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## Abstract

In aged skeletal muscle, impairments in regrowth and regeneration may be explained by a decreased responsiveness of muscle precursor cells (MPCs) to environmental cues such as growth factors. We hypothesized that impaired responsiveness to fibroblast growth factor 2 (FGF2) in MPCs from old animals would be explained by impaired FGF2 signalling. We determined that 5-bromo-2f-deoxyuridine (BrdU) incorporation and cell number increase less in MPCs from 32- compared with 3-month-old rats. In the presence of FGF2, we demonstrated that there were age-associated differential expression patterns for FGF receptor 1 and 2 mRNAs. Measurement of downstream signalling revealed that that mitogen-activated protein kinase/ERK kinase 1/2 (MEK1/2)-extracellular signal-regulated kinase 1/2, protein kinase C and p38 were FGF2-driven pathways in MPCs. Uniquely, protein kinase C signalling was shown to play the largest role in FGF2-stimulated proliferation in MPCs. c-Jun N-terminal kinase (JNK) signalling was ruled out as an FGF2-stimulated proliferation pathway in MPCs. Inhibition of JNK had no effect on FGF2 signalling to BrdU incorporation, and FGF2 treatment was associated with increased phosphorylation of p38, which inhibits, rather than stimulates, BrdU incorporation in MPCs. Surprisingly, the commonly used vehicle, dimethyl sulphoxide, rescued proliferation in MPCs from old animals. These findings provide insight for the development of effective treatment strategies that target the age-related impairments of MPC proliferation in old skeletal muscle.

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**In aged skeletal muscle, impairments in regrowth and regeneration may be explained by a decreased responsiveness of muscle precursor cells (MPCs) to environmental cues such as growth factors. We hypothesized that impaired responsiveness to fibroblast growth factor 2 (FGF2) in MPCs from old animals would be explained by impaired FGF2 signalling. We determined that 5-bromo-2<sup>f</sup>-deoxyuridine (BrdU) incorporation and cell number increase less in MPCs from 32- compared with 3-month-old rats. In the presence of FGF2, we demonstrated that there were age-associated differential expression patterns for FGF receptor 1 and 2 mRNAs. Measurement of downstream signalling revealed that that mitogen-activated protein kinase/ERK kinase 1/2 (MEK1/2)–extracellular signal-regulated kinase 1/2, protein kinase C and p38 were FGF2-driven pathways in MPCs. Uniquely, protein kinase C signalling was shown to play the largest role in FGF2-stimulated proliferation in MPCs. c-Jun N-terminal kinase (JNK) signalling was ruled out as an FGF2-stimulated proliferation pathway in MPCs. Inhibition of JNK had no effect on FGF2 signalling to BrdU incorporation, and FGF2 treatment was associated with increased phosphorylation of p38, which inhibits, rather than stimulates, BrdU incorporation in MPCs. Surprisingly, the commonly used vehicle, dimethyl sulphoxide, rescued proliferation in MPCs from old animals. These findings provide insight for the development of effective treatment strategies that target the age-related impairments of MPC proliferation in old skeletal muscle.**

Muscle precursor cells (MPCs), or satellite cells, are adult skeletal muscle stem cells that are located between the basal lamina and the plasmalemma (Mauro, 1961; Schultz & McCormick, 1994). They reside in a quiescent state and, when activated, MPCs undergo proliferation, migration and subsequent differentiation, whereby they fuse and contribute new myonuclei (Hawke & Garry, 2001; Roy *et al.* 1999). Proliferation of MPCs is required for the effective regrowth and regeneration of skeletal muscle (Wheldon *et al.* 1982). Importantly, both regrowth and regeneration are impaired in aged skeletal muscle (Brooks & Faulkner, 1990; Pattison *et al.* 2003), largely due to a decreased activation of MPCs (Carlson *et al.* 2008). One potential contributor to attenuated MPC proliferation in aged skeletal muscle could be a blunted responsiveness to proliferation-stimulating growth factors in skeletal muscle.

There are multiple growth factors associated with the local skeletal muscle milieu which stimulate the proliferation of MPCs (Gopinath & Rando, 2008). Fibroblast growth factor 2 (FGF2), previously known as basic FGF or bFGF, is a polypeptide growth factor that stimulates MPC proliferation (Allen & Boxhorn, 1989; Mezzogiorno *et al.* 1993). Fibroblast growth factor 2 binds to low-affinity (heparan sulphate proteoglycan, HSPG) and high-affinity receptors (FGFR), the latter having four isoforms. After FGF2 binds to its receptor, FGFR dimers are trans-phosphorylated, and downstream signalling is initiated to stimulate proliferation (Plotnikov *et al.* 1999; Ornitz & Itoh, 2001; Chen & Forough, 2006). Within muscle precursor cells, FGF2 is known to stimulate mitogen-activated protein kinase/ERK kinase 1/2 (MEK1/2) – extracellular signal-regulated kinase 1/2 (ERK1/2) signalling (Campbell *et al.* 1995; Kastner

*et al.* 2000; McFarland & Pesall, 2008), which in turn stimulates proliferation. Other evidence has shown that FGF2 treatment activates signal transduction and activation of transcription (STAT) or c-Jun N-terminal kinase (JNK) signalling (Megeney *et al.* 1996). However, the role of additional mitogen-activated protein kinase (MAPK) pathways in FGF2-stimulated proliferation, to our knowledge, has not been fully elucidated in MPCs.

The functionally associated outcome of increased FGF2 stimulation is muscle growth. In skeletal muscle, FGF2 is expressed at a high level during muscle regeneration (Anderson *et al.* 1995), and FGF2 injection has been shown to improve recovery in reinnervated (Iwata *et al.* 2006) and dystrophic muscle (Lefaucheur & Seville, 1995a). Neutralizing antibodies to FGF2 attenuate skeletal muscle repair (Lefaucheur & Seville, 1995b). Upon muscle damage, FGF2 is released from the extracellular matrix, macrophages and fibroblasts, which infiltrate into the muscle as part of the repair process. Anatomically, MPCs exist in close proximity to the capillaries, which are also likely to serve as a source of FGF2 via production from the endothelial cells and the circulation.

