Focal adhesion kinase is required for IGF-1-mediated growth of skeletal muscle cells via a TSC2-mTOR-S6K1-associated pathway

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ABSTRACT

Focal adhesion kinase (FAK) is an attachment complex protein associated with the regulation of muscle mass, through as of yet unclear mechanisms. We tested whether FAK is functionally important for muscle hypertrophy, with the hypothesis that FAK knockdown (FAK-KD) would impede cell growth associated with a trophic stimulus. C2C12 skeletal muscle cells harbouring FAK targeted (FAK-KD) or scrambled (SCR) shRNA were created using lentiviral transfection techniques. Both FAK-KD and SCR myotubes were incubated for 24 h with IGF-1 (10 ng.ml⁻¹) and additional SCR cells (± IGF-1) were incubated with a FAK-kinase inhibitor before assay of cell growth. Muscle protein synthesis (MPS) and putative FAK signalling mechanisms (immunoblotting and co-immunoprecipitation) were assessed. IGF-1-induced increases in myotube width (+41±7% vs. non-IGF-1-treated) and total protein (+44±6%) were, after 24 h, attenuated in FAK-KD cells while MPS was suppressed in FAK-KD vs. SCR after 4 h. These blunted responses were associated with attenuated IGF-1-induced FAK Tyr397 phosphorylation, and markedly suppressed phosphorylation of tuberous sclerosis complex 2 (TSC2) and critical downstream mTOR signalling (ribosomal S6 kinase, eIF4F assembly) in FAK shRNA cells (all P<0.05 vs. IGF-1-treated SCR cells). However, binding of FAK to TSC2 or its phosphatase Shp-2 was not affected by IGF-1 or cell phenotype. Finally, FAK-KD mediated suppression of cell growth was recapitulated by direct inhibition of FAK kinase activity in SCR cells. We conclude that FAK is required for IGF-1-induced muscle hypertrophy, signalling through a TSC2-mTOR-S6K1-dependent pathway via means requiring the kinase activity of FAK, but not altered FAK-TSC2 or FAK-Shp-2 binding.

Key words: focal adhesion kinase; hypertrophy; IGF-1; S6K1; skeletal muscle
Attachment complexes, or focal adhesion complexes, are macromolecular structures situated in the sarcolemma of muscle fibres, that link the extracellular matrix (ECM) to the cytoplasmic cytoskeleton, and consist of a variety of ECM receptors/integrins and intracellular cytoskeletal and signaling molecules (7, 30). Interactions of ECM proteins with integrin receptors stimulate intracellular signaling pathways important in cell growth and migration (51), and in adult skeletal muscle, focal adhesion complexes play a crucial part in the transmission of lateral forces during contraction (41). Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to focal adhesion complexes and represents a key component of integrin-mediated signaling (9). Engagement of integrin receptors induces phosphorylation of FAK at Tyr397, which correlates with its activation (8), and a growing body of evidence has associated FAK activation with the hypertrophic response to mechanical stress in skeletal muscle. Indeed, expression patterns of FAK have been reported to be load-dependent, i.e., phosphorylation of FAK was lowered following hind-limb suspension in rodents (28) and immobilization in humans (13), and increased in models of chronic overload (18, 28) and following hypertrophic resistance exercise training in humans (58). Finally, local overexpression of FAK (pCMV-FAK plasmid electroporation) \textit{in vivo} in rodents was shown to stimulate muscle hypertrophy (16) and induce S6K1 phosphorylation (37), suggesting FAK is a key component of muscle hypertrophy.

FAK is not only activated by integrin engagement, but also through stimulation by hormones and growth factors, including insulin and insulin-like growth factor-1 (IGF-1) (3, 10, 25). In fibroblasts, it was demonstrated that FAK physically interacted with the IGF-1 receptor, which was proposed to be important for the stabilization and phosphorylation of the IGF-1 receptor (2). This stabilization impacted on downstream AKT-extracellular signal-related kinase (ERK) signaling, indicating a direct association between FAK and IGF-1 receptor signaling. The phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) signaling pathway is a critical regulator of IGF-1-mediated
skeletal muscle hypertrophy (5, 48), and cardiac hypertrophy due to FAK overexpression in mouse hearts was associated with stimulation of PI3K-AKT signaling pathways (11). FAK was also reported to modulate the activity of this pathway through increased phosphorylation of S6K1 (37), which is a potential regulator of in vivo muscle protein synthesis (MPS) downstream of mTOR (47, 56). In a recent study (40), activation of mTOR signaling and subsequent hypertrophy in response to mechanical stress (stretch) in rat neonatal cardiomyocytes was reported to occur via activation of FAK, which was itself subject to negative regulation by the protein tyrosine phosphatase SH2 domain-containing protein tyrosine phosphatase (Shp-2). Thus, these data implicate a central regulatory role for FAK in both mechanical activity- and growth factor-induced hypertrophy in skeletal muscle; however, the degree of reliance on intact FAK signaling for hypertrophy-related signaling in skeletal muscle remains to be defined.

