IL-13 mediates the recruitment of reserve cells for fusion during IGF-1-induced hypertrophy of human myotubes

Virginie Jacquemin, Gillian Sandra Butler-Browne, Denis Furling* and Vincent Mouly*,‡

UMR S 787, Inserm/UPMC-Paris 6, Institut de Myologie, Paris, France

*These authors contributed equally to this work

‡Author for correspondence (e-mail: mouly@ext.jussieu.fr)

Accepted 7 December 2006
Journal of Cell Science 120, 670-681 Published by The Company of Biologists 2007
doi:10.1242/jcs.03371

Summary

Insulin-like growth factor-1 (IGF-1) has been shown to induce skeletal muscle hypertrophy, to prevent the loss of muscle mass with ageing and to improve the muscle phenotype of dystrophic mice. We previously developed a model of IGF-1-induced hypertrophy of human myotubes, in which hypertrophy was not only characterized by an increase in myotube size and myosin content but also by an increased recruitment of reserve cells for fusion. Here, we describe a new mechanism of IGF-1-induced hypertrophy by demonstrating that IGF-1 signals exclusively to myotubes but not to reserve cells, leading, under the control of the transcription factor NFATc2, to the secretion of IL-13 that will secondly recruit reserve cells for differentiation and fusion. In addition, we show that IGF-1 also signals to myotubes to stimulate protein metabolism via Akt by (1) activating the mTOR-p70S6K-S6 pathway and inhibiting GSK-3β, both involved in the control of protein translation, and (2) inhibiting the Foxo1–atrogin-1 protein degradation pathway.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/4/670/DC1

Key words: Insulin-like growth factor-1 (IGF-1), Skeletal muscle, Hypertrophy, Fusion, Reserve cells, IL-13

Introduction

Insulin-like growth factor-1 (IGF-1) has been shown to play a crucial role in the control of skeletal muscle growth both during development and regeneration and in the regulation of muscle mass (Florini et al., 1996). Its overexpression in the muscle of transgenic mice results in an increase in muscle weight and protein content (Coleman et al., 1995; Musaro et al., 2001). Some studies have shown beneficial effects of IGF-1 on the muscle phenotype of dystrophic mice, such as an increase in muscle mass and force (Barton et al., 2002; Zdanowicz et al., 1995). It has been also reported that the overexpression of IGF-1 in muscle prevented muscle atrophy observed during ageing (Barton-Davis et al., 1998). In vitro, IGF-1-induced hypertrophy has been demonstrated in rodent models and is characterized by an increase in both the area (Musaro and Rosenthal, 1999; Semsarian et al., 1999a) and the protein content of myotubes (Vandenburgh et al., 1991).

IGF-1 is unique among growth factors since it stimulates both the proliferation and the differentiation of myoblasts even though its actions are mediated by a single receptor, the type 1 IGF-1 receptor (R-IGF-1) (Coolican et al., 1997). IGF-1 binding to its receptor induces an autophosphorylation of the IGF-1 receptor that will activate two primary substrates: (1) IRS-1 (insulin receptor substrate), an upstream regulator of the phosphoinositide 3 kinase (PI3K)-Akt pathway and (2) Shc, an upstream regulator of the Raf–mitogen-activated protein kinase (MAPK) pathway (Florini et al., 1996). In myoblasts, the MAPK pathway, including the p38 MAPK and the p42 MAPK, has been mainly implicated in the control of myoblast proliferation, whereas the PI3K-Akt pathway has been described to control myoblast differentiation (Coolican et al., 1997; Kaliman et al., 1996) and survival (Lawlor and Rotwein, 2000). The p42 MAPK has been implicated in the induction of hypertrophy by IGF-1 in rodents although the results are somewhat contradictory (Haddad and Adams, 2003; Rommel et al., 1999; Wu et al., 2000). The role of Akt in muscle hypertrophy is more commonly accepted and Akt has been shown to increase protein synthesis in both in vitro and in vivo studies in mice by activating mTOR (mammalian target of rapamycin)-p70S6K-S6 pathway and by inhibiting GSK-3 (glycogen synthase kinase-3) involved in the regulation of protein translation (Bodine et al., 2001b; Park et al., 2005; Rommel et al., 2001). More recently, IGF-1 has been reported to decrease protein degradation via the inhibition of the transcription factor Foxo, which controls the expression of the muscle-specific ubiquitin ligase F-box protein 32 (Fbxo32, also known as atrogin-1), involved in protein degradation during muscular atrophy (Gomes et al., 2001; Sandri et al., 2004; Stitt et al., 2004).

Finally, calcineurin, a calcium-activated serine phosphatase, has also been proposed to be involved in IGF-1-induced hypertrophy. Once activated, calcineurin mediates the dephosphorylation of the NFAT (nuclear factor of activated T cells) transcription factors and their translocation into the nucleus (Graef et al., 2001). Although the roles of calcineurin and NFAT in myoblast fusion have been clearly demonstrated

(Abbott et al., 1998; Horsley et al., 2001), the data in the literature concerning the role of calcineurin in skeletal muscle hypertrophy are again often contradictory since some studies in rodent models show that IGF-1-induced hypertrophy can be suppressed using the calcineurin inhibitors cyclosporine A or FK506 (Musaro et al., 1999; Semsarian et al., 1999b), whereas other groups see no effect of these inhibitors on hypertrophy and no increase in calcineurin activity in the presence of IGF-1 (Bodine et al., 2001b; Rommel et al., 2001).

The ability of IGF-1 to act as an anabolic factor on skeletal muscle and to counterbalance the signalling pathways of muscle atrophy has led to the proposition that IGF-1 could be used as a therapeutic agent to combat muscle atrophy related to age (sarcopenia) or to various diseases. However all data available until now describing the mechanisms of IGF-1-induced hypertrophy have been obtained in rodent models, and very little is known about the effects and the signalling pathways of IGF-1 in human skeletal muscle. It is becoming increasingly evident that the results obtained in rodent models cannot always be directly transposed to man. For example, whereas a twofold increase was observed in the lifespan of myoblasts from transgenic mice overexpressing IGF-1 in muscle (Chakravarthy et al., 2000), we recently showed in human myoblasts that IGF-1 has no effect on the proliferative lifespan, suggesting a different mechanism of regulation in these two species (Jacquemin et al., 2004).

We previously developed an in vitro model of human myotube hypertrophy induced by IGF-1 where cultures were exposed to IGF-1 only 3 days after the induction of differentiation, a time when most of the myoblasts have already fused into myotubes and no more proliferation is observed. This model allows us to distinguish between the different effects of IGF-1 on proliferation, differentiation and hypertrophy (Jacquemin et al., 2004). In these conditions, IGF-1 is added to a differentiated culture that is composed of two cell populations: myotubes and reserve cells. Following serum deprivation, the majority of the myogenic cells differentiate: they irreversibly exit the cell cycle by the induction of the cell cycle inhibitors p21 or p57 (Parker et al., 1995; Zhang et al., 1999), express myogenin and finally fuse into myotubes. But there is always a small number of these desmin-expressing cells, called ‘reserve cells’, that remain mononucleated and do not fuse even though they have withdrawn from the cell cycle (Baroffio et al., 1995; Kitzmann et al., 1998; Lindon et al., 1998; Yoshida et al., 1998). We showed that, when IGF-1 was added to differentiated human muscle cultures, it acts not only on myotubes to increase their size but, more surprisingly, it induces an increase in the fusion index resulting from a decrease in the reserve cell compartment (Jacquemin et al., 2004). This would suggest that there is recruitment of reserve cells for a new wave of myogenic differentiation and fusion.

In the present study, we investigated the mechanisms by which IGF-1 induces hypertrophy of human myotubes and more specifically how the reserve cells are recruited for differentiation and fusion. We showed that IGF-1 treatment induced a re-expression of MyoD, myogenin and p57, corresponding to a new wave of myogenic differentiation. By differential trypsinization, we analyzed the effects of IGF-1 treatment on myotubes and reserve cells separately. We showed that IGF-1 exclusively induced Akt and p42 MAPK activation in myotubes but not in reserve cells, suggesting that myotubes recruit reserve cells by a secondary mechanism. The hypothesis that a soluble factor was responsible for reserve cell recruitment was confirmed by conditioned media experiments in which we showed that this factor was secreted by myotubes. We identified this factor to be IL-13 using neutralizing antibodies and confirmed its fusion-promoting activity by treating cultures with exogenous IL-13. We showed that the induction of IL-13 expression was mediated by NFATc2 that is translocated into the nucleus of myotubes in response to IGF-1 treatment. Finally, we showed that, in addition to increase cell fusion, IGF-1 stimulated the protein metabolism of myotubes, via the activation of Akt, by activating the mTOR-p70S6K pathway and inhibiting GSK-3β, and by inducing a downregulation of the forkhead box O1 (Foxo1)–atrogin-1 protein degradation pathway. Therefore, we propose a model of IGF-1/IL-13 induced hypertrophy in which IGF-1 exclusively signals to myotubes leading, (1) via Akt to induce an increase in protein content and (2) via nuclear factor of activated T-cells, cytoplasmic calcineurin-dependent 2 (NFATc2) to the secretion of IL-13 that will secondly activate and recruit reserve cells for fusion.

Results

IGF-1 exclusively signals to myotubes but not to reserve cells

In a previous study, we developed a model of IGF-1-induced hypertrophy of human myotubes characterized not only by an increase in the size and protein content of myotubes but also by a significant increase in the fusion index which was correlated with a decrease in the number of reserve cells. This finding was demonstrated on human primary myoblasts isolated from muscle biopsies from young, adult and old subjects. We proposed that IGF-1 induces the recruitment of reserve cells and stimulates their fusion and their differentiation into myotubes (Jacquemin et al., 2004). Reserve cells can be separated from myotubes by differential trypsinization and characterized by western blotting. As shown in Fig. 1A, reserve cells are desmin-positive cells that express Pax7 (up to 90% of the desmin-positive reserve cells expressed Pax7 as verified by immunocytochemistry; data not shown) but do not express myogenin or the cell cycle regulator p57, which has been shown to be involved in myogenic differentiation. MyoD was either not detectable or expressed at extremely low levels in reserve cells as compared with myotubes. Treatment of total cultures with IGF-1 for 24 hours at day 3 of differentiation (DM3) induced a significant increase in the level of MyoD (43.7±13.6%), myogenin (46±20%) and p57 (24±0.02%), confirming this new wave of myogenic differentiation (Fig. 1B).