The present study was performed to complete two objectives. The first objective was to determine the effect of FGF2 stimulation on MPC proliferation from 3-(young) and 32-month-old (aged) animals by assessing the proportion of cells in S phase in MPC cell culture using flow cytometric analysis separation of 5-bromo-2'-deoxyuridine (BrdU) pulse-labelled MPCs and increases in cell number. The second objective was to determine the FGF2-dependent signalling pathways in MPCs from young and aged animals and assess whether ageing differences in signalling to BrdU incorporation exist.

## Methods

### Animals

All animal procedures were approved by the Animal Care and Use Committee at the University of Missouri-Columbia. Young (3-month-old) and old (32-month-old) Fischer 344 × Brown Norway F1 hybrid rats were obtained from the National Institute of Aging (NIA). Animals were housed with a 12 h – 12 h light – dark cycle at 21°C and were provided with food and water *ad libitum*. At the time of cell isolation, animals were deeply anaesthetized with an intraperitoneal injection of ketamine (80 mg kg<sup>-1</sup>), xylazine (10 mg kg<sup>-1</sup>) and acepromazine (4 mg kg<sup>-1</sup>), and killed by exsanguination. Following exsanguination, gastrocnemius and plantaris muscles were dissected.

### Isolation of muscle precursor cells

Muscle precursor cells were isolated using the method developed by Allen *et al.* (1997), incorporating the modifications made by Lees *et al.* (2006). Cells were

isolated from gastrocnemius and plantaris muscles and transferred to a 150 mm tissue culture-treated plate (negative charge induced) for a 24 h preplate incubation. After 24 h, MPCs were transferred to Matrigel-coated (BD Biosciences, San Jose, CA, USA) 150 mm plates (0.1 mg ml<sup>-1</sup> Matrigel, 60 min at 37°C) and cultured for 3–5 days in growth medium (GM, composed of 20% fetal bovine serum, 100 units ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin and 40 µg ml<sup>-1</sup> gentamicin in Ham's F-10). This method has been shown previously to yield a population of cells with a high degree of myogenic purity (Lees *et al.* 2006). The MPCs were passaged once to Matrigel-coated cell culture plates for further experimentation at passage 1 unless indicated otherwise. The MPCs were cultured in a humidified incubator with ambient air and 5% CO<sub>2</sub> at 37°C.

### Cell proliferation

Muscle precursor cells were passaged to 100 mm plates (150 000 cells per plate) and grown for 24 h in GM. After 24 h, medium was changed to low-mitogen/serum medium [2% horse serum (HS) with 100 nM dexamethasone], and the MPCs were treated with FGF2 or vehicle. Medium was changed to low-mitogen/serum medium in order to test the specific effects of FGF2 on MPC proliferation by minimizing the influence of other mitogens.

At 23 h of treatment with FGF2, MPCs were pulse-labelled with BrdU (10 µM) for 1 h at 37°C in the humidified incubator. After 1 h, MPCs were trypsinized, fixed with ice-cold 70% EtOH, and stored at 4°C until further analysis. Immunodetection of BrdU using flow cytometry was performed as described by Lees *et al.* (2008). Briefly, cells were incubated in 2N HCl for 30 min, followed by two neutralizing washes with 0.1 M sodium borate (pH 8.5) and a final wash in Dulbecco's-phosphate-buffered saline (PBS). Cells were incubated with anti-BrdU – fluorescein (5 µg ml<sup>-1</sup> in 0.1% bovine serum albumin in PBS, Roche Applied Sciences, Indianapolis, IN, USA) for 30 min. Twenty thousand cells were analysed using a BD FAC-Scan and CellQuest Pro software (BD Biosciences), and the proportions of BrdU-positive (BrdU<sup>+</sup>) and BrdU-negative (BrdU<sup>-</sup>) nuclei were determined as described previously (Lees *et al.* 2008).

### Cell number

Muscle precursor cells were seeded onto to six-well plates (12 500 cells per well) and grown for 24 h in GM. After 24 h, the medium was changed to low-mitogen medium and the MPCs were treated with variable doses of FGF2 for 48 h; the medium was refreshed after 24 h. The MPCs were harvested from the six-well culture plates, and cell number was determined by measuring cells adherent to the cell

**Table 1. Forward and reverse primers for the FGF receptors**

Receptor type	Forward primer	Reverse primer
FGFR1	5 <sup>1</sup> -CAGGGCTACCAGCCAACAA-3 <sup>1</sup>	5 <sup>1</sup> -CACTGTACACCTTGACATGAACTC-3 <sup>1</sup>
FGFR2	5 <sup>1</sup> -GTGATGCCCGAGCCCCATA-3 <sup>1</sup>	5 <sup>1</sup> -AGCCCATCAGGTCCATATTTACTG-3 <sup>1</sup>
FGFR3	5 <sup>1</sup> -CAGAAGCTATTGGCATCGACAAG-3 <sup>1</sup>	5 <sup>1</sup> -TCGCATCATCTTTCAGCATCTT-3 <sup>1</sup>
FGFR4	5 <sup>1</sup> -TCCCGGCCAGACCAAAC-3 <sup>1</sup>	5 <sup>1</sup> -TCAGGTCTGCCAAATCCTTGT-3 <sup>1</sup>

culture plate using the CyQUANT assay kit (Invitrogen, Carlsbad, CA, USA).

