

# Focal adhesion kinase regulates insulin resistance in skeletal muscle

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## Abstract

**Aims/hypothesis** On the basis of our previous studies, we investigated the possible role of focal adhesion kinase (FAK) in the development of insulin resistance in skeletal muscle, a major organ responsible for insulin-stimulated glucose uptake.

**Materials and methods** Insulin-resistant C2C12 skeletal muscle cells were transfected with FAK wild-type or FAK mutant plasmids, knocked down using small interfering RNA (siRNA), and their effects on the levels and activities of insulin-signalling molecules and on glucose uptake were determined.

**Results** A significant decrease in tyrosine phosphorylation of FAK in insulin-resistant C2C12 cells was observed. A similar decrease was observed in skeletal muscle obtained

from insulin-resistant Sprague–Dawley rats fed a high-fat diet. Increased levels of FAK in insulin-resistant C2C12 skeletal muscle cells increased insulin sensitivity and glucose uptake. These effects were reversed by an increase in the level of kinase activity mutant FAK or suppression of endogenous FAK by siRNA. FAK was also found to interact downstream with insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase C and glycogen synthase kinase 3 $\beta$ , leading to translocation of glucose transporter 4 and resulting in the regulation of glucose uptake.

**Conclusions/interpretation** The present study provides strong evidence that the modulation of FAK level regulates the insulin sensitivity of skeletal muscle cells. The results demonstrate a direct role of FAK in insulin-resistant skeletal muscle cells for the first time.

**Keywords** FAK · Insulin resistance · siRNA · Skeletal muscle

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## Abbreviations

2-DOG	2-deoxy[ <sup>3</sup> H]glucose
FAK	focal adhesion kinase
GSK-3 $\beta$	glycogen synthase kinase 3 $\beta$
IR	insulin receptor
LDM	low-density microsome
MF	MCDB 201:Ham's F-12 serum-free medium (1:1)
MFI	MCDB 201:Ham's F-12 serum-free medium with insulin (1:1)
PDBu	phorbol 12,13-dibutyrate
PI3K	phosphatidyl inositol 3-kinase
PMSF	phenylmethylsulphonyl fluoride
PKC	protein kinase C
siRNA	small interfering RNA

## Introduction

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase and is known to play a key role in integrin signalling [1]. FAK can associate with multiple cellular components, including adhesion-associated proteins and signalling molecules [2]. We have previously reported regulation of myogenesis [3, 4] and cell spreading [5, 6] by FAK as a function of its tyrosine phosphorylation in C2C12 skeletal muscle cells due to insulin stimulation. Bianchi et al. [7] reported the regulation of FAK phosphorylation by glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) during cell spreading and migration. In addition, regulation of FAK phosphorylation by insulin stimulation in non-muscle cells has been reported [8–10]. A regulatory role of FAK in IRS-1 levels [11, 12] and hepatic insulin action [13, 14] has also been reported. Genetic studies have provided evidence for interaction between integrin and insulin signalling [15, 16]. The available literature also focuses on the role of FAK in insulin-mediated effects on the cytoskeleton [10, 17]. Recently, Huang et al. [18] have reported that a reduced level of FAK disrupts insulin action in skeletal muscle. However, the role of FAK in regulating insulin resistance in skeletal muscle, if any, and its possible pathway have not been investigated.

We had previously generated an insulin-resistant skeletal muscle model by differentiating mouse C2C12 cells in the chronic presence of insulin (100 nmol/l) in serum-free medium (MFI) [19–22]. The model was validated using glucose-lowering drugs, including pioglitazone [19], metformin [20] and gliclazide [21]. This model was used in the present study to investigate the role of FAK, if any, under conditions of impaired insulin action.

## Materials and methods

**Materials** Nutrient Mixture F-12 Ham, MCDB 201 medium and 2-deoxy[ $^3$ H]glucose (2-DOG) were from Sigma Chemical Company (St Louis, MO, USA). Anti-phospho Akt (Ser473), anti-Akt, anti-phospho (Ser) GSK-3 $\beta$  and anti-GSK-3 $\beta$  antibodies were from Cell Signaling Technology (MA, USA). Anti-FAK, anti-phosphotyrosine, anti-insulin receptor (IR) and anti-IRS-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine insulin and anti-GLUT4 (also known as SLC2A2) was purchased from Calbiochem (CA, USA). All other reagents were from Sigma unless attributed otherwise.

**Cell culture** C2C12 skeletal muscle cell lines (wild-type and transfectants) were maintained in DMEM supplemented with 15% FCS containing penicillin 100 IU/ml and streptomycin 100  $\mu$ g/ml in 5% CO $_2$  at 37°C [3]. Cells

at 70% confluence were differentiated in an equal mixture of MCDB 201 and Ham's F-12 medium as described previously [19] in the absence (MF, insulin-sensitive condition) or in the chronic presence of 100 nmol/l insulin (MFI, insulin-resistant condition) for 3 days.

**Subcellular fractionation** C2C12 myotubes, pretreated with or without insulin, were washed with ice-cold PBS, scraped into buffer A (mmol/l: HEPES 20, pH 7.4, sucrose 250, EDTA 1, phenylmethylsulphonyl fluoride [PMSF] 2) with 10  $\mu$ g/ml aprotinin and leupeptin and homogenised for 10 min at 4°C, after which low-density microsome (LDM) and plasma membrane fractions were isolated by differential centrifugation [23]. Protein samples (100  $\mu$ g) were subjected to SDS-PAGE and western immunoblotting.

**Transfection of plasmid vectors** C2C12 cells were stably transfected with FAK wild-type and mutant plasmids as described earlier [22].

**siRNA transfection** siRNA oligonucleotides against FAK targeted sequence 5'-TGCAATGGAACGAGTATTA-3' were designed and synthesised by Qiagen GmbH (Hilden, Germany). C2C12 cells were transiently transfected with FAK-specific and non-specific siRNA (400 nmol/l) using RNAiMax transfection reagent (Qiagen) in reduced-serum Opti-MEM media (Gibco BRL, Carlsbad, CA, USA) according to the manufacturer's instructions, with minor modifications.

**Preparation of cell lysates** Cells were washed with ice-cold PBS and lysed in lysis buffer (mmol/l: HEPES 50, pH 7.4, NaCl 150, MgCl $_2$  1.5, EGTA 1, sodium pyrophosphate 10, sodium fluoride 50,  $\beta$ -glycerophosphate 50, Na $_3$ VO $_4$  1, PMSF 2) with 1% Triton X-100, 10  $\mu$ g/ml each of leupeptin, aprotinin and soyabean trypsin inhibitor at 4°C for 30 min. Homogenates were centrifuged at 16,000 g for 15 min at 4°C [4]. Protein estimation was performed using the Bicinchoninic Acid Kit (Sigma) according to the manufacturer's instructions. Equal concentrations of lysates of all the samples were subjected to SDS-PAGE followed by western immunoblotting [3].

**Tissue preparation from rats fed a high-fat diet** Male Sprague–Dawley rats (Central Animal Facility, NIPER, India) were divided into two equal groups. One of the groups was fed a normal pellet diet, while the other group was fed a high-fat diet for 4 weeks. The high-fat diet comprised: powdered pellets (364 g), lard (310 g), casein (250 g), cholesterol (10 g), DL-methionine (3 g), Yee-sac powder (1 g), vitamin and mineral mix powder (60 g), sodium chloride (2 g). Biochemical analyses and tolerance tests were performed as described previously [24, 25]. Skeletal muscle from the thigh region was collected,

washed with PBS (pH 7.4) and lysed using the lysis buffer as described earlier [4]. The use of animals for the experiments was in compliance with the guidelines of our institutional animal ethics committee.

