In vivo inhibition of focal adhesion kinase causes insulin resistance

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Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, has recently been implicated in the regulation of insulin resistance *in vitro*. However, its *in vivo* validation has not been attempted due to lethality of FAK knockout. Hence, to ascertain the role of FAK in the development of insulin resistance *in vivo*, we have down-regulated FAK expression by delivering FAK-specific small interfering RNA (siRNA) in mice using hydrodynamic tail vein injection. Here, we show for the first time that FAK silencing ($57 \pm 0.05\%$ in muscle and $80 \pm 0.08\%$ in liver) exacerbates insulin signalling and causes hyperglycaemia (251.68 ± 8.1 mg dl^{-1}) and hyperinsulinaemia (3.48 ± 0.06 ng ml^{-1}) *in vivo*. FAK-silenced animals are less glucose tolerant and have physiological and biochemical parameters similar to that of high fat diet (HFD)-fed insulin-resistant animals. Phosphorylation and expression of insulin receptor substrate 1 (IRS-1) was attenuated by $40.2 \pm 0.03\%$ and $35.2 \pm 0.6\%$ in muscle and $52.3 \pm 0.04\%$ and $40.2 \pm 0.03\%$ in liver in FAK-silenced mice. Akt-Ser473-phosphorylation decreased in muscle and liver ($50.3 \pm 0.03\%$ and $70.2 \pm 0.02\%$, respectively) in FAK-silenced mice. This, in part, explains the mechanism of development of insulin resistance in FAK-silenced mice. The present study provides direct evidence that FAK is a crucial mediator of insulin resistance *in vivo*. Considering the lethality of FAK gene knockout the approach of this study will provide a new strategy for *in vivo* inhibition of FAK. Furthermore, the study should certainly motivate chemists to synthesize new chemical entities for FAK activation. This may shed light on new drug development against insulin resistance.

Insulin resistance of target organs, principally liver and muscle, is a major feature of the pathological manifestation associated with diabetes (Le Roith & Zick, 2001; Petersen & Shulam, 2006). Skeletal muscle accounts for 80% of total glucose disposal under insulin-stimulated conditions (Ryder *et al.* 2001). Defects in skeletal muscle insulin action precede clinical diagnosis of insulin resistance (Kruszynska & Olefsky, 1996). Focal adhesion kinase (FAK) is a cytoplasmic, non-receptor tyrosine-phosphorylated protein kinase present within focal adhesion contact sites in normal cells (Schlaepfer & Mitra, 2004). We have recently reported that modulation of FAK expression regulates insulin sensitivity of skeletal muscle cells (Bisht *et al.* 2007). The activity of FAK is abnormally decreased in insulin resistant skeletal muscle cells and inhibition of

FAK expression interferes with insulin action. In addition, recently Huang *et al.* (2006) has reported that FAK regulates insulin-mediated cytoskeletal rearrangement essential for normal glucose transport and glycogen synthesis in skeletal muscle cells. The role of FAK in regulation of glycogen synthesis in HepG2 cells and hepatic insulin signalling *in vitro* has also been reported (Cheung *et al.* 2000; Huang *et al.* 2002). Nevertheless, in order to establish the role of FAK as such in the *in vivo* situation, knowledge gained from *in vitro* studies needs to be extended to examine FAK action in animal models. Thus, considering the role of FAK in hepatocytes and skeletal muscle cells *in vitro*, we sought to determine whether FAK plays a crucial role in the development of insulin resistance *in vivo*. In this study, we demonstrate that FAK functions as a positive regulator of insulin action as inhibition of FAK causes impaired insulin signalling resulting to whole body insulin resistance generation.

This paper has online supplemental material.

The study therefore established a novel role of FAK in the regulation of glucose homeostasis *in vivo* for the first time and provides opportunities for developing new therapeutic strategies against insulin resistance.

Methods

Animals

Male Swiss albino mice, 5 weeks old, weighing 10–11 g, were procured from a central animal facility of the institute. The animals were randomly divided into four groups based on body weight, which were not significantly different. First group $(n = 6)$ was untreated control; the second group was subjected to injection of saline only;

the third group was injected with scrambled siRNA (in saline); and the fourth group was injected with siRNA (in saline) of FAK. The animals were kept in controlled environmental conditions (room temperature $22 \pm 2°C$, humidity $55 \pm 5\%$ and 12 h light–dark cycles). Mice were fed with commercially available rodent pellet diet (Pranav Agro Industries, New Delhi, India) and water was provided *ad libitum*. Body weight, food intake and water intake were monitored every 3 days for 2 weeks. Food was pre-weighed before feeding and the leftover feed was weighed 3 days after. Mice were killed by cervical dislocation. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and the animal experiments were carried out in accordance with guidelines of the Committee for the Purpose of Control
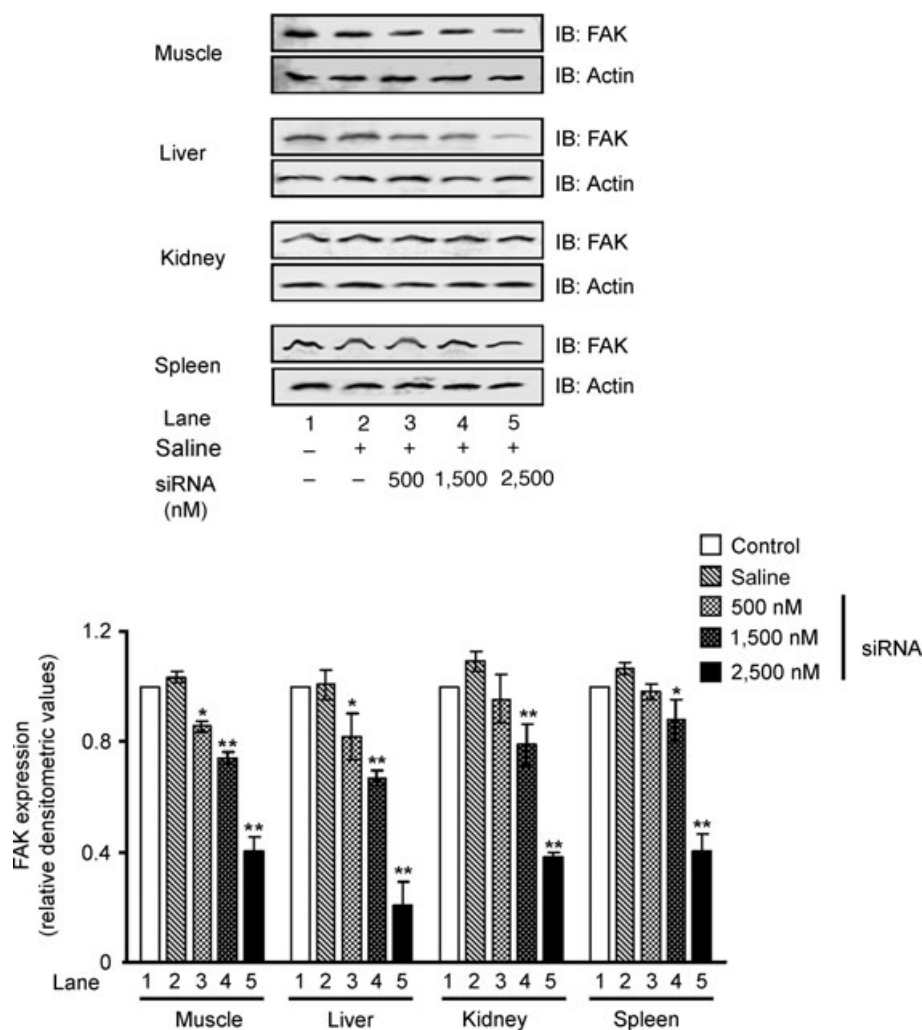
Figure 1. Inhibition of FAK expression by siRNA
Effect of dose on the interfering efficiency of siRNA: male mice (Swiss albino; 10–11 g) were injected with various amounts of siRNA in 1.2 ml saline within 5 s. The level of FAK expression was determined 24 h after injection in muscle, liver, kidney and spleen. Upper panel: Western immunoblots for FAK, stripped and reprobed with actin. Lower panel: relative densitometric values of FAK expression normalized to the amount of protein loaded. Data are shown as means $\pm$ S.E.M. ($n = 5$). *$P < 0.05$, **$P < 0.01$. IB, immunoblotting.