### Real-time PCR

Muscle precursor cells were passaged to 60 mm plates (55 000 cells per plate) and grown for 24 h in GM. After 24 h, the medium was changed to low-mitogen medium and the MPCs were treated with one dose of FGF2 (10 ng ml<sup>-1</sup>) for a 24 h treatment. The RNA was harvested from the cell culture plate using RLT lysis buffer (Qiagen, Valencia, CA, USA) with 1%  $\beta$ -mercaptoethanol and passed through a QIAshredder (Qiagen). The RNA was isolated and purified using RNeasy micro kit<sup>®</sup> (Qiagen), and reverse transcription was performed. Quantification of RNA was done using spectrophotometry by measuring the absorbance at 260 nm, and purity was determined by the ratio of absorbance at 260 and 280 nm. Verification of RNA integrity was determined by agarose gel electrophoresis and ethidium bromide staining. Levels of FGFR(1 – 4) mRNA were determined using real-time quantitative PCR using SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7000 (Applied Biosystems) using 25 ng cDNA. Primers for each FGF receptor are shown in Table 1. Data were analysed using the Ct method. Primers for 18S rRNA were obtained from Applied Biosystems. Standard curves for target mRNA and 18S rRNA were run in order to determine amplification efficiency. Each FGF receptor value was normalized to values for 18S ribosomal RNA from control samples. Each FGFR(1 – 4) mRNA level was determined in mitogen-rich (without FGF2) and in low-mitogen conditions with FGF2 stimulation in MPCs from 3- and 32-month-old animals.

### Western blot

Muscle precursor cells were seeded on 100 mm plates (150 000 cells per plate) and grown for 24 h in GM. After 48 h, the medium was changed to low-mitogen medium for 3 h. Then, the MPCs were treated with FGF2 (0, 0.5 or 10 ng ml<sup>-1</sup>) for 10 min. After 10 min treatment, radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) was used to lyse and harvest cells. Protein concentration was determined using DC Assay (BioRad, Hercules, CA, USA). Equal amounts of protein were loaded and separated via SDS-PAGE,

and protein was transferred to nitrocellulose membranes (Fischer-Scientific, Pittsburgh, PA, USA). To ensure equal loading, nitrocellulose membranes were stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA), which allows for both the qualitative visualization and the quantification of the amount of protein in a given lane (Klein *et al.* 1995). Antibodies for phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202, Tyr204), and phospho-p38 (Thr180/Tyr182) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit immunoglobulin G and anti-mouse immunoglobulin G secondary antibodies conjugated with horseradish peroxidase were obtained from Pierce Biotechnology (Rockford, IL, USA). Primary – secondary immunocomplexes were visualized using a chemiluminescent reaction mixture, and images were scanned. The signal bands were scanned using a Kodak Image Station 4000R Digital Imaging System (Eastman Kodak Company, Rochester, NY, USA) and quantified using Kodak molecular imaging software (version 4.0).

### Pathway inhibitors

Muscle precursor cells were passaged to 100 mm plates (150 000 cells per plate) and grown for 24 h in GM. After 24 h, the medium was changed to low-mitogen medium and chemical inhibitors were added. Inhibition of MEK1/2 was performed in MPCs treated with FGF2 using two chemical inhibitors: the more specific MEK1/2 inhibitor U0126 (10  $\mu$ M, Cell Signaling Technology) and the non-specific MEK1/2 inhibitor SL327 (40  $\mu$ M, Calbiochem, Gibbstown, NJ, USA). Previously, non-specific effects of the non-specific MEK1/2 inhibitor have been documented (Scherle *et al.* 2000).

A Western blot dose – response experiment was performed using both MEK1/2 inhibitors to determine the effective dose of inhibition. The p38 inhibitor, SB203580 (10  $\mu$ M, Calbiochem), the protein kinase C (PKC) inhibitor, bisindolylmaleimide I (BIS I, 10  $\mu$ M, Calbiochem), and the JNK inhibitor, SP600125 (10  $\mu$ M, Calbiochem), were used to determine the contribution of p38, PKC and JNK pathways, respectively, in FGF2-dependent signalling. Optimal doses for SB203580, BIS I and SP600125 were obtained from the literature (Kefaloyianni *et al.* 2006; Huang *et al.* 2007; Krejci *et al.* 2007). After a 1 h treatment with inhibitors, MPCs were treated with FGF2 (10 ng ml<sup>-1</sup>) for 24 h. After

23 h of treatment, the MPCs were pulse-labelled with BrdU ( $10 \mu\text{M}$ ) for 1 h and harvested as described above (see 'Cell proliferation'). For Western blot measurements, inhibitors were added to low-mitogen medium 1 h prior to treatment. The FGF2 was added to the medium for 10 min, followed by cell lysis (RIPA buffer).

## Statistics

Muscle precursor cells isolated from one animal were considered an  $n$  value of 1. Data are presented as

means  $\pm$  S.E.M. Comparisons between groups were made using ANOVA (SigmaStat, version 3.1, Systat Software, San Jose, CA, USA). Statistical significance was accepted at  $P < 0.05$ .

