Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells

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Summary

The process of cell migration is initiated by protrusion at the leading edge of the cell, the formation of peripheral adhesions, the exertion of force on these adhesions, and finally the release of the adhesions at the rear of the cell. Focal adhesion kinase (FAK) is intimately involved in the regulation of this process, although the precise mechanism(s) whereby FAK regulates cell migration is unclear. We have used two approaches to reduce FAK expression in fibroblasts. Treatment of cells with FAK-specific siRNAs substantially reduced FAK expression and inhibited the spreading of fibroblasts in serum-free conditions, but did not affect the rate of spreading in the presence of serum. In contrast with the wild-type cells, the FAK siRNA-treated cells exhibited multiple extensions during cell spreading. The extensions appeared to be inappropriately formed lamellipodia as evidenced by the localization of cortactin to lamellipodial structures and the inhibition of such structures by expression of dominant-negative Rac. The wild-type phenotype was restored by re-expressing wild-type FAK in the knockdown cells, but not by expression of FAK containing a point mutation at the autophosphorylation site (FAK Y397F). In wound-healing assays, FAK knockdown cells failed to form broad lamellipodia, instead forming multiple leading edges. Similar results were obtained using primary mouse embryo fibroblasts from FAK-flox mice in which Cre-mediated excision was used to ablate the expression of FAK. These data are consistent with a role for FAK in regulating the formation of a leading edge during cell migration by coordinating integrin signaling to direct the correct spatial activation of membrane protrusion.

Key words: FAK, Cell polarity, Cell migration, Lamellipodium

Introduction

The migration of cells is essential for a wide number of cellular processes including embryonic development, wound-healing, inflammation and cancer metastasis (Hood and Cheresh, 2002; Dormann and Weijer, 2003). Cell migration on solid substrates (two-dimensional migration) is an integrated process (Ridley et al., 2003). Cells form a ‘leading edge’ by preferentially protruding membrane in a single direction, usually in response to a directional or chemotactic signal. This is mediated by the localized polymerization of filopodia and cortical actin, a branched array of actin filaments, in a directional manner. As the leading edge comes into contact with the extracellular matrix, small adhesions are formed that adhere the membrane to the matrix. An internal complex of structural and signaling molecules form on the cytoplasmic side of these adhesions, termed ‘focal contacts’ or ‘nascent adhesions’, serving to link the extracellular matrix to the intracellular cytoskeleton (Sastry and Burridge, 2000). Following the formation of these adhesions, the cell is able to exert force on them by contraction of the actin cytoskeleton through activation of myosin II (Wakatsuki et al., 2003). It is this force that propels the cell forward. The newly formed adhesions either turnover or mature into larger focal adhesions, which are linked to stress fibers and accumulate at sites behind the leading edge (Webb et al., 2002). The final step of cell migration occurs when adhesions at the rear of the cell are disassembled and the cell retracts the trailing edge.

Although this model of cell migration is well accepted, the precise mechanisms that regulate these processes remain unclear. Evidence indicates that multiple signaling molecules, including focal adhesion kinase (FAK), paxillin, p130Cas and ERK are recruited to newly formed adhesions and contribute to the process of assembly and turnover (Cary et al., 1998; Huang et al., 2004; Ren et al., 2000; Webb et al., 2004). Furthermore, tyrosine phosphorylation of these molecules occurs on cell adhesion, and interference with the phosphorylation or dephosphorylation events results in impaired cell migration (Brennan et al., 1999; Manes et al., 1999; Petch et al., 1995). FAK is implicated in the initiation of a tyrosine phosphorylation signaling cascade because it is a tyrosine kinase that is localized to focal adhesions and activated on cell adhesion to fibronectin (Parsons, 2003). This activation results in the autophosphorylation of FAK on tyrosine 397, which then becomes a binding site for the SH2 domain of src family kinases (SFKs) (Schaller et al., 1994). The interaction between FAK and SFKs stabilizes the SFKs in their active state, allowing the phosphorylation of other focal adhesion proteins in a SFK-dependent manner (Schaller et al., 1999).
The importance of FAK in the process of cell adhesion and migration is illustrated by the embryonic lethality of FAK−/− mice (Ilic et al., 1995a). Immortalized cell lines derived from FAK−/− mice show defects in spreading and migration compared with the wild-type (FAK+/+) mice (Ilic et al., 1995a). However, owing to the clonal nature of FAK−/− cells and the continued propagation over time, mutations have accumulated in these cells as indicated by the increased expression of PYK-2, a FAK homolog, to compensate for the lack of FAK in these cells (Owen et al., 1999). The genetic instability of FAK−/− cells is underscored by the fact that FAK−/− cells also lack the cell cycle checkpoint protein p53 (Ilic et al., 1995a; Ilic et al., 1995b). Therefore, because additional factors may affect cell migration in the FAK−/− cells, it is important to examine other cell models in which FAK expression is attenuated to fully delineate the role of FAK in cell migration.