**Phosphatidyl inositol 3-kinase activity** Cells were differentiated under MF and MFI conditions and were stimulated with insulin for 10 min. Cells (500  $\mu$ g) were lysed and immunoprecipitated for IRS-1 [19]. IRS-1-associated activity of phosphatidyl inositol 3-kinase (PI3K) was determined with the PI3K ELISA Kit (Echelon Biosciences Inc., Salt Lake City, UT, USA) according to the manufacturer's instructions and as reported previously [26–28].

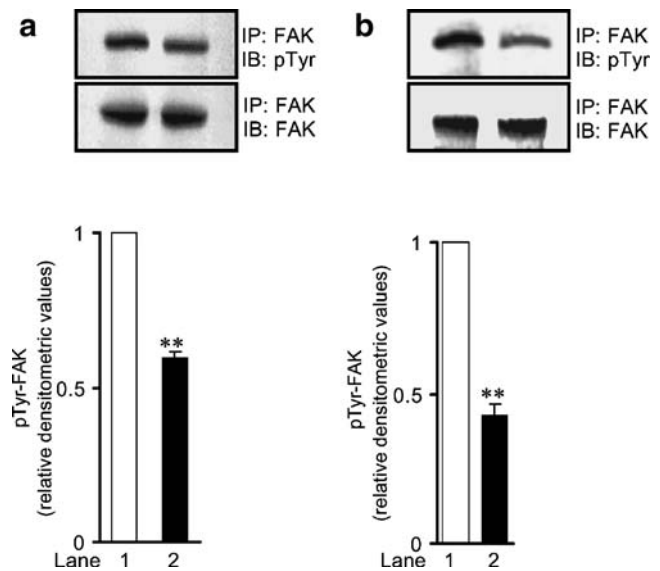
**Glucose uptake assays** Glucose uptake assays were performed using 2-DOG as described earlier [21].

**Densitometric analysis** Relative densitometric analyses of the samples were determined using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA) and Quantity One 1-D analysis software (Bio-Rad) by giving an arbitrary value of 1.0 to the respective control samples of each experiment, keeping the background value as 0.

**Statistical analysis** The data are expressed as mean $\pm$ SE. For comparison of two groups, *p* values were calculated using the two-tailed unpaired Student's *t* test. In all the cases *p*<0.05 was considered to be statistically significant.

## Results

To determine the role of FAK in insulin resistance, if any, C2C12 cells were differentiated under insulin-sensitive (MF) or insulin-resistant (MFI) conditions [19]. Cells were lysed, immunoprecipitated with anti-FAK and subjected to western immunoblotting with anti-phosphotyrosine or anti-FAK antibody (Fig. 1a). FAK tyrosine phosphorylation was found to be reduced by  $40.3\pm 0.01\%$  in insulin-resistant cells, with no change in its protein level (Fig. 1a). To determine whether FAK tyrosine phosphorylation is affected in insulin-resistant animals, skeletal muscles were isolated from the high-fat-diet-fed Sprague–Dawley rats [24, 25] and examined for FAK phosphorylation. Rats fed high-fat diets show significant elevations in body weight, plasma insulin, plasma glucose, plasma triacylglycerol and plasma cholesterol levels, resulting in insulin resistance [24, 25]. Skeletal muscle isolated from normal and high-fat-diet-fed Sprague–Dawley rats were immunoprecipitated with anti-FAK and subjected to western immunoblotting with anti-phosphotyrosine or anti-FAK antibody. A decrease ( $57.5\pm 0.04\%$ ) in tyrosine phosphorylation was observed



**Fig. 1** FAK tyrosine phosphorylation in insulin-resistant skeletal muscle. **a** C2C12 cells were differentiated in serum-free medium in the absence (MF) or chronic presence of 100 nmol/l insulin (MFI) for 3 days. Protein extracts (200  $\mu$ g each) were immunoprecipitated with anti-FAK antibody, subjected to western immunoblotting and probed with anti-phosphotyrosine or anti-FAK antibody. Bars represent relative densitometric values of pTyr-FAK. Lane 1, C2C12 cells differentiated in MF medium; lane 2, C2C12 cells differentiated in MFI medium. Experiments were repeated three times and a representative result is shown. Values are mean $\pm$ SE. **\*\****p*<0.01. Open bars, MF; filled bars, MFI. **b** Protein lysates (200  $\mu$ g) from skeletal muscle of normal (control) and high-fat diet-fed (insulin-resistant) rats were immunoprecipitated with anti-FAK antibody, subjected to western immunoblotting and probed with anti-phosphotyrosine or anti-FAK antibody. Bars represent relative densitometric values of pTyr-FAK. Lane 1; normal rat, lane 2; high-fat diet-fed rat. Experiments were repeated six times and a representative result is shown. Values are mean $\pm$ SE. **\*\****p*<0.01. Open bars, normal rats; filled bars, rats fed high-fat diet. IP, immunoprecipitated; IB, immunoblotted

in high-fat-diet-fed Sprague–Dawley rats compared with normal rats (Fig. 1b), with no change in its protein level (Fig. 1b). The data thus confirmed the *in vitro* findings and strongly emphasised the possible involvement of FAK in skeletal muscle insulin resistance.

The role of FAK could be shown either by generating FAK knockout mice or use specific inhibitors. However, FAK knockout studies could not be performed because of reported embryonic lethality [29, 30] and there is no inhibitor of FAK available [2]. Therefore, to address the issue we undertook several lines of study. We generated the C2C12 cell line, which overexpresses haemagglutinin (HA)-tagged plasmid constructs containing the wild-type gene encoding FAK (C2FAK<sup>wt/+</sup>) or a mutation in the major autophosphorylation site, tyrosine-397 (Y397F; mutant FAK gene, C2FAK<sup>mut/+</sup>) (Electronic supplementary material [ESM] Fig. 1). C2FAK<sup>wt/+</sup> and C2FAK<sup>mut/+</sup> cells showed  $52\pm 0.1$  and  $50\pm 0.1\%$  overexpression of the FAK<sup>wt</sup> and FAK<sup>mut</sup> genes, respectively, compared with untransfected

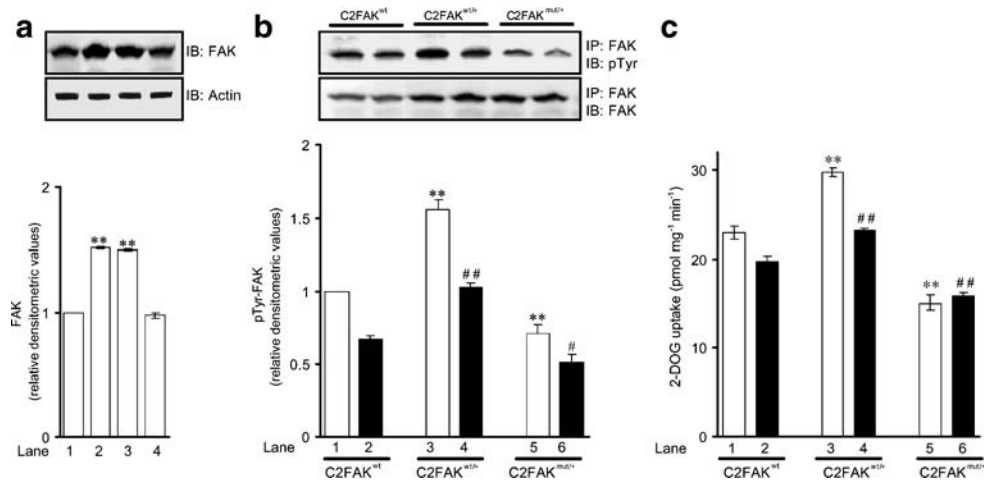
cells (Fig. 2a). No significant change in cellular morphology (ESM Fig. 2), growth rates and cell death due to transfection was observed, as shown by Trypan Blue exclusion and the TUNEL (terminal deoxyribonucleotide transferase-mediated dUTP nick end labelling) assay (data not shown).