and Supervision of Experiments on Animals (CPCSEA), Government of India.

siRNA treatment *in vivo*

siRNA was delivered into mice using a hydrodynamic tail-vein injection method, by which 2500 nM siRNA dissolved in 1.2 ml of saline were rapidly injected into the tail vein (Liu *et al.* 1999). Swiss albino mice (Ghosh *et al.* 1994; Brown *et al.* 2000) weighing 10–11 g were injected by a hydrodynamic-based procedure where increasing amounts of siRNA (500, 1500 and 2500 nM), against the FAK targeted sequence 5′-TGCAATGGA-ACGAGTATTAAA-3′, dissolved in 1.2 ml of saline were injected into the tail-vein over less than 5 s. siRNA-treated and control mice of similar body weights were monitored under the same conditions until the end of the experiments.

Preparation of tissue homogenates and Western immunoblotting

Animals were humanely sacrificed and various organs (liver, skeletal muscle, kidney and spleen) were collected.

Tissues were washed with ice-cold PBS and lysed in lysis buffer as previously described (Goel & Dey, 2000). Homogenates were centrifuged at 16 000 g at 4°C for 30 min. Protein estimations were performed using the Bicinchoninic Acid Kit (Sigma Chemical Company, USA) as per manufacturer's instructions. Lysates were boiled with Laemmli sample buffer (Tris-HCl 62.5 mM, pH 6.7, glycerol 10% (v/v), SDS 2% (w/v), bromophenol blue 0.002% (w/v) containing β-merceptoethenol 143 mM) for 5 min, resolved by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane. Membranes were blocked with BSA (5%) and incubated with the indicated primary antibodies for 12–16 h, followed by 1 h incubation with alkaline phosphatase-conjugated secondary antibody. The protein bands were visualized with 5-Bromo-4-chloro-3-indolol-phosphate/4-Nitro blue tetrazoilum chloride (BCIP/NBT) as substrates.

Immunoprecipitation

Tissues were lysed and the lysates were clarified by centrifugation at 60 000 g for 10 min at 4°C. Protein (300–500 μg) was immunoprecipitated with primary
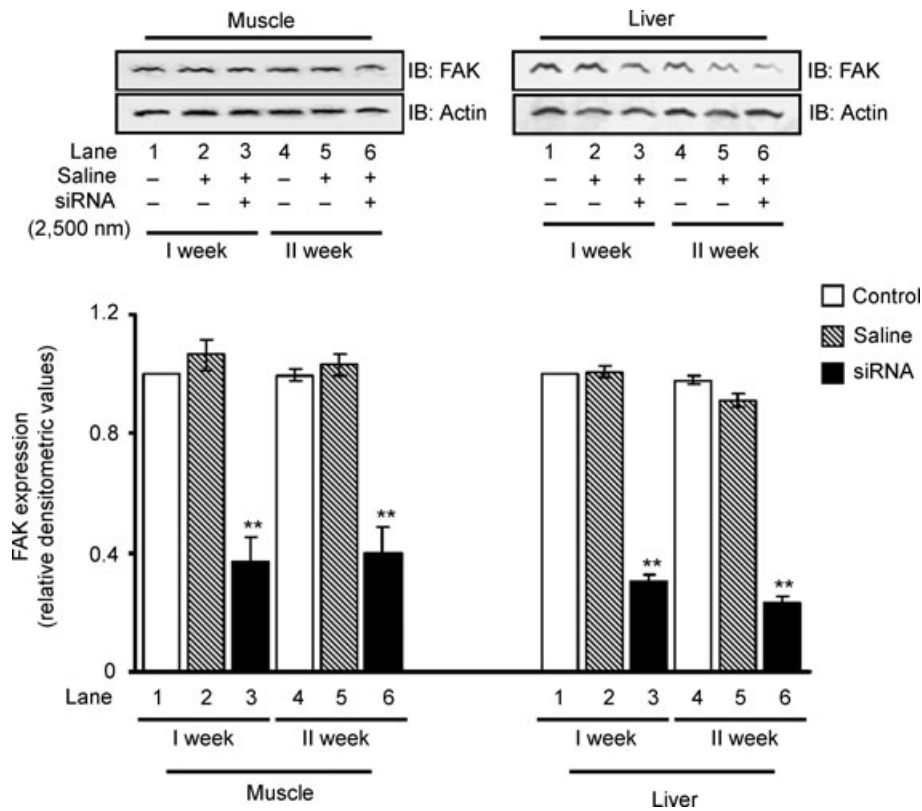
Figure 2. RNA interference at different time points
Three groups of mice (10–11 g) were injected with 2500 nM siRNA in 1.2 ml saline. Animals were sacrificed at first and second week after injection. Expression of FAK in the muscle and liver were examined. Upper panel: Western immunoblots of FAK stripped and reprobed with actin. Lower panel: relative densitometric values of FAK expression normalized with the amount of protein loaded. Data are shown as means ± S.E.M. ($n = 5$). **$P < 0.01$. IB, immunoblotting.

antibody and protein A agarose beads by incubating overnight at 4°C. Precipitates were washed 3 times with lysis buffer, supernatants were removed and pellet was resuspended in SDS-PAGE sample buffer. Proteins were separated in SDS-PAGE.

Biochemical measurements

Blood samples were collected from tail vein/retro orbital sinus into heparinized ($200\ IU\ ml^{-1}$) tubes and immediately centrifuged at $1000\ g$ for 10 min for the separation of plasma. Plasma was stored at $-20°C$ until

assayed. Plasma was used for estimation of glucose (AutoZyme blood glucose kit, Accurex Biomedical, India), triglyceride (Sigma) and insulin (mouse insulin ELISA kit, Linco research, St Charles, MO, USA) and TNF-α (mouse TNF ELISA Kit, BD Biosciences, San Diego, CA, USA) by commercial kits as per manufacturer's instructions.