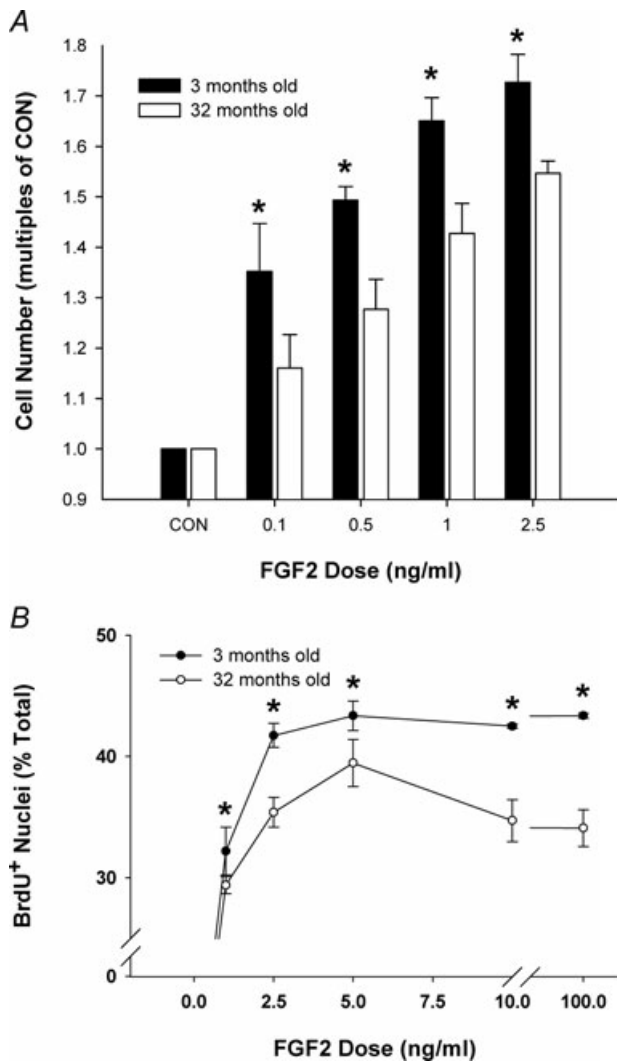
## Results

In aged skeletal muscle, impairment of MPC proliferation is likely to contribute to decrements in regrowth and regeneration (Conboy *et al.* 2003; Carlson *et al.* 2008). Since FGF2 is a potent stimulator of MPC proliferation, the hypothesis was tested that FGF2-stimulated proliferation was attenuated in MPCs isolated from aged animals compared with young animals. In order to determine the mitogenic effects on FGF2 on MPCs isolated from young and old animals, dose-response curves for FGF2-stimulated proliferation in low-mitogen culture conditions (2% HS) were obtained. In response to multiple doses of FGF2, MPCs from 32-month-old animals exhibited a lower number of cell (Fig. 1A) and reduced BrdU incorporation (Fig. 1B) compared with MPCs from 3-month-old animals.

We were interested in demonstrating that FGFR mRNAs are responsive to the removal of mitogens in GM and to the FGF2 concentration in the medium, and that the age-dependent decrease in FGF2-stimulated proliferation may be explained by differences in FGFR expression (Fig. 2A). Expressions of mRNA for the four FGFR isoforms in MPCs from 3- and 32-month-old animals were first determined in mitogen-rich GM, and no age differences were found (Fig. 2A). The relative expression levels of the FGFR isoform mRNAs were found to be present in the rank order of  $\text{FGFR4} \gg \text{FGFR1} = \text{FGFR2} = \text{FGFR3}$ . Replacement of the mitogen-rich GM medium with low-serum medium resulted in a decrease in mRNA content of all of the FGFRs (Fig. 2A).

Next, FGFR isoform mRNA expression was measured in the presence of FGF2 in the 2% HS (low-mitogen) culture conditions to minimize the effect of other mitogens found in high-mitogen serum. The mRNA for each FGFR isoform decreased in the presence of FGF2, compared to high-serum, demonstrating receptor downregulation (Fig. 2B). However, the mRNA for FGFR1 decreased more in MPCs from 32-month-old animals, while the mRNA for FGFR2 decreased more in MPCs from 3-month-old animals. There were no age differences in FGFR3 and FGFR4 mRNAs.

Since age differences did exist in both proliferation and the mRNA levels of FGFR1 and FGFR2 in the presence of FGF2 in low-mitogen medium (2% HS), downstream proliferation-specific signalling was measured. Figure 3A demonstrates that  $10 \text{ ng ml}^{-1}$  FGF2 caused a nearly 400% increase in the phosphorylation of MEK1/2 in MPCs from young and aged animals without any difference between the ages. An approximately 50 and 140% increase



**Figure 1. Fibroblast growth factor 2-stimulated MPC proliferation**

A, CyQUANT assay for cell number in MPCs from 3- and 32-month-old animals. The assay was performed after 48 h of FGF2 treatment ( $n = 4$ ). The cell number at each dose of FGF2 stimulation was normalized to control cell number (CON;  $0 \text{ ng ml}^{-1}$  FGF2).

B, BrdU incorporation data of MPCs from 3- and 32-month-old animals treated with different doses of FGF2 for 24 h ( $n = 4$  for 1.0, 2.5 and  $10 \text{ ng ml}^{-1}$ ,  $n = 6$  for  $5 \text{ ng ml}^{-1}$  and  $n = 2$  for  $100 \text{ ng ml}^{-1}$ ).

Values are means  $\pm$  S.E.M. \* Significant difference for cell number compared with 32-month-old animals ( $P < 0.05$ ).

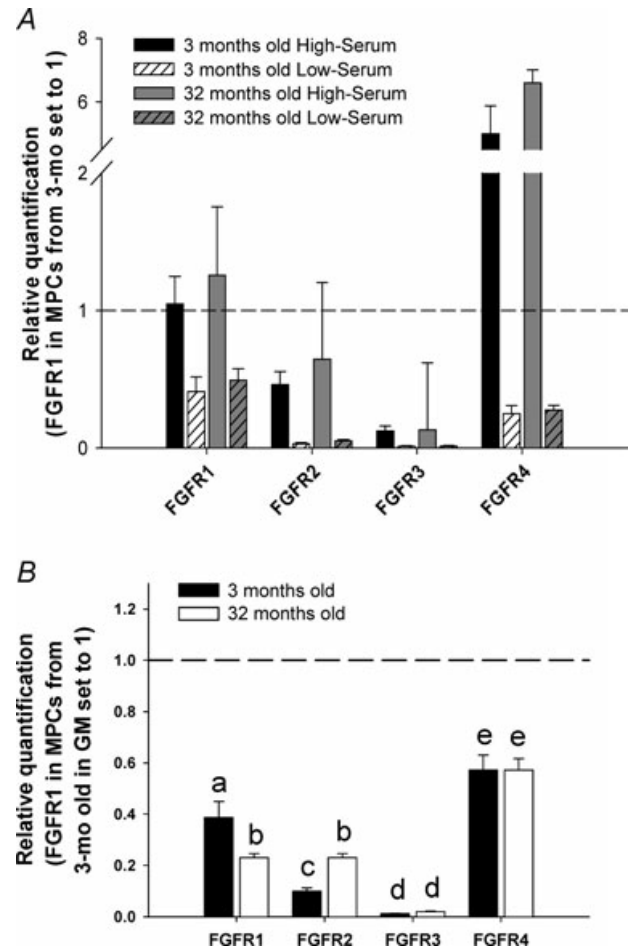
in ERK1/2 phosphorylation occurred in response to 0.5 and 10 ng ml<sup>-1</sup> FGF2, respectively (Fig. 3B), again with no difference between the ages.