However, the mechanism of recruitment of reserve cells by IGF-1 is not known. Since IGF-1 receptor was expressed by both myotubes and reserve cells (Fig. 1A), we investigated whether IGF-1 directly signals onto reserve cells to recruit them for fusion or if IGF-1 signalling is targeted only to myotubes, which then recruit the reserve cells through a secondary mechanism. The main pathways that have been described as mediators of skeletal muscle hypertrophy are the MAPK, the PI3K-Akt and the calcineurin pathways. To test the involvement of the latter pathways in the increase in cell fusion in response to IGF-1 during human myotube hypertrophy, the ability of specific inhibitors of these pathways to prevent the

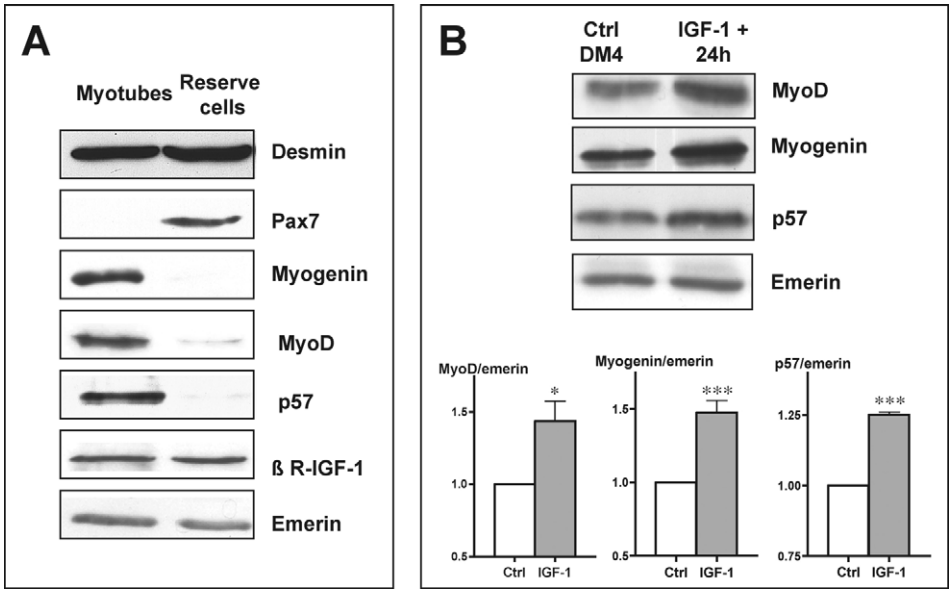

Fig. 1. IGF-1 induces a new wave of myogenic differentiation. (A) Desmin, Pax7, MyoD, myogenin, p57 and β subunit of IGF-1 receptor in myotubes and reserve cells were analyzed separately by western blot, after differential trypsinization at day 3 of differentiation. (B) Cultures were treated with IGF-1 at day 3 of differentiation. The content of MyoD, myogenin and p57 in total cultures (myotubes + reserve cells) was evaluated after 24 hours treatment with IGF-1, by western blot analysis. The amount of each factor relative to that of emerin is shown below. *P<0.05, ***P<0.001.

increase in fusion index induced by IGF-1 was tested. As shown in Fig. 2A, the p42 MAPK inhibitor PD098059 or the calcineurin inhibitor FK506, prevented the increase in fusion index, suggesting that both calcineurin and p42 MAPK are involved in the recruitment of reserve cells for fusion. As a control, cultures were treated with these inhibitors in the absence of IGF-1 and we observed no effect on the final fusion index (data not shown). These results are consistent with an increase of 49.2±17.4% in calcineurin activity observed after 15 minutes of treatment with IGF-1 (Fig. 2B) and with the activation of p42 MAPK as demonstrated by the increased phosphorylation of the Tyr204 residue of p42 MAPK, observed by western blot analysis (Fig. 2C). By contrast, when cultures were treated with SB203580, a specific inhibitor of p38 MAPK, prior to IGF-1 treatment, the fusion index was not significantly different from that observed in the IGF-1-treated cultures, suggesting that the p38 MAPK pathway is not required for reserve cell recruitment (Fig. 2A). This is consistent with the lack of p38 MAPK activation following IGF-1 treatment observed in Fig. 2C.

An activation of Akt was also observed after IGF-1 treatment as shown by the increased phosphorylation of the Ser473 residue of Akt followed by a later phosphorylation of Thr308 (Fig. 2C). Therefore, we tested the ability of LY294002 and rapamycin, specific inhibitors of PI3K and mTOR respectively, to prevent this increase in fusion index induced by IGF-1. However, in the presence of the latter inhibitors, a dramatic decrease in the fusion index as well as a dramatic effect on myotube morphology was observed (data not shown), suggesting that inhibition of the PI3K pathway not only prevented hypertrophy but also completely inhibited protein synthesis. Treatment with LiCl, a non competitive inhibitor of GSK-3β, mimicked the increase in fusion index observed in the presence of IGF-1 (Fig. 2A), suggesting that Akt and its target GSK-3β are also involved in the recruitment of reserve cells for fusion.

To investigate whether IGF-1 signals directly to both myotubes and reserve cells, the activation of Akt and p42 MAPK was next analyzed in myotubes and reserve cells separately. As shown in Fig. 2D, IGF-1 induces a phosphorylation of the Ser473 residue of Akt exclusively in myotubes but not in reserve cells although the same amount of total Akt was found in both populations. IGF-1 also induces a phosphorylation of Tyr204 of p42 MAPK exclusively in myotubes, suggesting that IGF-1 signals to myotubes but not to reserve cells. Therefore, a secondary signalling event must be triggered from myotubes to reserve cells to recruit them for fusion.

IL-13 is secreted in response to IGF-1 treatment and recruits reserve cells for fusion

To determine whether a soluble factor secreted by myotubes in response to IGF-1 could activate reserve cells and recruit them for fusion, we tested the effect of medium conditioned on IGF-1-treated cultures on non-treated cultures. Cultures were treated at day 3 of differentiation with IGF-1 for 90 minutes, then rinsed twice and incubated for 24 hours with fresh DMEM. This conditioned medium was collected and applied to non-treated cultures at day 4 of differentiation. At day 7 of differentiation, cultures were fixed and the fusion index was determined. As shown in Fig. 3A, we observed an increase in the fusion index of cultures treated with conditioned medium (66.7±1.5%), similar to that observed in IGF-1-treated cultures (70.0±1.4%). This increase was significant as compared to control cultures (58.7±1.0%). The fusion index of IGF-1-treated cultures and conditioned medium-treated cultures were not significantly different, confirming the presence of a secreted factor produced in response to IGF-1 treatment and responsible for the recruitment of reserve cells for fusion. To investigate whether this secreted factor was produced by myotubes or reserve cells, the ability of conditioned medium from IGF-1-treated reserve cell cultures to increase cell fusion was tested. Reserve cell cultures were obtained after myotube removal by brief trypsinization. As shown in Fig. 3A, no increase in fusion index was observed in the presence of the conditioned medium from reserve cells (59.9±2.2%), demonstrating that the soluble factor is secreted by the myotubes in response to IGF-1 treatment.

Among the possible candidates for fusion promoters, the cytokines IL-4 and IL-13 have recently been shown to be

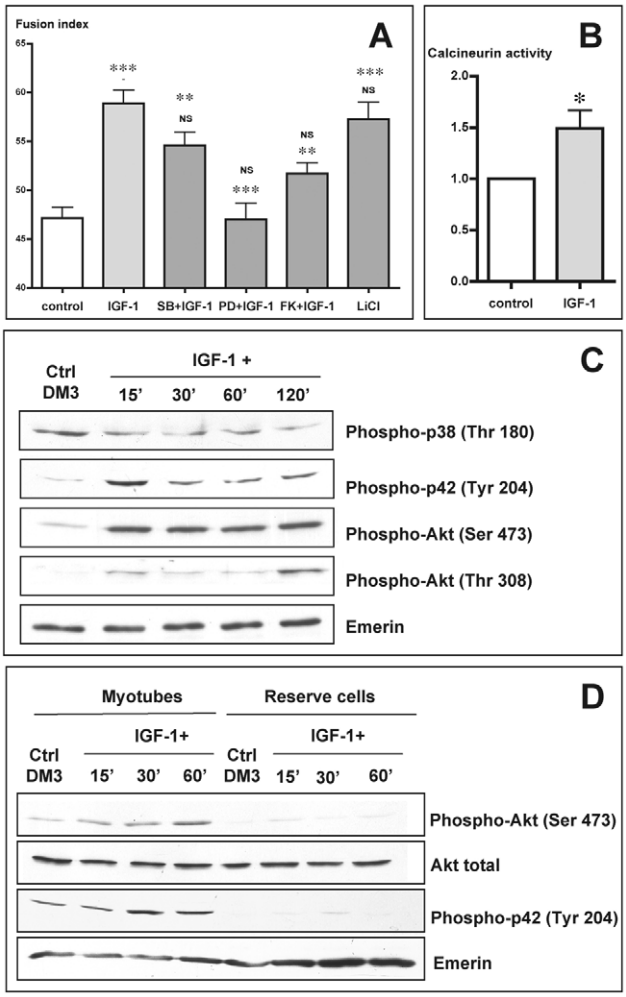

Fig. 2. IGF-1 exclusively signals to myotubes but not to reserve cells. (A) The ability of specific inhibitors of the p38 MAPK (SB203580; SB), p42 MAPK (PD098059; PD), and calcineurin (FK506; FK) to prevent the increase in cell fusion induced by IGF-1 was tested, as well as the ability of LiCl, inhibitor of GSK-3β, to mimic IGF-1-induced increase in fusion index. Above each column the significance of the sample versus the control (upper) and versus the IGF-treated sample (lower) is given; NS, non significant; $^{**}P<0.01$ and $^{***}P<0.001$. (B) Calcineurin activity was measured in total cultures at day 3 of differentiation after 15 minutes treatment with IGF-1. (C) Activation of p38 MAPK, p42 MAPK and Akt after treatment with IGF-1 was investigated in total cultures at day 3 of differentiation by western blot analysis using antibodies specific for the phosphorylated forms of the molecules. (D) The phosphorylation of Akt, normalized by total Akt, and p42 MAPK was analyzed in myotubes and reserve cells separately, at day 3 of differentiation, after differential trypsinization, by western blot.

responsible for the recruitment of myoblasts and the promotion of fusion in mice (Horsley et al., 2003). Another candidate, IL-15, has been shown to induce muscle hypertrophy of mouse and human myotubes (Furmanczyk and Quinn, 2003; Quinn et al., 2002). To examine whether these cytokines were responsible for the fusion promoting activity of the conditioned medium during IGF-1-induced hypertrophy in human cells, cultures were treated with neutralizing antibodies specific for human IL-4, IL-13 and IL-15, following IGF-1 treatment.