In this report, we use two approaches to reduce FAK expression in fibroblasts which yield cells deficient for FAK in a wild-type background: siRNA of FAK in fibroblast cell lines and the disruption of the FAK gene in primary fibroblasts by Cre-Lox mediated excision. The most obvious phenotype of the FAK-deficient cells is the inability to form broad well-organized lamellipodia on spreading and migration into a wound. In addition, FAK siRNA-treated cells exhibited an inhibition in the reorientation of the Golgi apparatus in response to migration into a wound. These data are consistent with a role for FAK in regulating the formation of a leading edge during cell migration by coordinating integrin signaling to result in the correct spatial activation of membrane protrusion and the reorientation of cellular organelles.

Materials and Methods
Cell culture
Rat embryo fibroblast cell lines (REF52 and Rat-2) were routinely passaged in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Mice bearing a single floxed allele of FAK (FAK-flox) (Beggs et al., 2003) were bred to obtain homozygous FAK-flox mice. Both wild-type fibroblasts and FAK-flox fibroblasts were isolated from 12-day-old embryos and cultured in DMEM containing 10% fetal bovine and supplemented with an additional 2 mM L-glutamine. Recombination of the floxed allele was carried out by infection of cells with an adenovirus containing both a Cre and GFP gene (Beggs et al., 2003). PCR-mediated genotyping of the cells revealed complete (greater than 95%) excision of the affected intron within 3-4 days post infection with the Cre adenovirus. Before each experiment, the absence of FAK in the adenovirus-treated cells was confirmed by PCR analysis (Beggs et al., 2003) and western blot. Under the culture conditions used, floxed FAK cells deficient for FAK did not undergo significant apoptosis and continued to grow at rates comparable to wild-type controls (data not shown).

Antibodies and reagents
Monoclonal anti-FAK, anti-paxillin, anti-GM130 and HRP-conjugated monoclonal anti-phosphotyrosine antibody (RC20) were purchased from BD Transduction Labs. Monoclonal anti-cortactin (4F11) was purified from ascites fluid (Wu et al., 1991). FITC-phalloidin was purchased from Molecular Probes (Eugene, OR). Polyclonal antibodies against phosphorylated FAK or paxillin were purchased from Biosource International. Secondary HRP-conjugated antibodies were purchased from Amersham.

siRNA and plasmid transfection
The siRNA used for the experiments was purchased from Dharmacon and targeted against the mRNA of human FAK. The siRNA oligonucleotide has a one base-pair mismatch with rat FAK mRNA, yet was still efficient in reducing FAK expression (see Fig. 1). The sequence for the siRNA oligonucleotides (sense strands) are as follows: Luciferase (GL1): CGUACCGGAAUACUUCGA; FAK: GCUAGUGACGUAGGAUGU. REF52 fibroblasts were plated at a density of 4.75x10^3 cells/cm² 24 hours before transfection with siRNA. The following day, siRNA was introduced into the cells by calcium phosphate precipitation. Briefly, the siRNA (1 pmol/6x10⁵ cells) was mixed with 0.25 M calcium chloride, followed by slowly adding the siRNA/calcium mixture to 2X HEPES buffered saline (HEBS, pH 7.1). The solution was incubated at room temperature for 30 minutes before addition to the cells. The culture media was changed 24 hours after the transfection. In some experiments, the cells were replated 24 hours following transfection with siRNA and then additionally transfected with either GFP-wtFAK or GFP-Y397F-FAK using Polyfect (Qiagen) according to the manufacturer’s instructions.

Analysis of cell morphology
Seventy-two hours post transfection with siRNA, the cells were plated on fibronectin (10 µg/ml)-coated coverslips for 1 hour either in the presence or absence of serum. The coverslips were then washed twice with PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. The cells were washed with PBS and permeabilized with 0.5% Triton-X in PBS for 2 minutes at room temperature. The coverslips were then blocked overnight at 4°C with 20% BSA and 20% goat serum in PBS. Immunofluorescence staining was performed at room temperature by incubating the primary antibody with the coverslips for 1.5 hours, washing three times with PBS, then incubating with the fluorescently labeled secondary antibody for 1 hour. The coverslips were washed twice with PBS and twice with water before being mounted on microscope slides and analyzed by fluorescence microscopy. Digital photographs were captured using a Nikon E600 upright fluorescent scope equipped with a Hamamatsu Orca CCD camera.

Wound-healing assays
Two days post siRNA treatment, cells were plated at near confluency on 35 mm Bioptechs delta-T dishes (Fisher Scientific) designed for live-cell microscopy. The following day, an artificial wound was made in the monolayer by scraping a 10 µl pipette tip across the bottom of the dish. The media was changed to Leibovitz L-15 media (Gibco) containing 10% FBS. Time-lapse movies of migrating cells were made by photographing the wound every 5 minutes for 7 hours using a Nikon TE200 inverted microscope with a 20x DIC objective and a Bioptechs heated stage. Images were captured with a Hamamatsu Orca camera and compiled using Improvision Openlab software.