Glucose tolerance test

All the animals were fasted overnight prior to performing glucose tolerance tests at the end of 2 weeks. Blood samples were collected at 0, 30, 60 and 120 min
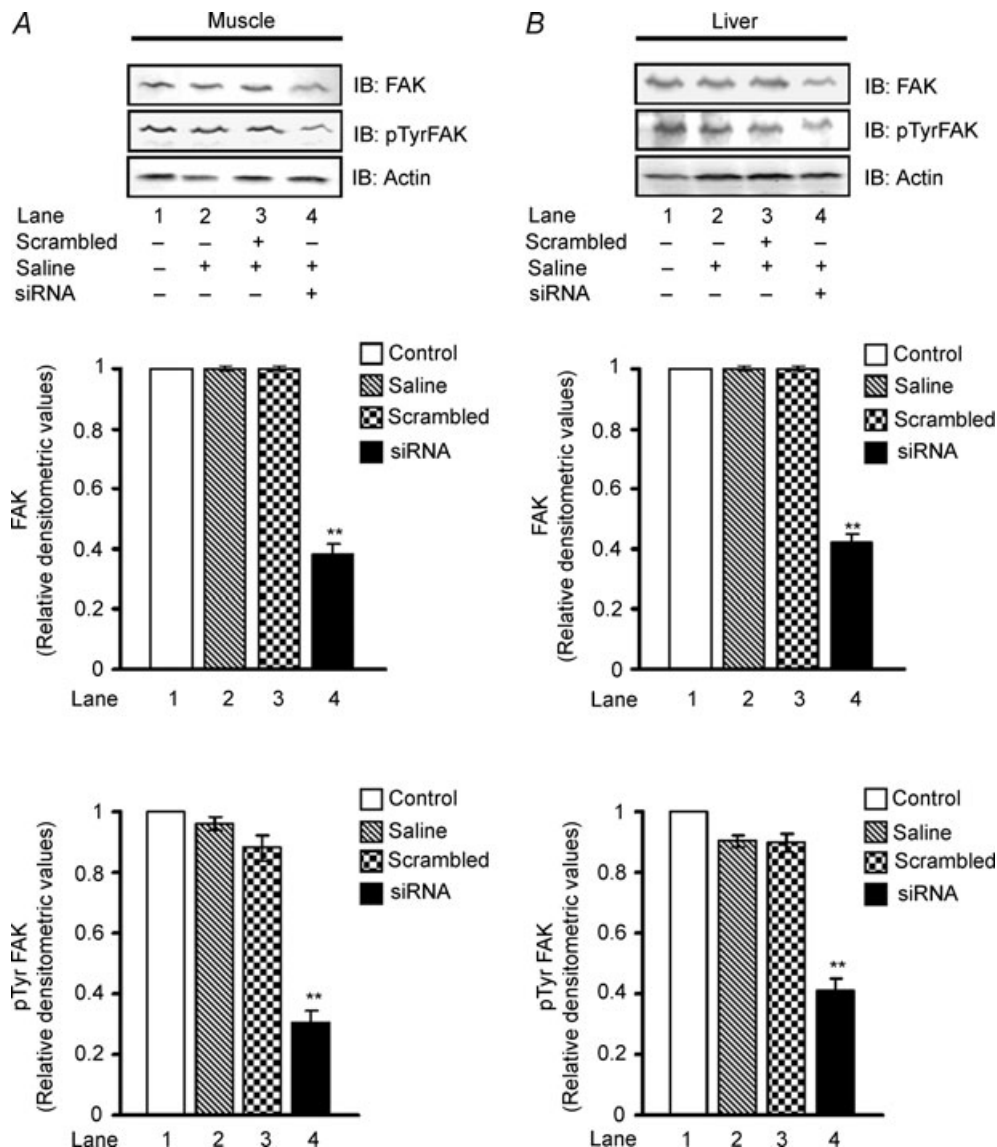
Figure 3. Specificity of FAK-specific siRNA
Four groups of mice (10–11 g) were injected with 2500 nM of FAK-specific and scrambled siRNA in 1.2 ml saline. Animals were killed after the second week of injection and were examined for FAK expression and tyrosine phosphorylation in the muscle (A) and liver (B). Upper panel: Western immunoblots of FAK stripped and reprobed with pTyr FAK and actin. Middle panel: relative densitometric values of FAK expression normalized with the amount of protein loaded. Lower panel: relative densitometric values of FAK phosphorylation normalized to the amount of protein loaded. Data are shown as means ± S.E.M. ($n = 5$). **$P < 0.01$. IB, immunoblotting.

after oral administration of glucose $(2\,\mathrm{g\,kg^{-1}}\,(10\,\mathrm{ml})^{-1})$ for the determination of plasma glucose and insulin concentration.

Densitometric analysis

Densitometric analyses of Western immunoblots were performed using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA) equipped with Quantity One 1-D analysis software. The relative values of samples were determined by giving

an arbitrary value of 1.0 to the respective control samples of each experiment, keeping background value as 0.