In C2C12 myotubes, IGF-1 incubation is a classical model for studying muscle hypertrophy (i.e., increases in myotube diameter) through activation of the canonical PI3K-AKT-mTOR signaling pathway (39, 48). Herein, we used this approach to assess whether FAK is required for myocyte growth and to define some of the mechanisms involved; we hypothesized that reducing FAK expression by short hairpin (sh)RNA would restrict growth in response to IGF-1. More specifically, we sought to explore whether the role of FAK in IGF-1-mediated growth of C2C12 myotubes was due to a structural property of FAK in the adhesion complex, or through its catalytic role as a kinase, using a reportedly specific inhibitor of FAK that targets its phosphorylation at Tyr397 (26).
MATERIALS AND METHODS

Cell Culture

Murine C2C12 myoblasts (ECACC, Salisbury, UK; passage number 6-9) were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Paisley, UK) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; Lonza BioWhittaker®, Verviers, Belgium), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 mg/ml; all from Sigma-Aldrich, Poole, UK) at 37°C and 5% CO₂ atmosphere. Proliferating myoblasts were seeded onto six-well plates (Nunclon™ Delta; Thermo Scientific) and after 2-4 days (at ~95% confluency), the media was switched to differentiation medium consisting of DMEM containing 2% (v/v) horse serum (Sigma-Aldrich) as well as the above mixture of antimycotics and antibiotics, to induce differentiation of the myoblasts into multinucleated myotubes. Differentiation medium was changed every 24 h and experiments were performed on day 4-5 post induction of differentiation. Experiments where signaling was measured were carried out more than 24 h following a media change.

shRNA Interference

The lentiviral plasmid used (pLKO.1-mFAK) was obtained from OpenBiosystems (Huntsville, AL, USA; Clone ID: RMM4534-NM_001130409) and targeted the mouse sequence 5’-CAA CCT TAA TAG AGA AGA AA-3’; the scrambled shRNA (SCR) was used as a negative control as previously reported (35) with a hairpin sequence: CCT AAG GTT AAG TCG CCC TCG CTC TAG CGA GGG CGA CTT AAC CTT AGG (Addgene plasmid 1864, Cambridge, MA, USA). The plasmids were transformed in DH5α cells and isolated. The actual DNA sequence was confirmed at the Pennsylvania State University College of Medicine DNA sequencing core facility. Packaging plasmids psPAX2 and envelope protein plasmid pMD2.G were a gift from Trono Lab (Addgene plasmids 12260 and 12259 respectively). HEK293FT cells (Invitrogen, Carlsbad, CA, USA) were grown in DMEM; 80–85% confluent plates were rinsed once with Opti-MEM (Invitrogen, Carlsbad, CA, USA) and then incubated with Opti-MEM for 4 h before transfections. psPAX2 and pMD2.G along with either scramble or pLKO.1-mFAK were added
after mixing with Lipofectamine 2000 as per the manufacturer’s instructions (Invitrogen). Opti-MEM media was changed after overnight incubation with DMEM containing 10% FBS without antibiotics to allow cells to take up the plasmids and recover. Culture media were collected at 36 and 72 h post-transfection for viral particles. Viral particles present in the supernatant were harvested after a 15-min spin at 1,500 g to remove cellular debris. The supernatant was further filtered using a 0.45-µm syringe filter. Supernatant-containing virus was stored at −80°C for long-term storage. C2C12 cells at 60% confluence were infected twice overnight with 3 ml of viral supernatant containing 8 µg.ml⁻¹ polybrene in serum-free–antibiotic-free DMEM. Fresh DMEM media containing 10% FBS, antibiotics and 2 µg.ml⁻¹ puromycin (Sigma, St. Louis, MO, USA) were added the next day. Cells were selected for 2-5 generations in puromycin and no selection was used in the generation where cells were to be experimentally used. Cells that survived under puromycin selection were either harvested (as stable cells) and stored or used as myotubes following differentiation. For FAK-KD, 3 independent shRNAs targeting FAK mRNA were designed and the one that gave the highest degree of knockdown was selected to proceed with experimentation.

**IGF-1 and FAK 14 inhibitor incubations**

Experiments using IGF-1 were carried out on day 4-5 post induction of differentiation. For acute experiments, the media was changed 24 h prior to treatments, after which cells were incubated in the presence of 10 ng.ml⁻¹ long R3 IGF-1 (Sigma-Aldrich) for 2 h, 4 h and 8 h. For chronic IGF-1 treatment, the media was changed immediately before incubating cells in 10 ng.ml⁻¹ of IGF-1 for 24 h. Long R3 IGF-1 was chosen due to its low affinity for IGF-1 binding proteins, and used at a dose previously shown to induce hypertrophy in C2C12 cells (38). For inhibitor experiments, cells were incubated for 30 min prior to IGF-1 administration with 1 µmol.l⁻¹ of FAK 14 inhibitor (F14; Tocris Bioscience, Bristol, UK). The dose of F14 was chosen based upon initial experiments demonstrating that 1 µmol.l⁻¹ of F14 effectively suppressed basal FAK Tyr397 phosphorylation after 2 h of treatment (data not shown). At the end of experiments, the media was retained and cells were washed twice in
ice-cold phosphate-buffered saline, before being harvested in sodium hydroxide for protein/DNA measurements or extraction buffer for immunoblotting (see below).

**Myotube Diameter**

Following 24 h of IGF-1 treatment, light microscope images were taken and myotube diameter was assessed using ImageJ software (NIH, Frederick, MD, USA), measuring 10 myotubes per field and using 5 random fields. Data were expressed relative to the 0 h time point in the SCR control group.

**Protein/DNA Measurements**

Total alkaline-soluble protein and DNA were measured in cells following 24 h of IGF-1 treatment. Cells were harvested in 0.3 mol.l\(^{-1}\) NaOH and incubated at 37°C for 30 min, before removing an aliquot for measurement of total protein using the Bradford assay. The remaining sample was incubated at 4°C for 10 min in the presence of 1 mol.l\(^{-1}\) perchloric acid (PCA), before centrifuging samples at 3000 \(g\) for 10 min. The resultant pellet was washed with 0.2 mol.l\(^{-1}\) PCA before being incubated at 70°C for 1 h in 2 mol.l\(^{-1}\) PCA. After further centrifugation of samples at 5,000 \(g\) for 10 min, the DNA-containing supernatant was collected, and DNA was quantified by spectrophotometric measurement of the absorbance at 260 and 280 nm (NanoVue; GE Healthcare, Little Chalfont, UK).