Golgi reorientation analysis
Analysis of Golgi reorientation was performed as described previously (Nobes and Hall, 1999), with minor modifications. Briefly, cells were fixed at the indicated times following artificial wounding on 35 mm Bioptechs delta-T dishes (Fisher Scientific) designed for live-cell microscopy. The following day, an artificial wound was made in the monolayer by scraping a 10 µl pipette tip across the bottom of the dish. The media was changed to Leibovitz L-15 media (Gibco) containing 10% FBS. Time-lapse movies of migrating cells were made by photographing the wound every 5 minutes for 7 hours using a Nikon TE200 inverted microscope with a 20x DIC objective and a Bioptechs heated stage. Images were captured with a Hamamatsu Orca camera and compiled using Improvision Openlab software.
be assigned a score of 1/3). This was performed for 50-100 cells per siRNA treatment, per time point. Percent cells with reoriented Golgi were determined by computing the ratio of properly reoriented cells to the total number of cells counted. Statistical analysis was carried out using Student’s t-test for analysis of paired means.

Results
Inhibition of FAK expression by siRNA
To provide a direct comparison of properties of cells before and after the loss of FAK expression, siRNAs specific for FAK were transfected into REF52 cells. Transfection of the FAK siRNA oligonucleotide resulted in a significant decrease in FAK expression (Fig. 1A,a) after 36 hours as detected by western blot analysis of total cell lysates. As a control for the nonspecific effects of siRNA oligonucleotide treatment, cells were transfected with an siRNA oligonucleotide designed against luciferase mRNA, a gene not expressed in mammalian cells. Such treatment had no effect on FAK expression as measured by western blot analysis (Fig. 1A,a). Similarly, siRNA knockdown of cortactin had no effect on FAK expression (data not shown).

To examine effects of loss of FAK expression on integrin-dependent signaling, cells were replated on fibronectin and the lysates were analyzed for global tyrosine phosphorylation, FAK phosphorylation (using anti-FAK pY397) and the adhesion-dependent phosphorylation of paxillin (Fig. 1A). On plating of either untreated or luciferase siRNA-treated cells on fibronectin, there was a significant increase in the phosphorylation of proteins with the relative molecular weights of 130 kDa, 125 kDa and 70 kDa, corresponding to the relative molecular weights of p130Cas, FAK and paxillin, respectively (Fig. 1A,b). These bands were diminished in the lysates from the cells treated with the FAK siRNA (Fig. 1A,b), whereas the levels of paxillin and p130Cas remained unchanged (Fig. 1A,f,g). To specifically detect paxillin phosphorylation, the blot was probed with phosphospecific antibodies directed against paxillin pY31 and paxillin pY118. The cells treated with the FAK siRNA showed an inhibition of paxillin phosphorylation (Fig. 1A,d,e), indicating that the phosphorylation of focal adhesion components is impaired in the cells that are lacking FAK, consistent with a model of FAK as an upstream modulator of focal adhesion component phosphorylation (Ruest et al., 2001).

The loss of FAK expression following FAK siRNA was also examined by immunofluorescence microscopy. The cells treated with the control siRNA showed FAK localized to focal adhesions in REF52 fibroblasts plated on fibronectin for 4 hours; however, the cells treated with the FAK siRNA showed a significant reduction in FAK staining (Fig. 1B), consistent with the loss of FAK expression as measured by western blot. Immunofluorescence staining of cells following FAK siRNA treatment revealed that >95% of the cells had undetectable FAK expression (data not shown).

Effect of FAK RNAi on cell spreading and morphology
To examine the effect of the inhibition of FAK expression by siRNA oligonucleotides, we analyzed the dynamics of the knockdown cells as they adhered and spread on fibronectin. Previous studies have shown that the spreading of FAK–/− cells on fibronectin is impaired (Ilic et al., 1995a; Owen et al., 1999). In agreement with these studies, FAK siRNA-treated REF52 cells showed delayed spreading on fibronectin, particularly at
10 and 30 minutes under serum-free conditions when compared with cells treated with the luciferase siRNA (Fig. 2A-F). At these earlier time points there was roughly 50% inhibition of spreading in the FAK siRNA-treated cells as measured by cell area (data not shown). After 60 minutes on fibronectin, however, the FAK siRNA-treated cells had spread to the same extent as the control cells (Fig. 2C,F).

In the presence of 10% serum, the cells treated with the luciferase siRNA exhibit the typical rounded shape of a spreading fibroblast (Fig. 2G-I). Interestingly, the presence of serum was able to partially compensate for the loss of FAK, in that the FAK siRNA-treated cells spread at the same rate as the control cells (Fig. 2J-L). This is in contrast with previous studies of the FAK –/– cells in which serum failed to rescue the spreading defect (Ilic et al., 1995a; Owen et al., 1999). Additionally, in the presence of serum, FAK siRNA-treated cells lacked the smooth rounded morphology typical of a spreading fibroblast and instead exhibited numerous membrane extensions after 60 minutes of spreading on fibronectin (Fig. 2L). These data confirm that FAK is important for cell spreading on fibronectin and further indicate that the reduced level of FAK expression in fibroblasts leads to altered protrusive events at the cell periphery.