Statistical analysis

Data are expressed as mean ± S.E.M. For comparison of two groups, P values were calculated by two-tailed Student's unpaired t test. Differences over time (glucose tolerance test (GTT) and time course), i.e. more than two groups, were compared by two-way ANOVA. In all cases, $P < 0.05$ was considered to be statistically significant.
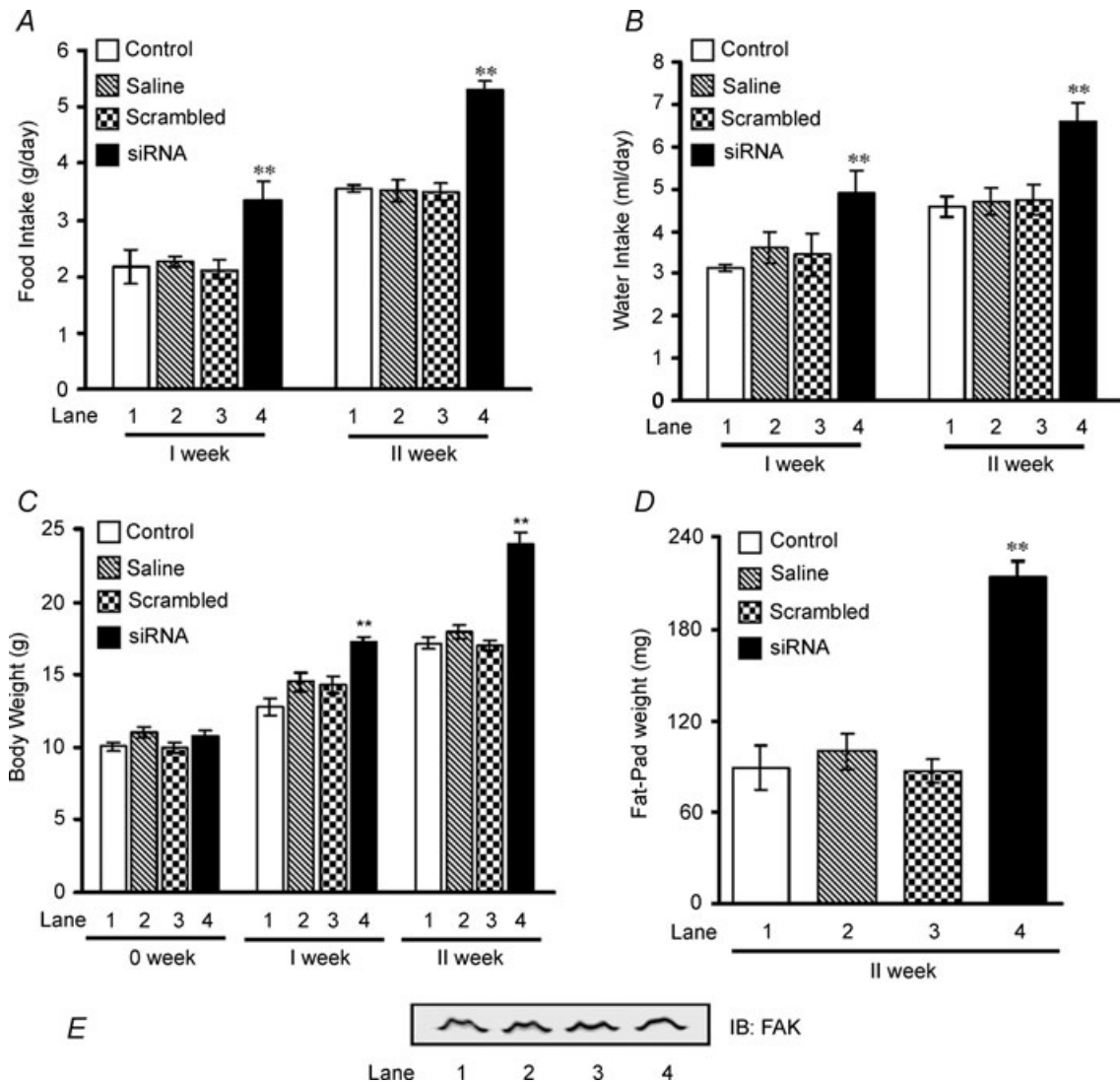
Figure 4. Insulin resistance in FAK-silenced mice
Male mice (Swiss albino; 10–11 g) were injected with 2500 nM of siRNA on day zero and then divided into two groups. The first groups of animals were sacrificed at the appropriate time (after 1 week) without receiving an additional injection. The rest of the animals received an additional injection of the same amount of siRNA on day 6 and were sacrificed 1 week there after (2 week). Effect of FAK silencing on food intake (*A*), water intake (*B*), body weight (*C*), epididymal fat-pad weight (*D*) and expression of FAK in adipose tissue (*E*) after 2 weeks of siRNA injection. Lane 1, control; lane 2, saline; lane 3, scrambled; and lane 4, siRNA. Data are shown as means ± S.E.M. ($n = 6$). **$P < 0.01$.

Results

In vivo silencing of FAK expression using siRNA

In order to reveal the role of FAK in the development of insulin resistance, FAK expression was silenced using the RNA interference (RNAi) approach. At first we determined the concentration of siRNA needed to induce RNAi *in vivo*. The expression of FAK was examined in major insulin target organs (liver, muscle, kidney and spleen) after 24 h of injection. A dose-dependent inhibition of FAK expression was observed with maximum inhibition in muscle ($57 \pm 0.05\%$) and liver ($80 \pm 0.08\%$) in mice treated with 2500 nM siRNA (Fig. 1, lane 5 *versus* lane 1, $P < 0.01$, respectively). FAK expression was also found to be significantly inhibited in other organs (Fig. 1). No significant inhibition was observed in mice injected

with vehicle alone (Fig. 1, lane 2 *versus* lane 1). No change in expression was observed in an unrelated protein, actin, under the same conditions (Fig. 1). As significant inhibition was observed at 2500 nM, a higher concentration of siRNA was not used to avoid pleotropic effects, as it would result in a FAK null lethal condition. Results indicate a dose-dependent inhibition of FAK expression by hydrodynamic tail vein injection of FAK siRNA.

Longevity of RNA interference induced by FAK-specific siRNA

To determine the longevity of RNAi induced by siRNA, mice were injected with 2500 nM of siRNA per animal on
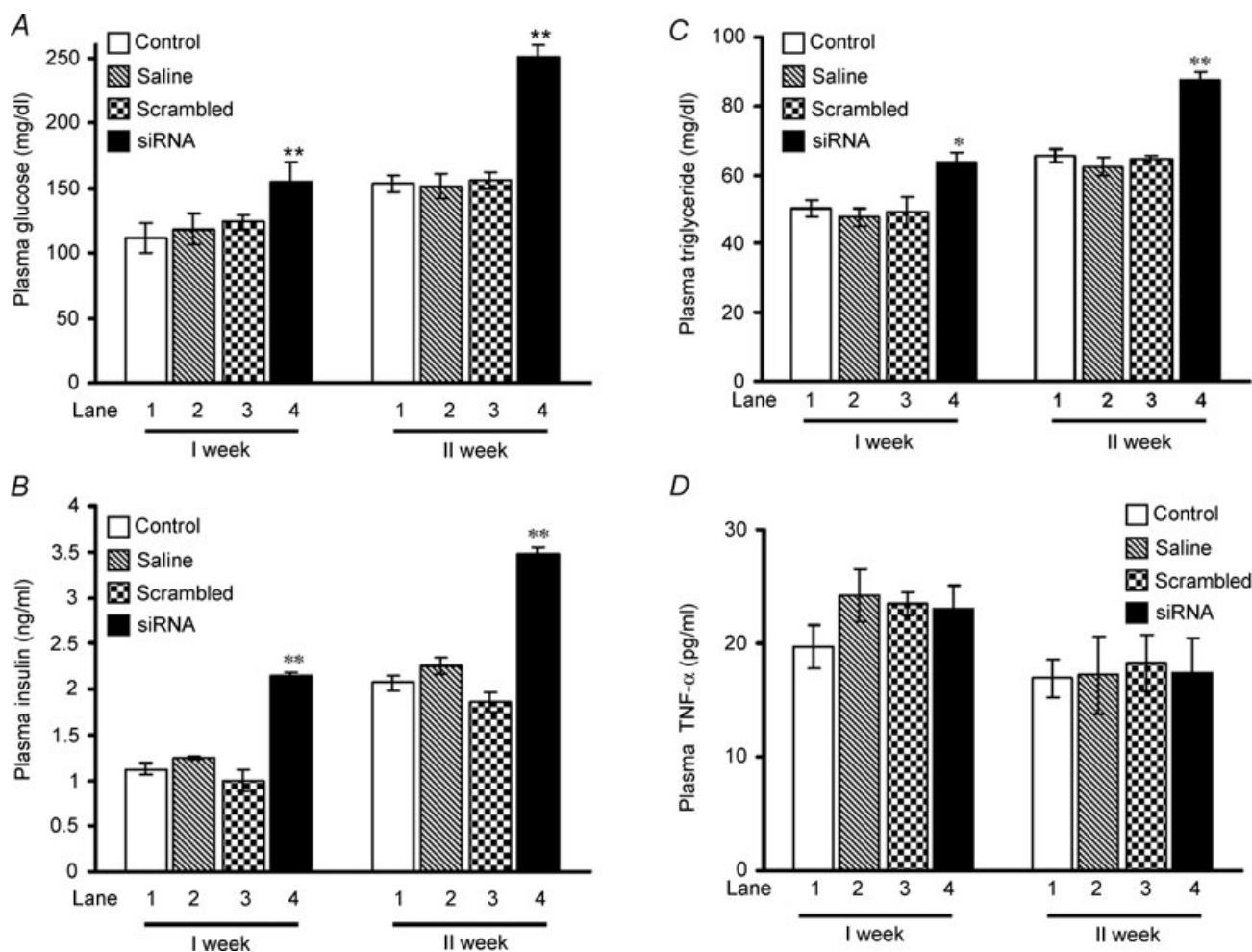
Figure 5. Effect of FAK silencing on insulin resistance generation in FAK-silenced mice
Male mice (Swiss albino; 10–11 g) were injected with 2500 nM of siRNA on day zero and then divided into two groups. The first groups of animals were sacrificed after 1 week without receiving an additional injection. The rest of the animals received an additional injection of the same amount of siRNA on day 6 and were killed 1 week there after (2 week). Biochemical measurements of plasma glucose (*A*), plasma insulin (*B*), plasma triglyceride (*C*) and plasma TNF-α (*D*) in mice that had been treated with 2 injections of FAK siRNA at a weekly interval for 2 weeks. Data are shown as means ± s.e.m. (*n* = 6). *$P < 0.05$, **$P < 0.01$.

