RESEARCH PAPER

Dose-dependent modulation of myogenesis by HGF: implications for c-Met expression and downstream signalling pathways

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Abstract
Hepatocyte growth factor (HGF) regulates satellite cell activation, proliferation, and differentiation. We analyzed the dose-dependent effects of HGF on myogenesis. Murine C2C12 and human donor-derived skeletal muscle myoblasts were treated with 0, 2, or 10 ng/ml HGF followed by assessment of proliferation and differentiation. HGF (2 ng/ml) significantly promoted cell division, but reduced myogenic commitment and fusion. Conversely, 10 ng/ml HGF reduced proliferative capability, but increased differentiation. c-Met expression analysis revealed significantly decreased expression in differentiating cells cultured with 2 ng/ml HGF, but increased expression in proliferating cells with 10 ng/ml HGF. Mitogen-activated protein kinase (MAPKs: ERK, JNK, or p38K) and phosphatidylinositol-3-kinase (PI3K) inhibition abrogated the HGF-stimulated increase in cell number. Interestingly, PI3K and p38 kinase facilitated the negative effect of HGF on proliferation, while ERK inhibition abrogated the HGF-mediated decrease in differentiation. Dose-dependent effects of HGF are mediated by changes in c-Met expression and downstream MAPK and PI3K signalling.

Keywords
- c-Met, hepatocyte growth factor, MAPK, myogenesis, myoblast, PI3K

Introduction
Skeletal muscle has the ability to regenerate and repair damage following injury or disease (Tedesco et al., 2010). Satellite cells, located between the basal lamina and sarcolemma of the myofiber, are the primary cells facilitating this regenerative process (Boldrin et al., 2010; Goetsch et al., 2013; O’Reilly et al., 2008). In response to skeletal muscle injury, these satellite cells become activated and enter the cell cycle to undergo a series of proliferative steps (Haley & Cantley, 2004). This is followed by migration to the site of injury and subsequent differentiation (Grounds et al., 2002). The activation of satellite cells, subsequent expansion, and terminal differentiation of the myoblast population is regulated by growth factors, such as fibroblast growth factor (FGF), insulin growth factor-1 (IGF-1), transforming growth factor beta (TGF-β), and hepatocyte growth factor (HGF) (Gal-Levi et al., 1998; Miller et al., 2000; O’Blenes et al., 2010; O’Reilly et al., 2008; Pownall & Isaacs, 2010; Yamada et al., 2010).

HGF, a heparin-binding protein, is sequestered in an inactive form in the extracellular matrix (ECM) of uninjured muscle fibres (Miller et al., 2000); however, upon tissue injury, it is cleaved and released to stimulate satellite cell activation (Birchmeier & Gherardi, 1998). HGF transduces its effects on satellite cells through specific interaction with the c-Met receptor (Humphrey et al., 1995). c-Met is a transmembrane tyrosine kinase cell surface receptor consisting of a 145 kDa and 50 kDa β- and α-chains (Sonnemberg et al., 1993). It has been shown to be essential during satellite cell activation, proliferation, migration, and differentiation (Organ & Tsao, 2011).

Upon binding of HGF to the c-Met receptor, the kinase becomes active (Bottaro et al., 1991). This results in the phosphorylation of two tyrosine residues (Tyr1349 and Tyr1356) in the carboxy-terminal tail of c-Met. The phosphorylated residues become docking sites for a range of adaptor proteins including phosphatidylinositol-3-kinase (PI3K), Grb2-associated adaptor protein (Gab1) and growth factor receptor-bound protein 2 (Grb2). These pathways proceed to mediate c-Met-dependent cell proliferation, migration, survival, and differentiation (Faria et al., 2011). Grb2 and GAB transduce signals through mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (ERK1 and ERK2), Jun amino-terminal kinases (JNK1, JNK2, and JNK3), and p38. It has also been shown that HGF regulates proliferation rates via signalling through SHP2, a protein tyrosine phosphatase that mediates MAPK activity; these proliferation rates were dependent on the HGF dose utilized (Chazaud, 2010; Li et al., 2009). MAPKs are thought to be more intensively involved in regulating cell proliferation, differentiation and cell migration, while signalling through PI3K mediates cell survival and resistance to apoptosis (Faria et al., 2011; Keren et al., 2005; Knight &
Kothary, 2011; Li et al., 2000; Lluis et al., 2006; Organ & Tsao, 2011).

Regulation of HGF:c-Met signaling can be achieved by a number of mechanisms. In hepatocytes, HGF-bound c-Met receptors are internalized and the c-Met cell surface concentration is observed to decrease within 30 min of exposure to elevated HGF levels (Naka et al., 1993). HGF is subsequently degraded and released by the cell. HGF signaling is also regulated by a number of protein tyrosine phosphatases (PTPs), which bind to the cytoplasmic domain of c-Met. These phosphatases can control HGF signaling by modifying the intracellular kinase activity or binding domains of c-Met. These phosphatases therefore play a role in determining the sensitivity of the c-Met cell surface concentration to changes in HGF levels. They also prevent binding of signal transducers to the tyrosine kinase domain of c-Met, leading to decreased HGF signaling.

