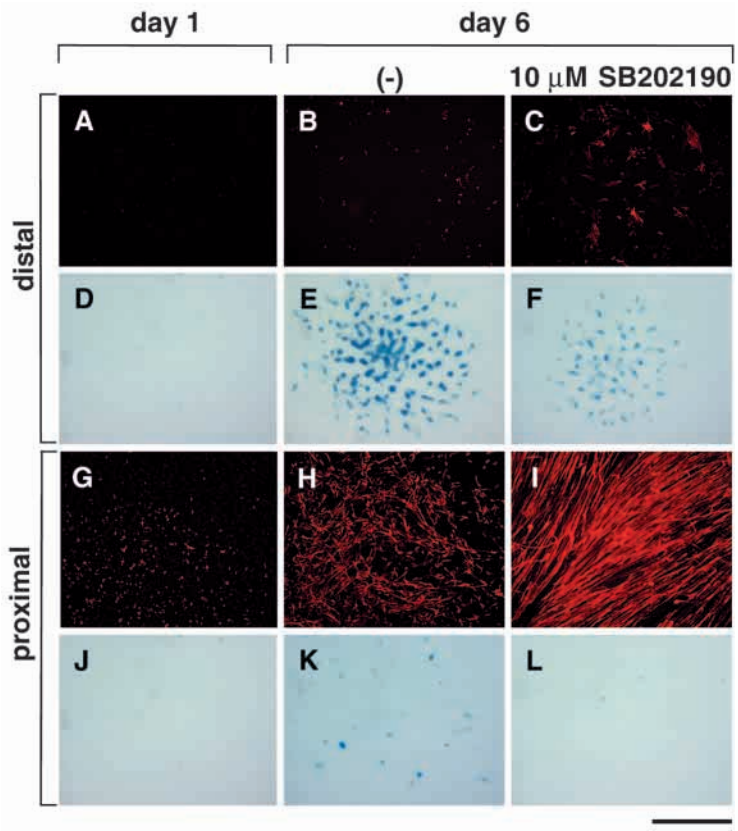

Fig. 5. Inhibition of p38 promotes myogenesis of pre-existing myoblasts. Cultures were established from distal limb mesenchyme (A-F) or proximal limb mesenchyme (G-L). Proximal cultures contain more MyHC-positive cells compared to distal cultures (compare G with A). After 6 days, there are fewer detectable muscle cells in untreated distal cultures (B) compared with proximal cultures (H), but many more cartilage nodules form from distal mesenchyme (E) than from proximal mesenchyme (K). Following 6 days of treatment with SB202190, in both distal and proximal cultures, there is a decrease in nodule formation and an increase in the formation of foci of MyHC-positive cells. The muscle cells in treated proximal cultures (I), however, are much more prevalent, stain more intensely and are more highly organized into parallel arrays compared with muscle cells of distal cultures (C), and there are more cartilage nodules in the distal cultures (F) compared with the proximal cultures (L). Bars, fluorescence images (A-C, G-I) 750 μm; bright-field images (D-F, J-L) 1.5 mm.