day zero. The groups were sacrificed at different days (as indicated) up to 1 week, after injection, with five mice per time point. FAK expression remained significantly silenced up to 6 days after injection in muscle and liver of mice (Supplementary Fig. 1, lane 7 *versus* lane 1); however, expression started restoring after 6 days (data not shown). Data indicate that the effect of RNAi significantly persists for 6 days after siRNA delivery.

However, to determine whether expression of FAK can be kept down-regulated by repeated treatment of siRNA, the sixth day after the first injection, a second injection was given and the expression of FAK was studied. Data showed that FAK expression remained inhibited as a result of the second injection of siRNA in liver and muscle (Fig. 2). Data demonstrate that FAK silencing *in vivo* in insulin-sensitive tissues remained up to at least 2 weeks by repeated injections.

In order to determine the specificity of FAK siRNA, similar injections (2500 nM) were given to animals for 2 weeks with non-specific scrambled siRNA (5′-CGUACGCGGAAUACUUCGATT-3′, 5′-UCGAA-GUAUUCCGCGUACGTT-3′) and were examined for FAK expression in muscle and liver. No change in FAK expression was observed in animals injected with scrambled siRNA against the inhibition observed in animals injected with FAK specific siRNA (Fig. 3*A* and *B*; lane 3 *versus* lane 1). Further, we investigated whether the reduced protein concentration is associated with reduced activity of the kinase. The tyrosine phosphorylation of FAK was examined after 2 weeks of FAK-specific and scrambled siRNA injection. A decreased tyrosine phosphorylation of FAK was observed in both muscle and liver of FAK-silenced mice, concomitant to its expression (Fig. 3*A* and *B*, lane 4 *versus* lane 1). No significant inhibition of FAK was observed in animals injected with scrambled siRNA indicating specificity of FAK silencing (Fig. 3*A* and *B*, lane 3 *versus* lane 1).

In vivo silencing of FAK expression affects physiological parameters of FAK-silenced animals

To investigate the effect of *in vivo* FAK silencing on development of whole body insulin resistance, two groups of mice ($n = 6$) were injected with 2500 nM of FAK-specific or scrambled siRNA per animal on day zero. The first group ($n = 6$) was sacrificed after 6 days. The second group ($n = 6$) was injected with the same amount of siRNA for the second time on day 6. After 2 weeks, mean food and water intake of FAK-silenced mice was significantly more ($51.1 \pm 0.28\%$ and $46.6 \pm 0.20\%$, $P < 0.01$, respectively) than their control counterparts (Fig. 4*A*, lane 4 *versus* lane 1 and Fig. 4*B*, lane 4 *versus* lane 1). Up to the first week of FAK silencing, mice gained $35.8 \pm 0.75\%$ more weight than controls (Fig. 4*C*, lane 4 *versus* lane 1, $P < 0.01$),

which further increased to $40.3 \pm 1.75\%$ by the end of the second week (Fig. 4*C*, lane 4 *versus* lane 1, $P < 0.01$). No change in food and water intake or body weight was observed in animals injected either with saline (Fig. 4*A*, *B* and *C*, lane 2 *versus* 1) or with scrambled siRNA (Fig. 4*A*, *B* and *C*, lane 3 *versus* 1) as compared to normal control group.

We next assessed whether differences in weight gain were related to alterations in adiposity. The epididymal fat-pad weights, which are highly representative of body fat, measured at 2 weeks, were higher in the FAK-silenced group (Fig. 4*D*) than controls and could also be visualized morphologically (Supplementary Fig. 2). To examine the possibility, if any, of FAK silencing in adipose tissue as a
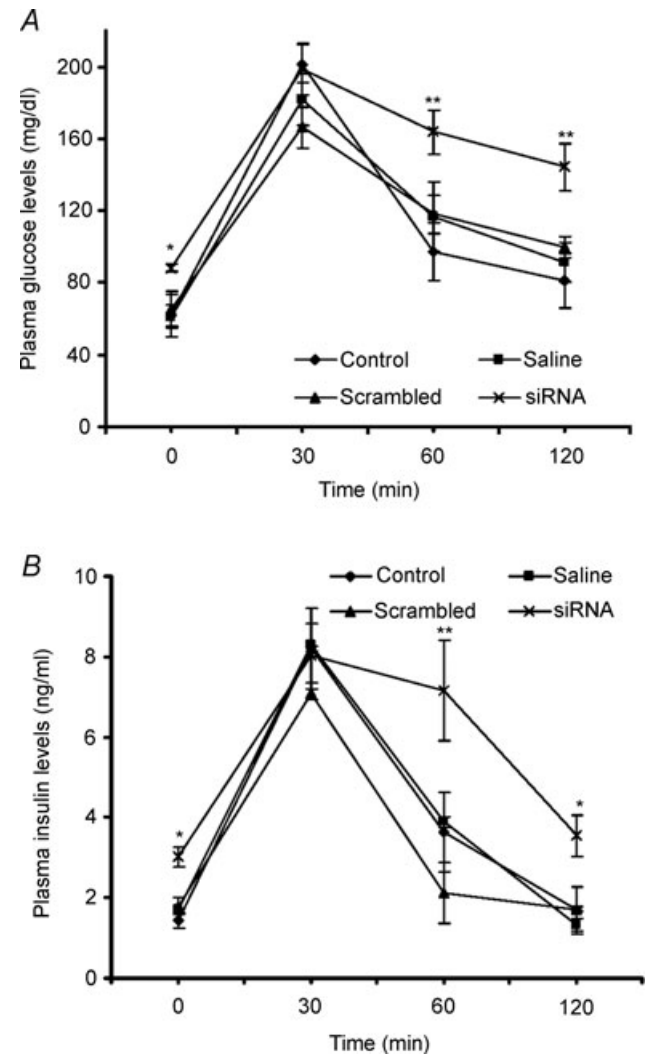
Figure 6. Effect of FAK silencing on glucose tolerance
Male mice (Swiss albino, 10–11 g weight) were injected with 2500 nM of siRNA for 2 weeks. The glucose tolerance tests (GTT) were performed after 16 h of starvation. (*A*), blood plasma glucose levels after glucose administration at different time intervals. (*B*), blood plasma insulin levels after glucose administration at different time intervals. Data are shown as means ± S.E.M. ($n = 6$). *$P < 0.05$, **$P < 0.01$.