Rescue of the FAK RNAi phenotype with wild-type FAK, but not FAK Y397F

An important caveat to siRNA studies is the possibility that the siRNA oligonucleotide may be affecting the expression of a gene other than the target, owing to cross-reaction with an additional mRNA sequence. To ensure that the observed phenotype in the siRNA-treated cells is in fact specifically due to a loss of FAK, we transiently transfected GFP-tagged wild-type FAK (GFP-wtFAK) 2 days following siRNA treatment. This FAK construct is derived from chicken FAK and is therefore able to escape the RNAi machinery in the cell because it has five base-pair mismatches with the siRNA oligonucleotide. As shown in Fig. 3A,B expression of GFP-wtFAK in the FAK knockdown cells plated in the presence of serum results in the reversion of the cellular phenotype to a rounded shape typical of a fibroblast spreading on fibronectin (compare to Fig. 2G-I). Interestingly, expression of GFP-tagged FAK containing a point mutation at tyrosine 397 does not rescue the phenotype (Fig. 3E-H), and these cells still exhibit the irregular morphology of the knockdown cells. This observation indicates that the autophosphorylation site of FAK is an important part of the signaling process that maintains proper cellular morphology during cell adhesion to fibronectin.

The morphology of the FAK knockdown cells is the result of improper lamellipodia formation

Actin polymerization at the periphery of a cell is necessary for spreading and migration on fibronectin. Integrin engagement induces organized actin polymerization resulting in the broad extension of the membrane (lamellipodium) around the periphery of a spreading cell, or the leading edge of a migrating cell (Small et al., 2002). The lack of a smooth round peripheral membrane in the FAK siRNA-treated cells upon spreading on fibronectin in serum suggests that the cells lacking FAK are unable to form proper lamellipodia. To gain further insight into the nature of the protrusions in the FAK knockdown cells, the spreading dynamics of both the control and the knockdown cell types were monitored by time-lapse microscopy. The control cells were able to form ruffles at the periphery as they spread (Fig. 4A). However, the FAK knockdown cells, in lieu of ruffles, formed pointed projections as they spread on fibronectin (Fig. 4B). These projections were present in >90% of the FAK siRNA-treated cells (data not shown). The projections contained focal adhesions, as determined by staining with anti-paxillin antibodies (Fig. 4C). Thus, these structures behave like lamellipodia in that they extend from the body of the cell and adhere to the substrate by forming paxillin-rich focal adhesions.

The similarities between the projections and lamellipodia are further illustrated upon staining for the Arp2/3-binding protein cortactin (Fig. 5) (Weed et al., 2000). In REF52 cells, cortactin normally localized to the edges of protruding lamellipodia where cortical actin polymerization occurs (Fig. 5A). The cells treated with the FAK siRNA contained cortactin...
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Exclusive in the numerous extensions at the periphery of the cells (Fig. 5B). Therefore, the altered lamellar extensions appear to be analogous to lamellipodia, we expressed dominant-negative Rac (dnRac) in cells following FAK siRNA. The cells expressing dnRac were unable to form the numerous extensions that were typically seen in the knockdown cells (Fig. 5E,F), indicating that these extensions are Rac-dependent. Together with the enrichment of cortactin in these structures, these data indicate that the extensions are altered or malformed lamellipodia.

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Fig. 3. Wild-type FAK, but not FAK Y397F, rescues the phenotype resulting from FAK knockdown. Cells treated with siRNAs for FAK were transiently transfected with either wtFAK-GFP (A-D) or FAK Y397F-GFP (E-H) and plated on fibronectin for 1 hour. The images show GFP-FAK (A-B, E-F) and the corresponding phase photographs (C-D, G-H). Arrows indicate cells that are expressing the GFP-FAK constructs.

exclusively in the numerous extensions at the periphery of the cells (Fig. 5B). Therefore, the altered lamellar extensions appear to be analogous to lamellipodia of wild-type cells. In addition, upon re-expression of GFP-wtFAK, cortactin returned to the typical localization along the length of the cellular edge, similar to untreated cells (Fig. 5C,D).

The small GTPase Rac is responsible for lamellipodium formation in migrating cells, and overexpression of Rac in fibroblasts results in continual actin polymerization at the periphery of adhered cells (Ridley et al., 1992). In addition to lamellipodium formation, Rac activity results in the translocation of cortactin to the periphery of spreading cells (Weed et al., 1998). As both cortactin translocation to the periphery and lamellipodia formation are dependent on the activity of Rac, and the numerous membrane extensions appear to be analogous to lamellipodia, we expressed dominant-negative Rac (dnRac) in cells following FAK siRNA. The cells expressing dnRac were unable to form the numerous extensions that were typically seen in the knockdown cells (Fig. 5E,F), indicating that these extensions are Rac-dependent. Together with the enrichment of cortactin in these structures, these data indicate that the extensions are altered or malformed lamellipodia.