Transfected cells were then subjected to the MF and MFI conditions and tyrosine phosphorylation of FAK was assayed (Fig. 2b). Under both conditions, tyrosine phosphorylation of C2FAK<sup>wt/+</sup> cells increased by  $56\pm 0.06$  and  $42.2\pm 0.03\%$  over their respective controls (Fig. 2b, lane 3 vs lane 1,  $p<0.01$ ; lane 4 vs lane 2,  $p<0.01$ ). Although tyrosine phosphorylation of FAK in the MFI condition was always less than in the MF condition, insulin-resistant C2FAK<sup>wt/+</sup> cells exhibited increased tyrosine phosphorylation, and the level of phosphorylation was similar to that of insulin-sensitive C2FAK<sup>wt</sup> cells (Fig. 2b, lane 4 vs lane 1). Tyrosine phosphorylation of FAK in C2FAK<sup>mut/+</sup> cells under the MF or MFI condition was less (by  $29.4\pm 0.06$  and  $25.8\pm 0.05\%$ , respectively) than in their respective controls (Fig. 2b, lane 5 vs lane 1,  $p<0.01$ ; lane 6 vs lane 2,  $p<0.05$ ). The data therefore suggest correlation between FAK phosphorylation and insulin resistance.

To assess the functional contribution of altered FAK protein level and/or its activation in the pathogenesis of insulin resistance, glucose uptake was measured in the above-mentioned cell lines cultured under the MF or MFI condition. Insulin-sensitive C2FAK<sup>wt/+</sup> cells showed a  $40.5\pm 0.48\%$

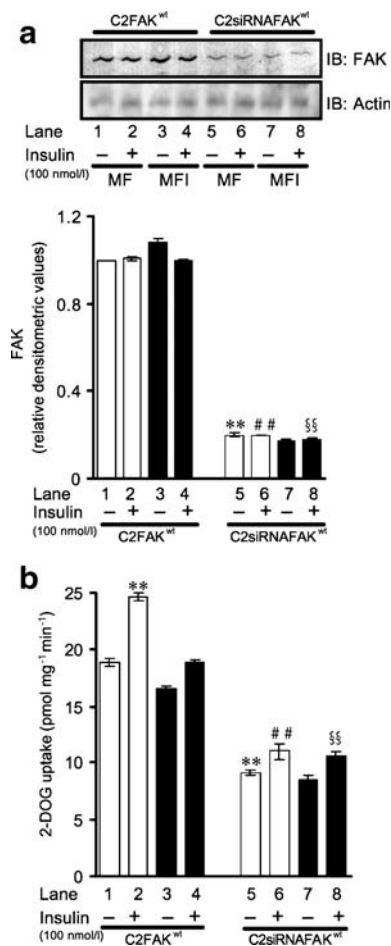
increase in insulin-stimulated 2-DOG uptake compared with C2FAK<sup>wt</sup> cells (Fig. 2c, lane 3 vs lane 1,  $p<0.01$ ). For detailed data with and without insulin stimulation see ESM Fig. 3. Under insulin-resistant conditions, C2FAK<sup>wt/+</sup> cells exhibited increased glucose uptake ( $28.6\pm 0.61\%$ ) compared with C2FAK<sup>wt</sup> cells (Fig. 2c, lane 4 vs lane 2,  $p<0.01$ ), comparable with that observed in insulin-sensitive C2FAK<sup>wt</sup> cells (Fig. 2c, lane 4 vs lane 1). Cells with increased levels of FAK were thus protected from the development of hyperinsulinaemia-induced insulin resistance. However, C2FAK<sup>mut/+</sup> cells developed insulin resistance as there was no insulin-stimulated increase in glucose uptake (Fig. 2c, lane 5 vs lane 1,  $p<0.01$  and lane 6 vs lane 2,  $p<0.01$ ).

To further establish the role of FAK, the siRNA approach was used. Maximum downregulation ( $84.6\pm 0.01\%$ ) of FAK was observed at 400 nmol/l of siRNA (ESM Fig. 4a), specific to FAK (ESM Fig. 4b), without affecting cellular morphology (ESM Fig. 5), viability and growth rate (data not shown). We next studied the effect of FAK downregulation under the MF and MFI conditions with and without insulin stimulation. The level and phosphorylation of FAK in siRNA-transfected cells remained downregulated under all the culture conditions (Fig. 3a, ESM Fig. 6). Next, we examined the effect of FAK silencing on insulin-stimulated glucose uptake in siRNA-transfected cells (C2siRNAFAK<sup>wt</sup>) differentiated under the MF and MFI conditions (Fig. 3b). Basal and



**Fig. 2** Effect of modulation of FAK level on FAK tyrosine phosphorylation and glucose uptake. **a** C2C12 cells were transfected with HA-tagged FAK<sup>wt</sup> and FAK<sup>mut</sup> containing vector pCDNA3.1, differentiated and lysed, and protein lysates (30  $\mu$ g each) were subjected to western immunoblotting with anti-FAK antibody. Equal loading was confirmed by stripping and reprobing the blot with anti-actin antibody. Bars represent relative densitometric values of FAK. Lane 1, C2FAK<sup>wt</sup> cells; lane 2, C2FAK<sup>wt/+</sup> cells; lane 3, C2FAK<sup>mut/+</sup> cells; lane 4, cells transfected with vector. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p<0.01$  compared with lane 1. **b** Transfected cells were differentiated in serum-free medium in the absence (MF) or chronic presence of 100 nmol/l insulin (MFI) for 3 days. Protein extracts (200  $\mu$ g each) were immunoprecipitated with

anti-FAK antibody, subjected to western immunoblotting and probed with anti-phosphotyrosine or anti-FAK antibody. Bars represent relative densitometric values of pTyr-FAK. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p<0.01$  compared with lane 1; ## $p<0.01$  and # $p<0.05$  compared with lane 2. **c** Cells differentiated in serum-free medium in the absence (MF) or chronic presence of insulin (MFI) for 3 days were stimulated with 100 nmol/l insulin for 15 min. Uptake of 2-DOG was measured in 40  $\mu$ g cell lysates. Uptake was measured in duplicate. Data are pmol mg<sup>-1</sup> min<sup>-1</sup>. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p<0.01$  compared with lane 1, ## $p<0.01$  compared with lane 2. Open bars, MF; filled bars, MFI. IP, immunoprecipitated; IB, immunoblotted



**Fig. 3** Effect of FAK downregulation on glucose uptake. **a** Cells transfected with siRNA against FAK were differentiated under MF or MFI media with (+) or without (-) insulin (100 nmol/l) stimulation. Lysates were subjected to western immunoblotting and probed with anti-FAK antibody. Blots were stripped and again probed with anti-actin antibody. Bar represents relative densitometric values of FAK expression. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ## $p$ <0.01 compared with lane 2; §§ $p$ <0.01 compared with lane 4. **b** Effect of FAK knock-down on glucose uptake. siRNA-transfected cells were differentiated under MF and MFI conditions and analysed for insulin-mediated glucose uptake. Uptake was measured in duplicate. Data are shown as pmol mg<sup>-1</sup> min<sup>-1</sup>. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ## $p$ <0.01 compared with lane 2; §§ $p$ <0.01 compared with lane 4. Open bars, MF; filled bars, MFI