Similarly, CD148 prevents binding of signal transducers to c-Met, and LAR counteracts Met beta (RPTP-beta) activity. Lastly, phospholipase C (PLC) can bind to the carboxy-terminal tail of c-Met and activate protein kinase C (PKC), which acts as a negative regulator of c-Met activity (Organ & Tsao, 2011).

In vitro, it has been demonstrated that HGF activates primary culture satellite cells at concentrations as low as 2.5 ng/ml (Tatsumi et al., 1998). Studies in primary rat skeletal myoblasts and the C2C12 murine cell line have also demonstrated a promotion of proliferation in response to HGF at concentrations ranging from 3 ng/ml to 50 ng/ml (Allen et al., 1995; Anastasi et al., 1997; O’Blenes et al., 2010). In contrast, however, a separate study utilising rat satellite cells demonstrated a suppression of proliferation in response to HGF at concentrations greater than 10 ng/ml (Yamada et al., 2010). A reduction in activation and proliferation was also observed in both chicken skeletal muscle and mouse C2 cells in response to addition of exogenous HGF at 20 and 50 ng/ml (Gal-Levi et al., 1998). These conflicting results suggest potential dose-dependent sensitivity regarding the effect of HGF on cellular proliferation.

Skeletal myogenesis is regulated by several myogenic regulatory factors (MRFs) including MyoD, Myf-5, MRF-4, and myogenin (Gal-Levi et al., 1998; Halevy & Cantley, 2004). MRFs are proteins expressed in cells committed to differentiation; together with Pax7, they are implicated in specification of the myogenic lineage (Buckingham et al., 2006; McFarlane et al., 2008; Seale et al., 2004). Differentiation studies published to date suggest an inhibitory effect of HGF (2.5–50 ng/ml) on myoblast differentiation (Gal-Levi et al., 1998; Halevy et al., 2004; Leshem et al., 2000; Yamane et al., 2004; Zeng et al., 2002). Accumulating evidence implies that HGF may influence myogenesis via its regulation of MyoD, Myf-5, and myogenin (Charge & Rudnicki, 2004; Halevy et al., 2004; Rosen et al., 1990).

In addition, some studies provide evidence of a role for p27 (a cyclin-dependent kinase) and Twist (a basic helix-loop-helix transcription factor) in the mediation of HGF on differentiation (Leshem et al., 2000).

HGF therefore plays a central role during skeletal muscle myogenesis; however, the effect of different doses on cellular activities is unclear and requires further examination. In the present study, we culture both the C2C12 murine cell line and donor-derived human myoblasts to compare the effects of different HGF concentrations on proliferation and differentiation. Furthermore, we investigate whether dose-dependent sensitivities are related to changes in the total protein expression of the c-Met receptor and utilise both MAPK and PI3K inhibitors in an attempt to understand the signaling mechanisms at play.

Materials and methods

Cell culture

The C2C12 murine cell line was donated by Prof Anna-Mart Engelbrecht (Department of Physiological Sciences, University of Stellenbosch, Stellenbosch, South Africa). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (Highveld Biological, Lyndhurst, South Africa, cat. CN3193-9) supplemented with 10% (v/v) Fetal Bovine Serum (Biowest, Nuailles, France, cat. S181H-500), 2% (v/v) L-glutamine (Lonza, Basel, Switzerland, cat. BE17-605E) and 2% (v/v) penicillin–streptomycin (Lonza, Basel, Switzerland, cat. DE17-602E). For differentiation studies, cells were cultured to a confluence of 80% after which media were changed to differentiation media (DMEM supplemented with 2% (v/v) horse serum) (Biowest, Nuailles, France, cat. S090H-100).

Human primary culture skeletal muscle (HSkM) myoblasts were purchased from Lonza (Basel, Switzerland, cat. CC-2561). Cells were cultured in HAMS-F10 (Life Technologies, Carlsbad, CA, cat. 31550-015) supplemented with 20% (v/v) fetal bovine serum (Biowest, Nuailles, France, cat. S181H-500), 2% (v/v) L-glutamine (Lonza, Basel, Switzerland, cat. BE17-605E), 2% (v/v) penicillin–streptomycin (Lonza, Basel, Switzerland, cat. DE17-602E), and 2.5 ng/ml FGF (Promega, Madison, WI, cat. G507A). For differentiation studies, cells were cultured to a confluence of 80% on coverslips after which media was changed to differentiation media (DMEM supplemented with 2% (v/v) horse serum).

Human HGF (PeproTech, Rocky Hill, NJ, cat.100-39) was used at a final concentration range of 0, 2, and 10 ng/ml and media changed every 48 h during differentiation studies. Cells were incubated at 37 °C in a humidified incubator at 5% CO2; HSKM cells were used at passages 2 and 3. All experiments were carried out under sterile conditions in a Class II Biohazard Safety Cabinet.