The loss of FAK results in a defect in leading edge formation during migration

Cell migration requires the extension of a lamellipodium at the leading edge, followed by adhesion of the lamellipodium to the substrate and contraction of the cell body (Ridley et al., 2003). Therefore, because proper organization and orientation of the lamellipodium plays a crucial role in directional movement, we next tested whether the loss of FAK would result in a defect in cell migration. To examine the dynamics of the cells during migration, confluent monolayers of either control or FAK knockdown REF52 cells were artificially ‘wounded’ by scraping them with a pipette tip and filmed by time-lapse microscopy as they migrated into the wound. Control cells, treated with the luciferase siRNA, exhibited typical broad lamellipodia at their leading edges as they migrated into the wound (Fig. 6A, see Movie 1 in supplementary material). The cells that were treated with the FAK siRNA migrated at a rate similar to that of the control cells; however, they lacked broad lamellipodial structures and instead exhibited multiple leading edges as they migrated (Fig. 6B, see Movie 2 in supplementary material). In contrast to the control cells which moved as a sheet perpendicular to the wound, the knockdown cells did not move as a single sheet, and they had the tendency to migrate over neighboring cells. In addition, some cells achieved a more elongated morphology as they either appeared to be unable to release their trailing edges or they simultaneously moved in multiple directions (Fig. 6B, see Movie 2 in supplementary material).

While the wound-healing assays are ideal for stimulating migration in the REF52 cells, they may not provide a true test for directionality since the cells can only move in a limited number of directions (i.e. into the wound). Therefore, to further examine the effect of improper lamellipodium formation on cell migration, fibroblasts were plated on fibronectin at low densities and in the presence of serum, and their random migrations were monitored by time-lapse microscopy. When plated under such conditions, various cells have been observed to spontaneously polarize and migrate in the absence of a chemotactic gradient (Chung et al., 2001). Because wild-type REF52 cells failed to spontaneously polarize at low densities (data not shown), we examined the effects of FAK knockdown on polarity in Rat-2 fibroblasts. As shown in Fig. 7A, Rat-2 cells treated with the luciferase siRNA and plated at low density spontaneously polarized, forming distinct broad leading edges (Fig. 7A, arrows; see also Movie 3 in supplementary material) and narrow trailing edges. Conversely, Rat-2 cells treated with FAK siRNA failed to form broad leading edges when plated on fibronectin and instead formed multiple processes extending from the cell (Fig. 7B, arrowheads; see also Movie 4 in supplementary material).
alteration in cell morphology was consistently observed in >90% of the Rat-2 cells following FAK knockdown (data not shown). Together, these observations indicate an alteration in global cell polarity in the FAK knockdown cells, as these cells appear to be unable to establish a functional lamellipodium at their leading edges, and they appear to lack the signal at the rear of the cell that is responsible for the release of adhesion to the substrate.

The subcellular location of the Golgi apparatus is believed to be determined by the organization of the microtubule network, and the Golgi is usually located between the nucleus and the leading edge of a migrating cell (Kupfer et al., 1982). Therefore, Golgi localization to the front of the nucleus has been shown to be a marker for global cell polarity during migration (Nobes and Hall, 1999). To specifically determine alterations in cellular polarity in the FAK knockdown cells, control and knockdown Rat-2 cells were subjected to a wound-healing assay and analyzed for Golgi reorientation. Fig. 8A shows cells at the edge of a wound 4 hours following wounding of the monolayer. The Golgi in the control cells (Fig. 8A) were consistently compact, and the majority of the Golgi were located between the nucleus and the leading edge (Fig. 8C). By contrast, the cells that were treated with FAK siRNA exhibited disorganized Golgi (Fig. 8B), which showed a delayed reorientation towards the leading edge (Fig. 8C). At later time points, the Golgi of the FAK knockdown cells began to show more significant reorientation towards the wound compared with the control cells (at 6 hours, \( P = 0.14 \)). These data suggest that FAK is important for proper cellular organelle orientation during cell migration.

Fig. 4. Aberrant lamellipodia formation in cells treated with FAK siRNAs. Cells treated with either luciferase siRNA (A) or FAK siRNA (B) were allowed to spread on fibronectin and analyzed by time-lapse microscopy. Arrows in A indicate ruffling edge of the control cells; arrowheads in B indicate the formation of a projection in the FAK knockdown cells. (C) FAK knockdown cells were allowed to spread on fibronectin for 1 hour, then fixed and stained for paxillin. The white outline in the enlarged inset represents the plasma membrane.

Fig. 5. The projections in the cells treated with FAK siRNAs are rich in cortactin and are dependent on functional Rac. Cells treated with either luciferase siRNA (A) or FAK siRNA (B-F) were allowed to spread on fibronectin for 1 hour. (A) Control cells stained for cortactin. (B) FAK knockdown cells stained for cortactin. Arrowheads indicate cortactin staining along the cell periphery in A and in the multiple cellular extensions in B. (C) Cortactin staining in FAK knockdown cells following re-expression of GFP-wtFAK. Arrow indicates cell transfected with GFP-wtFAK. D is the corresponding GFP image for C. (E-F) FAK knockdown cells expressing GFP-dnRac. E shows the GFP image and F shows the corresponding phase image. Arrows point to cells expressing GFP-dnRac.
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Cre/Lox-mediated loss of FAK in primary fibroblasts yields a phenotype similar to that of the FAK siRNA treated cells

To ensure that the phenotype that was observed in the FAK knockdown cells was exclusively due to a loss of FAK, we performed experiments on primary fibroblasts in which Cre-mediated excision was used to delete the second exon of the kinase domain of FAK, rendering FAK inactive (Beggs et al., 2003). Either wild-type cells or cells homozygous for the floxed FAK gene (floxed FAK) were infected with adenovirus containing the Cre recombinase gene (adeno-Cre). In the floxed FAK cells, there was a complete loss of FAK in the cellular genome and at the protein level, as analyzed by PCR and western blot, respectively, whereas FAK levels in the wild-type cells that were infected with adeno-Cre were unaffected (Fig. 9A,B).