**MPS measurements**

For acute measures of MPS, the surface sensing of translation (SUnSET) technique was used (27, 52). This method involves incubation of cells with the antibiotic puromycin (a tyrosyl-tRNA analog) and subsequent immunoblotting using anti-puromycin antibodies to assess levels of incorporation of puromycin into newly synthesized polypeptide chains. Puromycin (1 µmol.l\(^{-1}\)) was added to cells in the last 30 minutes of IGF-1 treatment, and cells were harvested in extraction buffer for measurement of puromycin-labelled peptides by immunoblotting (see below), using mouse monoclonal puromycin antibody (12D10).
Chronic (24h) measures of MPS were performed using the stable isotope tracer deuterium oxide (D$_2$O); a method specifically adapted for assessing longer-term rates of MPS (22). Media was changed immediately prior to incubating cells either with or without 10 ng.ml$^{-1}$ of IGF-1 for 24h, and replaced with fresh media enriched with 5% D2O. Following incubations, 1 ml of media was sampled from each well and cells were harvested in 1 ml of PCA (1 mol.l$^{-1}$). Following homogenization, samples were centrifuged (10,000 rpm, 10 min). The mixed muscle pellet was washed twice in 70% ethanol and hydrolysed overnight at 110°C in 1 ml of HCl (0.1 mol.l$^{-1}$) and 1 ml ³H dowex resin. Hydrolysed AA were eluted into 2 mol.l$^{-1}$ NH$_4$OH and evaporated to dryness. Deuterium labelling of protein-bound alanine was determined using GC-MS following conversion to its tBDMS derivative and single ion monitoring (SIM) of m/z 260 and 261. Media sampled from each well was analysed for D$_2$O enrichment using a modification of the acetone exchange method (59). Briefly, 2 µl of 10 N NaOH was added to 100 µl of media, and following a 15 s vortex mix, 1 µl of acetone was added. This was incubated for 24 h to allow full hydrogen/deuterium exchange. The acetone was extracted into 200 µl of n-heptane, the n-heptane layer was transferred to an autosampler vial, and 0.5 µl was injected into the GC-MS. D$_2$O enrichment was determined via SIM of m/z 58 and 59 with reference to a standard curve of known D$_2$O enrichments. Fractional synthesis rate (FSR) was calculated using the following equation:

$$FSR(\% / h) = \frac{\left[(MPE_{Ala})/\left[3.7 \times (MPE_{MW}) \times t\right]\right]}{100}$$

(22)

Where MPE$_{Ala}$ represents protein bound alanine enrichment, MPE$_{MW}$ represents media water enrichment and t represents time in hours. From this the absolute rate of protein synthesis (ASR) was calculated by:

$$ASR(\mu g \text{ protein per 24h}) = \left(\frac{FSR}{100}\right) \times \text{total protein per well} \times t$$

**Immunoblotting**

Cells were homogenized in extraction buffer (50 mM Tris-HCl, pH7.5, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaF and complete protease inhibitor tablet (Roche, West Sussex,
UK)) by repeated pipetting using gel-loading tips and were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was used for immunoblotting. Protein was quantified by Bradford assays, and samples were diluted and boiled in 1x laemmli loading buffer to the same concentration. Samples (5 µg of total protein) were loaded onto Criterion XT Bis-Tris 4-12% SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200 V for 1 h. Samples were transferred to PVDF membranes for 45 min at 100 V. Membranes were subsequently blocked in 2.5% low-fat milk (diluted in Tris-buffered saline (TBS) and 0.1% Tween-20) for 1 h at room temperature, then incubated overnight at 4°C in the presence of the following primary antibodies diluted 1:2000: FAK Tyr397 (#8556), total FAK (#3285), AKT Ser473 (#4058) and Thr308 (#4056), ERK 1/2 Thr202/Tyr204 (#4377), proline-rich AKT substrate of 40 kDa (PRAS40) Thr246 (#2640), tuberous sclerosis complex 2 (TSC2) Thr1462 (#3611), S6K1 Thr389 (#9205) and Thr421/Ser424 (#9204), eukaryotic initiation factor (eIF) 4E binding protein 1 (4EBP1) Thr37/46 (#2855), eukaryotic elongation factor 2 (eEF2) Thr56 (#2331), and eIF4G Ser1108 (#2441) (all from Cell Signaling Technology, Beverly, MA, USA). The following day, membranes were washed 3 x 5 min with TBS-T and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (New England Biolabs, Hitchin, UK). Membranes were washed 3 x 5 min in TBS-T and incubated for 5 min with enhanced chemiluminescence reagent (Millipore, Watford, UK) before being visualized using a Chemidoc XRS system (Bio-Rad, Hemel Hempstead, UK). Bands were quantified by measurement of peak density and normalization against Coomassie Brilliant Blue staining of the membrane (57). Since our principal objective was to compare the effects of FAK-KD on hypertrophic responses to IGF-1 incubation, the full time-course of immunoblotting data for each cell phenotype were run on separate gels and quantified relative to the 0 h time point in corresponding SCR or FAK-KD groups. Nonetheless, additional experiments indicated that there were no significant differences in basal phosphorylation of selected targets (S6K1 Thr389, AKT Ser473 and 4EBP1 Thr37/46) between FAK-KD and scrambled shRNA cells when samples from each cell phenotype were run on the same gel (data not shown).
m⁷GTP-Sepharose eIF4E affinity purification

m⁷GTP-Sepharose affinity purification was used to assess interactions between components of the eIF4F complex, namely associations between eIF4G and eIF4E and between 4EBP1 and eIF4E. Protein lysate (300 μg) was incubated with 30 μl of m⁷GTP-Sepharose 4B bead slurry (GE Healthcare, Little Chalfont, UK) at 4°C with continuous rotation overnight. Beads were subsequently collected by centrifugation (1,000 g, 2 min) and washed twice with buffer (50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM EGTA, 0.5 mM NaVO₄, 50 mM NaF and complete protease inhibitor tablet (Roche, West Sussex, UK)). Bound proteins were eluted by boiling samples at 100°C for 7 min in the presence of laemmlI buffer. Samples were centrifuged to recover protein and were loaded onto 4-12% polyacrylamide gels for immunoblotting as described above, with measurement of total levels of eIF4E (sc-13963), 4EBP1 (sc-6936) and eIF4G (sc-11373) (all from Santa Cruz biotechnology).