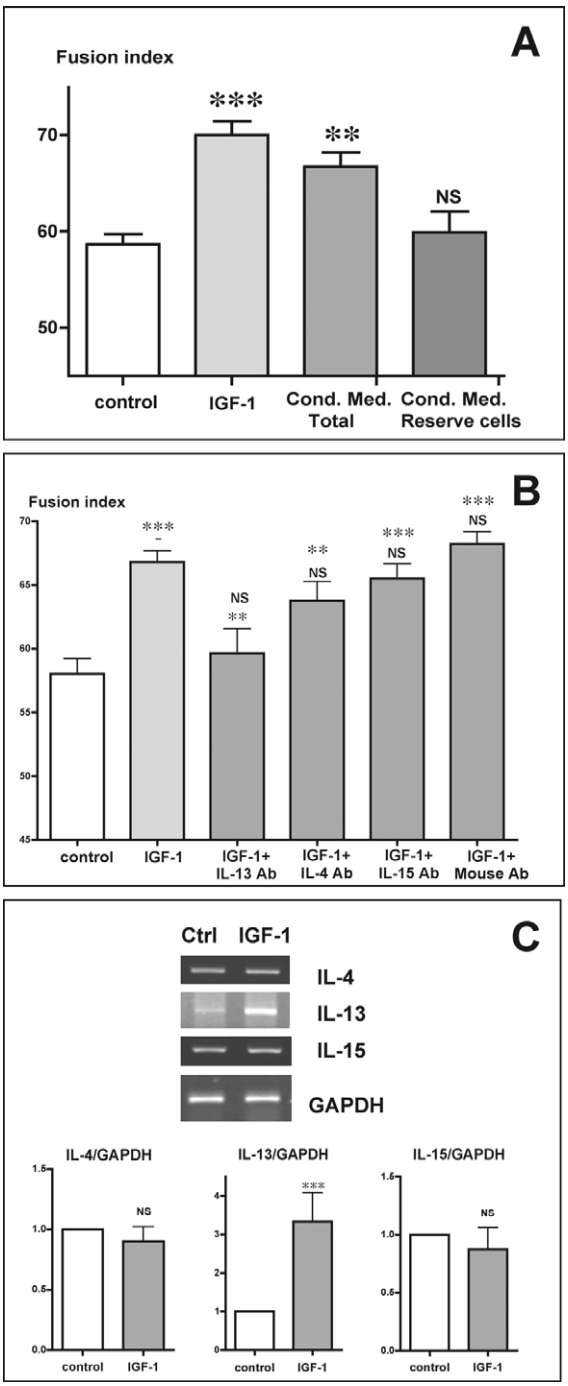

Fig. 3. A soluble factor is secreted by myotubes in response to IGF-1 treatment and recruits reserve cells for fusion. (A) The ability of conditioned medium from total cultures (myotubes + reserve cells) or reserve cell cultures (obtained after myotube removal by brief trypsinization) treated with IGF-1 to induce an increase in the fusion index was measured. (B) The ability of neutralizing antibodies directed against IL-13, IL-4, IL-15 to prevent the increase in cell fusion induced by IGF-1 was tested. Mouse immunoglobulins were used as a control. Above each column the significance of the sample versus the control (upper) and versus the IGF-treated sample (lower) is given; NS, non significant; $^{**}P<0.01$ and $^{***}P<0.001$. (C) The effects of IGF-1 on the expression of IL-13, IL-4 and IL-15 was investigated in total cultures, by RT-PCR analysis, and normalized with GAPDH.

Treatment with a neutralizing antibody against IL-13 prevented the increase in fusion index induced by IGF-1, whereas treatment with antibodies against IL-4, IL-15 or non specific mouse immunoglobulins had no significant effect (Fig. 3B). In addition, in the presence of the neutralizing antibody directed against IL-13, we also observed an inhibition of the increase in the mean number of nuclei by myotubes in response to IGF-1 treatment (see Fig. S1 in supplementary material). These results strongly suggest that IL-13 is the secreted factor responsible for the recruitment of reserve cells for fusion.

To confirm the involvement of IL-13 in the increase in fusion index, the expression of IL-4, IL-13 and IL-15 was examined by RT-PCR performed on total cultures. As shown in Fig. 3C, RT-PCR analysis reveals that IL-4, IL-13 and IL-15 were expressed following IGF-1 treatment. No significant increase in the expression of IL-4 and IL-15 was detected whereas a significant increase of 200±69% in IL-13 expression was detected 3 hours after IGF-1 treatment. The levels of IL-4, IL-13 and IL-15 proteins in the conditioned media were also measured by ELISA, and according to the results obtained by RT-PCR analysis, we detected an increase of 162% in IL-13 protein in the conditioned medium from IGF-1-treated cultures as compared to non treated cultures, whereas IL-4 could not be detected and no change in IL-15 expression was observed following IGF-1 treatment (data not shown).

To confirm that IL-13 was responsible for the increase in fusion index, we tested the effects of exogenous treatment by recombinant IL-13 or IL-4. Addition of as little as 5 ng/ml of exogenous IL-13 to cultures at day 3 of differentiation mimicked the increase in fusion index observed in IGF-1-treated cultures (Fig. 4A,C) whereas IL-4 treatment had no effect on the fusion index even at 50 ng/ml. The same results were observed when these cytokines were added at the same time as differentiation was induced (data not shown). To estimate myotube size, the mean number of nuclei per myotube in IL-13-treated cultures was also determined. As shown in Fig. 4B, treatment with 5 ng/ml IL-13 induced the same significant increase in the mean number of nuclei per myotube as in IGF-1-treated cultures. In addition, treatment by 5 ng/ml of IL-13 also mimics the increase in myosin content induced with IGF-1 (see Fig. S2 in supplementary material). Finally we confirmed by RT-PCR analysis that the two subunits of the IL-13 receptor, IL-13Rα1 and IL-4Rα, are expressed in both reserve cells and myotubes at 3 days of differentiation (data not shown). Taken together, our results clearly demonstrate a new mechanism of IGF-1-induced hypertrophy: IGF-1 signals to myotubes leading to the secretion of IL-13, which is responsible, in a second step, for the increased recruitment of reserve cells for fusion.

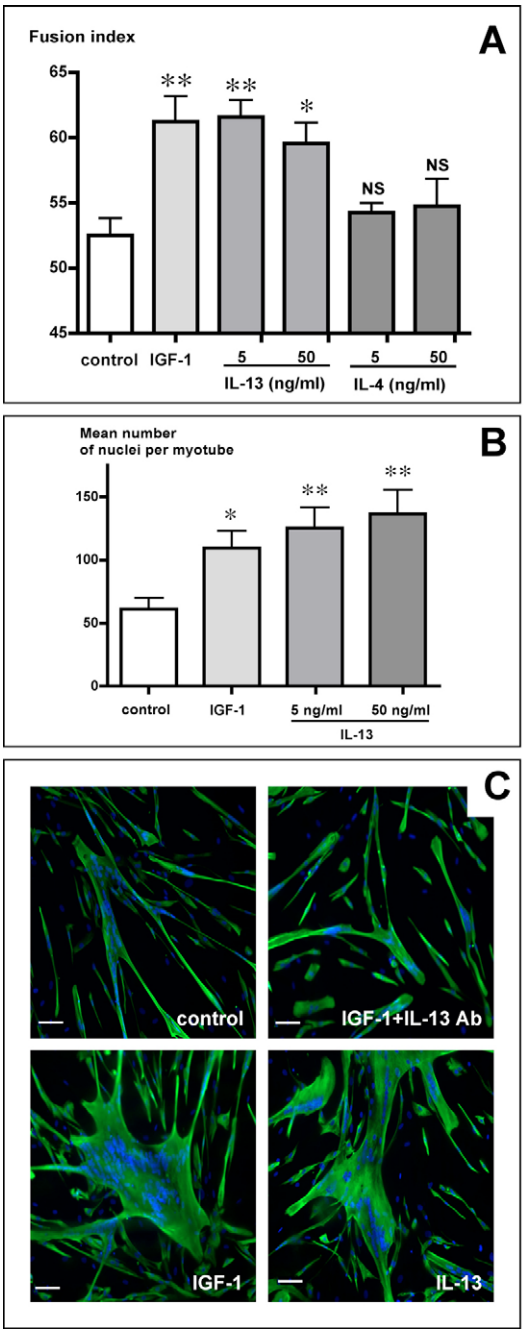

Fig. 4. Treatment with exogenous IL-13 mimics the increase in fusion index and myotube size observed during IGF-1 induced human myotube hypertrophy. (A) The effects of exogenous treatment with IGF-1, IL-4 or IL-13 on the fusion index were determined. (B) The effects of exogenous treatment with IGF-1 and IL-13 at 5 ng/ml on the mean number of nuclei per myotube was measured. NS, non significant; *P<0.05; **P<0.01. (C) Immunostaining using desmin antibody was performed on cultures treated with IGF-1, IL-13 at 10 ng/ml or neutralizing antibody against IL-13. Nuclei were counterstained with Hoechst. Bar, 100 μm.