Wild-type and FAK-deleted cells were plated on fibronectin, and wound-healing assays were performed to determine the effects of FAK deletion on cell migration (Fig. 9C,D). The wild-type fibroblasts exhibited broad lamellipodia and migrated as a single sheet into the wound (Fig. 9C, see Movie 5 in supplementary material). By contrast, the FAK deleted cells migrated in a chaotic fashion, and the monolayer separated into single cells as the wound closed. The FAK-deleted cells also had a tendency to migrate in multiple directions at once and showed an inability to release their trailing edges (Fig. 9D, see Movie 6 in supplementary material). This phenotype mirrors the migratory pattern of the cells treated with FAK siRNA (see Fig. 6B). Thus, both the cells deficient in the expression of FAK, either by recombinational deletion of FAK or by siRNA treatment, exhibit similar defects in cell migration and organization of lamellipodia. These data indicate that FAK plays a central role in organizing and propagating signals required for directional migration.

![Fig. 6. Treatment of cells with FAK siRNAs results in a loss of directional movement. REF52 cells were treated with either luciferase siRNA (A) or FAK siRNA (B). A confluent culture of REF52 cells was wounded using a pipette tip, and cell migration into the wound was monitored by time-lapse microscopy. Images correspond to photographs taken at the indicated times following the scraping of the monolayer. In A, the cells develop broad lamellipodia (arrows) as they move into the wound. In B, cell ‘a’ has migrated into the wound yet did not release its trailing edge, and cell ‘b’ eventually has become elongated as it develops two leading edges, one moving toward the wound and the other away from it. Both cells lack broad lamellipodia. See Movies 1 and 2 in supplementary material.](image)

![Fig. 7. Treatment of Rat-2 cells with FAK siRNAs inhibits spontaneous polarization. Rat-2 cells were either transfected with luciferase siRNA (A) or FAK siRNA (B) and allowed to spread on fibronectin for 10 minutes before video time-lapse microscopy. Images were taken at the indicated timepoints after the initiation of the recording. The arrows in A indicate the broad lamellipodia at the leading edge of the migrating cells; the arrowheads in B indicate the multiple extensions that form in the FAK knockdown cells. See Movies 3 and 4 in supplementary material.](image)
Discussion

In this report we have shown that the inhibition of FAK expression in rat or mouse fibroblasts, either by siRNA treatment or the generation of primary FAK null cells by Cre-mediated excision of a floxed-FAK allele, resulted in altered lamellipodia formation and an aberrant migrational phenotype. Treatment of REF52 fibroblasts with siRNAs specific for FAK results in the loss of FAK expression and inhibition of integrin-mediated tyrosine phosphorylation of the FAK substrates paxillin and p130Cas. In REF52 cells, reduced FAK expression slowed cell spreading in the absence of serum; however, in the presence of serum the FAK knockdown cells exhibited multiple extensions instead of a typical smooth lamellipodial structure characteristic of control cells. The wild-type phenotype was restored in FAK-deficient cells by re-expressing GFP-tagged FAK, but not FAK Y397F. Expression of dnRac blocked the formation of cellular extensions, consistent with these lamellipodia being Rac-dependent and rich in cortactin. The loss of FAK expression also resulted in defects in directional migration, and the cells were unable to establish proper polarity at the onset of migration. Overall, these data suggest that FAK plays an important role in directional migration by establishing a proper leading edge and maintaining polarity in a moving cell.

siRNA treatment has been successfully used to inhibit protein expression over 48-72 hours. Therefore, it provides a valuable tool for determining the function of a protein in a wild-type cellular background, as opposed to gene knockout studies which involve propagation of a clonal selection of cells over many passages. Similarly, transient expression of the Cre recombinase in cells bearing a floxed-FAK allele allowed for the conditional knockout of FAK expression in primary fibroblasts and assessment of these cells with 6-10 days post Cre treatment. The morphology of the fibroblasts obtained following either of these procedures differs from that of the fibroblasts originally obtained from FAK knockout mice, which have been typically described as more round and less spread than wild-type fibroblasts (Ilic et al., 1995a; Sieg et al., 1999). Other groups who have recently applied these techniques to inhibit FAK expression have also failed to obtain the typical rounded FAK knockout cell morphology (Beggs et al., 2003; Yano et al., 2004). This suggests that the FAK knockout cells contain additional mutations that reflect on their morphology. In addition, in both siRNA treated cells or FAK-deficient floxed fibroblasts, the expression of the FAK-related protein PYK2 was not altered (data not shown). This is in contrast to the traditional FAK knockout cells, which express high levels of PYK2, perhaps to compensate for the loss of FAK (Owen et al., 1999). Also, in siRNA treated cells, there was a substantial reduction in paxillin and p130Cas tyrosine phosphorylation, consistent with a role for FAK in directing integrin-mediated phosphorylation of these proteins. This also differs from the traditional FAK knockout cells, which show a slight increase in the phosphorylation of focal adhesion proteins (Ilic et al., 1995a). The observation that FAK autophosphorylation mutant does not rescue the siRNA-induced phenotype suggests that the recruitment of Src to focal adhesions is important for the establishment of lamellipodia in a migrating cell.