Inhibitors

PI3K inhibitor (LY294002, 2.5 μM, Santa Cruz Technology, Santa Cruz, CA, SC-201426), ERK inhibitor (PD98059, 12.5 μM, Santa Cruz Technology, Santa Cruz, CA, SC-3532), p38 inhibitor (SB203580, 5 μM, Santa Cruz Technology, Santa Cruz, CA, SC-3533), and JNK inhibitor (SP600125, 5 μM, Santa Cruz Technology, Santa Cruz, CA, SC-200635) were reconstituted in DMSO (Sigma, St. Louis, MO, D2650). Inhibitors were added to growth or differentiation media and replenished every 48 h with media change. Final DMSO concentrations never exceeded 0.04% and were not found to be toxic to C2C12 cells (Supplementary Figure 1); this is in agreement with previous studies where DMSO was shown to be non-toxic in C2C12 cells at concentrations up to 0.1% (Moorwood et al., 2011).
Assessment of cell number

The cell counts were carried out as previously described (Taylor et al., 2001). Briefly, C2C12 or HSkM cells were seeded in T25 tissue culture flasks (50,000 cells/flask for HSkM; 100,000 cells/flask for C2C12) containing growth media and incubated at 37°C (5% CO2) for 24 h. HGF (0, 2, and 10 ng/ml) was then added and cells incubated for a further 24 h. Thereafter, cells were trypsinized and counted using the automated BioRad TC-20 Cell Counter; viability was also assessed using 10 μl trypan blue (BioRad, Hercules, CA, 145-0021).

Immunocytochemistry

Cells were differentiated to day 5 (C2C12) and day 7 (HSkM) in the presence or absence of HGF (0, 2, or 10 ng/ml), fixed in 4% paraformaldehyde containing 0.1% Triton X-100 (Sigma, St. Louis, MO, cat. T9284) for 20 min and blocked with 5% donkey serum for 1 h. For MyoD and Myosin Heavy Chain (MyHC) detection, coverslips were incubated with a rabbit polyclonal anti-MyoD primary antibody (Santa Cruz Technology, Santa Cruz, CA, cat. sc-760; dilution 1/100) or a mouse monoclonal MF20 primary antibody, respectively (Developmental Studies Hybridoma Bank; dilution 1/200) overnight at 4°C. This was followed by 4 × 5 min PBS wash steps prior to incubation with secondary antibodies for 1 h at room temperature. The secondary antibodies (all at 1/1000 dilution) were DyLight 488-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, cat. CN-715-545-151) and DyLight 488- or 594-conjugated AffiniPure donkey anti-mouse IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA, cat. CN-715-485-151). Hoechst (Sigma, St. Louis, MO, B2267; 10 mg/ml stock; 1/2000 dilution) was added to all the cells for detection of nuclei. Moviol was used as mounting agent. All images were captured using the Zeiss 710 LSM confocal microscope (Zeiss, Jena, Germany).

Quantification of percentage MyoD + myoblasts

Images of differentiating cells were captured using the Zeiss 710 LSM confocal microscope (Zeiss, Jena, Germany) and analyzed with ImageJ software (http://rsbweb.nih.gov/ij/) (NIH, Bethesda, MD). The number of MyoD-expressing (nuclear expression) myoblasts were counted and divided by the total number of nuclei per field of view; a percentage was then determined. Images were captured on day 1 post-induction of differentiation. Five random fields of view (minimum 30 cells per field of view) were analyzed per experimental.

Fusion Index

The fusion index was calculated as described previously (Micheli et al., 2011). Briefly, myoblasts were differentiated on coverslips in the presence or absence of HGF (2 ng/ml or 10 ng/ml) and fixed at day 5 (C2C12 cells) or day 7 (HSkM cells) of differentiation. Immunocytochemistry was carried out to detect the expression of MyHC in the differentiating cells. Five random fields of view of cells were captured using the Zeiss 710 LSM confocal microscope (Zeiss, Jena, Germany). ImageJ software (NIH, Bethesda, MD) was utilized to determine formation of myotubes. The fusion index was calculated as the number of nuclei within MyHC-labeled myotubes (two or more nuclei per myotube) divided by the total number of nuclei per field of view, multiplied by 100.

Western blotting

Myoblasts were cultured in either growth or differentiatation media in the presence or absence of HGF (2 ng/ml or 10 ng/ml). Proliferating cells were harvested at day 1, while differentiating cells were harvested at days 1, 2, and 5 of differentiation. During differentiation, media (supplemented with or without HGF) were changed every 2 d. Cell lysates were prepared in 100 μl RIPA buffer (Sigma-Aldrich, St. Louis, MO, cat. R0278) containing 1 μl Protease Inhibitor Cocktail (PIC) (Sigma-Aldrich, St. Louis, MO, cat. P8340) for 1 h on ice, followed by centrifugation at 12,000 rpm for 5 min. Lysates were sonicated using the Ultrasonic cleaner (Shalam Laboratory Suppliers, Durban, South Africa) and protein concentrations determined via the Bradford Assay (Bradford, 1976). Total protein (30 μg) was loaded onto 12.5% SDS-PAGE, and following separation, was transferred onto a Nitrocellulose membrane (Life Sciences, Boston, MA, cat. PN 66485) using Western Blotting. The membrane was then incubated with primary antibodies overnight. Primary antibody dilutions: rabbit anti-c-Met 1:500 (Invtrogen, Grand Island, NY, cat. 182257); mouse anti-alpha-tubulin 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, B-7, sc-5286); and mouse anti-MyHC 1:1000 (Developmental Hybridoma Bank, MF20-S). Secondary antibody dilutions: goat anti-rabbit (Dako, Carpinteria, CA, PO448) 1:12,000 for c-Met detection; rabbit anti-mouse (Dako, Carpinteria, CA, PO260) 1:2,000 for alpha-tubulin detection and 1:20,000 for MyHC detection for 1 h. HRP activity was visualized using Enhanced Chemiluminescence (ECL, Bio-Rad, Hercules, CA, cat. 170-5070) and GeneSys Image Acquisition software (Vacutec, South Africa). Densitometric analysis was carried out using the Versa Doc Imaging System and Quantity One 2.6 (Bio-Rad, Hercules, CA).