Co-immunoprecipitation

Protein lysate (100 μg) from scrambled shRNA cells treated with IGF-1 for 2 and 4 h were used for immunoprecipitation with FAK antibody and Protein G agarose according to the manufacturers protocol (Sigma-Aldrich). Immunoprecipitated samples were used for immunoblotting (see above) with antibodies against Shp-2 (#3752) and TSC2 (#3612) (Cell Signaling Technology).

Statistical analysis

Results were analysed by unpaired Student’s t-test for two group comparisons, one-way analysis of variance (ANOVA) with Tukey’s post-hoc testing for multiple group comparisons, and two-way repeated measures ANOVA with Bonferroni post-tests for multiple groups with multiple time points, with statistical significance set at P<0.05 (GraphPad Prism version 5.0, GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± SEM and represent 6 replicates (wells) per experiment.
RESULTS

Efficacy of lentiviral-mediated knockdown of FAK protein

As shown in Figure 1A, efficacy of lentiviral-mediated knockdown of FAK was confirmed at the protein level in myotubes on day 4 post induction of differentiation (-87 ± 1% vs. scrambled shRNA cells; \( P < 0.0001 \)). The average myotube diameter of FAK-KD cells was consistently lower than scrambled shRNA cells (-35 ± 9%; \( P < 0.05 \); Figure 1B & C).

Effect of 24 h IGF-1 treatment on myotube diameter and protein/DNA content in FAK vs. scrambled shRNA C2C12 cells

IGF-1 treatment for 24 h increased average myotube width (+41±7%; \( P < 0.01 \) vs. non-IGF-1 treated controls; Figure 1B & C), as well as total protein (+44±6%; \( P < 0.001 \); Figure 1C) and DNA content (+54±9%; \( P < 0.001 \); Figure 1E) per well in scrambled shRNA cells. IGF-1-mediated increases in myotube diameter, total protein, and DNA in FAK-KD cells (all \( NS \) versus non-IGF-1 treated controls) were significantly attenuated compared with scrambled shRNA cells (Figure 1C, D & E).

FAK and AKT-mTOR signaling after IGF-1 treatment in FAK versus scrambled shRNA C2C12 cells

Initial experiments indicated that baseline phosphorylation of measured targets was similar between FAK-KD and scrambled shRNA cells (data not shown). Phosphorylation of FAK at Tyr397 was significantly increased after 2 h (+30±5%) and 4 h (+28±6%) of IGF-1 treatment in scrambled shRNA cells, returning to basal levels by 8 h (Figure 2A). Predictably, FAK Tyr397 phosphorylation was not increased in FAK-KD cells following IGF-1 administration (Figure 2A). Total FAK protein expression was unaltered with IGF-1, except for at the 8 h time point, where total FAK was similarly reduced in FAK-KD and scrambled shRNA cells (-22±3%; Figure 2B). At all time points, IGF-1 significantly increased AKT Ser473 phosphorylation, with a peak increase at 2 h (+306±6%; Figure 2C). Increases in AKT Ser473 were similar in FAK knockdown cells, with the exception of the 2 h time point where the increase was somewhat attenuated (+212±7; Figure 2C). Similarly, IGF-1 increased AKT Thr308 phosphorylation.
phosphorylation at each time point in scrambled shRNA cells, with the largest increase observed at 2 h (+460±22%) and 8 h (+438±17%; Figure 2D). Increases in AKT Thr308 phosphorylation with IGF-1 at 8 h in FAK-KD cells were significantly blunted compared with control cells (+271 ± 27%), although still significantly elevated above non-IGF-1-treated controls.

Phosphorylation of ERK1/2 at Thr202/Tyr204 was induced following IGF-1 treatment similarly in both scrambled and FAK-KD cells (+40±5% at 2 h; Figure 2E). In scrambled shRNA cells, PRAS40 Thr246 phosphorylation was increased after 2 h (+209±15%) and 4 h (+172±10%) IGF-1 treatment. PRAS40 phosphorylation was increased in FAK-KD cells in response to IGF-1, however the increase at 2 h was significantly attenuated compared to the scrambled shRNA group (+135±7%; Figure 2F). At all time points, IGF-1 significantly increased phosphorylation of TSC2 at Thr1462 in scrambled shRNA cells (+384±17% at 2 h; Figure 3A), and this was markedly attenuated in FAK knockdown cells, with only a significant increase observed at 2 h (+189±12%). As shown in Figure 3B, in scrambled shRNA myotubes, S6K1 Thr389 phosphorylation was elevated at all time points with IGF-1, (+187±2% at 2 h; Figure 3B), but there was no change in S6K1 Thr389 in FAK knockdown cells. Similarly, IGF-1 increased S6K1 Thr421/Ser424 in scrambled shRNA cells (+265±16% at 2 h; Figure 3C), whereas this increase was significantly lower in FAK-KD cells. Phosphorylation of 4EBP1 at Thr37/46 was increased at all time points with IGF-1 (+60±3% at 2 h), whereas in FAK-KD cells there was a reduction in 4EBP1 phosphorylation with IGF-1 (Figure 3D). In both scrambled and FAK-KD cells, IGF-1 resulted in a significant reduction in eEF2 Thr56 phosphorylation (-23±2% at 2 h; Figure 3E), returning to control levels by 8 h. Finally, eIF4G Ser1108 phosphorylation was increased after 2 h IGF-1 treatment in both scrambled (+76±12%) and FAK (+60±3%) shRNA cells (Figure 3F), and levels remained elevated in both groups at subsequent time points.