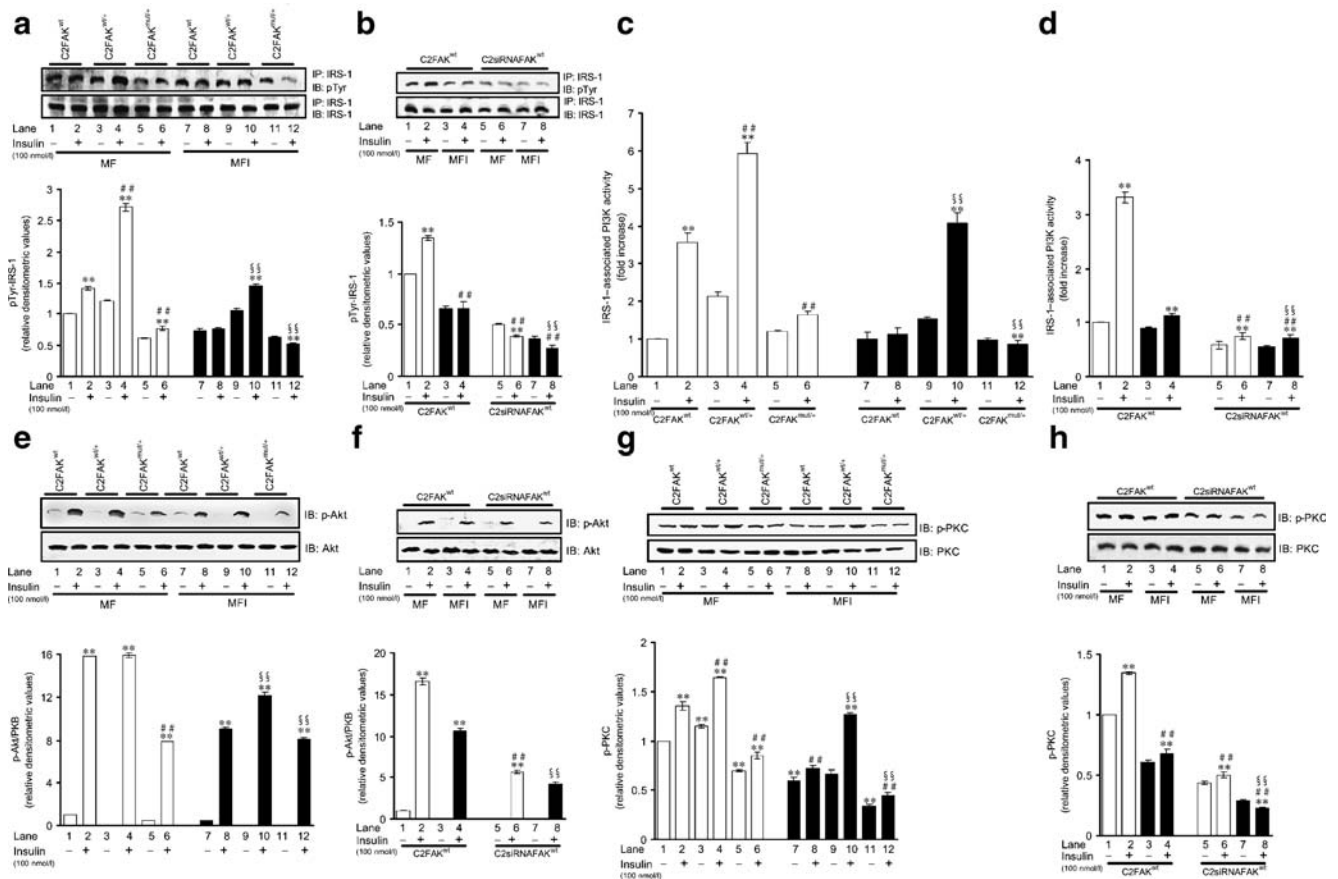
insulin-stimulated glucose uptake were reduced by 52.1 $\pm$ 0.23 and 55.2 $\pm$ 0.70% in C2siRNAFAK<sup>wt</sup> cells compared with C2FAK<sup>wt</sup> cells, respectively (Fig. 3b, lane 5 vs lane 1,  $p$ <0.01; lane 6 vs lane 2,  $p$ <0.01). Similar decreases were observed under the insulin-resistant condition. No change in insulin-stimulated glucose uptake was observed in cells transfected with non-specific siRNA (ESM Fig. 7). siRNA knock-down further supports the idea that elimination of FAK decreases cellular glucose uptake, which might be a consequence of impaired insulin signalling. To further substantiate our results, FAK was silenced in L6E9 cells, which are known to have a greater concentration of glucose

transporter than C2C12 cells, and the cells were used in experiments similar to those described above. Basal and insulin-stimulated glucose uptake was both substantially reduced in FAK-silenced L6E9 cells (L6siRNAFAK<sup>wt</sup>) compared with non-silenced wild-type cells (L6FAK<sup>wt</sup>) (ESM Fig. 8). The data suggest that the FAK is necessary for insulin-stimulated glucose uptake in L6E9 cells, as observed in the C2C12 cell line.

We next examined the potential molecular mechanisms that might underlie the protection from insulin resistance conferred by an increased level of FAK and corresponding tyrosine phosphorylation. The possible mechanisms include increased insulin-mediated activation of the IR and/or its downstream molecules. Therefore, we examined IR- $\beta$  tyrosine phosphorylation and production in C2FAK<sup>wt</sup>, C2FAK<sup>wt/+</sup>, C2FAK<sup>mut/+</sup> and C2siRNAFAK<sup>wt</sup> cells under the MF and MFI conditions. No significant change in insulin-mediated activation or level of IR- $\beta$  due to modulation of FAK activation was observed (data not shown).

Previously, FAK was reported to interact with IRS-1 but not with IR [31–33]. Therefore, IRS-1 tyrosine phosphorylation and protein level in C2FAK<sup>wt</sup>, C2FAK<sup>wt/+</sup> and C2FAK<sup>mut/+</sup> cells under MF and MFI conditions were examined. C2FAK<sup>wt/+</sup> cells in the MF condition showed increased (92.8 $\pm$ 0.05%) insulin-stimulated IRS-1 phosphorylation (Fig. 4a, lane 4 vs lane 2,  $p$ <0.01), whereas a decrease (46.4 $\pm$ 0.03%) was seen in C2FAK<sup>mut/+</sup> cells (Fig. 4a, lane 6 vs lane 2,  $p$ <0.01), in which phosphorylation was similar to that in insulin-resistant C2FAK<sup>wt</sup> cells (Fig. 4a, lane 6 vs lane 8). C2FAK<sup>wt/+</sup> cells under the MFI condition showed an 80.7 $\pm$ 0.03% increase in insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 4a, lane 10 vs lane 8,  $p$ <0.01), and the level of phosphorylation was similar to that in C2FAK<sup>wt</sup> cells (Fig. 4a, lane 10 vs lane 2), whereas it decreased (by 24.9 $\pm$ 0.01%) in C2FAK<sup>mut/+</sup> cells (Fig. 4a, lane 12 vs lane 8,  $p$ <0.01). No significant change was observed in the level of IRS-1. To confirm these results, IRS-1 tyrosine phosphorylation was measured in C2siRNAFAK<sup>wt</sup> cells. Decreases in insulin-stimulated IRS-1 phosphorylation of 71.0 $\pm$ 0.02% in the MF condition and 41.5 $\pm$ 0.02% in the MFI condition (Fig. 4b, lane 6 vs lane 2,  $p$ <0.01; lane 8 vs lane 4,  $p$ <0.01) were observed; these results were similar to those observed in C2FAK<sup>mut/+</sup> cells. The data therefore suggest that at least one of the ways in which FAK regulates insulin resistance in C2C12 skeletal muscle is through IRS-1 tyrosine phosphorylation. FAK functions upstream of IRS-1, as inhibition of FAK abrogates IRS-1 phosphorylation.