Fibroblast growth factor 2 is known to increase p38 activation (Lee *et al.* 2002; Matsumoto *et al.* 2002; Khalil *et al.* 2005). Further, it has been reported that inhibition of p38 MAPK induces cell proliferation of H9c2 rat cardiac myoblasts in differentiation medium and that mice lacking p38 $\alpha$  exhibit increased myoblast proliferation (Lee *et al.* 2002; Perdiguero *et al.* 2007). Therefore, p38 signalling in response to FGF2 stimulation was tested next. In response to FGF2, there was an approximately 80% increase in the phosphorylation of p38 protein in MPCs from 3- and 32-month-old animals, with no age difference observed (Fig. 4). Thus, Figs 3 and 4 indicate that MEK1/2 – ERK1/2 and p38 signalling are both FGF2-responsive pathways for MPC proliferation.

In order to expand the investigation of FGF2-induced signalling and BrdU incorporation in MPCs, MEK1/2 – ERK1/2, p38, JNK and PKC pathway inhibitors were next used. By inhibiting the signalling pathway, we would theoretically be able to test whether age-dependent differences exist downstream of the phosphorylation of ERK1/2 and p38. However, preliminary experiments using these inhibitors revealed that the age-associated phenotype in FGF2-dependent proliferation was eliminated. The commonly used vehicle, DMSO, was used in each pathway inhibition experiment; we therefore remarkably found that the addition of DMSO in the presence of FGF2 rescued the impaired proliferation in MPCs isolated from 32-month-old animals (Fig. 5).

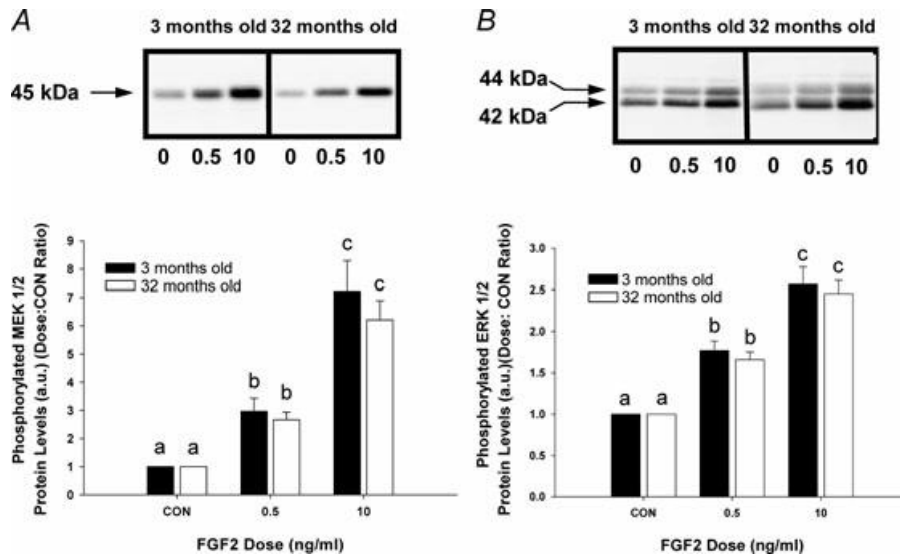
Based on the finding that MPCs from 32-month-old animals differentially increased their proliferation response in the presence of the vehicle (DMSO) for the pathway inhibitors, ageing differences could not be tested, and remaining experiments were performed on MPCs isolated from young animals only. Figure 6A and B demonstrates inhibition of FGF2-stimulated ERK1/2 phosphorylation in the presence of both MEK1/2 – ERK1/2 inhibitors, U0126 and the non-specific MEK1/2 inhibitor. Based on these experiments, the doses which corresponded to a 100% inhibition of FGF2-stimulated phosphorylation were used for proliferation experiments. In the presence of U0126, FGF2-stimulated BrdU incorporation was reduced by ~20%, but remained greatly elevated above untreated control cultures (Fig. 6C). In the presence of the non-specific MEK1/2 inhibitor, the FGF2-stimulated incorporation of BrdU was reduced to values similar to those without FGF2 (control). However, the non-specific MEK1/2 inhibitor is not considered to be as specific as U0126 and has been shown to inhibit PKC in addition to MEK1/2 – ERK1/2 signalling (Scherle *et al.* 2000). To test the potential role of PKC in FGF2-stimulated BrdU incorporation, the PKC inhibitor, bisindolylmaleimide I

(BIS I), was next used. Inhibition of PKC reduced the FGF2-stimulated BrdU incorporation by ~40% (Fig. 6C). Therefore, the two inhibitors, U0126 and BIS I, were used together. The combined use of U0126 and BIS I did not reduce the FGF2-stimulated BrdU incorporation any further than BIS I alone (Fig. 6C). These data suggest that ~60% of the FGF2-stimulated BrdU incorporation is a result of an additional signalling pathway. Since others have suggested that p38 or JNK signalling are activated by FGF2 (Megeny *et al.* 1996), p38 and JNK were next examined.