Changes in translational regulators, MPS and binding of FAK to Shp-2 and TSC2 following IGF-1 treatment
Reduced binding of 4EBP1 to eIF4E was seen following IGF-1 treatment in scrambled shRNA cells (-474 ± 40% at 4 h; Figure 4A), and in FAK-KD cells, a reduction was observed but this was significantly less than in scrambled shRNA cells (-163 ± 49% at 4 h). IGF-1 increased binding of eIF4G to eIF4E in scrambled shRNA cells, with the largest increase detected at 4 h (+547 ± 28%; Figure 4B). Increased binding of eIF4G to eIF4E was observed in FAK-KD cells, but was significantly lower than in control cells (+194 ± 35% at 4 h). IGF-1-induced increases in protein synthesis over 24 h (+73 ± 20% in scrambled shRNA cells), as measured by changes in D2O-bound alanine, were suppressed in FAK shRNA cells (Figure 4C). Additionally, IGF-1 significantly increased MPS after 4 h in scrambled shRNA cells (+135 ± 6%), as measured by incorporation of puromycin into newly synthesized peptide chains, but this was attenuated in FAK-KD cells (Figure 4D & E). Although Shp-2 and TSC2 both co-immunoprecipitated with FAK, there were no significant changes in binding of Shp-2 or TSC2 to FAK following 2 or 4 h of IGF-1 treatment in scrambled shRNA cells (Figure 4F).

Changes in protein, DNA and AKT-mTOR signaling with inhibition of FAK-kinase activity following IGF-1 treatment

IGF-1-induced increases in total protein (+34±3%) and DNA (+23±4%) after 24 h were significantly attenuated when scrambled shRNA cells were incubated in the presence of F14, an inhibitor of FAK Tyr397 phosphorylation (Figure 5A & B). IGF-1 treatment for 2 h (a time point where signaling responses to IGF-1 were predominantly at their largest) induced an increase in FAK Tyr397 (+28±9%), and this was significantly suppressed with inhibition of FAK phosphorylation (Figure 5C). Total FAK protein expression was unchanged with IGF-1 or F14 (Figure 5D). IGF-1 induced increases in AKT Ser473 (+528±49%), S6K1 Thr389 (+110±9%) and 4EBP1 Thr37/46 (+88±7%), and these were significantly suppressed with inhibition of FAK phosphorylation (Figure 5E – G).
DISCUSSION

Integrins are cell surface receptors that interact with ECM molecules and mediate intracellular signal transduction through the formation of multi-protein complexes known as focal adhesion complexes, and FAK is thought to be a key player this process (34). Integrin-mediated signaling is a central mediator of mechanical load-induced hypertrophy, and there are several lines of evidence implicating activation of FAK in load-mediated induction of protein synthesis and hypertrophy in rodent skeletal muscle in vivo (16, 37). Moreover, FAK can also be modulated by non-integrin stimuli such as insulin and IGF-1 (3, 25) and may be involved in PI3K/AKT activation (2). However, no studies to date have elucidated the requirement or molecular mechanisms by which FAK mediates growth in skeletal muscle. In the present study, we demonstrated that shRNA-mediated stable knockdown of FAK in C2C12 cells was associated with a marked attenuation of the growth response associated with IGF-1, and this was associated with FAK-mediated stimulation of the TSC2-mTOR-pathway (Figure 6). Critically, this appears to be related to the catalytic action of FAK rather than to its structural properties.

Skeletal muscle unloading has been associated with reduced levels of FAK phosphorylation, both in rodent soleus muscle after 7 d tail suspension (28), and following leg immobilization in humans (13, 24). Furthermore, increased FAK phosphorylation and protein content has been observed after training in animal models (18, 28) and in humans (54), and has been associated with the activation of anabolic signaling pathways (37) and hypertrophy in muscle (16). In this study, we demonstrated that IGF-1-induced cell growth (as estimated by changes in myotube diameter and total protein; Figure 2) was markedly suppressed in C2C12 myotubes harbouring FAK-targeted shRNA. Furthermore, untreated FAK-KD myotubes were generally thinner than control cells (Figure 1), suggesting FAK is also required for normal cell growth. IGF-1 also significantly increased DNA content in scrambled shRNA cells, and this increase was consistently observed whether cells were treated with IGF-1 at the onset of differentiation, or when myotubes were fully formed (data not shown).
The observed increases in DNA content with IGF-1 treatment could potentially have been due to IGF-1 forcing remaining quiescent undifferentiated myoblasts into the cell cycle, highlighting that IGF-1 represents a potent growth factor on a population of quiescent myoblasts amongst differentiated myotubes. However, it has been previously shown (43) that treatment with DNA synthesis inhibitor arabinofuranosyl cytidine (Ara-C) did not affect the ability of IGF-1 to induce increases in myotube diameter in C2C12 cells, indicating that the hypertrophic effects of IGF-1 may have been independent of its mitogenic effects. Nevertheless, increases in total protein and DNA with IGF-1 appeared to be dependent upon FAK, suggesting governance over both post-mitotic and mitotic cell growth.