To explore the role(s) of FAK in regulating downstream molecules, IRS-1-associated PI3K activity was determined. In C2FAK<sup>wt/+</sup> cells, insulin-stimulated PI3K activity increased 1.7 $\pm$ 0.29-fold in the MF condition (Fig. 4c, lane 4

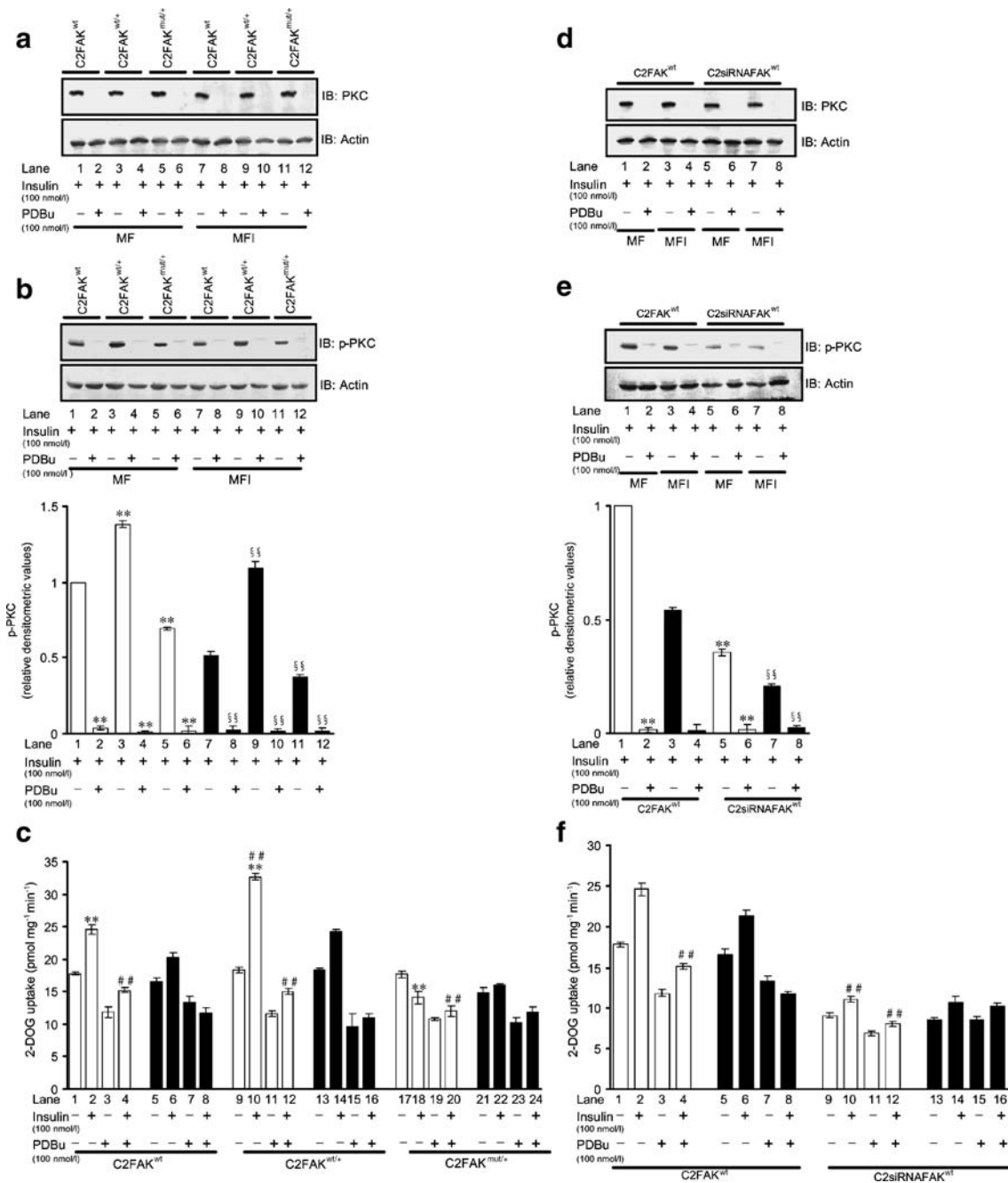


**Fig. 4** Effect of modulation of FAK on insulin signalling. Transfected and untransfected cells were differentiated in serum-free medium in the absence (MF) or chronic presence of insulin (MFI) for 3 days and stimulated with or without insulin (100 nmol/l) for 10 min. **a** Cell lysates (500  $\mu$ g) were immunoprecipitated with anti-IRS-1 antibody, subjected to western immunoblotting and probed with anti-phosphotyrosine antibody or anti-IRS-1 antibody. Bars represent relative densitometric values of pTyr-IRS-1 normalised to protein levels. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 8. **b** siRNA-transfected myotubes were stimulated with or without insulin (100 nmol/l) for 10 min and lysed. Lysate (500  $\mu$ g) was immunoprecipitated with anti-IRS-1 antibody, subjected to western immunoblotting and probed with anti-phosphotyrosine or anti-IRS-1 antibody. Bars represent relative densitometric values of pTyr-IRS-1 normalised to protein levels. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 4. **c** Cell lysates (500  $\mu$ g) were immunoprecipitated with anti-IRS-1 antibody and PI3K activity was measured. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 8. **d** siRNA-transfected myotubes were lysed, the lysate (500  $\mu$ g) was immunoprecipitated with anti-IRS-1 antibody, and PI3K activity was tested in the immunoprecipitates. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with

lane 4. **e** Cell lysates (30  $\mu$ g) were subjected to western immunoblotting and probed with anti-phospho Akt (Ser73) antibody or anti-Akt antibody. Bars represent relative densitometric values of pAkt (Ser73) normalised to protein levels. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 8. **f** siRNA-transfected differentiated cells were lysed, and the lysates (30  $\mu$ g) were subjected to western immunoblotting and probed with anti-phospho Akt (Ser73) antibody or anti-Akt antibody. Bars represent relative densitometric values of pAkt (Ser73) normalised to protein levels. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 4. **g** Cell lysates (30  $\mu$ g) were subjected to western immunoblotting and probed with anti-phospho pan-PKC antibody (pPKC) or anti-pan-PKC antibody. Bars represent relative densitometric values of pPKC normalised to protein levels. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 8. **h** siRNA-transfected differentiated cells were lysed, and the lysates (30  $\mu$ g) were subjected to western immunoblotting and probed with anti-phospho pan-PKC antibody and anti-pan-PKC antibody. Bars represent relative densitometric values of pPKC normalised to protein levels. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2; \$\$\$ $p$ <0.01 compared with lane 4. Open bar, MF; filled bar, MFI. IP, immunoprecipitated; IB, immunoblotted

vs lane 2,  $p$ <0.01), whereas it decreased 2.18 $\pm$ 0.08-fold in C2FAK<sup>mut/+</sup> cells (Fig. 4c, lane 6 vs lane 2,  $p$ <0.01), and in the mutant cells the PI3K activity was same as that of insulin-resistant C2FAK<sup>wt</sup> cells (Fig. 4c, lane 6 vs lane 8).