The lamellipodium at the leading edge of migrating cells is formed in response to both growth factor factor stimulation and integrin engagement. Along these lines, previous reports have implicated FAK in transducing signals to the cytoskeleton from both integrin-mediated and growth factor receptor-mediated signaling networks (Wang et al., 2001; Sieg et al., 2000). In the case of integrin-mediated signal transduction, FAK has been implicated in mechanosensing during cell migration (Wang et al., 2001). While wild-type fibroblasts respond to pushing forces by forming lamellipodia on the opposite side of the cell, FAK −/− cells (or FAK −/− cells expressing Y397F FAK) lack this response, suggesting that FAK plays a role in re-orienting the cell by directing the formation of a new leading edge in response to changes in the structure and/or plasticity of the extracellular matrix. In addition to its role in adhesion-dependent signaling, FAK is required for cells to efficiently migrate in response to epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which implicates FAK in a role that links growth factor signaling to sites of integrin engagement (Sieg et al., 2000). This is particularly interesting in the context of our findings in which both the FAK knockdown cells and the conditional FAK knockout cells exhibited defects in the establishment of a lamellipodium at their leading edges, as lamellipodium formation is regulated by signals from both integrins and growth factor receptors (Small
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et al., 2002). Thus, our data assigns a physiological function (the establishment and/or stabilization of the leading edge of migrating cells) to previous reports that implicate FAK as a mediator of integrin and growth factor receptor signaling.

The lamellipodium is an important structure for the establishment of polarity during migration. It is this structure that constitutes the leading edge of a moving cell, and the strongest traction forces are within the lamellipodium (Beningo et al., 2001). The FAK knockdown cells lack a single dominant lamellipodium; rather, they exhibit multiple narrow projections. These projections are characterized as lamellipodium-like because they are Rac-dependent and cortactin is exclusively localized to these structures. Cortactin has several actin-binding domains and is consistently found at sites of active cortical actin polymerization, such as the leading edge of migrating cells (Weaver et al., 2001; Weed et al., 2000). Because of the presence of several protrusions instead of a single lamellipodium, the FAK knockdown cells had a tendency to form multiple leading edges. As a result, Rat-2 cells were unable to form spontaneous polarized structures (i.e. a single broad leading edge and a narrow trailing edge).

Because adhesions were still able to form within these structures (as determined by the presence of paxillin-containing focal adhesions), it can be hypothesized that the observed phenotype is not merely due to a lack of adhesion formation per se; rather, FAK activity is required within these adhesions to properly link adhesive events to the cortical cytoskeleton. It is interesting to note that the FAK knockdown cells showed an inability to organize and re-orient their Golgi in the direction of migration. The Golgi has been shown to be intimately associated with microtubules (Kupfer et al., 1982). Thus, the lack of Golgi spatial organization may also reflect a lack in orientation of the microtubule network, which plays an important role in establishing cell polarity during migration by mediating transport of vesicles to the leading edge of the cell (Schmoranzer et al., 2003).

Since a typical migrating fibroblast is polarized with respect
to a broad leading edge and a narrow trailing edge, the observation that inhibition of FAK expression results in aberrant leading edge formation suggests that FAK plays an important role in the establishment of cellular polarity. There are several potential mechanisms that would implicate the FAK-Src complex in this process. First, focal adhesions dissociate (‘turnover’) at the front of a migrating cell, and both FAK and Src are important for focal adhesion turnover (Ren et al., 2000; Webb et al., 2004; Fincham and Frame, 1998). The lack of FAK in focal adhesions in the knockdown cells would inhibit focal adhesion turnover at the front of the cell, thereby disrupting polarity with respect to adhesion formation and dissociation. This is supported by the observation that the knockdown cells were unable to release their trailing edges. Second, the phosphorylation of focal adhesion proteins at the leading edge of a migrating cell would result in the recruitment of further signaling molecules such as Crk (Abassi and Vuori, 2002), which is a binding partner for Dock180/ELMO, a complex that functions as a guanine exchange factor (GEF) for Rac (Lu et al., 2004). Because Rac is important for the polymerization of the cortical actin meshwork that constitutes lamellipodia, FAK would be important for actin-mediated lamellipodium formation. Interestingly, a recent report by Yano et al. showed that RNAi of FAK or paxillin in HeLa epithelial cells results in an upregulation of Rac activity at areas of cell-cell contact (Yano et al., 2004). This conflicts with previous reports which, as noted above, suggest that FAK promotes Rac activity. However, if FAK does indeed inhibit Rac activity through its interaction with paxillin, this does not exclude the possibility that Rac is still activated in a FAK/p130Cas-dependent manner. In addition, the apparent upregulation of Rac at cell-cell contacts could be the result of a mislocalization (as opposed to an actual upregulation) of Rac activity, especially since the global levels of Rac activity remained unchanged after FAK knockdown (Yano et al., 2004). Nonetheless, the formation of multiple protrusions in fibroblasts following loss of FAK may be due to unregulated Rac activity at certain points along the cell periphery. The precise signaling pathways that link FAK to protrusive events remain to be established.