IGF-1-mediated myotube growth in vitro occurs via activation of anabolic signaling pathways, particularly the PI3K-AKT-mTOR pathway (5, 48). Recent studies suggested that the integrin-associated protein FAK could be an important component of the IGF-1-PI3K-AKT pathway in muscle (37, 40). In the current study, IGF-1 induced an acute, transient increase in FAK Tyr397 phosphorylation, which has been linked with its activation (8). Increases in AKT Ser473 and Thr308 phosphorylation with IGF-1 were similar in both scrambled and FAK shRNA cells, apart from at the 2 h time point for Ser473 (residue under regulation by mTORc2 (50)) and at 8 h for Thr308 (residue downstream of PI3K (1)), where there was a suppression in FAK knockdown cells. Whether the suppression of AKT in FAK-KD cells, at the time points observed in the present study, were important in the blunted anabolic response to IGF-1 remain to be established. However, there is evidence that whilst AKT Thr308 may be important for mTOR regulation, Ser473 phosphorylation might be more important in the regulation of Forkhead Box O (FOXO) transcription factors (29, 33). Thus the suppression in AKT Ser473 in FAK-KD cells after 2 h IGF-1 treatment is unlikely to have impacted on downstream mTOR signaling. Nevertheless, the suppressed phosphorylation of AKT Ser473 at 2 h paralleled changes in AKT substrate PRAS40 Thr246 phosphorylation in FAK-KD cells. Phosphorylation at this residue is thought to relieve PRAS40 inhibition of mTOR, which in non-stimulated conditions occurs through binding to raptor, preventing Ras homologue enriched in brain
(Rheb)-mediated activation of mTOR (49). It has previously been demonstrated that activation of mTORc1 can cause feedback inhibition of IRS-1, resulting in reduced AKT phosphorylation at Thr308 (54). This was not observed in the present study, although AKT phosphorylation was not measured beyond 8 h of IGF-1 treatment. Nonetheless, it is feasible that feedback inhibition from mTORC1 activation to proximal IGF-1 signaling is not active, at least during IGF-1-induced muscle growth. In contrast to the changes in AKT phosphorylation, increased ERK1/2 Thr202/Tyr204 phosphorylation in response to IGF-1 did not differ in FAK-KD and control shRNA cells. These data contrast with previous studies showing that ERK1/2 activation in cardiac myocytes paralleled activation of FAK in response to mechanical stress (15, 19), suggesting a role for ERK1/2 signaling in the hypertrophic effects of FAK in response to mechanical signals as opposed to growth factor stimulation, and/or in cardiac but not skeletal myocytes.

Following stimulation by growth factors, AKT inhibits the GTPase activating protein (GAP) activity of TSC2 through phosphorylation at Thr1462, leading to activation of the GTPase Rheb, a positive regulator of mTOR (21, 31, 60). In this study, IGF-1 stimulation of TSC2 Thr1462 was ablated in FAK knockdown cells, suggesting that the mechanism by which FAK mediated IGF-1-induced growth occurred in a TSC2-dependent manner. It is not clear whether FAK was directly responsible for the increased phosphorylation at this residue on TSC2, as it is thought to be downstream of AKT (as described above). There is evidence to suggest that FAK may positively regulate anabolic signaling pathways through direct interaction with TSC2, albeit in non-muscle cells (20), but whilst in the present study co-immunoprecipitation experiments revealed binding of FAK to TSC2 in scrambled shRNA cells, there were no differences in interactions between IGF-1-treated and control cells (Figure 6). Furthermore, there was no evidence of tyrosine phosphorylation of TSC2 under any of the present conditions (data not shown), suggesting that the blunting of IGF-1-induced TSC2 Thr1462 phosphorylation in FAK-KD cells was independent of TSC2 tyrosine phosphorylation.
Concurrent with an inhibition of TSC2 phosphorylation in FAK-KD cells, IGF-I induced increases in S6K1 phosphorylation at both Thr389 and Thr421/Ser424 (both required for the full activation of p70 kinase activity (46) and an indirect marker of mTOR activity (32)) were completely suppressed in FAK-KD cells. Moreover, increases in Thr37/46 phosphorylation of 4EBP1 (another substrate of mTOR (6)) seen with IGF-1 treatment in control cells were completely attenuated in FAK-KD cells. S6K1 regulates multiple targets involved in protein translation, including eEF2 (56), and its activation has been correlated with increases in MPS in human subjects (38). 4EBP1 is also important for cap-dependent protein translation, since phosphorylation of 4EBP1 results in the release of translation factor eIF4E (36). Thus, FAK appeared to be important for full activation of anabolic signaling targets downstream of mTOR, namely S6K1 and 4EBP1. This was associated with inhibition of TSC2, and potentially occurred via an AKT-independent mechanism (see Figure 6).

In contrast to the differences in S6K1 and 4EBP1 between FAK knockdown and control cells, decreases in eEF2 Thr56 and increases in eIF4G Ser1108 after IGF-1 were similar in both FAK and scramble shRNA cells. It is thought that eEF2 catalyses translocation of peptidyl-tRNA during elongation and is regulated by S6K1, while phosphorylation at Thr56 inactivates eEF2 via eEF2 kinase (56). In light of other results however, these changes may not represent important indicators of translation under the current conditions. Indeed, the observed changes in TSC2 and S6K1 strongly agree with our findings that increased association of eIF4E and eIF4G with IGF-1, and reduced association of eIF4E with 4EBP1, were attenuated in FAK knockdown cells. A crucial component of protein translation initiation is the binding of mRNA to the 43S pre-initiation complex, and this is mediated by formation of the eIF4F complex (42). Binding of eIF4E to eIF4G enables 40S ribosomal subunit to associate with the m^7GTP cap structure on mRNA, and the ability of eIF4E to interact with eIF4G is dependent on the extent of its binding to 4EBP1. Phosphorylation of 4EBP1 reduces interactions between eIF4E and 4EBP1, enabling formation of eIF4F and activating protein translation initiation. Thus, attenuation of increased eIF4E•eIF4G and reduced eIF4E•4EBP1 interactions is consistent with impairment in activation of protein translation following IGF-1
stimulation in FAK knockdown cells. The lack of differences in eIF4G phosphorylation between FAK-KD and control groups suggests that impairment of FAK activation was not important for activation of eIF4G, and that the impairment of IGF-1-mediated activation of translation initiation through FAK occurred through impaired activation of eIF4E and upstream factors. We also measured changes in MPS after 2 and 4 h IGF-1 treatment (time points where signaling responses were generally at their greatest). Increases in MPS after 4 h IGF-1 were significantly suppressed in FAK knockdown cells. The lack of detectable changes in MPS during the first 2 h of IGF-1 treatment, despite activation of multiple anabolic signaling targets in control cells, indicates that there may have been some delay in the onset of IGF-1-induced increases in MPS. Nevertheless, increases in MPS over 24 h, as assessed by D2O incorporation into protein-bound alanine, were attenuated in FAK-KD cells.