C2FAK<sup>wt/+</sup> cells under the MFI condition showed a 3.6 $\pm$ 0.25-fold increase in PI3K activity (Fig. 4c lane 10 vs lane 8,  $p$ <0.01), and the extent of activation was same as that of insulin-sensitive C2FAK<sup>wt</sup> cells (Fig. 4c, lane 10 vs lane 2).



**Fig. 5** Effect of PKC inhibition on FAK-mediated glucose uptake. Transfected and untransfected cells were differentiated in serum-free medium in the absence (MF) or chronic presence (MFI) of insulin and PDBu (100 nmol/l) for 3 days, and stimulated with or without insulin (100 nmol/l) for 10 min. **a, d** Cell lysates (30  $\mu$ g) of insulin-stimulated cells differentiated in the presence of PDBu were subjected to western immunoblotting and probed with anti-pan-PKC antibody, stripped and reprobed with anti-actin antibody. **b** Cell lysates (30  $\mu$ g) of insulin-stimulated cells differentiated in presence of PDBu were subjected to western immunoblotting, probed with anti-phospho pan-PKC antibody (pPKC), stripped and reprobed with anti-actin antibody. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ## $p$ <0.01 compared with lane 2; §§ $p$ <0.01 compared with

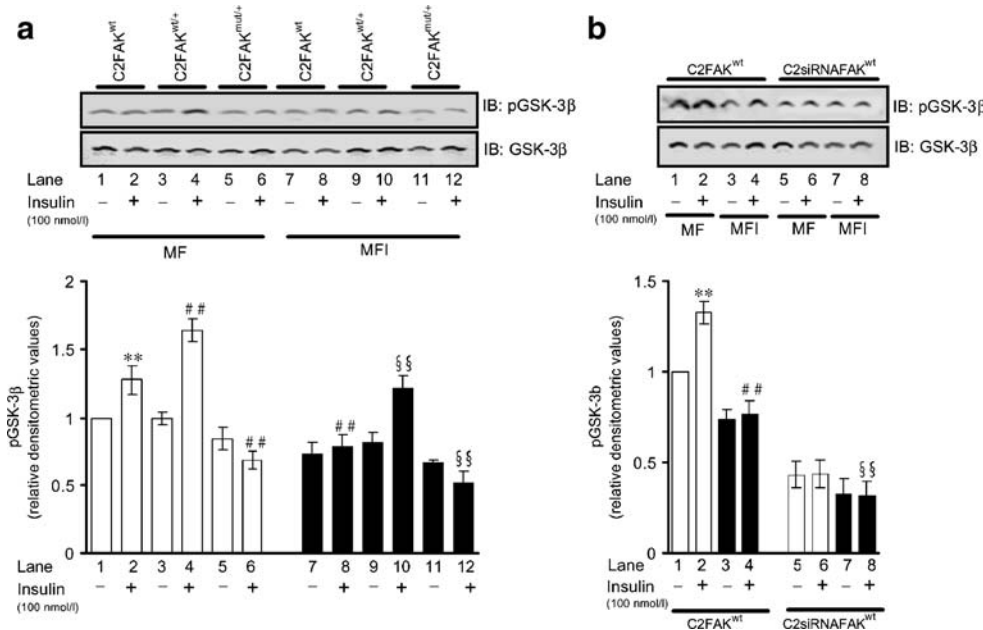
lane 8. **c, f** Cells differentiated in serum-free medium in the absence (MF) or chronic presence of insulin (MFI) and PDBu (100 nmol/l) for 3 days were stimulated with 100 nmol/l insulin for 15 min. Uptake of 2-DOG was measured in 40  $\mu$ g cell lysates. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ## $p$ <0.01 compared with lane 2; §§ $p$ <0.01 compared with lane 4. *Open bars*, MF; *filled bars*, MFI

Insulin-stimulated IRS-1-associated PI3K activity was unaltered in C2FAK<sup>mut/+</sup> cells under the MFI condition (Fig. 4c, lane 12 vs lane 11), but decreased 4.4±0.01-fold and 1.2±0.01-fold under the MF and MFI conditions, respectively (Fig. 4d, lane 6 vs lane 2, *p*<0.01; lane 8 vs lane 4, *p*<0.01) in C2siRNAFAK<sup>wt</sup> cells.

PI3K activates several PIP3-dependent serine/threonine kinases, e.g. Akt (also known as protein kinase B [PKB]) and protein kinase C (PKC) [34, 35]. Therefore, insulin-stimulated activation, if any, of Akt was examined in all the cell lines (Fig. 4e). No additional insulin-stimulated effect of Akt phosphorylation (Ser473) was observed in C2FAK<sup>wt/+</sup> cells (Fig. 4e, lane 4 vs lane 2). This may have been due to saturable activation of Akt. Although under the insulin-resistant condition C2FAK<sup>wt/+</sup> cells showed additional insulin-stimulated Akt phosphorylation, the extent of activation was not equivalent to that seen in insulin-sensitive C2FAK<sup>wt</sup> cells (Fig. 4e, lane 10 vs lane 2). C2FAK<sup>mut/+</sup> cells displayed decreased Akt activation in both the MF and the MFI condition (Fig. 4e, lane 6 vs lane 2, *p*<0.01; lane 12 vs lane 8, *p*<0.01). Similar decreases were observed in C2siRNAFAK<sup>wt</sup> cells (66.2±0.2 and 58±0.13% in the MF and MFI conditions, respectively) (Fig. 4f, lane 6 vs lane 2, *p*<0.01; lane 8 vs lane 4, *p*<0.01). This

indicates possible signal divergence under the insulin-resistant condition.

PKC is reported to regulate glucose uptake downstream of PI3K [34, 35]. Therefore, insulin-stimulated activation of PKC, if any, was tested in all the cell lines by probing with anti-phospho pan-PKC antibody. Insulin-stimulated PKC phosphorylation of C2FAK<sup>wt/+</sup> cells increased (by 21.4±0.05%) under the MF condition (Fig. 4g, lane 4 vs lane 2, *p*<0.01) but decreased (by 37.4±0.03%) in C2FAK<sup>mut/+</sup> cells (Fig. 4g, lane 6 vs lane 2, *p*<0.01), and the level of activation was similar to that in insulin-resistant C2FAK<sup>wt</sup> cells (Fig. 4g, lane 6 vs lane 8). Insulin-stimulated PKC phosphorylation increased (76.3±0.03%) in C2FAK<sup>wt/+</sup> cells under the MFI condition (Fig. 4g, lane 10 vs lane 8, *p*<0.01), and the extent of activation was as observed in insulin-sensitive C2FAK<sup>wt</sup> cells (Fig. 4g, lane 10 vs lane 2), whereas it was further decreased (37.5±0.01%) in C2FAK<sup>mut/+</sup> cells (Fig. 4g, lane 12 vs lane 8, *p*<0.01) without affecting its protein level (Fig. 4g). PKC phosphorylation of C2siRNAFAK<sup>wt</sup> cells decreased by 62.6±0.02 and 56±0.01% under the MF and MFI conditions, respectively (Fig. 4h, lane 6 vs lane 2, *p*<0.01 and lane 8 vs lane 4, *p*<0.01) without altering the protein level. The data confirm the involvement of PKC and suggest its