Statistical analysis

The results from each experiment were expressed as a mean ± SEM. The ANOVA statistical test was performed on all data followed by the Tukey’s pairwise post hoc test on all qualifying data sets. Samples were considered to be statistically significant if they produced a p value of less than 0.05. The number of experimental repeats is specified in each figure legend.

Results

C2C12 myoblast differentiation is regulated by HGF

To determine the downstream effect of HGF on terminal myogenic differentiation, we first assessed C2C12 myotube fusion and sarcomeric myosin heavy chain (MyHC) expression at day 5 of differentiation. Myotube formation occurred in the presence of all doses of HGF with the highest levels of densely packed, aligned myotubes observed in response to
10 ng/ml HGF (Figure 1A). Fewer MyHC-expressing myotubes were observed in response to 2 ng/ml HGF, along with reduced alignment and reduced MyHC expression (Figure 1A). Under control conditions, the fusion index for C2C12 was 42% (Figure 1B), in line with independently published studies (Velica & Bunce, 2011). Treatment with 10 ng/ml HGF significantly (*p < 0.05) increased tube formation by 12%; in contrast 2 ng/ml HGF significantly reduced myotube formation by 17% compared to the control (*p < 0.05) (Figure 1B). These data were supported by a 30% increase in total MyHC expression in C2C12 cells treated with 10 ng/ml HGF when compared to control (*p < 0.05; Figure 1C). Furthermore, a 20% reduction in MyHC production was observed when C2C12 cells were treated with 2 ng/ml HGF during differentiation compared to control (Figure 1C).

MyoD, a basic helix-loop-helix transcription factor, is expressed in satellite cells that have been activated to differentiate (Zammit et al., 2006). To determine whether the observed effect of HGF on terminal differentiation is mediated via MyoD, we analyzed the frequency of MyoD+ cells at day 1 post-induction of differentiation. We found a reduction in the percentage MyoD+ cells from 33% in control samples to 25% in samples treated with 2 ng/ml HGF (Figure 1D). In contrast, 10 ng/ml HGF stimulated a significant increase in the percentage MyoD+ C2C12 cells (*p < 0.05; Figure 1D) to 58%. This increase in commitment to differentiation would support the subsequent observed increase in terminal differentiation.

**Human myoblast differentiation is also regulated by HGF**

To determine whether differentiating human myoblasts respond to HGF in a similar dose-dependent manner, we assessed HSkM myotube fusion at day 7 of differentiation. HSkM myoblasts were provided an additional 2 d due to their reduced rate of myogenesis in culture (Boldrin et al., 2010). HGF promoted myotube formation at 10 ng/ml. This was comparable to the C2C12 observations (Figure 2A). Fewer MyHC-expressing myotubes were observed in response to 2 ng/ml HGF, along with reduced alignment, and reduced MyHC expression (Figure 2A). Under control conditions, the fusion index for human myoblasts was 31% (Figure 2B). Similar to C2C12 cells, HSkM cells showed a significant increase in fusion in response to 10 ng/ml HGF (53%; *p < 0.005 compared with control), but a significant decrease when treated with 2 ng/ml (25%; *p < 0.005 compared with control) (Figure 2B).

Analysis of percentage MyoD+ cells at day 1 of differentiation revealed a similar significant reduction following incubation with 2 ng/ml HGF with 26% MyoD+ cells compared with 32% under control conditions (*p < 0.005; Figure 2C). In contrast, 10 ng/ml HGF stimulated a significant increase at
day 1 of differentiation with 39% MyoD+ HSkM cells compared to control ($p < 0.005$; Figure 2C); as with the C2C12 cells, indicative of increased differential commitment.

Taken together, the dose-dependent effects of HGF on myoblast fusion were similar in mouse C2C12 and human donor-derived myoblasts. This suggests a high degree of similarity across these species in their response to changing concentrations of HGF to such an extent that different doses of HGF can elicit either positive or negative effects during myogenesis.