IGF-1 induces an increase in the DNA binding activity of the NFATs and a nuclear translocation of NFATc2

NFAT (nuclear factor of activated T-cells) transcription factors have been shown to control the expression of many secreted cytokines including IL-13 (Dolganov et al., 1996). Therefore, we investigated whether NFATs were activated in response to IGF-1 treatment during human myotube hypertrophy. The effect of IGF-1 on the DNA binding activity of NFAT transcription factors was analyzed by electrophoretic mobility shift assay (EMSA) using a sequence from the human IL-13 promoter (Dolganov et al., 1996). As shown in Fig. 5A, in the presence of IGF-1, a significant increase of 87.5±38.9% in the DNA binding activity of NFATs was observed after 1 hour of treatment. The specificity of NFAT binding was confirmed by competition with the unlabelled competitor oligo, using a

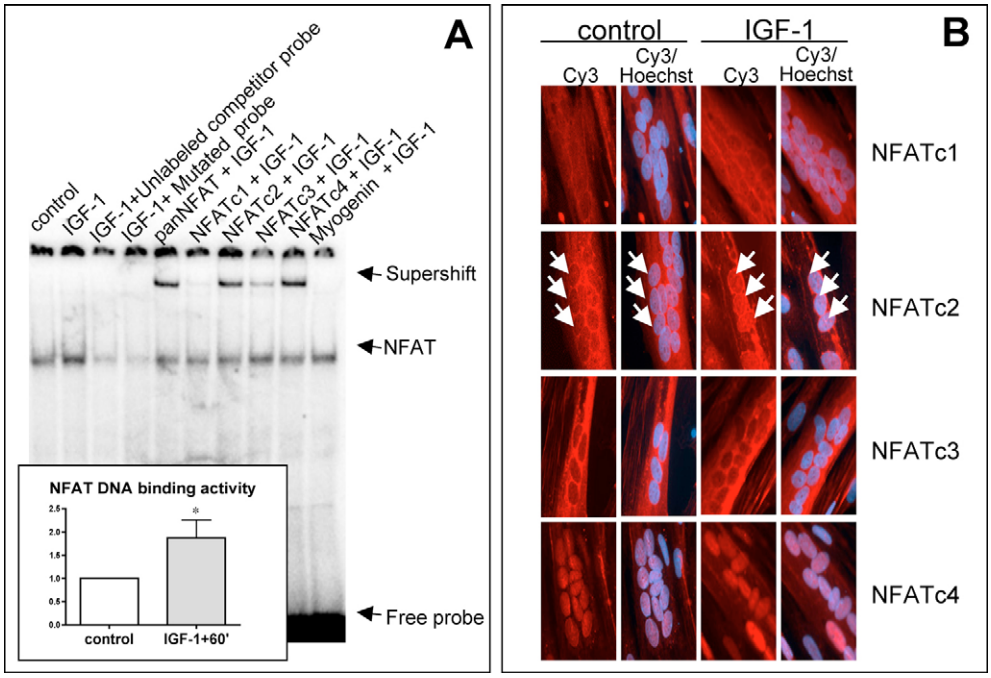

Fig. 5. IGF-1 induces an increase in the DNA binding activity of the NFAT transcription factors and a nuclear translocation of NFATc2. (A) The NFAT DNA binding activity of the cultures treated by IGF-1 was investigated by EMSA using the consensus NFAT sequence from the IL-13 promoter. A competition with the unlabelled competitor oligo, a mutated probe and supershift using panNFAT antibody or antibodies directed against NFATc1, NFATc2, NFATc3 or NFATc4 were performed. (B) Immunostaining using sera directed against NFATc1, NFATc2, NFATc3 or NFATc4 isoforms was performed after IGF-1 treatment. Nuclei were counterstained with Hoechst. **P*<0.05.

mutated probe and by supershift using a panNFAT antibody. At least four NFAT isoforms have been described in the literature including NFATc1, NFATc2, NFATc3 and NFATc4. All these isoforms were detected by RT-PCR and western blot analysis (data not shown) in cultures after 3 days of differentiation. To determine which NFAT isoform was responsible for this increase in NFAT DNA binding activity in the presence of IGF-1, we tested the ability of specific antibodies for the four NFAT isoforms to induce a supershift. As shown in Fig. 5A, antibody against NFATc1 failed to induce a supershift, excluding a role of NFATc1 in the increase in NFAT DNA binding activity in the presence of IGF-1. On the other hand, in the presence of antibodies directed against NFATc2 and NFATc4, and NFATc3 to a less extent, the mobility of NFAT was shifted, suggesting that these three isoforms may be activated in the presence of IGF-1.

The localization of the different NFAT isoforms was next examined by immunocytochemistry in the presence and absence of IGF-1. As shown in Fig. 5B, no changes in NFATc1, 3 and 4 localization were observed following IGF-1 treatment, whereas a nuclear translocation of NFATc2 was observed. It can be noted that NFATc1 and NFATc3 remained localized in the cytoplasm in the presence and in the absence of IGF-1, whereas NFATc4 is nuclear in both conditions. These data, in accordance with the results of the EMSA, show that in response to IGF-1, NFATc2 previously localized in myotube cytoplasm will translocate to the nucleus, leading to an increase in the NFAT DNA binding activity that may be responsible for the increase in IL-13 expression.

IGF-1 induces an Akt dependant stimulation of protein translation and inhibition of the Foxo–atrogin-1 protein degradation pathway

In addition to activating the NFATc2–IL-13 pathway leading to an increase in cell fusion, IGF-1 also induced a twofold increase in the myosin content of myotubes (Fig. 6A) measured by the ratio of myosin to emerin as described previously (Jacquemin et al., 2004). To investigate the pathways involved in this increase in protein content, we tested the ability of specific inhibitors for the MAPKs, Akt and calcineurin pathways to prevent the increase in the myosin:emerin ratio observed in the presence of IGF-1. In the same way as for the fusion index, inhibition of either p42 MAPK by PD098059 or calcineurin by FK506 induced a decrease in the myosin:emerin ratio that reached the level observed in the control cultures, whereas p38 MAPK inhibition by SB203580 had no effect (Fig. 6A), suggesting a role for p42 MAPK and calcineurin in the regulation of protein content during human myotube hypertrophy induced by IGF-1.

Akt has been shown to control protein metabolism during muscle hypertrophy in rodents by acting on mTOR/p70S6K and GSK-3β, both involved in protein translation. As shown in Fig. 6B, IGF-1 treatment induces an increase in the phosphorylation of the residues Thr389 of p70S6K and Ser9 of GSK-3β, providing evidence of p70S6K activation and GSK-3β inhibition (Harwood, 2001). These increased phosphorylations were PI3K dependent since they were inhibited in the presence of LY294002 (data not shown). We also showed that GSK-3β inhibition by LiCl mimicked the increase in myosin content observed in the presence of IGF-1 (Fig. 6A), confirming that GSK-3β negatively regulates hypertrophy.

In addition to controlling protein translation, Akt has been shown to be involved in the control of protein degradation by regulating the transcription factor Foxo (Zhang et al., 2002). To investigate whether IGF-1 treatment acts on this protein degradation pathway during human myotube hypertrophy, we tested the effects of IGF-1 on the activity of the transcription factor Foxo. IGF-1 treatment induces an increase in the phosphorylation of the residue Ser256 of Foxo1 (Fig. 6B), which has been shown to inhibit its activity (Brunet et al., 1999). The DNA binding activity of Foxo was next investigated

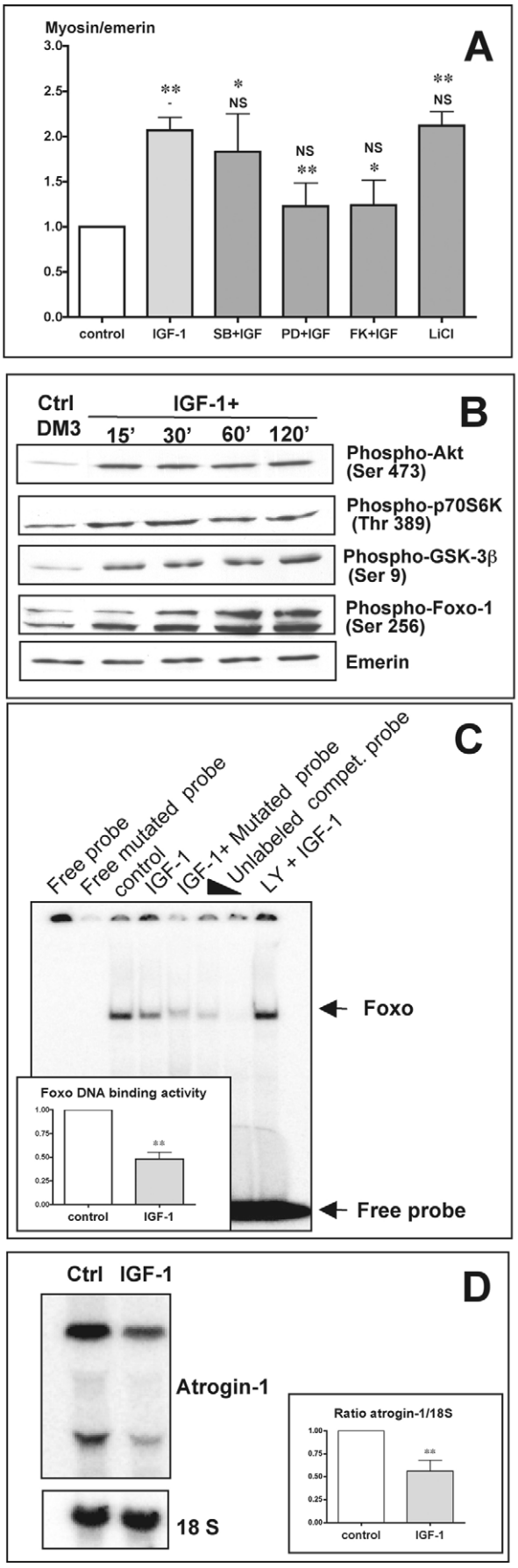

Fig. 6. IGF-1 stimulates protein metabolism via Akt (A) The ability of specific inhibitors of the p38 MAPK (SB=SB203580), p42 MAPK (PD=PD098059), the calcineurin (FK=FK506) and GSK-3β (LiCl) to prevent the increase in myosin content induced by IGF-1 was tested. Above each column the significance of the sample versus the control (upper) and versus the IGF-treated sample (lower) is given; NS, non significant; **P*<0.05; ***P*<0.01. (B) The phosphorylation of Foxo1, p70S6K, GSK-3β and Akt was investigated by western blot analysis after treatment by IGF-1. (C) The effect of IGF-1 on the Foxo DNA binding activity of the cultures was investigated by EMSA. A competition with the unlabelled competitor oligo and a mutated probe were used to assess signal specificity. (D). The effect of IGF-1 on the expression of atrogin-1 was tested by northern blot analysis after a 3-hour treatment. Two mRNA forms of 2.4 kb and 6.5 kb were detected for atrogin-1, as described previously (Li et al., 2005).

using EMSA. As shown in Fig. 6C, a significant decrease of 51.5±2.5% in the DNA binding activity of Foxo was observed 1 hour after IGF-1 treatment, consistent with the increased phosphorylation of Foxo1 observed on western blots (Fig. 6B). Treatment by LY294002 prior to IGF-1 treatment prevented this decrease in Foxo DNA binding activity, confirming that Foxo was downregulated following the activation of the PI3K-Akt pathway (Fig. 6C).