result of siRNA injection, FAK expression was examined in adipose tissues. No change in FAK expression was observed in epididymal adipose tissue as a result of FAK silencing (Fig. 4*E*). Data indicate an increase in body weight of mice due to increased fat mass resulting from enhanced food intake.

It has been postulated that a high level of triglycerides in the circulation due to peripheral insulin resistance leads to fatty liver (Nagle *et al.* 2007). Examination of liver from FAK-silenced mice reveals marked hepatomegaly (Supplementary Fig. 3*A*), concomitant with increased liver weight (Supplementary Fig. 3*B*). The observation supports development of fatty liver; however, further studies are warranted. Thus, observations suggest plausibility of generation of insulin resistance in FAK-silenced mice.

In vivo silencing of FAK expression affects biochemical parameters of FAK-silenced animals

Insulin resistance is characterized by hyperglycaemia, due to reduced glucose utilization, and compensatory hyperinsulinaemia. Hence, we next examined the role of endogenous FAK in glucose homeostasis. A significantly higher plasma glucose concentration ($64.1 \pm 5.0\%$) and

plasma insulin level ($68.3 \pm 0.12\%$) was observed in FAK-silenced mice than control littermates after the second week of siRNA injection (Fig. 5*A*, lane 4 *versus* lane 1, $P < 0.01$ and Fig. 5*B*, lane 4 *versus* lane 1, $P < 0.01$) suggesting the development of hyperglycaemia and hyper-insulinaemia. Increased body weight with adiposity and the existence of mild hyperglycaemic levels along with hyperinsulinaemia in siRNA-treated mice clearly indicates development of insulin resistance as a consequence of FAK silencing. According to Randel's glucose–fatty acid cycle, the body favours excess lipid stores to glucose for metabolic oxidation in insulin resistance (Randel *et al.* 1963). To further establish this fact, the triglyceride levels in blood plasma of FAK-silenced mice were examined. The triglyceride level of FAK-silenced mice exhibited a $3.5 \pm 0.11\%$ increase as compared to controls (Fig. 5*C*, lane 4 *versus* lane 1, $P < 0.01$). No significant change in plasma glucose, insulin or triglyceride levels in animals injected with saline (Fig. 5*A*, *B* and *C*, lane 2 *versus* 1) or with scrambled siRNA (Fig. 5*A*, *B* and *C*, lane 3 *versus* 1) was observed. Data indicate that due to the inability of peripheral tissue to undertake glucose uptake, triglyceride was stored in the form of fat that was later mobilized to provide energy to these tissues. Data prove FAK silencing causes insulin resistance.
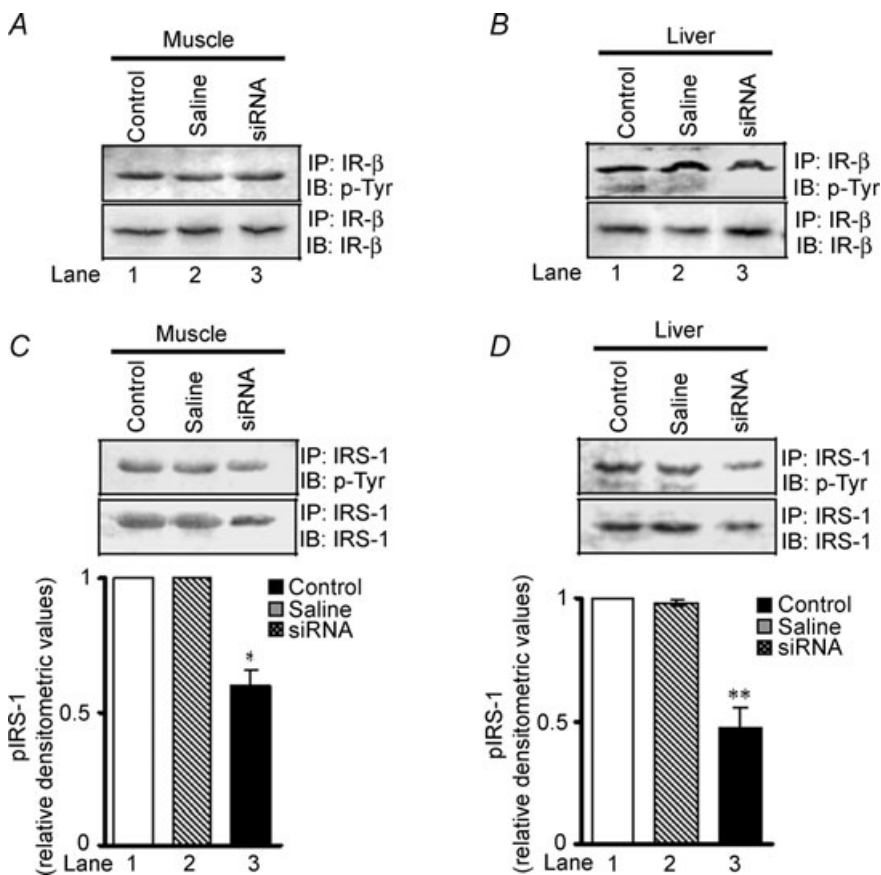
Figure 7. Effect of FAK silencing on insulin signalling
After treatment with FAK siRNA, cell extracts were obtained from insulin target tissues (muscle and liver) in mice; then expression and phosphorylation of IR-β (*A* and *B*), IRS-1 (*C*, *D*, *E* and *F*) and Akt (*G* and *H*) in muscle and liver after second week of injection were measured. Akt blots were stripped and reprobed with actin. Upper panels: IR-β phosphorylation and expression in muscle and liver. Middle panels: IRS-1 phosphorylation in muscle and liver. Lower panels: IRS-1 expression in muscle and liver. Data are shown as means $\pm$ S.E.M. ($n = 6$). *$P < 0.05$, **$P < 0.01$. IP, immunoprecipitated; IB, immunoblotted.