HGF regulates c-Met receptor expression during myoblast differentiation

In order to gain insight into the mechanism by which HGF may be mediating its effect on myogenesis, we analysed c-Met expression in differentiating C2C12 cells. A correlation between c-Met expression and HGF concentration was observed. In response to 2 ng/ml, c-Met expression was significantly suppressed by 30% at day 1 of differentiation ($p < 0.05$; Figure 3A). However, cells treated with 10 ng/ml HGF demonstrated a 20% increase in c-Met expression at day 1 (Figure 3A). The significant decrease in c-Met expression in response to 2 ng/ml HGF is maintained through day 2 ($p < 0.005$; Figure 3B). By day 5 of differentiation, c-Met receptor expression levels had equalised and there was no significant difference between control and HGF-treated cells (Figure 3C). These data suggest that the observed dose-dependent effect of HGF on myoblast differentiation may, at least in part, be mediated by changes in c-Met receptor expression levels.

Inhibition of ERK, modulates the dose-dependent effect of HGF on C2C12 myoblast differentiation

Intracellular pathways known to mediate HGF signalling include the MAPK’s as well as PI3K. In an effort to begin to clarify the intracellular mechanisms underlying the observed dose-dependent response of differentiating myoblasts to HGF, we utilised the PI3K inhibitor (LY294002), p38 inhibitor (SB205380), JNK inhibitor (SP600125), and ERK inhibitor (PD98059).

LY294002, SB205380, and SP600125, but not PD98059, were observed to reduce C2C12 differentiation in the presence or absence of HGF (Figure 4). Myosin heavy chain (MyHC) expression and the presence of fused myotubes were greatly reduced in cells treated with these inhibitors regardless of HGF exposure (Figure 4). Interestingly, PD98059 was observed abrogate the negative effect of 2 ng/ml HGF on MyHC expression, suggesting that the ERK signaling pathway may be responsible, at least in part, for the inhibitory effect of HGF on myoblast differentiation.

Figure 2. HSKM myoblast differentiation in response to HGF. HSKM myoblasts were incubated with 0, 2, and 10 ng/ml HGF in differentiation media for up to 7 d. (A) Representative images of differentiated C2C12 myoblasts (day 7) showing MyHC expression (white arrows). (B) Fusion index calculated as the percentage HSKM myotube formation at day 7. (C) Percentage MyoD positive HSKM myoblasts at day 1. Data represent three independent experiments. All immunofluorescence images were taken with the Zeiss 710 LSM confocal microscope (Zeiss, Jena, Germany) with the 20× objective. Scale bar = 20 μm. Data are presented as mean ± SEM. ** $p < 0.005$. DOI: 10.3109/08977194.2015.1058260
HGF regulates myoblast proliferation in a dose-dependent manner

Next we tested whether the dose-dependent effect of HGF was restricted to differentiation, or whether it could be extended to myoblast proliferation. Myoblasts were seeded at 100,000 cells (C2C12) per well, or 50,000 per T25 flask (HSkM), and were treated with either 0, 2, or 10 ng/ml HGF for 24 h prior to cell counts. Compared with control, C2C12 and HSK myoblast numbers were significantly increased in response to 2 ng/ml HGF (Figure 5A and B). Cell counts revealed a significant 21% increase, from 39 × 10^4 cells to...

Figure 3. Effect of HGF on c-Met expression in differentiating C2C12 myoblasts. Western blot and densitometric analysis of c-Met expression levels in differentiating C2C12 myoblasts treated with 0, 2, or 10 ng/ml HGF. Media were changed every two days and cell lysates harvested at days 1 (A), 3 (B) and 5 (C) post-induction of differentiation. Alpha-tubulin was used as a loading control and c-Met expression was calculated relative to alpha-tubulin expression. Data represent six independent experiments. Images were assessed by Quantity One image analysis software (Bio-Rad, Hercules, CA). Data are presented as mean ± SD. *p < 0.05 and **p < 0.005.

Figure 4. The role of MAPK’s and PI3K in mediating the dose-dependent effect of HGF on differentiation. Differentiating C2C12 myoblasts were incubated with 0, 2, and 10 ng/ml HGF in the presence or absence of PD98059 (12.5 μM), SP600125 (5 μM), LY294002 (2.5 μM), or SB203580 (5 μM). Representative images were taken at day 5 of differentiation using confocal microscopy (left panel of HGF each set) and phase contrast microscopy (right panel of each HGF set). Confocal microscopy shows MyHC expression (white arrows) and nuclei; images were taken with a Zeiss 710 LSM confocal microscope (Zeiss, Jena, Germany) using the 40× objective. Scale bar = 40 μm and 100 μm for confocal and phase contrast images, respectively.
47 × 10^4 cells (C2C12 cells; Figure 5A; p < 0.05) and 35% increase from 10 × 10^4 cells to 14 × 10^4 cells (HSkM cells, Figure 5B; p < 0.005) in response to 2 ng/ml HGF compared with the untreated control. However, following incubation with 10 ng/ml HGF, a significant reduction in C2C12 and HSkM myoblast number was observed. Cell counts revealed a significant 40% decrease, from 39 × 10^4 cells to 24 × 10^4 cells (C2C12 cells; Figure 5A; p < 0.005) and 20% decrease from 10 × 10^4 cells to 8 × 10^4 cells (HSkM cells, Figure 5B; p < 0.005) in response to 10 ng/ml HGF. Analysis of c-Met expression in proliferating C2C12 cells revealed a significant increase in receptor levels in cells treated with 10 ng/ml HGF, but not 2 ng/ml HGF (Figure 5C; p < 0.005). This suggests that the anti-proliferative effect of HGF could be mediated by an increase in c-Met receptor expression.