Increased phosphorylation of FAK at Tyr397 correlates with increased catalytic activity in response to mechanical stretch of muscle (40). We thus questioned whether the kinase activity of FAK was required for IGF-1 mediated growth in muscle cells, or whether the effects were a result of its structural role in the costamere. Scrambled shRNA myotubes were incubated with F14, an ATP-dependent competitive inhibitor of FAK phosphorylation at Tyr397, such that FAK was present but unable to be phosphorylated at its key tyrosine residue, and so become activated. Inhibition of FAK Tyr397 phosphorylation using F14 suppressed IGF-1-mediated growth as well as acute (2 h) IGF-1-induced increases in AKT and S6K1 phosphorylation, providing strong evidence that FAK kinase activity is required for IGF-1-induced cell growth in skeletal muscle cells.

It is not clear how IGF-1 activates FAK, however it has emerged that FAK may be subject to regulation by the protein phosphatase Shp-2. Indeed, during muscle differentiation, Shp-2-mediated dephosphorylation of FAK is required for myoblasts to withdraw from the cell cycle and undergo differentiation into myotubes (14). Also, in cardiac myocytes, activation of mTOR signaling and hypertrophic growth in response to cyclic stretch was associated with activation of FAK, secondary to reduced inhibition by Shp-2. Thus, mTOR and S6K1 appear to be critical downstream effectors of
anabolic signaling mediated by FAK following IGF-1 stimulation, most likely via direct binding to TSC2, and potentially secondary to reduced inhibition by Shp-2. However, although co-immunoprecipitation analysis in the present study revealed direct binding of Shp-2 to FAK in scramble shRNA myotubes, there were no changes in the amount of binding in response to IGF-1. Thus, the mechanism by which IGF-1 induced activation of FAK appeared not to have occurred via reduced binding/dephosphorylation by SHP-2.

We have identified that FAK represents a key component of IGF-1-mediated growth in C2C12 cells. Whether FAK plays an important part in human skeletal muscle mass regulation in vivo remains to be determined, but increasing evidence suggests its central role (13, 58). Furthermore, we hypothesize that dysregulation of the attachment complex could represent a crucial feature of pathological muscle wasting conditions beyond genetic dystrophy disorders e.g. in sarcopenia. Indeed, dystrophic phenotypes have been observed in rodents deficient in various attachment proteins in muscle, including talin (12) and various integrin subunits (23, 53). Furthermore, acute knockdown, by RNAi, of various attachment proteins in fully developed worm (17) and fly (44) muscle yields dystrophic phenotypes. However, it has also been reported that the costamere-associated protein integrin-linked kinase (ILK) is an important component of activation of IGF-1 receptor and AKT signaling in response to mechanical stress in skeletal muscle (55), demonstrating that molecular redundancy will almost certainly exist in vivo. Finally it should also be highlighted that recent studies have demonstrated a complex relationship between mTORc1 signaling and skeletal muscle growth in vivo, where more mTORc1 activity does not necessarily translate into more growth (4, 45) such that while FAK represents a key component of IGF-1 via mTORC1, this is just one component of a complex network of genes regulating hypertrophy, in vivo. In conclusion, the findings of the present study provide support for the importance of attachment complex proteins such as FAK in muscle growth, although further investigation into their potential role in ageing and in wasting diseases in human skeletal muscle in vivo is warranted.
ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

AAK, PJA, NJS, HC, and PP contributed to conception and design of the experiments. AAK, HC, PJA, NJS, DJW, KS, CHL and JAT performed all data collection, analysis and interpretation. HC and PJA drafted the manuscript. All authors contributed to revisions of the manuscript and approved the final version of the manuscript.

DISCLOSURES

The authors declare they have no conflict of interest.
REFERENCES


**FIGURES LEGENDS**

**Figure 1.** Protein expression of total focal adhesion kinase (FAK) in non-treated scrambled short hairpin (sh)RNA versus FAK-KD C2C12 cells (A), and light microscope images (B), mean myotube diameter (C), total protein per well (D) and total DNA per well (E) following chronic insulin-like growth factor (IGF)-1 treatment in FAK versus scrambled shRNA cells. Protein expression and myotube width data are presented as relative arbitrary units ± SEM (n=6-12 replicates per group). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs. corresponding non-IGF-1 treated or scrambled shRNA groups.

**Figure 2.** Changes in phosphorylation of focal adhesion kinase (FAK; A), total FAK protein expression (B), and phosphorylation of AKT (C & D), extracellular signal-related kinase 1/2 (ERK 1/2; E), and proline-rich AKT substrate of 40 kDa (PRAS40; F) in response to insulin-like growth factor (IGF)-1 treatment in FAK versus scrambled short hairpin (sh)RNA C2C12 cells. Data are presented as relative arbitrary units (RAU) ± SEM normalized to Coomassie staining of the membrane (n=12 replicates per group). a; P<0.05 vs. respective basal group. b; P<0.05 between groups at that time point. c; P<0.05 from basal at that time point for both groups.