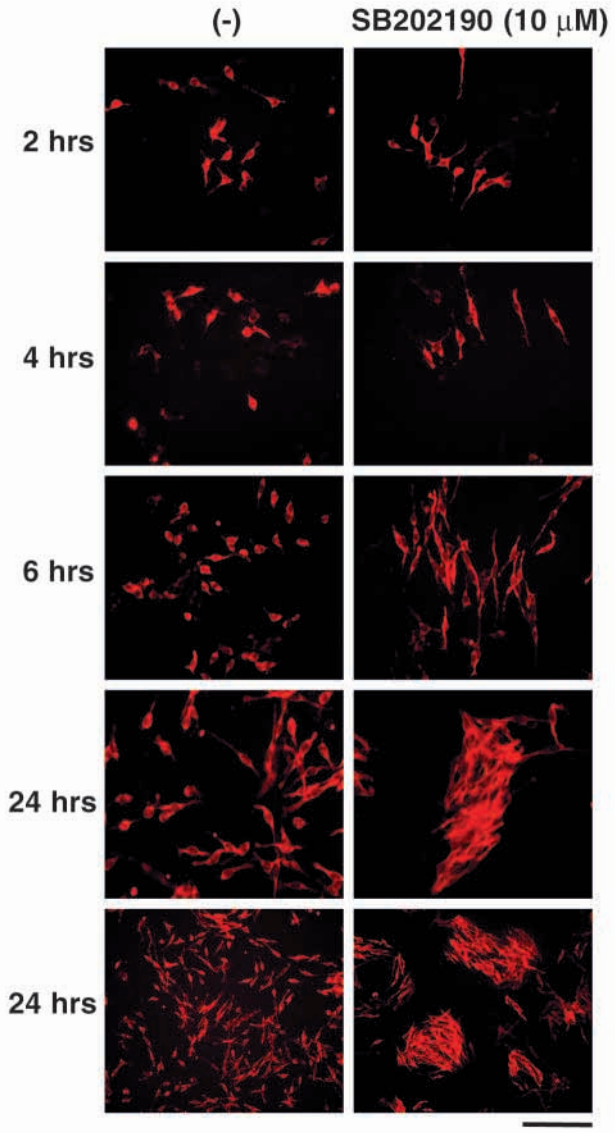

Fig. 6. Inhibition of p38 signaling rapidly alters morphology of myoblasts. SB202190-treated and untreated PLM cultures were fixed and analyzed for MyHc expression at various times over 24 hours. In control cultures, at all times examined, MyHc-positive cells are only slightly bipolar, with small cellular extensions, are randomly oriented and are distributed throughout the culture. Within only a few hours of SB202190 addition, these cells acquire an enhanced bipolarity, with the bipolar cellular extensions oriented in the same direction. These bipolar cells are also in very close proximity to each other. As early as 6 hours after treatment, this polarization and aggregation is pronounced, as indicated by the presence of foci of myoblasts that become much more discernible by 24 hours. Bar, top eight panels, 125 μm; bottom panel, 500 μm.

of muscle formation by facilitating the polarization, fusion and aggregation of myocytes.

Discussion

Our results demonstrate, unambiguously, that treatment of primary limb mesenchymal cultures with p38 inhibitors enhances muscle formation in a system where myogenic cells are normally lost. The continued progression of myogenesis by SB202190 is not solely due to an inhibition of chondrogenesis (and hence the absence of factors normally provided by cartilage), as similar effects have never been reported for other factors that attenuate cartilage formation, including retinoic acid, the bone morphogenetic protein (BMP) antagonist Noggin, trichostatin A and others. Moreover, myogenic cells within the primary cultures are not normally maintained and do not progress to form muscle. Thus, p38 inhibition actively promotes the maintenance and progression of these cells, rather than simply providing a permissive environment for the myogenic program to proceed. The ultimate fate of myoblasts in the untreated cultures is not yet clear; however, these results strongly suggest that the muscle-promoting effects of p38 inhibition require factors provided in the limb mesenchymal environment.

Importance of the extracellular environment in myogenesis

The ability of p38 inhibition to activate myogenic markers in limb mesenchymal cultures is surprising given that in a variety of cell types the exact opposite was found. Moreover, here we demonstrate activation of MEF2A and C by p38 inhibition, despite the well-documented phosphorylation and subsequent activation of these factors by p38 in other systems (Yang et al., 1999; Zetser et al., 1999; Zhao et al., 1999). Thus, the role of p38 signaling in activating the myogenic program within the primary cultures used here seems exactly opposite to that described for other populations of homogeneous myogenic cells. Given the ability of SB202190 to enhance muscle formation of G8 and C2C12 cells only after they are co-cultured with the primary limb mesenchymal cultures, it appears to be important to study myogenesis in the context of other factors that are non-myogenic in origin. A major difference between the primary limb mesenchymal cultures and the C2C12 and G8 clonal populations is the heterogeneity of the primary cultures, resulting in the production of a number of factors by non-myogenic cells that are absent from clonal populations but probably important for myogenesis. The contribution of signals from non-myogenic cells is well documented and includes such factors as WNTs, sonic hedgehog (SHH), fibroblast growth factors (FGFs) and BMPs (reviewed in Blagden and Hughes, 1999).

In general, limb development relies on the concerted action of multiple factors secreted from local signaling centers to direct the commitment and differentiation of precursor cells. This is especially true for myogenic cells since, despite their somitic origin, limb muscle progenitors enter the skeletal myogenic program only after they reach the limb bud and are under the influence of local extrinsic factors that control the specification, differentiation and patterning of these cells. The importance of the local cellular environment in mediating muscle development and patterning in the limb was recently further highlighted in a study that utilized retroviral vectors to analyze the fate of somitic-derived myogenic precursors in the chick (Kardon et al., 2002). To study myogenesis in the context of embryonic development, heterogeneous limb mesenchymal cultures may provide a more relevant in vitro model system, in that they contain factors that are normally present during in vivo development of muscle.