Interestingly, cells incubated in the presence of HGF (2 ng/ml) showed a small, but significant increase in C2C12 cell viability from 85% (control) to 89% (p < 0.05; Figure 5D). HGF at 10 ng/ml did not, however, significantly affect cell viability when compared with control (Figure 5D). It is therefore possible that at least part of the ability of HGF to promote cell growth is due to a pro-survival rather than a pro-proliferative mechanism.

**ERK and JNK mediate pro-proliferative effects of HGF**

PD98059 and SP600125 (ERK and JNK inhibitors, respectively) significantly reduced the pro-proliferative effect of 2 ng/ml HGF (p < 0.005) such that when treated with 2 ng/ml HGF and either PD98059 (12.5 μM) or SP600125 (5 μM), the ability of HGF to significantly increase C2C12 cell numbers was abolished (Figure 6A and B). This suggests that the pro-proliferative effect of 2 ng/ml may be facilitated, at least in part, by the ERK and JNK pathways; however, these pathways seem to play little role in mediating the anti-proliferative effect of 10 ng/ml HGF in C2C12 cells. Interestingly, inhibition of the JNK pathway also significantly (p < 0.05) reduced cell viability in the presence of 2 ng/ml HGF (Figure 6D, p < 0.05). Therefore, in addition to mediating myoblast proliferation, HGF may also act as a pro-survival factor via the JNK pathway (Figure 6D).

**Inhibition of the PI3K and p38 pathways negates the effect of HGF on myoblast proliferation**

LY294002 and SB203580 (PI3K and p38 inhibitors, respectively) were observed to neutralize both the pro- and anti-proliferative effects of HGF (Figure 7A and B). LY294002 (2.5 μM) and SB203580 (5 μM) significantly reduced the pro-proliferative effect of 2 ng/ml HGF (p < 0.05) such that, when treated with 2 ng/ml HGF and either LY294002 or SB203580, the ability of HGF to significantly increase C2C12 cell numbers was abolished (Figure 7A and B). LY294002 (2.5 μM) and SB203580 (5 μM) also significantly decreased the anti-proliferative effect of 10 ng/ml HGF.

Figure 5. Effect of HGF on C2C12 and HSkM myoblast cell number and c-Met expression. Cells were incubated for 24 h with growth media containing 0, 2, and 10 ng/ml HGF. C2C12 (A) and HSkM (B) cell numbers were determined using the Bio-Rad TC-20 Cell Counter (Bio-Rad, Hercules, CA). Dashed line represents initial cell number seeded. (C) Western blot and densitometric analysis of c-Met expression levels in proliferating C2C12 myoblasts treated with 0, 2, or 10 ng/ml HGF. Alpha-tubulin was used as a loading control and c-Met expression was calculated relative to alpha-tubulin expression. (D) C2C12 cell viability determined via Trypan Blue using the BioRad TC-20 Cell Counter. 50,000 cells/flask for HSkM; 100,000 cells/flask for C2C12. Data represent three (B), four (A), six (C) or sixteen (D) independent experimental repeats. Data are presented as mean ± SEM. *p < 0.05 and **p < 0.005.
Situation cell activation, proliferation, and subsequent skeletal myoblast differentiation are critical for successful myogenesis following muscle injury in adult mammals. In this regard, the relationship between growth factors and myogenesis has been extensively investigated in vitro using primary culture myoblasts and myogenic cell lines (Coolican et al., 1997; Florini et al., 1991; Florini et al., 1996; Jimenez-Amilburu et al., 2013; Linkhart et al., 1981; Nadal-Ginard, 1978; Zanou & Gailly, 2013). It is also well established that myogenesis in tissue culture is accompanied by an irreversible withdrawal from the cell cycle resulting in commitment of post-mitotic myoblasts to fusion and formation of multinucleated myotubes (Zammit et al., 2006). The local concentration of growth factors supplied endogenously (by interacting cells) or exogenously (recombinant protein addition) is, therefore, critical for maintaining cells in a proliferative state or, alternatively, inducing differentiation (Florini et al., 1991). HGF, a key growth factor during myogenesis, has been shown to regulate satellite cell activation, proliferation and differentiation (Anastasi et al., 1997; Gal-Levi et al., 1998; O’Blenes et al., 2010; Yamada et al., 2010). However, several discrepancies have arisen with regard to the effect of particular HGF doses on myogenesis (Anastasi et al., 1997; Bandow et al., 2004; Gal-Levi et al., 1998; O’Blenes et al., 2010; Yamada et al., 2010). This may be due to differences in the cell species used or in divergent extracellular environments, leading to changes in the type of intracellular signalling pathway activated. Furthermore, very few studies have investigated the expression of the c-Met receptor in response to HGF during myoblast proliferation and differentiation. In the present study, we have compared the effect of HGF on proliferation and differentiation in murine C2C12 myoblasts and human donor-derived skeletal muscle (HSkM) myoblasts. Furthermore, we determined the expression of c-Met in response to HGF under these conditions. We then attempted to understand the signaling mechanisms at play by selectively inhibiting specific pathways downstream of c-Met.