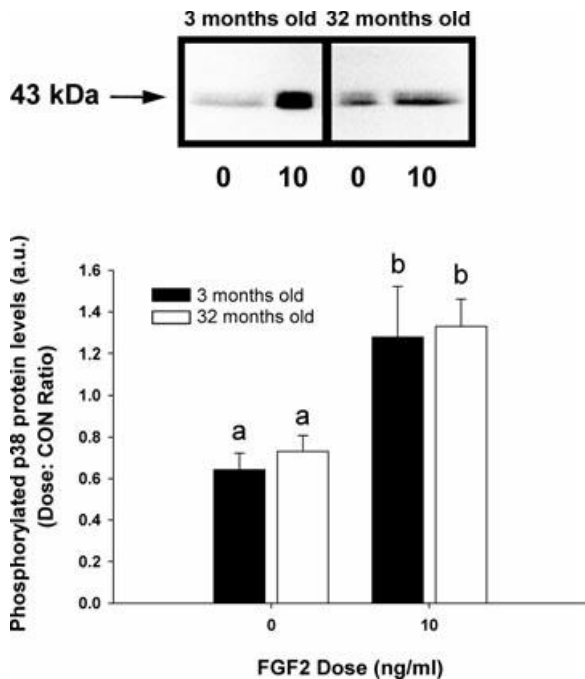
**Figure 2. Quantification of mRNA for FGFR isoforms**

A, mRNA for FGFRs determined in MPCs from 3- and 32-month-old animals after 24 h in GM ( $n = 4$  for young and old). Relative quantities for each isoform were determined based on the relative quantity value for FGFR1 from 3-month-old animals set to 1. B, quantification of mRNA in MPCs from 3- and 32-month-old animals after 24 h of FGF2 treatment ( $n = 3$  for young and old). Relative quantities for each isoform were determined based on the relative quantity of FGFR1 from 3-month-old animals in GM set to 1. All relative quantities of FGFR1–4 mRNAs in low-serum conditions were significantly less than their respective high-serum values. The relative expression levels of the FGFR isoform mRNAs were significantly different in the rank order of FGFR4  $\gg$  FGFR1 = FGFR2 = FGFR3. Different letters denotes a significant difference between groups.



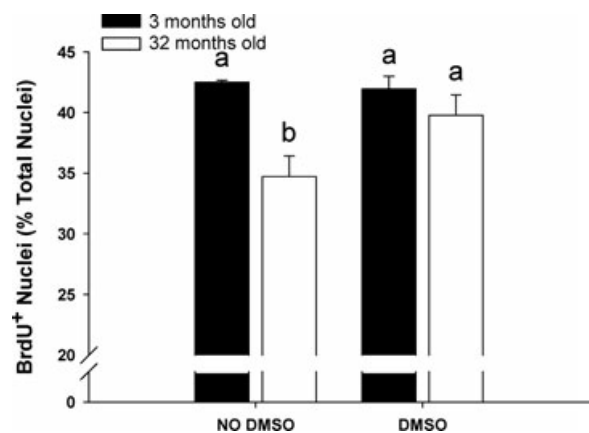
**Figure 3. Cell signalling measured by Western blot**

A, Western blot analysis of the increase in phosphorylated MEK1/2 (Ser217/Ser221) in MPC cell lysates with FGF2 treatment (0, 0.5 or 10 ng ml<sup>-1</sup>) with a representative Western blot; *n* = 8 for young and *n* = 7 for old. Muscle precursor cells were grown for 48 h in GM. Then, the medium was changed to low-serum medium for 3 h. After 3 h, the MPCs were treated with FGF2 for 10 min. B, Western blot analysis of the increase in phosphorylated ERK1/2 (Thr202/Tyr204) in MPC cell lysates with FGF2 treatment (0, 0.5 or 10 ng ml<sup>-1</sup>) with a representative Western blot; *n* = 9 for young and *n* = 8 for old. Cell culture conditions were same as in A. Different letters denote a significant difference between FGF2 doses (*P* < 0.05).



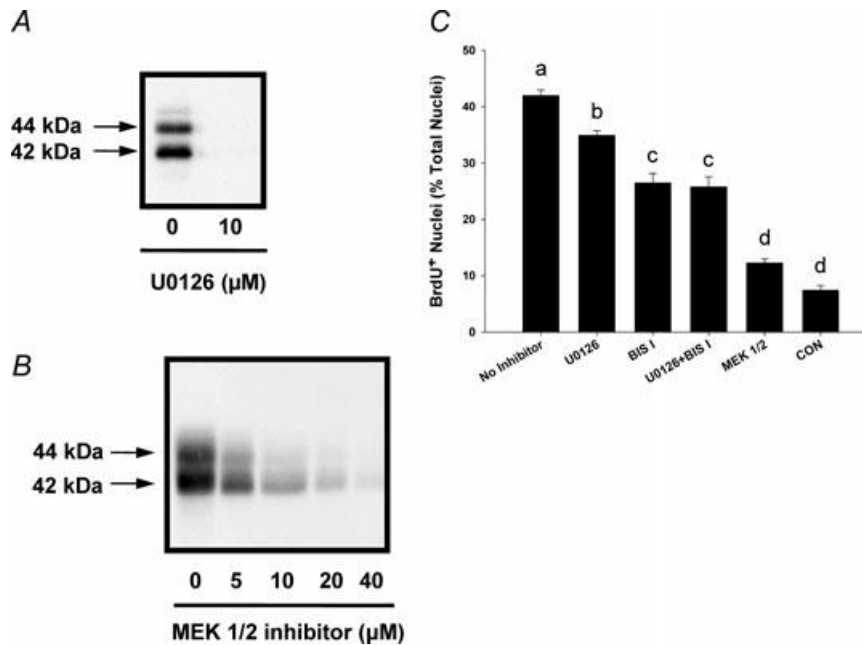
**Figure 4. Representative Western blot showing levels of phosphorylated p38 protein (Thr180/Tyr182) in MPC cell lysates** The figure shows quantitative Western blot data (*n* = 6 for young and old) from MPCs treated with FGF2 (0 or 10 ng ml<sup>-1</sup>). Muscle precursor cells were grown in GM for 24 h. After 24 h, GM was changed to low-serum medium for a 3 h equilibration period. After 3 h of treatment, FGF2 was added to the medium for a 10 min treatment. Data were analysed by two-way ANOVA. Different letters denote statistical significance (*P* < 0.05).