In rodents, Foxo1 has been shown to regulate the expression of the ubiquitin-ligase atrogin-1 (MAFbx). We analyzed, by northern blot, the effect of IGF-1 treatment on the expression of the atrogin-1. Two forms of atrogin-1 mRNA were detected at 2.4 kb and 6.5 kb, as described previously (Li et al., 2005). A significant decrease of 44±11.85% in atrogin-1 mRNA expression was observed after a 3-hour treatment with IGF-1 on both forms (Fig. 6D). Taken together, these data show that during human myotube hypertrophy IGF-1 acts on myotubes via Akt to stimulate protein metabolism by (1) activating the mTOR-p70S6K pathway and inhibiting GSK-3β, both being involved in protein synthesis, and (2) inhibiting the Foxo1–atrogin-1 pathway involved in protein degradation (Fig. 7).

Discussion

Given the ability of IGF-1 to counterbalance the signalling pathways involved in atrophy, to induce muscle hypertrophy even in aged muscle, and to improve the muscular phenotype of several dystrophic models, IGF-1 has been proposed as a potential therapeutic tool for the treatment of muscle atrophy and neuromuscular diseases. This potential therapeutic application requires a better understanding of the mechanisms by which IGF-1 induces hypertrophy, particularly in humans. To investigate the effects of IGF-1 on differentiated human muscle cells, we developed a model of human myotube hypertrophy in which cultures were treated with IGF-1 after 3 days of differentiation. Hypertrophy was characterized not only by an increase in myotube size but also by an increase in the fusion index resulting from the increased recruitment of reserve cells for differentiation and fusion (Jacquemin et al., 2004). This latter finding is consistent with in vivo studies of hypertrophy where it has been shown that the destruction of satellite cells by gamma radiation prevented, at least in part, the hypertrophy induced by IGF-1 (Barton-Davis et al., 1999) or by overload (Rosenblatt and Parry, 1992). This requirement

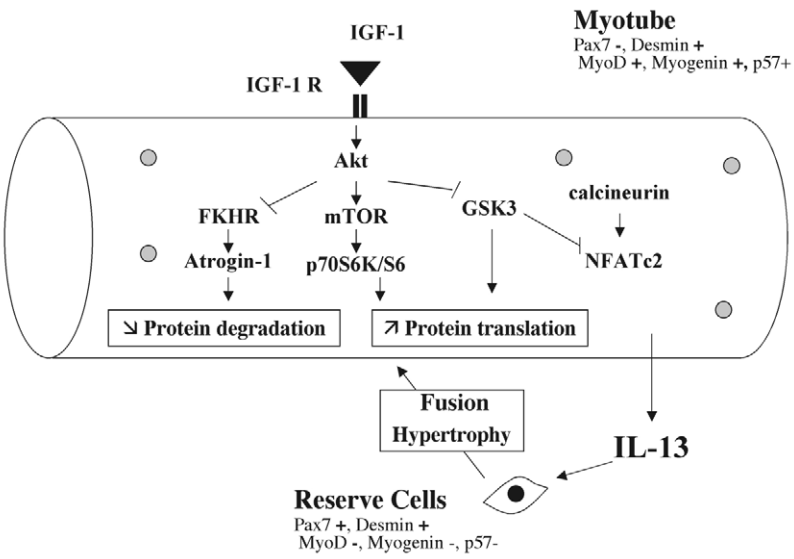

Fig. 7. Mechanisms of IGF-1-induced human myotube hypertrophy. (1) IGF-1 induces an increase in the protein content of myotubes via Akt. Within Akt targets, IGF-1 acts on the mTOR/p70S6K and GSK-3β pathways, both of which are involved in the control of protein translocation. IGF-1 also downregulates the Foxo/atrogin-1 protein degradation pathway. (2) IGF-1 induces an increase in the recruitment of reserve cells for fusion via the secretion of IL-13 by myotubes under the control of NFATc2.

of satellite cells for fusion during hypertrophy reflects the necessity of keeping the ratio between the nuclear content and cytoplasmic volume, also called the 'myonuclear domain', constant during hypertrophy in vivo (Allen et al., 1995).

However, the mechanism by which reserve cells are recruited by IGF-1 to fuse to myotubes is not yet understood. In the present study, we demonstrate a new mechanism of IGF-1-induced human myotube hypertrophy. We show that IGF-1 induces the secretion of IL-13 by myotubes that will mediate, in a second step, the recruitment of reserve cells for fusion. This finding resulted from the fact that conditioned medium from IGF-1-stimulated total cultures (myotubes + reserve cells), but not from IGF-1-stimulated reserve cell cultures, was able to induce the same increase in fusion index as IGF-1 does, proving the existence of a soluble factor secreted by myotubes. This was consistent with the fact that activation of either the p42 MAPK or Akt following IGF-1 treatment was not detected in reserve cells, suggesting that IGF-1 signals exclusively to myotubes that secondly signal to reserve cells. Given the recent data that the different IGF-1 isoforms can induce distinct signals through the IGF-1 receptor (Barton, 2006), we cannot exclude that the reserve cells will be responsive to other IGF-1 isoforms. Since the ability of the conditioned medium to recruit reserve cells for fusion was inhibited in the presence of neutralizing antibodies against IL-13 but not against IL-4, IL-15 or in the presence of non-specific mouse immunoglobulins, we identified the soluble factor to be IL-13. In addition, we show that IGF-1 induces an increase in IL-13 but not in IL-4 and IL-15 expression, as evidenced at both at the RNA and protein levels. Moreover, exogenous treatment with as little as 5 ng/ml of recombinant IL-13 mimicked the increase in fusion index and in the mean number of nuclei per myotube induced by IGF-1, whereas IL-4 did not induce any change in the fusion index even at 50 ng/ml.

Preliminary studies have demonstrated a role for IL-13 in the control of fusion in human monocyte/macrophage (DeFife et al., 1997) and mouse myoblasts (Horsley et al., 2003). In contrast to mice where IL-4 has been demonstrated to be more potent than IL-13 in inducing myoblast fusion (Horsley et al., 2003), we show that IL-13 and not IL-4 is responsible for the recruitment of reserve cells during human myotube hypertrophy induced by IGF-1. It should be noted that homologies have been described between these two cytokines: they share 30% sequence homology, their genes are closely linked in both the human and mouse genomes suggesting a coregulation of these two genes (Smirnov et al., 1995), and they have redundant functions in immune cells (Minty et al., 1993) due to the common subunit shared by their receptors (Callard et al., 1996). It was recently shown in human and mouse muscle cell cultures that IL-4 is involved in the control of myotube size during myotube formation following differentiation (Horsley et al., 2003; Lafreniere et al., 2006). Since no induction of IL-4 expression was detected following IGF-1 treatment in human myotube cultures, we propose that IL-4 and IL-13 could share the redundant ability to recruit mononucleated cells for fusion but not toward the same cell populations: IL-4 being responsible for the 'normal' myoblast fusion into nascent myotubes during myogenic differentiation, and IL-13 for additional recruitment of reserve cells during IGF-1-induced hypertrophy. This hypothesis was reinforced by the fact that exogenous treatment by IL-13 at the time of differentiation induction induces the same increase in fusion index as when it was added after 3 days of differentiation (data not shown); on the contrary, we observed that IL-4 did not induce any change in the final fusion index when added either at the time of differentiation induction (data not shown) or after 3 days of differentiation, in accordance with previous studies on human and mice myoblasts (Horsley et al., 2003; Lafreniere et al., 2006).

We also tested the involvement of IL-15 as a candidate for the activation of reserve cells, since it has been shown to be involved in myotube hypertrophy in mouse, bovine and human myoblasts (Quinn et al., 2002). Although we were able to confirm the expression of IL-15 in human myotubes, we did not observe any increase in IL-15 expression following IGF-1 treatment. This is consistent with the fact that IL-15 has been shown to induce hypertrophy by an increase in protein metabolism, and particularly myosin content, but not in cell fusion (Quinn et al., 2002) and that IL-15 and IGF-1 can act additively on protein accumulation (Quinn et al., 1995) suggesting that differential mechanisms are used by these factors to induce hypertrophy.

The NFATs have been shown to be involved in the regulation of IL-13 expression in human T cells (Dolganov et al., 1996). Since IGF-1 induces an increase in the DNA binding activity of the NFATs, measured by EMSA using the described sequence from the IL-13 human promoter (Dolganov et al., 1996), as well as a nuclear translocation of the NFATc2

isoform, we proposed that IL-13 expression is induced following IGF-1 treatment under the control of NFATc2. Our finding that IGF-1 induces myotube hypertrophy by activating NFATs are consistent with a previous study in rodents (Musaro et al., 1999). However, in the present study, we show that only NFATc2, but not NFATc1, translocates to the nucleus in response to IGF-1, suggesting that in human myotubes, NFATc2 mediates IGF-1-induced hypertrophy. The NFATc2 isoform has already been shown to be involved in myoblast fusion to nascent myotubes in mice (Horsley et al., 2001; Horsley et al., 2003). Previous studies showed that several signalling pathways, including calcineurin, GSK-3β (Beals et al., 1997; Neal and Clipstone, 2001) and p42 MAPK (Sanna et al., 2005), could control the activity of the NFAT transcription factors. Given the prevention of reserve cell recruitment observed in the presence of FK506 and PD098059 and the ability of LiCl to mimic IGF-1-induced increase in the fusion index, we propose that these pathways may cooperate to activate NFATc2 during IGF-1-induced hypertrophy of human myotubes.