In order to investigate the possibility that some of the observed effects in FAK-silenced mice were due to the inflammation caused by siRNA injections, we measured the level of tumour necrosis factor (TNF)-α, a pro-inflammatory cytokine and an important contributor to the development of insulin resistance (Shoelson *et al.* 2006; Solinas *et al.* 2007) under FAK-silenced conditions. No significant change in the level of plasma TNF-α was observed under any conditions tested (Fig. 5*D*), thus eliminating the possibility of inflammatory responses.

In vivo silencing of FAK expression results in impaired insulin sensitivity

To determine whole body sensitivity to insulin in FAK-deficient mice, the glucose tolerance test (GTT) was carried out. GTT revealed that mice deficient in FAK were moderately glucose intolerant as compared to controls. The plasma glucose and corresponding insulin levels remained significantly elevated at 60 min (139.5 mg dl^{-1}, $P < 0.01$ and 7.1 ng ml^{-1}, $P < 0.01$) and

Figure 7. *Continued.*
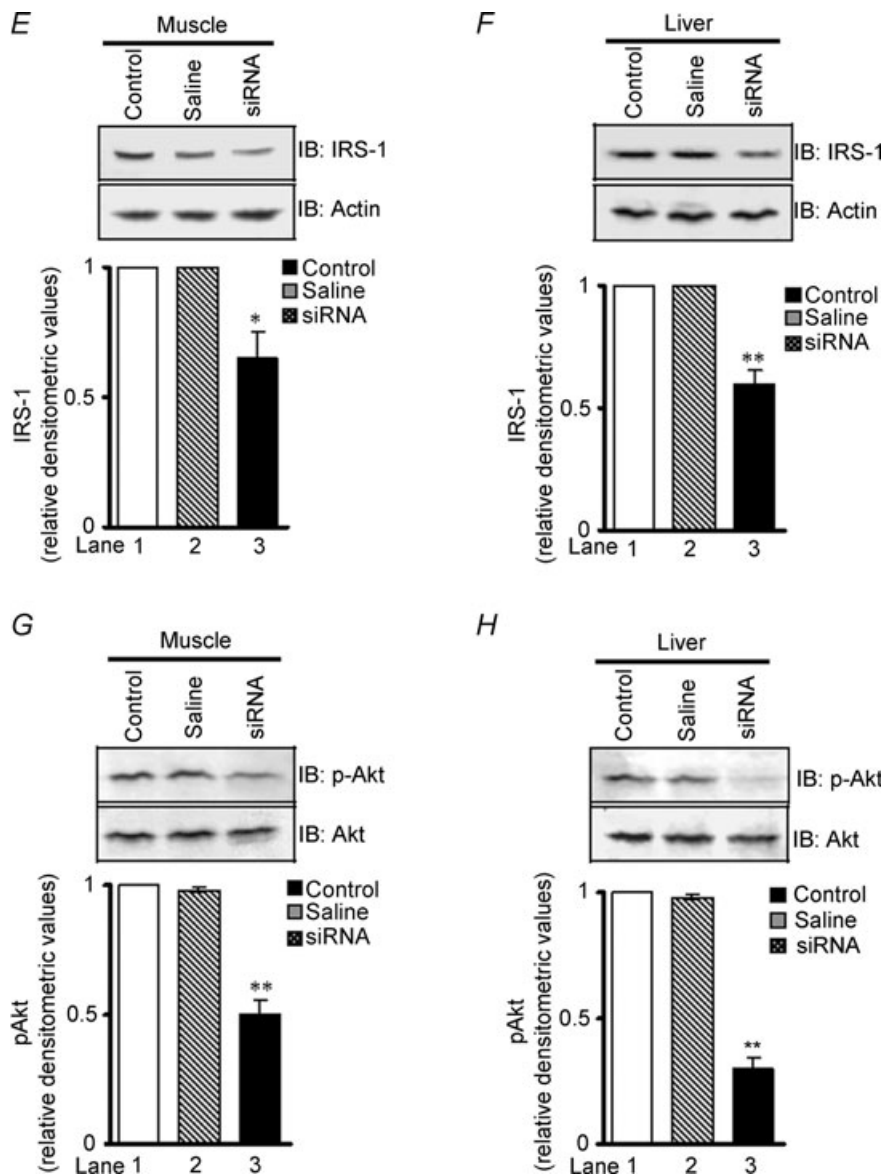
120 min (106.04 mg dl^{-1}, $P < 0.01$ and 3.55 ng ml^{-1}, $P < 0.05$) post-glucose administration as compared to control levels (Fig. 6*A* and *B*). Fasting glucose and insulin levels in FAK-silenced mice before a glucose challenge at 0 min were observed to be significantly elevated as compared to control ($P < 0.05$). However, no significant differences were observed in animals injected with either saline or scrambled siRNA (Fig. 6*A* and *B*) as compared to controls.

To determine the possible pathway of FAK-regulated insulin resistance, we evaluated insulin receptor-β (IR-β) tyrosine phosphorylation and expression in muscle and liver, of control and FAK-silenced mice. No significant change in activation and expression of IR-β due to down-regulation of FAK was observed (Fig. 7*A* and *B*, upper panels). This observation correlates with a previous report that suggests FAK interacts with IRS-1 but not with IR (Annabi *et al.* 2001). Therefore, IRS-1 tyrosine phosphorylation and expression was examined. Phosphorylation of IRS-1 was attenuated by $40.2 \pm 0.03\%$ in muscle (Fig. 7*C*, lane 3 *versus* 1, $P < 0.05$, middle panel) and $52.3 \pm 0.04\%$ in liver (Fig. 7*D*, lane 3 *versus* 1, $P < 0.01$, middle panels) in FAK-silenced mice as compared with controls. Interestingly, expression of IRS-1 was found to be decreased by $35.2 \pm 0.6\%$ and $40.2 \pm 0.03\%$ in muscle and liver, respectively (Fig. 7*E*, lane 3 *versus* 1, $P < 0.05$ and Fig. 7*F*, lane 3 *versus* 1, $P < 0.01$, lower panels). Concomitantly, in FAK-silenced mice a decrease in Akt-Ser473-phosphorylation was seen in both muscle and liver ($50.3 \pm 0.03\%$ and $70.2 \pm 0.02\%$, lane 3 *versus* 1, $P < 0.01$, respectively) compared with control mice (Fig. 7*G* and *H*).

Discussion

In the present study we have reported a novel role of FAK in the development of insulin resistance *in vivo*. Novelty of the study is that significant inhibition of FAK expression in liver and muscle results in whole body insulin resistance.

Knockout studies have proved very useful to break down pathophysiology and genetics of insulin resistance (Mauvais-Jarvis *et al.* 2002; Kahn, 2003). Unfortunately, FAK-deficient mice are embryonicaly lethal (Zachary, 1997), which precludes the study of a role of FAK in the mammalian insulin signalling pathway in a whole animal setting. Recently, an endothelial cell (EC)-specific knockout of FAK using a Cre-loxP approach has been reported (Shen *et al.* 2005). Hakim *et al.* (2007) has also reported another conditional knockout of FAK in *nkx2–5*-expressing cells 'termed FAK^{nk} mice'. However, FAK^{nk} mice died shortly after birth. In contrast to total FAK knockouts, conditional knockouts to some extent facilitate the exploration of the unaddressed issues regarding FAK. However, due to lethality of FAK-null mice understanding its biological functions, activation and its interactions with

other signalling molecules *in vivo* remains unaddressed and offers an exciting challenge.