A potential dual role for p38 in myogenesis

Despite the differences between our culture system and those used by others, the opposing effects seen in response to p38 inhibition may be explained by a dual role for p38 signaling at distinct stages of myogenesis, much like the factor MyoD was recently shown to regulate discrete subprograms of gene expression during muscle formation (Bergstrom et al., 2002). It is interesting to note that the same study reveals only a subset of MyoD-regulated genes required p38 kinase activity. Thus, the possibility that p38 signaling differentially modulates subsets of the myogenic program is worth pursuing. Our analysis thus far has focused on the effects of SB202190 on differentiated muscle cells (e.g. cells already expressing MyHC) and thus do not preclude a possible requirement for p38 signaling in steps leading up to myocyte formation.

It is possible that p38 signaling is required early on to induce myocyte differentiation, but also acts to prevent the premature progression of those cells. In this context, p38 signaling would be active during the differentiation stage, but would subsequently be suppressed for those differentiated cells to elongate, polarize, aggregate, and fuse. Very little is currently known about these later stages of muscle development. Given the dramatic and rapid phenotypic changes observed after addition of SB202190, it seems likely that p38 is important in these post-differentiation events. Further study of the phenotypic changes caused by SB202190 would improve our current understanding of the mechanisms of polarization, aggregation and fusion of myocytes. In this respect, treatment of the limb mesenchymal cultures with p38 inhibitors provides an excellent model system for characterizing these aspects of myogenesis.

The authors would like to thank I. Skerjanc and H. Petropoulos for the cardiac actin promoter, pGL2-E4-Luc, myogenin and Mef2c probes, MKK6E expression vector, and S. P. Yee for a plasmid containing the myogenin promoter. We are also grateful to B. de Crombrugghe for the 4X48-p89 luciferase construct, A. Sharrocks for the GAL4-MEF2A and GAL4-MEF2C constructs, R. Davis for pcDNA3-p38α, and J. Han for pcDNA3.1-p38β. All experiments with animals were conducted in accordance with a University of Western Ontario Animal Use Subcommittee protocol. A.D.W. and A.V.S. were supported by scholarships from the Canadian Institutes of Health Research (CIHR), and A.G.R. was funded by a scholarship from the National Science and Engineering Research Council (NSERC). T.M.U is a CIHR New Investigator, and this work was funded by a grant to T.M.U. from CIHR.

References

Bader, D., Masaki, T. and Fischman, D. A. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* **95**, 763-770.

Bergstrom, D. A., Penn, B. H., Strand, A., Perry, R. L., Rudnicki, M. A. and Tapscott, S. J. (2002). Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. *Mol. Cell* **9**, 587-600.

Blagden, C. S. and Hughes, S. M. (1999). Extrinsic influences on limb muscle organisation. *Cell Tissue Res.* **296**, 141-150.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8**, 701-709.

Braun, T., Bober, E., Winter, B., Rosenthal, N. and Arnold, H. H. (1990). Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. *EMBO J.* **9**, 821-831.

Cabane, C., Englaro, W., Yeow, K., Ragno, M. and Derijard, B. (2003). Regulation of C2C12 myogenic terminal differentiation by MKK3/p38alpha pathway. *Am. J. Physiol. Cell Physiol.* **284**, C658-C666.

Cash, D. E., Bock, C. B., Schughart, K., Linney, E. and Underhill, T. M. (1997). Retinoic acid receptor alpha function in vertebrate limb skeletogenesis: a modulator of chondrogenesis. *J. Cell Biol.* **136**, 445-457.

Chang, L. and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature* **410**, 37-40.

Christian, C. N., Nelson, P. G., Peacock, J. and Nirenberg, M. (1977). Synapse formation between two clonal cell lines. *Science* **196**, 995-998.

Conejo, R. and Lorenzo, M. (2001). Insulin signaling leading to proliferation, survival, and membrane ruffling in C2C12 myoblasts. *J. Cell Physiol.* **187**, 96-108.

Cuenda, A. and Cohen, P. (1999). Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J. Biol. Chem.* **274**, 4341-4346.

Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95-105.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Edmondson, D. G. and Olson, E. N. (1989). A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* **3**, 628-640.

Gallea, S., Lallemand, F., Atfi, A., Rawadi, G., Ramez, V., Spinella-Jaegle, S., Kawai, S., Faucheu, C., Huet, L., Baron, R. et al. (2001). Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. *Bone* **28**, 491-498.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A. and Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol. Cell Biol.* **9**, 5022-5033.

Kardon, G., Campbell, J. K. and Tabin, C. (2002). Local extrinsic signals determine muscle and endothelial cell fate and patterning in the vertebrate limb. *Dev. Cell* **3**, 533-545.

Kaushal, S., Schneider, J. W., Nadal-Ginard, B. and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science* **266**, 1236-1240.

Kyriakis, J. M. and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **81**, 807-869.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. and Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58**, 823-831.

Lefebvre, V., Zhou, G., Mukhopadhyay, K., Smith, C. N., Zhang, Z., Eberspaecher, H., Zhou, X., Sinha, S., Maity, S. N. and de Crombrugghe, B. (1996). An 18-base-pair sequence in the mouse proalpha1(II) collagen gene is sufficient for expression in cartilage and binds nuclear proteins that are selectively expressed in chondrocytes. *Mol. Cell Biol.* **16**, 4512-4523.

Li, Y., Jiang, B., Ensign, W. Y., Vogt, P. K. and Han, J. (2000). Myogenic differentiation requires signalling through both phosphatidylinositol 3-kinase and p38 MAP kinase. *Cell Signal.* **12**, 751-757.

Martin, P. (1990). Tissue patterning in the developing mouse limb. *Int. J. Dev. Biol.* **34**, 323-336.

Miner, J. H. and Wold, B. (1990). Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA* **87**, 1089-1093.

Molkentin, J. D., Black, B. L., Martin, J. F. and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**, 1125-1136.

Obata, T., Brown, G. E. and Yaffe, M. B. (2000). MAP kinase pathways activated by stress: the p38 MAPK pathway. *Crit. Care Med.* **28**, N67-N77.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K. and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22**, 153-183.

Perry, R. L. and Rudnicki, M. A. (2000). Molecular mechanisms regulating myogenic determination and differentiation. *Front Biosci.* **5**, D750-D767.

Puri, P. L., Wu, Z., Zhang, P., Wood, L. D., Bhakta, K. S., Han, J.,

Feramisco, J. R., Karin, M. and Wang, J. Y. (2000). Induction of terminal differentiation by constitutive activation of p38 MAP kinase in human rhabdomyosarcoma cells. *Genes Dev.* **14**, 574-584.

Rhodes, S. J. and Konieczny, S. F. (1989). Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev.* **3**, 2050-2061.

Ridgeway, A. G., Wilton, S. and Skerjanc, I. S. (2000). Myocyte enhancer factor 2C and myogenin up-regulate each other's expression and induce the development of skeletal muscle in P19 cells. *J. Biol. Chem.* **275**, 41-46.

Wang, X. Z. and Ron, D. (1996). Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* **272**, 1347-1349.

Weston, A. D., Rosen, V., Chandraratna, R. A. and Underhill, T. M. (2000). Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. *J. Cell Biol.* **148**, 679-690.

Weston, A. D., Chandraratna, R. A., Torchia, J. and Underhill, T. M. (2002). Requirement for RAR-mediated gene repression in skeletal progenitor differentiation. *J. Cell Biol.* **158**, 39-51.

Wright, W. E., Sassoon, D. A. and Lin, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56**, 607-617.

Wu, Z., Woodring, P. J., Bhakta, K. S., Tamura, K., Wen, F., Feramisco, J. R., Karin, M., Wang, J. Y. and Puri, P. L. (2000). p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Mol. Cell. Biol.* **20**, 3951-3964.

Yaffe, D. and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727.

Yang, S. H., Galanis, A. and Sharrocks, A. D. (1999). Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol. Cell. Biol.* **19**, 4028-4038.

Yee, S. P. and Rigby, P. W. (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.

Zetser, A., Gredinger, E. and Bengal, E. (1999). p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. *J. Biol. Chem.* **274**, 5193-5200.

Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., di Padova, F., Olson, E. N., Ulevitch, R. J. and Han, J. (1999). Regulation of the MEF2 family of transcription factors by p38. *Mol. Cell. Biol.* **19**, 21-30.