We show here that p38 MAPK is not required for IGF-1-induced hypertrophy of human myotubes, neither is it activated by IGF-1 in these conditions; our data obtained by western blot analyses (Fig. 1C) even suggest that IGF-1 induces a decrease in p38 MAPK activity. This is consistent with the studies of Booth and colleagues which showed that p38 phosphorylation increased during atrophy induced by immobilization in young and old rats, suggesting a role for p38 MAPK in protein loss during muscle atrophy (Childs et al., 2003; Morris et al., 2004). Such a role of p38 MAPK in muscle atrophy would seem to be in contradiction with in vivo studies showing an increase in p38 MAPK phosphorylation after exercise or during muscle overload (Carlson et al., 2001; Nader and Esser, 2001) although it can be postulated that in these conditions, p38 MAPK is activated in response to stress (Kyriakis and Avruch, 2001) and does not participate to the hypertrophic signals. Moreover, it has been shown recently that TNFα, which induces a loss of protein content in myotubes, stimulates the expression of the muscle atrophy related ubiquitin ligase atrogin-1 in murine models via the activation of p38 MAPK (Li et al., 2005). One should keep in mind that p38 MAPK has also been involved in the induction of myogenic differentiation in in vitro studies (Lluis et al., 2006). Even if the role of p38 MAPK in the control of muscle mass remains unclear, and varies depending on the nature of the stimuli and of the model, here we clearly show that p38 MAPK is not required for IGF-1-induced hypertrophy in human muscle cells.

By contrast, we show an inhibition of hypertrophy in the presence of the p42 MAPK inhibitors PD098059 and UO126. These results are in agreement with a previous study showing that serum-induced hypertrophy of C2C12 myotubes was mediated by p42 MAPK and prevented by PD098059 (Wu et al., 2000) and with an in vivo study which showed that concomitant treatment with PD098059 and IGF-1 in rats prevented IGF-1-induced hypertrophy (Haddad and Adams, 2003). In the present study, we show that p42 MAPK is phosphorylated in myotubes that are postmitotic, suggesting that, in this model, p42 MAPK has a role distinct from the one usually described in the control of proliferation, such as a role in the control of protein synthesis as suggested previously (Wang et al., 2001), or in the control of the NFAT transcription factors as shown in cardiac muscle (Sanna et al., 2005). Connections between the PI3K-Akt and the MAPK pathways, positive (Wang et al., 2001) and negative (Rommel et al., 1999; Tiffin et al., 2004), have been described. In human myotubes, we show that IGF-1 induces an activation of p42 MAPK and Akt pathways in an independent manner, consistent with previous studies (Foulstone et al., 2004; Halevy and Cantley, 2004). We do not have evidence of connections between these two pathways, because (1) the p42 MAPK inhibitor UO126 has no effect on the phosphorylation of Akt, GSK-3β or S6, and (2) the PI3K inhibitor LY294002 has no effect on p42 MAPK phosphorylation (data not shown). These apparent contradictions can be resolved as being dependent on the cellular context, suggesting that the same signalling mechanisms can be interpreted differently according to differentiation stage, cell types and species.

The PI3K-Akt pathway has been widely studied in rodent models of IGF-1-induced muscle hypertrophy. Among the targets of Akt, mTOR/p70S6K and GSK-3β have been described as the two main mediators of this hypertrophy (Park et al., 2005; Rochat et al., 2004; Vyas et al., 2002). In human myotubes, we confirm that IGF-1 induces, via Akt, the activation of mTOR/p70S6K and the inhibition of GSK-3β, that have been previously shown to be involved in the control of protein synthesis via the eukaryotic initiation factor eIF2B (Rommel et al., 2001; Welsh et al., 1998). In addition, we show that the p70S6K-S6 pathway that has been widely implicated in the control of cell size in animals ranging from *Drosophila* (Montagne et al., 1999) to mice (Ohanna et al., 2005) also plays a key role in the control of cell size in human. Another target of Akt is the transcription factor Foxo, recently shown to be involved in protein degradation during muscle atrophy. In atrophic conditions, the activity of the PI3K-Akt pathway decreases, leading to an increase in Foxo1 and Foxo3 activity and a subsequent increase in the expression of the ubiquitin ligase atrogin-1 (Bodine et al., 2001a; Sandri et al., 2004). As shown in C2C12 where IGF-1 has been shown to prevent this Foxo-dependent expression of atrogin-1 via the activation of the PI3K-Akt pathway (Latres et al., 2005; Stitt et al., 2004), we demonstrate that IGF-1 phosphorylates Foxo1 and decreases its DNA binding activity during IGF-1-induced human skeletal muscle hypertrophy. We also observed a decrease by half in the expression of atrogin-1, confirming the inhibition of this protein degradation pathway by IGF-1 during human myotube hypertrophy. Taken together these data clearly show that IGF-1 acts via Akt on protein metabolism during human myotube hypertrophy (1) by increasing protein translation via the activation of the mTOR-p70S6K pathway and the inhibition of GSK-3β and (2) by inhibiting protein degradation via a down regulation of Foxo and atrogin-1.

In conclusion, this study demonstrates a new mechanism of IGF-1-induced hypertrophy of human myotubes. In the proposed model (Fig. 7), IGF-1 acts exclusively on myotubes and induces hypertrophy by two separate mechanisms: (1) IGF-1 induces the secretion of IL-13 by myotubes under the control of NFATc2 which will then recruit reserve cells for fusion, and (2) IGF-1 induces, via Akt, an increase in protein content by stimulating protein synthesis via mTOR/p70S6K and GSK-3β, and by inhibiting protein degradation via the downregulation of Foxo/atrogin-1. These in vitro mechanisms can be related to in vivo studies which show that even if the

major part of IGF-1-induced hypertrophy requires the activation and the addition of new satellite cells to the fibre, IGF-1 can still induce an increase in muscle size independently of satellite cells by acting on protein metabolism (Barton-Davis et al., 1999). In the present study, IGF-1 induces an increase in cell fusion in the absence of proliferation, which will result in a decrease in the number of reserve cells. Therefore, an increased recruitment of cells for fusion could deplete the pool of quiescent cells in vivo leading to a decrease in the long term in the regenerative capacity of muscle. One could hypothesize that, given the different environments of reserve cells in vitro and satellite cells in vivo, satellite cells would most probably proliferate before fusing to the muscle fibres in vivo allowing the pool of satellite cells to remain intact. However, if conditions of growth factors or matrix were to change during aging or disease, this process could then be disturbed and IGF-1 treatment could deplete the pool. Potential therapeutic strategies directed to specific targets of IGF-1, which may act on protein metabolism rather than on satellite cell recruitment, based upon the results of the present study, could avoid a decrease in the long term regenerative capacity of the muscle.

Materials and Methods

Human skeletal muscle culture

Satellite cells were isolated as described previously (Edom et al., 1994) from the quadriceps muscle of a 5-day-old infant in accordance with the French legislation on ethical rules. Cells were grown in Ham's F10 supplemented with 20% foetal bovine serum and 5 μg/ml of gentamycin in 5% CO_2. For differentiation, subconfluent cultures were switched to a serum-free Dulbecco's modified Eagle's (DME) medium. Culture reagents were purchased from Invitrogen. Myogenic purity, calculated as described previously (Jacquemin et al., 2004), was always more than 75%. The fusion index was determined at day 7 of differentiation by the number of nuclei in differentiated myotubes (>2 myonuclei) as a percentage of the total number of nuclei. For the isolation of reserve cells, plates were rinsed twice and incubated in 1× trypsin-EDTA for 1 minute at 37°C. Myotubes that detached first (Kitzmann et al., 1998) were rapidly collected. Plates were then rinsed and incubated again intrypsin-EDTA to allow reserve cells to detach. These cells were then filtered through a 100 μm cell strainer (BD Falcon, Erembodegem, Belgium) before being pelleted.

Treatments by IGF-1 and inhibitors

Exogenous treatment by human recombinant IGF-1 (Sigma) was carried out at the optimal concentration of 50 ng/ml as previously described (Jacquemin et al., 2004). Cultures were treated with each inhibitor 1 hour prior to IGF-1 treatment at the following concentrations: 15 μM SB 203580 (Sigma), 30 μM PD098059 (Sigma), 10 mM LiCl (Sigma), 100 ng/ml FK506 (Fujisawa Pharmaceutical, Osaka, Japan).

Western blot analysis

Cell pellets were extracted in RIPA buffer (150 mM NaCl, 50 mM Hepes pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 20 μg/ml PMSF) in the presence of a protease inhibitor cocktail (Roche) and sonicated. Equal amounts of proteins were loaded in Laemmli buffer and resolved by 10% SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes. After blocking, the membranes were incubated with the following antibodies: phospho-Akt (Ser473) and (Thr308), Akt, phospho-p42 (Tyr204), phospho-p38 (Thr180), phospho-Foxo1 (Ser256), phospho-p70S6K (Thr389) and phospho-GSK-3β (Ser9) from Cell Signaling Technology; desmin (D33) and MyoD (clone 5.8A) from Dako; Emerin from Novocastra (Newcastle Upon Tyne, UK); p57 and R-IGFβ from Santa Cruz; myogenin (clone F5D), MF20 and Pax7 from the Developmental Studies Hybridoma Bank. The ratio of myosin to emerin was determined as previously described (Jacquemin et al., 2004). The secondary antibody coupled to HRP was revealed using the ECL kit (Pierce) and band intensities were quantified using NIH Image software.