In response to HGF we observed similar dose-dependent effects in both the mouse C2C12 and human skeletal myoblasts. In essence, the lower HGF concentration (2 ng/ml) significantly increased cell number (promoting a proliferative response) while decreasing myogenic commitment and subsequent differentiation and fusion. In contrast, a five-fold higher concentration of HGF (10 ng/ml) had the opposite effect, with a decrease in cell number and an increase in both the percentage MyoD+ cells and MyHC expression, resulting in increased fusion. This is the first study to compare the dose-dependent effect of HGF on both proliferation and differentiation in mouse and human skeletal myoblasts and
demonstrates a complementary dose-dependent regulatory system co-ordinated by HGF.

The premise that HGF plays a dual role in regulating myogenic proliferation and differentiation is not new. HGF has been well documented in activating quiescent satellite cells to proliferate and differentiate (Allen et al., 1995; Miller et al., 2000; Tatsumi et al., 1998). In a study by Yamada et al. (2010), satellite cells were observed to respond to high concentrations of HGF (10–500 ng/ml) by increasing myostatin protein expression and secretion; this was accompanied by a decreased cell proliferation and MyoD expression in primary cultures indicating a re-entry into a quiescent state. Yamada et al. (2010) hypothesize that this re-entry into quiescence could be via the observed increase in myostatin expression. This was proposed as neutralising antibodies (against myostatin) allowed cells to re-express MyoD and myogenin, even when incubated with higher concentrations of HGF (Yamada et al., 2010). In response to lower HGF concentration (2.5 ng/ml), BrdU incorporation was observed to increase myogenic commitment and terminal differentiation as seen by the increase in percentage MyoD+ cells (day 1) and subsequent increased Myosin Heavy Chain expression (day 5). This has not previously been demonstrated. Gal-Levi et al. (1998) observed a decrease in MyHC expression levels with increasing HGF concentrations in chicken satellite cells and a myogenic C2 cell line. This is not in agreement with our current study; however, expression was determined over a 2-d period with HGF concentrations of 20 and 50 ng/ml, exceeding our highest HGF dose. It can, therefore, be hypothesized that a temporal increase in extracellular concentrations of HGF (as would be experienced post-injury) is key in the modulation of satellite cell activation and subsequent differentiation. Together, these results suggest an intriguing dose-dependent regulatory effect of HGF on the balance between quiescence and activation as well as proliferation and differentiation.

HGF binds to the c-Met receptor, a membrane-bound, disulfide-linked heterodimer with an intracellular tyrosine kinase domain, found on both quiescent and activated satellite cells (Cornelison & Wold, 1997; Giordano et al., 1989). Results by Lesch et al. (1998) have shown that coupling of c-Met with Grb2 is required for inhibition of muscle differentiation mediated by HGF. This inhibition occurred only when Phosphatidylinositol 3-Kinase (PI3K) signalling downstream of c-Met was low, suggesting that increased coupling of PI3K to c-Met would lead to an up-regulation of muscle regulatory factors, such as MyoD, thereby promoting
cell differentiation (Leshem et al., 2002; Maina et al., 2001). This supports our observed increase in c-Met expression levels at the onset of myogenic commitment (day 1) in response to 10 ng/ml HGF. Our findings suggest that subsequent dose-dependent regulation of differentiation by HGF is possibly correlated with total c-Met receptor level expression with an observed significant drop in c-Met receptor levels in samples incubated with 2 ng/ml HGF at day 1 and day 2. Although HGF is the dominant binding factor to c-Met, there are a multitude of signalling adaptors and cell surface co-receptors, such as CD44, ICAM-1, and several integrin’s, which mediate biological responses unique to c-Met (Organ & Tsao, 2011). Studies in epithelial and cancer cells have shown that in this way, despite constant c-Met expression levels, HGF can elicit differential effects via downstream signalling mediators (Hammond et al., 2010; Organ et al., 2011).

Potential downstream pathways that have been suggested to mediate the dose-dependent effect of HGF on myogenesis include PI3K, p38, JNK, and ERK (Haley & Cantley, 2004). We attempted to glean a better understanding of the mechanisms involved in the dose-dependent effect of HGF on C2C12 proliferation and differentiation by utilizing specific inhibitors against some of the above-mentioned pathways. LY294002 is a potent, reversible inhibitor of phosphoinositide 3-kinases (PI3K) (Maira et al., 2009). The PI3K pathway signals through Akt and has been shown to be important in proliferation, differentiation and survival of muscle stem cells (Ceci et al., 2004; Guttridge, 2004). Akt has been observed to be vital in the IGF-induced increase of myogenin expression in C2C12 cells (Xu & Wu, 2000). We showed that the addition of LY294002 decreased C2C12 myoblast fusion, irrespective of HGF dose. Our findings were supported by Jiang et al. (1999) and Sumitani et al. (2002) who reported LY294002, and resulting inhibition of Akt, interfered with myotube formation and the expression of muscle-specific proteins such as MyoD and MyHC (Jiang et al., 1999; Li et al., 2000; Sumitani et al., 2002). Furthermore, we showed that addition of 2.5 mM LY294002 to proliferating C2C12 cells treated with either 2 ng/ml HGF or 10 ng/ml HGF abrogated the effect of this growth factor on cell number. This result was expected in part due to the known involvement of the PI3K/AKT pathway in myoblast proliferation, survival and regulation of apoptosis (Mandll et al., 2007).