**Fig. 6** Effect of modulation of FAK on GSK-3β. Transfected and untransfected cells were differentiated in serum-free medium in the absence (MF) or chronic presence (MFI) of insulin for 3 days, and stimulated with or without insulin (100 nmol/l) for 10 min. **a** Cell lysates (30 μg) were subjected to western immunoblotting and probed with anti-phospho (Ser) GSK-3β antibody or anti-GSK-3β antibody. Bars represent relative densitometric values of pGSK-3β normalised to protein levels. Experiments were repeated three times. Values are mean±SE. \*\**p*<0.01 compared with lane 1; ##*p*<0.01 compared with

lane 2; §§*p*<0.01 compared with 8. **b** siRNA-transfected myotubes were lysed, and the lysates (30 μg) were subjected to western immunoblotting and probed with anti-phospho (Ser) GSK-3β antibody or anti-GSK-3β antibody. Bars represent relative densitometric values of pGSK-3β normalised to protein levels. Experiments were repeated three times. Values are mean±SE. \*\**p*<0.01 compared with lane 1; ##*p*<0.01 compared with lane 2; §§*p*<0.01 compared with lane 4. Open bars, MF; filled bars, MFI



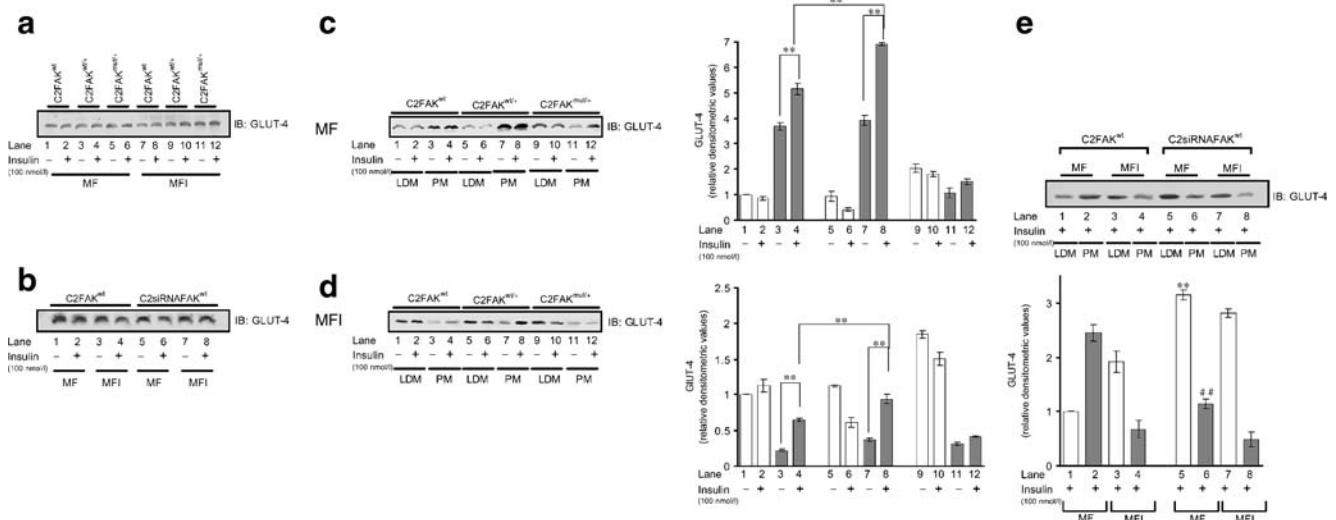
possible involvement in signal divergence in FAK-mediated insulin signalling.

To evaluate the contribution of PKC to glucose uptake, we downregulated the level of PKC by differentiating C2FAK<sup>wt</sup>, C2FAK<sup>wt/+</sup> and C2FAK<sup>mut/+</sup> cells under prolonged exposure (3 days) to 100 nmol/l phorbol 12,13-dibutyrate (PDBu) [4, 36]. PKC protein level (Fig. 5a) and phosphorylation (Fig. 5b) were completely inhibited under all the conditions examined. A significant decrease in insulin-stimulated glucose uptake was observed in all the cell lines treated with PDBu (Fig. 5c). Similar effects on PKC level/phosphorylation (Fig. 5d,e) and glucose uptake (Fig. 5f) were observed in C2siRNAFAK<sup>wt</sup> cells treated with PDBu. The data suggest that PKC is the downstream molecule responsible for FAK-mediated glucose uptake.

FAK was previously reported to regulate the activity of GSK-3 $\beta$  (a regulatory enzyme upstream of glycogen synthase) in hepatocytes, [7, 13]. To determine whether the observed effect of an increased level of FAK was accompanied by GSK-3 $\beta$  activation, insulin-stimulated Ser-9 phosphorylation of GSK-3 $\beta$  was examined. C2FAK<sup>wt/+</sup> cells increased insulin-stimulated GSK-3 $\beta$  phosphorylation under the MF condition (by 27.8 $\pm$ 0.05%; Fig. 6a, lane 4 vs lane 2,  $p$ <0.01), whereas phosphorylation was decreased in C2FAK<sup>mut/+</sup> cells (by 32.4 $\pm$ 0.03%; Fig. 6a, lane 6 vs lane 2,  $p$ <0.01) compared with insulin-resistant C2FAK<sup>wt</sup> cells (Fig. 6a, lane 6 vs lane 8). C2FAK<sup>wt/+</sup> cells showed increased (by 49.3 $\pm$ 0.03%) GSK-3 $\beta$  phosphorylation under the MFI condition (Fig. 6a, lane 10 vs lane 8,  $p$ <0.01), and the level of phosphorylation was similar to that observed in insulin-

sensitive C2FAK<sup>wt</sup> cells (Fig. 6a, lane 10 vs lane 2), whereas it was further decreased (by 21.5 $\pm$ 0.01%) in C2FAK<sup>mut/+</sup> cells (Fig. 6a, lane 12 vs lane 8,  $p$ <0.01). An acute decrease in GSK-3 $\beta$  phosphorylation was observed in C2siRNAFAK<sup>wt</sup> cells under the MF and MFI conditions (Fig. 6b, lane 6 vs lane 2,  $p$ <0.01 and lane 8 vs lane 4,  $p$ <0.01). No change in GSK-3 $\beta$  level was observed under any condition. The data suggest anabolism between enhanced glucose uptake and glycogen synthesis as a result of increased GSK-3 $\beta$  phosphorylation due to FAK overproduction.

Since no change in the level of GLUT4 was observed in any of the cell lines under any condition (Fig. 7a,b), insulin-stimulated translocation of GLUT4 was examined in subcellular fractions (LDM and plasma membrane). We observed a 32.0 $\pm$ 0.12% increase in GLUT4 translocation to the plasma membrane fraction of C2FAK<sup>wt/+</sup> cells under the MF condition (Fig. 7c, lanes 7 and 8 vs lanes 3 and 4), indicating enhanced insulin-stimulated GLUT4 translocation due to an increased level of FAK. In contrast, GLUT4 molecules remained in the LDM fraction in insulin-sensitive C2FAK<sup>mut/+</sup> cells (Fig. 7c, lanes 9 and 10 vs lanes 1 and 2). Under the MFI condition, maximum translocation was observed in C2FAK<sup>wt/+</sup> cells (Fig. 7d, lanes 7 and 8 vs lanes 3 and 4) and almost no translocation was observed in C2FAK<sup>mut/+</sup> cells (Fig. 7d, lanes 11 and 12 vs lanes 1 and 2). Insulin-stimulated incremental GLUT4 translocation was significantly impaired in C2siRNAFAK<sup>wt</sup> cells compared with C2FAK<sup>wt</sup>. In contrast to C2FAK<sup>wt</sup> cells, the maximum number of GLUT4 molecules in C2siRNAFAK<sup>wt</sup> cells remained in the LDM fraction under