Conditioned media experiments, antibody neutralization, ELISA and cytokine treatment

For conditioned media experiments, total cultures (myotubes + reserve cells) or reserve cell culture were treated with IGF-1 at day 3 of differentiation for 90 minutes and then rinsed twice with DMEM before being incubated with fresh DMEM for 24 hours. After 24 hours, conditioned medium was collected and added to non-treated cultures at day 4 of differentiation. For neutralization experiments, antibodies were added at day 3 of differentiation at three times the concentration of ND50, defined as the concentration of antibody required to yield one-half maximal inhibition of the activity of 10 ng/ml of each cytokine: 7.5 μg/ml for IL-4 antibody (R&D, 6 μg/ml for IL-13 antibody (Sigma) and 16.5 μg/ml for IL-15 (R&D) and 6 μg/ml mouse immunoglobulins (Dako) as a control. ELISA for IL-4 and IL-15 were performed by SearchLight Multiplex Sample Testing by Endogen (Perbio, Aalst, Belgium). ELISA for IL-13 was performed with IL-13 ELISA kit from Biosource. All ELISA measurements were performed after concentration of the conditioned media using Amicon Ultra columns (Millipore). Recombinant IL-13 and IL-4 were purchased from Sigma.

Reverse transcriptase-polymerase chain reaction

RNA was extracted using Trizol Reagent (Invitrogen). Reverse transcription and PCR were performed according to the manufacturer's protocol (Invitrogen and ABGene, respectively) using the following primers: GAPDH-F 5′-GAT GAC AAG CTT CCC GTT CTC AGC C-3′, GADPH-R 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′, IL-4-F 5′-TGT TCT TGG AGG CAG CAA AGA-5′ IL-4-R 5′-TGT CCA CGG ACA CAA GTG CGA-3′, IL-13-F 5′-GCG CTT TTG TTG ACC ACG GTC ATT-3′, IL-13-R 5′-TTG AAC TGT CCC TCG CGA-5′, IL-15-F 5′-TAA AAC AGA AGC CAA CTG-3′, IL-15-R 5′-CAA GAA GTG TTG ATG AAC AT-3′. Thirty-five cycles (IL-4, IL-13 and IL-15) or 20 cycles (GAPDH) of amplification were performed, each consisting of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, followed by a final 10-minute extension at 72°C.

Whole cell extracts (WCE) and electrophoretic mobility shift assay (EMSA)

Whole cells were extracted as described previously (Neal and Clipstone, 2001). For EMSA, 20 μg of WCE were incubated with 0.2 μg/μl of poly(dI-dC), 100 mM EDTA, 8% glycerol, 25 mM Hepes, 5 mM $MgCl_2$, 34 mM KCl, 1 mM DTT and 2 ng of ^{32}P-labeled oligonucleotide probe at 4°C for 30 minutes. The resulting protein-DNA complexes were resolved by electrophoresis on a 5% native polyacrylamide gel, dried, and exposed to Phosphor Screen (Kodak). For supershift, 1 μl of sera directed against either panNFAT, NFATc1, NFATc2, NFATc3, NFATc4 (Lyakh et al., 1997) (from Nancy Rice, NCL-Frederick Cancer Research and Development Center, Frederick, MD) or myogenin (as a control) was added for 30 minutes at 4°C before incubation with the probe. Quantification was performed using the Bio-Rad MolecularImagerFX and the QuantityOne software. The double-stranded oligonucleotide probes used in EMSA were for NFAT, 5′-TG**T GGA AA**A TCC AGT G-3′ from the human IL-13 promoter (Dolganov et al., 1996) and the mutated probe TG**T GGT CA**A TCC AGT G, and for Foxo, 5′-CTA GAT G**GT AAA CAA** CTG TGA CTA GTA GAA CAC GG-3′ and the mutated probe 5′-CTA GAT G**GT CGG TGA** CTG TGA CTA GTA GAA CAC GG-3′ (Zhang et al., 2002).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde 0.1% Triton X-100. After blocking in 5% bovine serum, cells were incubated with Desmin antibody (Dako) or sera specific for NFAT isoforms (see EMSA) for 1 hour at room temperature at a dilution of 1:50. Secondary antibodies used were Alexa 488 (Molecular Probes) or Cy3 (Jackson ImmunoResearch). Nuclei were counterstained with Hoechst 33258 (Sigma).

Calcineurin phosphatase activity

Phosphatase activity was measured by using the calcineurin assay kit (Biomol, Plymouth Meeting, PA, USA) according to the manufacturer's instructions, as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide) in the presence or absence of EGTA. The amount of phosphate released was determined photometrically using the Biomol Green reagent.

Northern blot analysis

20 μg of RNA were separated by electrophoresis through a 1% agarose-MOPS gel containing 0.66 M formaldehyde and transferred onto a Biodyne B membrane (Pall Corp., East Hills, NY). Atrogin-1 cDNA (from A. Goldberg, Harvard Medical School, Boston, MA) was labelled with [^{32}P]dCTP by random priming and used as probe. The membranes were hybridized overnight at 68°C in 1× SSPE, 2× Denhart's, 10% dextran sulphate, 2% SDS and 100 μg/ml ssDNA and washed in 0.1× SSC, 0.5% SDS. Membranes were exposed to a PhosphorScreen (Kodak; for quantification, see EMSA). Expression of atrogin-1 was normalized to 18S rRNA signal as described previously (Furling et al., 2001).

Statistics

Results are expressed as the mean ± s.e.m. of at least three separate experiments. Significance was tested by Student's unpaired *t*-test or ANOVA-1, and a P value <0.05 was considered as significant (NS non significant, *P<0.05, **P<0.01 and ***P<0.001).

The authors would like to thank N. Rosenthal and J. Dhawan for fruitful discussions, Alfred L. Goldberg for the atrogin-1 cDNA and Nancy Rice and Mimi Ernst for the NFAT antibodies. This work was

supported by the MYORES Network of Excellence (contract 511978) from the European Commission 6th Framework Programme, the CPLD (Conseil de Prévention et de Lutte contre le Dopage), the CNRS, the INSERM, the University Pierre and Marie Curie Paris 6 and the AFM (Association Française contre les Myopathies). V. J. was supported by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche (University Denis Diderot) and by the FRM (Fondation pour la Recherche Médicale).

References

Abbott, K. L., Friday, B. B., Thaloor, D., Murphy, T. J. and Pavlath, G. K. (1998). Activation and cellular localization of the cyclosporine A-sensitive transcription factor NF-AT in skeletal muscle cells. *Mol. Biol. Cell* **9**, 2905-2916.

Allen, D. L., Monke, S. R., Talmadge, R. J., Roy, R. R. and Edgerton, V. R. (1995). Plasticity of myonuclear number in hypertrophied and atrophied mammalian skeletal muscle fibers. *J. Appl. Physiol.* **78**, 1969-1976.

Baroffio, A., Bochaton-Piallat, M. L., Gabbiani, G. and Bader, C. R. (1995). Heterogeneity in the progeny of single human muscle satellite cells. *Differentiation* **59**, 259-268.

Barton, E. R. (2006). Viral expression of insulin-like growth factor-I isoforms promotes different responses in skeletal muscle. *J. Appl. Physiol.* **100**, 1778-1784.

Barton, E. R., Morris, L., Musaro, A., Rosenthal, N. and Sweeney, H. L. (2002). Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice. *J. Cell Biol.* **157**, 137-148.

Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N. and Sweeney, H. L. (1998). Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. *Proc. Natl. Acad. Sci. USA* **95**, 15603-15607.

Barton-Davis, E. R., Shoturma, D. I. and Sweeney, H. L. (1999). Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. *Acta Physiol. Scand.* **167**, 301-305.

Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P. and Crabtree, G. R. (1997). Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-1934.

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K. et al. (2001a). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-1708.

Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J. et al. (2001b). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.* **3**, 1014-1019.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-868.

Callard, R. E., Matthews, D. J. and Hibbert, L. (1996). IL-4 and IL-13 receptors: are they one and the same? *Immunol. Today* **17**, 108-110.

Carlson, C. J., Fan, Z., Gordon, S. E. and Booth, F. W. (2001). Time course of the MAPK and PI3-kinase response within 24 h of skeletal muscle overload. *J. Appl. Physiol.* **91**, 2079-2087.

Chakravarthy, M. V., Abraha, T. W., Schwartz, R. J., Fiorotto, M. L. and Booth, F. W. (2000). Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3′-kinase/Akt signaling pathway. *J. Biol. Chem.* **275**, 35942-35952.

Childs, T. E., Spangenburg, E. E., Vyas, D. R. and Booth, F. W. (2003). Temporal alterations in protein signaling cascades during recovery from muscle atrophy. *Am. J. Physiol. Cell Physiol.* **285**, C391-C398.

Coleman, M. E., DeMayo, F., Yin, K. C., Lee, H. M., Geske, R., Montgomery, C. and Schwartz, R. J. (1995). Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. *J. Biol. Chem.* **270**, 12109-12116.

Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J. and Florini, J. R. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J. Biol. Chem.* **272**, 6653-6662.

DeFife, K. M., Jenney, C. R., McNally, A. K., Colton, E. and Anderson, J. M. (1997). Interleukin-13 induces human monocyte/macrophage fusion and macrophage mannose receptor expression. *J. Immunol.* **158**, 3385-3390.

Dolganov, G., Bort, S., Lovett, M., Burr, J., Schubert, L., Short, D., McGurn, M., Gibson, C. and Lewis, D. B. (1996). Coexpression of the interleukin-13 and interleukin-4 genes correlates with their physical linkage in the cytokine gene cluster on human chromosome 5q23-31. *Blood* **87**, 3316-3326.

Edom, F., Mouly, V., Barbet, J. P., Fiszman, M. Y. and Butler-Browne, G. S. (1994). Clones of human satellite cells can express in vitro both fast and slow myosin heavy chains. *Dev. Biol.* **164**, 219-229.

Florini, J. R., Ewton, D. Z. and Coolican, S. A. (1996). Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr. Rev.* **17**, 481-517.

Foulstone, E. J., Huser, C., Crown, A. L., Holly, J. M. and Stewart, C. E. (2004). Differential signalling mechanisms predisposing primary human skeletal muscle cells to altered proliferation and differentiation: roles of IGF-I and TNFalpha. *Exp. Cell Res.* **294**, 223-235.

Furling, D., Lemieux, D., Taneja, K. and Puymirat, J. (2001). Decreased levels of myotonic dystrophy protein kinase (DMPK) and delayed differentiation in human myotonic dystrophy myoblasts. *Neuromuscul. Disord.* **11**, 728-735.