RNAi has been used *in vivo* for target validation studies (Dorsett & Tuschl, 2004; Zhou *et al.* 2004). This allows the transient knockdown of lethal genes that would otherwise prevent the investigation of their functions in postnatal animals and also prevent pleiotropic side-effects that could occur in global-knockout animals. Therefore, to elucidate the biological function of FAK in the development of insulin resistance, the RNAi approach has been used to transiently knock-down the lethal FAK gene in mice by hydrodynamic tail vein injection. After McCaffrey *et al.* (2002) provided the first proof-of-concept for gene silencing by siRNAs and shRNAs *in vivo*, several reports have appeared using this technique to deliver siRNA to various organs in mice and rats for more than half a decade now (Liu *et al.* 1999; Yang *et al.* 2002; Xia *et al.* 2002; Dagnæs-Hansen *et al.* 2002; McCaffrey *et al.* 2002; He *et al.* 2004; Braasch *et al.* 2004; Bradley *et al.* 2005; Xu *et al.* 2005; Dykxhoorn *et al.* 2006; Zhu *et al.* 2006; De Souza *et al.* 2006; Lewis & Wolff, 2007; Fukushima *et al.* 2007). The technique is elegant and effective and does not require any specialized equipment or surgical expertise. The hydrodynamic tail vein injection has been used successfully in mouse models for various metabolic diseases (Dagnæs-Hansen *et al.* 2002; He *et al.* 2004; Xu *et al.* 2005; Zhu *et al.* 2006; De Souza *et al.* 2006; Lewis & Wolff, 2007). Bradley *et al.* (2005) reported successful introduction of siRNA against the insulin 2 (Ins2) gene directly into pancreatic islets cells. This may be advantageous in scenarios designed to treat diabetes. Fukushima *et al.* (2007) reported hydrodynamic tail vein-based delivery of adiponectine to treat type I diabetes. Similarly, against hepatitis B virus (HBV), the technique has been utilized to deliver siRNA against the HBV genome (Yang *et al.* 2002). Among different approaches used for siRNA delivery, viral vectors though effective in cell line based experiments (Xia *et al.* 2002), may generate immune responses in the animal model. Therefore, considering the efficiency and ease of use, hydrodynamic tail-vein injection involving rapid injection of a large volume was used to facilitate siRNA delivery *in vivo* (Lewis *et al.* 2002). This enables siRNA to get into cells in highly vascularized organs (Liu *et al.* 1999; Dykxhoorn *et al.* 2006).

Utilizing this technique we were able to significantly knock down FAK expression in both liver and muscle, key organs in the regulation of whole body glucose homeo-stasis. We were able to achieve as high as ∼80% knock down at a concentration of 2500 nM siRNA dissolved in 1.2 ml of saline. As reported in the literature, for effective delivery the volume of the vehicle should be 10–12% of the body weight (Liu *et al.* 1999; Lewis *et al.* 2002). Since significant inhibition was already observed with 2500 nM, a higher concentration of siRNA was not used, to avoid pleotropic effects as it would result in

a FAK-null-lethal condition. We did not observe any negative effects after the hydrodynamic injection, even after multiple injections. No morbidity or mortality was observed due to tail vein injection. As reported previously, mice or rats fed a high-fat diet for 2 weeks resulted in the development of insulin resistance (Zierath *et al.* 1997; Srinivasan *et al.* 2005); in the present study insulin resistance was developed within 2 weeks, without any dietary interventions.

In vivo FAK silencing affects physiological parameters significantly. The food and water intake and gained weight of FAK-silenced mice were significantly more than the wild type littermates. It has been previously reported that hypothalamus lesions of an electrical, chemical or gene deletion in nature produces an almost immediate increase in food intake and circulating insulin, which increases lipid deposition and causes peripheral insulin resistance (Bergen *et al.* 1998; Butler & Cone, 2002). However, in the present study no change between control and animals injected with vehicle or scrambled siRNA was observed. Moreover, hydrodynamic tail vein injection is reported to transfect cells of vascularized organs near to the inferior vena cava, e.g. liver and spleen (Dykxhoorn *et al.* 2006). No report on transfection of brain cells has been reported. This eliminates the possibility that hydrodynamic siRNA delivery had a direct impact at the level of the hypothalamus but might be consequent upon impaired insulin signalling or insufficient glucose utilization at the periphery by FAK inhibition.

There are reports indicating that activation of the inflammatory pathway may lead to the development of insulin resistance (Arkan *et al.* 2005; Cai *et al.* 2005). However, compared to wild type littermates, no significant change in either physiological or biochemical parameters were observed in mice injected with scrambled siRNA. Moreover, no change in TNF-α concentration was observed. These data therefore indicate that no inflammatory response was activated in animals due to siRNA injection. Hence inflammation or an immune response did not contribute to insulin resistance.

In order to explore the molecular mechanism whereby FAK silencing develops insulin resistance leading to hyperglycaemia and hyperinsulinaemia, FAK was observed to regulate IRS-1 and Akt activity; however, IR-β remained unaffected. The data are in accordance with our *in vitro* study (Bisht *et al.* 2007). Interestingly, IRS-1 expression was also found to be inhibited in FAK-silenced animals. This observation was supported by the study of Lebrun *et al.* (2000), according to which no IRS-1 expression was observed in FAK$^{-/-}$ fibroblasts. However, in our previous study on skeletal muscle cells, we did not observe such a change (Bisht *et al.* 2007). This could be due to differences in *in vivo* regulation. Hence, impaired insulin signalling, increased body weight, food and water intake, elevated glucose, insulin and triglyceride levels under physiological insulin levels, in two major insulin-sensitive organs due to

FAK silencing reveals the rationale for the development of insulin resistance.

The present study provides strong evidence for an important role of FAK in glucose homeostasis. This occurs possibly via key insulin signalling proteins *in vivo*. Interestingly, development of insulin resistance and hyperglycaemia in mice substantially lacking in endogenous FAK correlates well with that of high-fat-diet-induced resistant animals (Zierath *et al.* 1997). We suggest that selective activation/overexpression of FAK presents an attractive opportunity for the treatment of insulin resistance, an ever increasing epidemic of the 21st century.

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Author's contributions

B.B. designed and executed experiments, analysed data and drafted the manuscript. K.S. (veterinarian) performed the hydrodynamic tail vein injection, glucose tolerance test, blood/organ collection and animal care. C.S.D. conceived the study, designed, coordinated experiments, wrote and finalized the manuscript.

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Supplemental material

Online supplemental material for this paper can be accessed at: http://jp.physoc.org/cgi/content/full/jphysiol.2008.157107/DC1