PD98059 is a potent, selective inhibitor of ERK kinase (Braun et al., 2013). It binds the ERK-specific MAP kinase MEK and prevents phosphorylation of ERK1/2 by MEK1/2 downstream of c-Met, thus effecting its inhibitory action (Rehman et al., 2013). Myostatin-stimulated activation of the ERK pathway has been shown to negatively regulate myogenin in C2C12 cells, and this effect can be reversed with the addition of PD98059 (Yang et al., 2006). We observed that the addition of PD98059 reduced the negative effect of 2 ng/ml HGF, on differentiation, confirming that the ERK signaling pathway may mediate, at least in part, the inhibitory effect of HGF on myogenesis (Yang et al., 2006). Our study also showed that PD98059 modulated the pro-proliferative effect of 2 ng/ml HGF on C2C12 myoblasts. IGF-1 has been observed to activate the ERK pathway and lead to increased proliferation of mouse primary culture myoblasts (Madhala-Levy et al., 2012). This, together with our results strongly, suggests that the pro-proliferative effect of 2 ng/ml HGF is regulated, at least in part, by the ERK pathway. It has also been demonstrated that PI3K activity is required for HGF-induced MAPK activation; this adds an additional layer of complexity and may explain why LY294002 is able to affect both pro- and anti-proliferative pathways in myoblasts (Haley & Cantley, 2004).

SB203580 is a highly specific inhibitor of p38 MAPK (Saklatvala et al., 1996). Activation of the p38 pathway by 5 mM creatine has been shown to increase C2C12 fusion and MyHC expression in vitro (Deldicque et al., 2007). Inhibition of p38 by SB203580 has been shown to inhibit sarcomeric myosin expression in human embryonal rhabdomyosarcoma cells, indicating a pivotal role for p38 during myogenesis (Mauro et al., 2002). Inhibition using PD98059 and SB205380 allowed Kook et al. (2008) to determine that mechanical stretch-induced C2C12 proliferation was not due to an ERK-related mechanism, but due to p38 kinase. They also showed that 3 μM SB203580 prevented myogenin expression, suggesting that p38 activation was essential for efficient C2C12 differentiation (Kook et al., 2008). Li et al. (2000) had also previously shown that p38 inhibition prevents myogenic differentiation (Li et al., 2000). Similarly, our results showed that SB203580 reduced myotube formation further supporting the notion that p38 is vital for effective C2C12 differentiation. However, as with LY294002, the inhibition was independent of HGF. In our proliferation studies, SB203580 was able to negate both the respective positive and negative effects of 2 ng/ml HGF and 10 ng/ml HGF on cell number. In support of our findings, Jones et al. (2005) found that p38 is vital to the activation of primary culture satellite cells and their subsequent proliferation. Inhibition of p38 with SB203580 drove MM14 cells towards a quiescent-like state where they exit the cell cycle, but fail to differentiate (Jones et al., 2005). These results underscore the importance of keeping in mind that changes in cell number are determined by numerous contributing factors, not proliferation alone.

SP600125 is a specific and potent reversible inhibitor of Jun N-terminal kinase (JNK) (Bennett et al., 2001). Mauro et al. (2002) showed that the activation of JNKs induced growth arrest and differentiation in human embryonal rhabdomyosarcoma cells. JNK has also been shown to be vital in the proliferation and cell survival of a range of cell types, including myoblasts (Hong et al., 2001; Liu & Lin, 2005; Papa et al., 2004; Stathopoulou et al., 2008; Svensson et al., 2011). We showed that inhibition of JNK with SP600125 abolished the pro-proliferative effect of 2 ng/ml HGF and prevented C2C12 fusion, regardless of HGF treatment. Interestingly, cell viability was reduced when JNK was inhibited with SP600125, further supporting JNKs’ well-documented role in cell survival. These results suggest that not only does the inhibition of JNK mediate HGF-induced proliferation, but also that this effect is compounded by a reduction in cell viability.

Conclusions

In conclusion, our findings support a role for HGF in regulating both myoblast cell number and terminal differentiation in a dose-dependent manner. However, we further
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suggest that not only does HGF regulate these processes, but that proliferation and differentiation in both mouse and human myoblasts can be either promoted or inhibited in response to changing exogenous HGF concentrations. Changes in c-Met receptor expression were noted in response to HGF and the dose-dependent effects of the growth factor on proliferation and differentiation could be correlated to expression levels of this receptor. Furthermore, our results suggest that P13K and p38 mediate the anti-proliferative effect of the higher dose HGF, whereas the ERK signalling pathway is, at least in part, p38 mediated the anti-proliferative effect of the higher dose HGF.

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Declaration of interest

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Supplementary material available online
Supplementary Figure 1