Furmanczyk, P. S. and Quinn, L. S. (2003). Interleukin-15 increases myosin accretion in human skeletal myogenic cultures. *Cell Biol. Int.* **27**, 845-851.

Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A. and Goldberg, A. L. (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. USA* **98**, 14440-14445.

Graef, I. A., Chen, F. and Crabtree, G. R. (2001). NFAT signaling in vertebrate development. *Curr. Opin. Genet. Dev.* **11**, 505-512.

Haddad, F. and Adams, G. R. (2003). Inhibition of MAP/ERK kinase prevents IGF-I induced hypertrophy in rat muscles. *J. Appl. Physiol.* **96**, 203-210.

Halevy, O. and Cantley, L. C. (2004). Differential regulation of the phosphoinositide 3-kinase and MAP kinase pathways by hepatocyte growth factor vs. insulin-like growth factor-I in myogenic cells. *Exp. Cell Res.* **297**, 224-234.

Harwood, A. J. (2001). Regulation of GSK-3: a cellular multiprocessor. *Cell* **105**, 821-824.

Horsley, V., Friday, B. B., Matteson, S., Kegley, K. M., Gephart, J. and Pavlath, G. K. (2001). Regulation of the growth of multinucleated muscle cells by an NFATC2-dependent pathway. *J. Cell Biol.* **153**, 329-338.

Horsley, V., Jansen, K. M., Mills, S. T. and Pavlath, G. K. (2003). IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. *Cell* **113**, 483-494.

Jacquemin, V., Furling, D., Bigot, A., Butler-Browne, G. S. and Mouly, V. (2004). IGF-1 induces human myotube hypertrophy by increasing cell recruitment. *Exp. Cell Res.* **299**, 148-158.

Kaliman, P., Vinals, F., Testar, X., Palacin, M. and Zorzano, A. (1996). Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. *J. Biol. Chem.* **271**, 19146-19151.

Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N. J. and Fernandez, A. (1998). The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J. Cell Biol.* **142**, 1447-1459.

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.

Lafreniere, J. F., Mills, P., Bouchentouf, M. and Tremblay, J. P. (2006). Interleukin-4 improves the migration of human myogenic precursor cells in vitro and in vivo. *Exp. Cell Res.* **312**, 1127-1141.

Latres, E., Amini, A. R., Amini, A. A., Griffiths, J., Martin, F. J., Wei, Y., Lin, H. C., Yancopoulos, G. D. and Glass, D. J. (2005). Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. *J. Biol. Chem.* **280**, 2737-2744.

Lawlor, M. A. and Rotwein, P. (2000). Insulin-like growth factor-mediated muscle cell survival: central roles for Akt and cyclin-dependent kinase inhibitor p21. *Mol. Cell. Biol.* **20**, 8983-8995.

Li, Y. P., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D. L. and Reid, M. B. (2005). TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J.* **19**, 362-370.

Lindon, C., Montarras, D. and Pinset, C. (1998). Cell cycle-regulated expression of the muscle determination factor Myf5 in proliferating myoblasts. *J. Cell Biol.* **140**, 111-118.

Lluis, F., Perdiguero, E., Nebreda, A. R. and Munoz-Canoves, P. (2006). Regulation of skeletal muscle gene expression by p38 MAP kinases. *Trends Cell Biol.* **16**, 36-44.

Lyakh, L., Ghosh, P. and Rice, N. R. (1997). Expression of NFAT-family proteins in normal human T cells. *Mol. Cell. Biol.* **17**, 2475-2484.

Minty, A., Chalon, P., Derocq, J. M., Dumont, X., Guillemot, J. C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B. et al. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* **362**, 248-250.

Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. and Thomas, G. (1999). Drosophila S6 kinase: a regulator of cell size. *Science* **285**, 2126-2129.

Morris, R. T., Spangenburg, E. E. and Booth, F. W. (2004). Responsiveness of cell signaling pathways during the failed 15-day regrowth of aged skeletal muscle. *J. Appl. Physiol.* **96**, 398-404.

Musaro, A. and Rosenthal, N. (1999). Maturation of the myogenic program is induced by postmitotic expression of insulin-like growth factor I. *Mol. Cell. Biol.* **19**, 3115-3124.

Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N. and Rosenthal, N. (1999). IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* **400**, 581-585.

Musaro, A., McCullagh, K., Paul, A., Houghton, L., Dobrowolny, G., Molinaro, M., Barton, E. R., Sweeney, H. L. and Rosenthal, N. (2001). Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. *Nat. Genet.* **27**, 195-200.

Nader, G. A. and Esser, K. A. (2001). Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J. Appl. Physiol.* **90**, 1936-1942.

Neal, J. W. and Clipstone, N. A. (2001). Glycogen synthase kinase-3 inhibits the DNA binding activity of NFATc. *J. Biol. Chem.* **276**, 3666-3673.

Ohanna, M., Sobering, A. K., Lapointe, T., Lorenzo, L., Praud, C., Petroulakis, E., Sonenberg, N., Kelly, P. A., Sotiropoulos, A. and Pende, M. (2005). Atrophy of S6K1(–/–) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control. *Nat. Cell Biol.* **7**, 286-294.

Park, I. H., Erbay, E., Nuzzi, P. and Chen, J. (2005). Skeletal myocyte hypertrophy requires mTOR kinase activity and S6K1. *Exp. Cell Res.* **309**, 211-219.

Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W. and Elledge, S. J. (1995). p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. *Science* **267**, 1024-1027.

Quinn, L. S., Haugk, K. L. and Grabstein, K. H. (1995). Interleukin-15: a novel anabolic cytokine for skeletal muscle. *Endocrinology* **136**, 3669-3672.

Quinn, L. S., Anderson, B. G., Drivdahl, R. H., Alvarez, B. and Argiles, J. M. (2002). Overexpression of interleukin-15 induces skeletal muscle hypertrophy in vitro: implications for treatment of muscle wasting disorders. *Exp. Cell Res.* **280**, 55-63.

Rochat, A., Fernandez, A., Vandromme, M., Moles, J. P., Bouschet, T., Carnac, G. and Lamb, N. J. (2004). Insulin and wnt1 pathways cooperate to induce reserve cell activation in differentiation and myotube hypertrophy. *Mol. Biol. Cell* **15**, 4544-4555.

Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D. and Glass, D. J. (1999). Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* **286**, 1738-1741.

Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., Yancopoulos, G. D. and Glass, D. J. (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* **3**, 1009-1013.

Rosenblatt, J. D. and Parry, D. J. (1992). Gamma irradiation prevents compensatory hypertrophy of overloaded mouse extensor digitorum longus muscle. *J. Appl. Physiol.* **73**, 2538-2543.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S. H. and Goldberg, A. L. (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**, 399-412.

Sanna, B., Bueno, O. F., Dai, Y. S., Wilkins, B. J. and Molkentin, J. D. (2005). Direct and indirect interactions between calcineurin-NFAT and MEK1-extracellular signal-regulated kinase 1/2 signaling pathways regulate cardiac gene expression and cellular growth. *Mol. Cell. Biol.* **25**, 865-878.

Semsarian, C., Sutrave, P., Richmond, D. R. and Graham, R. M. (1999a). Insulin-like growth factor (IGF-I) induces myotube hypertrophy associated with an increase in anaerobic glycolysis in a clonal skeletal-muscle cell model. *Biochem. J.* **339**, 443-451.

Semsarian, C., Wu, M. J., Ju, Y. K., Marciniec, T., Yeoh, T., Allen, D. G., Harvey, R. P. and Graham, R. M. (1999b). Skeletal muscle hypertrophy is mediated by a Ca2+-dependent calcineurin signalling pathway. *Nature* **400**, 576-581.

Smirnov, D. V., Smirnova, M. G., Korobko, V. G. and Frolova, E. I. (1995). Tandem arrangement of human genes for interleukin-4 and interleukin-13: resemblance in their organization. *Gene* **155**, 277-281.

Stitt, T. N., Drujan, D., Clarke, B. A., Panaro, F., Timofeyva, Y., Kline, W. O., Gonzalez, M., Yancopoulos, G. D. and Glass, D. J. (2004). The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell* **14**, 395-403.

Tiffin, N., Adi, S., Stokoe, D., Wu, N. Y. and Rosenthal, S. M. (2004). Akt phosphorylation is not sufficient for insulin-like growth factor-stimulated myogenin expression but must be accompanied by down-regulation of mitogen-activated protein kinase/extracellular signal-regulated kinase phosphorylation. *Endocrinology* **145**, 4991-4996.

Vandenburgh, H. H., Karlisch, P., Shansky, J. and Feldstein, R. (1991). Insulin and IGF-I induce pronounced hypertrophy of skeletal myofibers in tissue culture. *Am. J. Physiol. Cell Physiol.* **260**, C475-C484.

Vyas, D. R., Spangenburg, E. E., Abraha, T. W., Childs, T. E. and Booth, F. W. (2002). GSK-3beta negatively regulates skeletal myotube hypertrophy. *Am. J. Physiol. Cell Physiol.* **283**, C545-C551.

Wang, L., Gout, I. and Proud, C. G. (2001). Cross-talk between the ERK and p70 S6 kinase (S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. *J. Biol. Chem.* **276**, 32670-32677.

Welsh, G. I., Miller, C. M., Loughlin, A. J., Price, N. T. and Proud, C. G. (1998). Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett.* **421**, 125-130.

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.

Yoshida, N., Yoshida, S., Koishi, K., Masuda, K. and Nabeshima, Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J. Cell Sci.* **111**, 769-779.

Zdanowicz, M. M., Moyse, J., Wingertzahn, M. A., O'Connor, M., Teichberg, S. and Slonim, A. E. (1995). Effect of insulin-like growth factor I in murine muscular dystrophy. *Endocrinology* **136**, 4880-4886.

Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W. and Elledge, S. J. (1999). p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev.* **13**, 213-224.

Zhang, X., Gan, L., Pan, H., Guo, S., He, X., Olson, S. T., Mesecar, A., Adam, S. and Unterman, T. G. (2002). Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J. Biol. Chem.* **277**, 45276-45284.

Journal of Cell Science