Inhibition of JNK had no effect on FGF2 signalling to BrdU incorporation (data not shown), whereas p38 inhibition (10 μM SB203580) in the presence of FGF2 resulted in an approximately 30% increase in BrdU incorporation compared with FGF2-treated MPCs with



**Figure 5. Bromodeoxyuridine-positive nuclei with MEK1/2 and PKC inhibition in the presence of FGF2 (10 ng ml<sup>-1</sup>) and without or with DMSO (0.1%)**

Muscle precursor cells were grown for 24 h in GM. After 24 h, the medium was changed to low-serum medium with or without DMSO (*n* = 4 without DMSO and *n* = 6 with DMSO). After 1 h of exposure to DMSO, FGF2 (10 ng ml<sup>-1</sup>) was added to the medium. At 23 h of treatment with FGF2, MPCs were pulse-labelled with BrdU for 1 h. Muscle precursor cells were harvested and fixed after 24 h of treatment. Dissimilar letters denote significant differences between young and old without DMSO (*P* < 0.05).



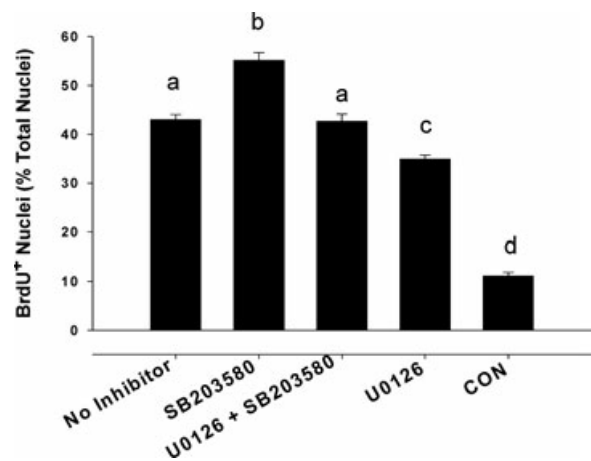
**Figure 6. MEK1/2 inhibitor Western blot and proliferation data**

A, representative Western blot for phosphorylated ERK1/2 (Thr202/Tyr204) in MPC cell lysates treated with FGF2 ( $10 \text{ ng ml}^{-1}$ ) in the presence or absence of U0126. Muscle precursor cells were grown for 24 h in GM. After 24 h, the medium was changed to 2% HS, and chemical inhibitors were added to the medium (0.4% DMSO) 1 h prior to a 10 min FGF2 treatment. B, representative Western blot for phosphorylated ERK1/2 (Thr202/Tyr204) in MPC cell lysates treated with FGF2 ( $10 \text{ ng ml}^{-1}$ ) in the presence of MEK1/2 inhibitor SL327 with the same cell culture conditions as in A. C, BrdU-positive nuclei with MEK1/2 and PKC inhibition in the presence of FGF2;  $n=6$  for young and old. CON = no FGF2 + DMSO (0.1%). Passage 2 MPCs were grown for 24 h in GM. After 24 h, the medium was changed to low-serum medium, and MEK1/2 inhibitors ( $10 \mu\text{M}$  U0126 and  $40 \mu\text{M}$  MEK1/2 inhibitor) and PKC inhibitor ( $10 \mu\text{M}$  bisindolylmaleimide I) were added for 1 h, prior to treatment with FGF2 for 23 h. Different letters denote significant differences between inhibitor types or conditions ( $P < 0.05$ ).

no inhibitor (Fig. 7). The addition of U0126 together with SB203580 resulted in proliferation values that were 20% higher than U0126 alone (Figs 6C and 7). These findings suggest that p38 and MEK1/2 - ERK1/2 signalling could be modulated independently in FGF2-stimulated MPCs.

## Discussion

Several significant and important findings are presented. In response to increasing doses of FGF2, cell number and BrdU incorporation increased less in single-passaged MPCs from 32-month-old animals compared with 3-month-old animals. However, in the presence of FGF2 in low-mitogen medium, ageing differences in the downregulation of mRNA levels for FGFR1 and FGFR2 were revealed. Signalling via MEK1/2 - ERK1/2 was confirmed as a mechanism of FGF2 signalling to increase BrdU incorporation. Importantly, though, new observations of PKC signalling pathways to assist FGF2 signalling of BrdU incorporation in MPCs were documented. Further, inhibition of JNK had no effect on FGF2 signalling to BrdU incorporation and FGF2-induced phosphorylation of p38 inhibited BrdU incorporation in MPCs. To our knowledge, this is the first report to



**Figure 7. Bromodeoxyuridine-positive nuclei with MEK1/2 and p38 pathway inhibition in the presence of FGF2 ( $10 \text{ ng ml}^{-1}$ )**  
CON = No FGF2 + DMSO (0.4%). Passage 2 MPCs were grown for 24 h in GM. After 24 h, the medium was changed to low-serum medium containing 0.4% DMSO and with two different chemical inhibitors alone and in combination ( $10 \mu\text{M}$  U0126 and  $10 \mu\text{M}$  SB203580). After 1 h of treatment with inhibitors, FGF2 was added to the media. After 23 h of treatment with FGF2, MPCs were pulse-labelled with BrdU ( $10 \mu\text{M}$ ) for 1 h. Muscle precursor cells were harvested and fixed after 24 h of treatment. Different letters denote significant differences between inhibitor types or conditions ( $P < 0.05$ ).  $n=3$  for no inhibitor,  $n=6$  for U0126,  $n=2$  for SB203580 + U0126 and CON and  $n=4$  for SB203580.



demonstrate this relationship between FGF2, p38, PKC and proliferation in MPCs. Lastly, we have determined that the addition of DMSO to our culture media restored FGF2-stimulated BrdU incorporation values in MPCs from old animals to those of young animals.

The combined inhibition of MEK1/2 – ERK1/2 and PKC signalling did not reduce FGF2-stimulated proliferation any more than PKC inhibition alone. These data imply that another signalling pathway(s) must facilitate FGF2-stimulated proliferation. In support of this observation, the non-specific MEK1/2 inhibitor totally prevented FGF2-stimulated proliferation. Putatively, this was the result of the non-specific MEK1/2 inhibitor acting on multiple other signalling pathways. The lack of an ageing difference in phosphorylation of MEK1/2 – ERK1/2 or p38 suggests that these pathways probably do not explain the ageing impairment of FGF2-stimulated proliferation of MPCs.