**Figure 3.** Changes in phosphorylation of tuberous sclerosis 2 (TSC2; A), ribosomal protein S6 kinase (S6K1; B & C), eIF4E binding protein 1 (4EBP1; D), eukaryotic elongation factor 2 (eEF2; E) and eukaryotic initiation factor 4G (eIF4G; F) in response to insulin-like growth factor (IGF)-1 in focal adhesion kinase (FAK) versus scrambled short hairpin (sh)RNA C2C12 cells. Data are presented as relative arbitrary units (RAU) ± SEM normalized to Coomassie staining of the membrane (n=12 replicates per group). a; P<0.05 vs. respective basal group. b; P<0.05 between groups at that time point. c; P<0.05 from basal at that time point for both groups.
Figure 4. Changes in binding of eukaryotic initiation factor (eIF) 4E binding protein 1 (4EBP1) to eIF4E (A) and eIF4G to eIF4E (B), changes in protein synthesis as measured by puromycin (C & D) and deuterium oxide (E) in response to insulin-like growth factor (IGF)-1 in focal adhesion kinase (FAK) versus scrambled short hairpin (sh)RNA C2C12 cells, and changes in binding of FAK to SH2 domain-containing protein tyrosine phosphatase (Shp)-2 and tuberous sclerosis (TSC) 2 (F) in response to IGF-1 in scrambled shRNA cells. Data are presented as relative arbitrary units (RAU) ± SEM (n=6 replicates per group). a; different from respective basal group. b; different between groups at that time point. *P<0.05; **P<0.01 vs. respective control group. ASR = absolute synthesis rate.

Figure 5. Protein content (A), DNA content (B), and changes in phosphorylation of focal adhesion kinase (FAK; C), total FAK protein expression (D) and phosphorylation of AKT (E), ribosomal protein S6 kinase (S6K1; F) and eIF4E binding protein 1 (4EBP1; G) in response to insulin-like growth factor (IGF)-1 and FAK inhibitor 14 (F14) in scrambled short hairpin (sh)RNA C2C12 cells. Data are presented as relative arbitrary units (RAU) ± SEM (n=6 replicates per group). *P<0.05; **P<0.01; ***P<0.001 vs. respective control group.

Figure 6. Proposed mechanism of focal adhesion kinase (FAK)-mediated growth of C2C12 cells in response to insulin-like growth factor 1 (IGF-1). (A) IGF-1 induces phosphorylation/activation of FAK. FAK facilitates in activation of the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway by inhibiting tuberous sclerosis (TSC) 2, ultimately resulting in activation of mammalian target of rapamycin (mTOR), and subsequent activation of ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), two key regulators of muscle protein synthesis. In FAK-
knockdown cells (B), cell growth is inhibited through reduced activation of mTOR, 4EBP1 and S6K1, secondary to reduced inhibition of TSC2.
Figure 1

A

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FAK / Coomassie (RAU)

B

CTRL 24 h IGF-1

C

Myotube Diameter (Relative Arbitrary Units)

D

Alkaline Soluble Protein (μg/well)

E

DNA (μg/well)
Figure 2

**A**

CTL 2 h 4 h 8 h

FAK<sup>Y397</sup> SCR
Coomassie

FAK<sup>Y397</sup> FAK-KD
Coomassie

**B**

CTL 2 h 4 h 8 h

FAK SCR
Coomassie

FAK FAK-KD
Coomassie

**C**

CTL 2 h 4 h 8 h

AKT<sup>Tyr397</sup> SCR
Coomassie

AKT<sup>Tyr397</sup> FAK-KD
Coomassie

**D**

CTL 2 h 4 h 8 h

AKT<sup>Thr308</sup> SCR
Coomassie

AKT<sup>Thr308</sup> FAK-KD
Coomassie

**E**

CTL 2 h 4 h 8 h

ERK1/2<sup>Thr202/Tyr204</sup> SCR
Coomassie

ERK1/2<sup>Thr202/Tyr204</sup> FAK-KD
Coomassie

**F**

CTL 2 h 4 h 8 h

PRAS40<sup>Thr246</sup> SCR
Coomassie

PRAS40<sup>Thr246</sup> FAK-KD
Coomassie
Figure 3

A

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S6K1Thr389

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C

S6K1Thr421/Ser424

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4EBP1Thr37/46

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E

eEF2Thr56

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F

eIF4GSer1108

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Graphs showing the effects of IGF-1 incubation on protein expression levels:

- TSC2Thr1462
- S6K1Thr389
- S6K1Thr421/Ser424
- 4EBP1Thr37/46
- eEF2Thr56
- eIF4GSer1108
Figure 4

A

4EBP1 SCR
4EBP1 FAK-KD
eIF4E

Time after IGF-1 incubation (h)

B

eIF4G SCR
eIF4G FAK-KD
eIF4E

eIF4G

eIF4E

Time after IGF-1 incubation (h)

C

ASR (μg protein per day)

SCR shRNA
FAK-KD

D

CTL 2 h 4 h

SCR shRNA
FAK-KD

E

Puro/mycin / Coomassie (RAU)

Time after IGF-1 incubation (h)

F

IB: Shp-2
IB: TSC2
IP: FAK

FAK Immunoprecipitation (RAU)

Time after IGF-1 incubation (h)
Figure 6

A Control

IGF-1 receptor

PI3K → AKT

TSC1, TSC2 → mTOR

4EBP1, eIF4F → S6K1

Protein synthesis & cell growth

B FAK-KD

IGF-1 receptor

PI3K → AKT

TSC1, TSC2 → mTOR

4EBP1, eIF4F → S6K1

Protein synthesis & cell growth