**Fig. 7** Effect of modulation of FAK levels on GLUT4 translocation. Transfected and untransfected cells were differentiated in serum-free medium in the absence (MF) or chronic presence of insulin (MFI) for 3 days, then stimulated with or without insulin (100 nmol/l) for 10 min. **a, b** Cell lysates (50  $\mu$ g) of insulin-stimulated differentiated cells were subjected to western immunoblotting and probed with anti-GLUT4 antibody. **c–e** Cells were differentiated in MF or MFI medium

and then stimulated with 100 nmol/l insulin for 10 min. The LDM and plasma membrane fractions were isolated, subjected to western immunoblotting and probed with anti-GLUT4 antibody. Experiments were repeated three times. Values are mean $\pm$ SE. \*\* $p$ <0.01 compared with lane 1; ### $p$ <0.01 compared with lane 2. Open bars, LDM fraction; filled bars, plasma membrane fraction. IP, immunoprecipitated, IB, immunoblotted

the MF as well as under the MFI condition (Fig. 7e, lane 5 vs lane 1 and lane 7 vs lane 3). However, in the plasma membrane fraction there was a significant decrease in the translocation of GLUT4 in C2siRNAFAK<sup>wt</sup> cells compared with C2FAK<sup>wt</sup> cells, which was further decreased under the MFI condition (Fig. 7e, lane 6 vs lane 2 and lane 8 vs lane 4). These results confirm the regulation of glucose uptake via GLUT4 translocation in skeletal muscle cells *in vitro* by FAK.

## Discussion

In the present study a direct correlation was observed between FAK protein level/phosphorylation and glucose uptake in the presence of insulin, suggesting insulin-dependent regulation of insulin resistance in C2C12 skeletal muscle cells by FAK. Huang et al. [18] reported that downregulation of FAK affects only insulin-stimulated glucose uptake, unlike the observations in our study (Fig. 3b). One of the possible explanations for this could be that Huang et al. reported glucose uptake when there was 53% downregulation in the level of FAK, leaving approximately 47% of the endogenous FAK available, whereas in our study 85% of the FAK was downregulated, thus eliminating any significant contribution of the endogenous protein. Under our experimental conditions, the mechanism of FAK-mediated regulation of insulin resistance could be the interaction of FAK with unidentified substrate(s) that becomes activated only in the presence of insulin and is necessary for the interaction between FAK and insulin signalling molecules. The observed increase in tyrosine phosphorylation in the basal condition of overexpressed FAK might be due to activation by other stimuli.

Like us, Annabi et al. [31] and Huang et al. [14] also observed no interaction between FAK and the IR in their studies, using CHO and HepG2 cells. However, we observed that up- or downregulation of FAK activity correspondingly affected insulin-stimulated glucose uptake via the IRS-1/PI3K pathway. Therefore, our results indicate that FAK regulates insulin signalling upstream of IRS-1 and then regulates the cascade downstream via PI3K. Insulin signalling without involving the IR, via IRS-1, has been reported previously in other non-receptor tyrosine kinases, pp59Lyn in 3T3-adipocytes [37]. FAK is also reported to interact with cytosolic tyrosine kinase Src, which can regulate IRS-1 tyrosine phosphorylation in CHO cells [33]. In human embryo kidney cells, Lebrun et al. [33] have reported that FAK can phosphorylate IRS-1 without the participation of Src.

In our study, the mechanism whereby mutant FAK inhibits the ability of insulin to induce phosphorylation of Akt remains unclear. It is possible that endogenous FAK

interacts with unidentified protein(s) necessary for interaction with Akt. Mutant FAK possibly exerts a dominant negative effect on endogenous FAK. A similar dissociation between these two proteins has been reported previously in hepatocytes [14]. However, an increased level of FAK<sup>wt</sup> did not restore Akt phosphorylation under insulin-resistant conditions even though tyrosine phosphorylation of IRS-1 and PI3K activation was restored. A possible explanation includes additional regulation of Akt independently of PI3K in the insulin-resistant condition. Lin et al. [38] found that PKC activator decreased the ability of insulin to phosphorylate both Akt and GSK-3 $\beta$  in rat skeletal muscle, whereas PI3K activation was unaffected.

Decreased glucose uptake irrespective of an increased or decreased level of FAK under conditions of PKC inhibition indicates a markedly smaller contribution of Akt. Therefore, in the insulin-resistant condition the signal travels mainly through activated PI3K to PKC rather than to Akt. Earlier reports have suggested that phosphatidylinositol (3,4,5)-triphosphate (PIP3) produced by PI3K, responsible for both Akt and PKC phosphorylation, has more affinity for PKC than Akt [34]. Moreover, in the insulin-resistant condition PKC downregulates Akt and this is unaccompanied by a change in PI3K activity in skeletal muscle cells [37]. Therefore, it is possible that FAK differentially regulates insulin signalling under normal and insulin-resistant conditions. Sajan et al. [39] recently reported a differential role of the atypical PKC isoforms  $\zeta$  and  $\lambda$  in insulin-stimulated glucose transport. Regulation of GLUT4 translocation in skeletal muscle of high-fat-diet-fed rats by PKC ( $\zeta/\lambda$ ) has also been reported [40]. Therefore, there is a possibility of FAK-regulated activity of PKC  $\zeta/\lambda$ , or any other isoform(s), in regulating insulin resistance. Further studies are in progress to determine the specific isoform(s) that may be responsible for interacting with FAK to regulate the pathway.

It has been reported that modulation of FAK level in hepatocytes regulates glycogen synthesis [7, 14]. We also observed that up- or downregulation of FAK correspondingly phosphorylates GSK-3 $\beta$ , implying the regulation of glycogen synthesis by FAK in these cells.

Kotliar and Pilch [41] observed that the increased level of GLUT4 fails to reconstitute insulin-stimulated glucose transporter in C2C12 cells and postulated the requirement for additional gene(s) to form functional translocating vesicles. Microtubules have been reported to be required for the translocation of GLUT4 [18, 42–46]. FAK is required for the stabilisation of microtubules in fibroblasts [47] and its tyrosine phosphorylation is essential for the insulin-stimulated rearrangement of actin fibres in CHO/IR cells [10]. Huang et al. [18] reported that FAK regulates insulin-mediated cytoskeletal rearrangement essential for normal glucose transport and glycogen synthesis. There-

fore, the observed increase in glucose uptake as a result of increased FAK level with no change in expression in our study is possibly due to the enhanced recruitment of actin filament and microtubules responsible for the GLUT4 translocation. Further studies are in progress to demonstrate interaction between GLUT, microtubules and FAK.

In summary, the present study demonstrates a novel role for FAK as a positive regulator of the insulin signalling pathway, leading to glucose transport and insulin sensitisation. This conclusion is based on the following findings: (1) in the insulin-resistant condition FAK was always found to be less active; (2) downregulation of FAK activity resulted in impaired insulin signalling; (3) decreased glucose uptake was a consequence of impaired insulin signalling due to downregulation of FAK; and (4) increased level of FAK in the insulin-resistant condition reversed all the above-mentioned observations. The stimulatory effect of FAK on insulin-stimulated glucose transport involves the IRS-1/PI3K/PKC arm of the insulin signalling pathway. Interestingly, IR and Akt do not seem to contribute to the pathway. Since an increase in FAK and its phosphorylation sensitises C2C12 skeletal muscle cells to insulin stimulation of GLUT4 translocation and glucose transport, these results further support a role for an insulin-stimulated FAK/IRS-1/PI3K/PKC signalling pathway as a physiologically important mediator of the action of insulin. Since increased levels of FAK leads to cellular insulin sensitisation, these results also raise the possibility that interfering with FAK production or activation may provide opportunities for developing new therapeutic strategies against insulin resistance. Chemical activators of FAK would be expected to act as insulin sensitisers, which could have beneficial effects in insulin resistance induced by hyperinsulinaemia. This opens up the possibility of new therapeutic intervention.

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