The intended use of pathway inhibitors was to dissect the relative contribution of various signalling pathways in the age-dependent decrease in FGF2-stimulated proliferation; however, DMSO itself rescued impaired proliferation in MPCs isolated from old animals. Dimethyl sulphoxide is the most commonly used organic solvent in cell culture experiments in which intracellular signalling inhibitors are used. It has been studied extensively, and numerous articles have been published about its chemical, biological and medical properties (Jacob & Herschler, 1986; Yu & Quinn, 1994; Santos *et al.* 2003; Brien *et al.* 2008). Dimethyl sulphoxide is known to have numerous biological properties; however, one property which could provide a potential explanation for the findings described here is its ability to act as a free radical scavenger. It is possible that the antioxidant effects of DMSO may provide protection to MPCs from 32-month-old animals and improve proliferation.

The mRNA for each FGFR isoform decreased in the presence of low-serum, demonstrating receptor downregulation (Fig. 2B), and an interesting expression shift was observed when the cells were switched to FGF2-stimulated cells in low-mitogen medium. The FGFR1 mRNA was suppressed less in low-mitogen medium, while FGFR2 mRNA was suppressed more in MPCs isolated from young animals after 24 h of FGF2 stimulation compared with suppression in MPCs from old animals. The physiological significance of FGFR1 and FGFR2 isoform switching was not pursued owing to the lack of specific receptor inhibitors or antibodies to verify receptor knockdown. Since the signalling and biological responses, including mitogenic potential, elicited by different FGFRs differ substantially (Wang *et al.* 1994), it is possible that in MPCs from old animals, different outcomes of BrdU incorporation could occur.

The redundancy of signalling pathways employed by FGF2 could imply that FGF2 evolved as a critical

regulator of MPC proliferation. Fibroblast growth factor 2 can transduce downstream signalling through binding to the FGFR, a tyrosine kinase which activates both PKC and growth factor receptor-bound protein-son-of-sevenless (Grb – SOS) signalling that, in turn, activates Ras – Raf – MEK1/2 (Simons, 2004). Moreover, FGF2 is known also to bind to low-affinity heparan sulphate proteoglycans (HSPG) at the cell surface (Simons, 2004) in addition to the aforementioned high-affinity (FGFR) isoforms. One such example is that FGF2 can activate downstream signalling through syndecan-4, a type of HSPG, which activates PKC through its interaction with phosphatidylinositol 4,5-bisphosphate (Horowitz & Simons, 1998). Therefore, FGF2 activation of PKC results in downstream signalling that is dependent (Ras – Raf) and independent (syndecan-4) of MEK1/2. Our BrdU incorporation data suggest that FGF2-stimulated proliferation acts partly through PKC. However, since an additive effect was not observed by combining MEK1/2 – ERK1/2 inhibition with PKC inhibition, it is possible that FGF2 stimulation of PKC in the current conditions is dependent on Ras – Raf activation.

In MPCs, FGF2 stimulation of p38 was associated with an inhibition of BrdU incorporation. These findings may be explained by previous literature showing that its four isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) exhibit variable or unknown effects on proliferation (Perdiguero *et al.* 2007). The p38 inhibitor that was used in our study is known to be a highly specific inhibitor of p38 $\alpha$  and p38 $\beta$ . These isoforms may act as negative regulators of proliferation in the presence of FGF2, while  $\gamma$  and  $\delta$  may have no role or may potentiate proliferation.

Previous literature investigating the response of skeletal muscle cells to FGF2 treatment has been equivocal. Johnson & Allen (1993) have demonstrated that cells from young rats (3 – 4 weeks old) responded to FGF2 at 18 h postplating, while cells from adult rats (9 – 12 months old) responded 48 h after plating. In addition, Mezzogiorno *et al.* (1993) reported a reduced mitogenic response in MPCs isolated from 22-month-old mice. In contrast, experiments using the single myofibre culture model showed no differences in FGF2-stimulated proliferation in growing (3-week-old), young adult (8- to 10-week-old) and old (9- to 11-month-old) rats (Yablonka-Reuveni *et al.* 1999). Importantly, in the study of Yablonka-Reuveni *et al.* (1999), 9- to 11-month-old animals were designated as old, but this age group is probably better described as adult in terms of sarcopenia research. Furthermore, Shefer *et al.* (2006) demonstrated that in single-fibre cultures, isolated from fast-twitch mouse flexor digitorum brevis muscle, there was no difference in FGF2-stimulated proliferation. However, in these experiments, cells that did not stain positive for ERK1/2 (used as a marker for satellite cells) were excluded from the analyses and it was stated that more cells were excluded from the analyses from muscle fibre

cultures of old mice. Therefore, our present findings are important because they demonstrate that MPCs isolated from sarcopenic animals exhibit a decreased proliferative response to FGF2 at all doses tested. Notably, the purity of the MPC culture used in the present study has been verified (Lees *et al.* 2006) using myogenic markers, and cell culture duration did not exceed 1 week after isolation from the animal.

In this report, our data demonstrate the novel finding that there are age-associated impairments of FGF2-stimulated BrdU incorporation in MPCs from 32-month-old animals. Also, an ageing-associated difference exists in the mRNA expression of FGFR1 and FGFR2 in the presence of FGF2. In MPCs from 3-month-old animals, we have documented that FGF2 signals to a greater extent via PKC rather than by the previously identified MEK1/2 – ERK1/2 signalling. In an attempt to determine the contribution of an additional signalling in FGF2-driven proliferation, we have also documented an increase in proliferation in the presence of p38 inhibition. The primary finding of decreased FGF2-stimulated proliferation in MPCs from old animals could be explained by unmeasured components of the measured signalling pathways or potential cross-talk between pathways that may be influenced via speculated antioxidant effects of DMSO.

In future experiments, it will be necessary to determine the specific pathway(s) that account for the ageing phenotype in FGF2-stimulated proliferation, the intracellular signalling pathways controlled by FGF2 and the potentially different roles for the various isoforms of PKC and p38. These findings will facilitate the development of treatment strategies aimed at improving age-related deficiencies in skeletal muscle repair and sarcopenia.

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