The conditional knockout of FAK addresses an important caveat in RNAi experiments, which is the possibility that the siRNA is silencing a target other than the intended mRNA. Also, RNAi is temporary and does not completely repress the expression of the target protein. For these reasons, we performed experiments using primary fibroblasts that have lost FAK expression in vitro due to Cre/Lox excision of the FAK gene from the cellular genome. These cells showed defects in polarity and migrated in a similar fashion to the FAK knockdown cells, thus supporting the RNAi data.

Overall, our results implicate FAK as a mediator of cellular polarity by establishing a proper leading edge during cell migration. This provides insight into the mechanism whereby FAK is important in the integration of signals from growth factor receptors and integrins. FAK → cells are defective in EGF- or PDGF-stimulated migration, and re-expression of FAK rescues this defect. Thus, FAK-mediated linkage between growth factor receptor and integrin signaling is crucial during chemotactic migration where the cell becomes oriented in the direction of the chemotactic stimulus. The loss of FAK would disrupt this coordinated signaling and result in defects in membrane protrusion and polarized adhesion turnover. Further studies will elucidate the signaling pathway that links FAK to the actin machinery that mediates protrusion at the front of a migrating cell.

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References

Abassi, Y. A. and Vuori, K. (2002). Tyrosine 221 in Crk regulates adhesion-dependent membrane localization of Crk and Rac and activation of Rac signaling. EMBO J. 21, 4571-4582.

Begg, H. E., Schahin-Reed, D., Zang, K., Goebbels, S., Nave, K. A., Gorski, J., Jones, K. R., Sretavan, D. and Reichardt, L. F. (2003). FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. Neuron 40, 501-514.

Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V. and Wang, Y. L. (2001). Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J. Cell Biol. 153, 881-888.

Brennan, H., Smith, S. and Stoker, A. (1999). Phosphotyrosine signalling as a regulator of neural crest cell adhesion and motility. Cell Motil. Cytoskeleton 42, 101-113.

Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K. and Guan, J. L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. J. Cell Biol. 140, 211-221.

Chung, C. Y., Funamoto, S. and Firtel, R. A. (2001). Signaling pathways controlling cell polarity and chemotaxis. Trends Biochem. Sci. 26, 557-566.

Dormann, D. and Weijer, C. J. (2003). Chemotactic cell movement during development. Curr. Opin. Genet. Dev. 13, 338-346.

Fincham, V. J. and Frame, M. C. (1998). The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. 17, 81-92.

Hood, J. D. and Cheresh, D. A. (2002). Role of integrins in cell invasion and migration. Nat. Rev. Cancer 2, 91-100.

Huang, C., Jacobson, K. and Schaller, M. D. (2004). A role for JNK-paxillin signaling in cell migration. Cell Cycle 3, 4-6.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995a). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377, 539-544.

Ilic, D., Furuta, Y., Suda, T., Atsumi, T., Fujimoto, J., Ikawa, Y., Yamamoto, T. and Aizawa, S. (1995b). Focal adhesion kinase is not essential for in vitro and in vivo differentiation of ES cells. Biochem. Biophys. Res. Commun. 209, 300-309.

Kupfer, A., Louvard, D. and Singer, S. J. (1992). Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. Proc. Natl. Acad. Sci. USA 79, 2603-2607.

Lu, M., Kinchen, J. M., Rossman, K. L., Grimesly, C., de Bakker, C., Brugnera, E., Tosello-Trampont, A. C., Haney, L. B., Klingele, D., Sondek, J. et al. (2004). PH domain of ELMO functions in trans to regulate Rac activation via Dock180. Nat. Struct. Mol. Biol. 11, 756-762.

Manes, S., Mira, E., Gomez-Mouton, C., Zhao, Z. J., Lacalle, R. A. and Martinez, A. C. (1999). Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. Mol. Cell Biol. 19, 3125-3135.

Nobes, C. D. and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144, 1235-1244.

Owen, J. D., Ruest, P. J., Fry, D. W. and Hanks, S. K. (1999). Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. Mol. Cell. Biol. 19, 4806-4818.

Parsons, J. T. (2003). Focal adhesion kinase: the first ten years. J. Cell Sci. 116, 1409-1410.

Petch, L. A., Bockholt, S. M., Bouton, A., Parsons, J. T. and Burridge, K. (1995). Adhesion-induced tyrosine phosphorylation of the p130 src substrate. J. Cell Sci. 108, 1371-1379.
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Small, J. V., Stradal, T., Vignal, E. and Rottner, K. (2002). The lamellipodium: where motility begins. Trends Cell Biol. 12, 112-120.

Wakatsuki, T., Wysolmerski, R. B. and Elson, E. L. (2003). Mechanics of cell spreading: role of myosin II. J. Cell Sci. 116, 1617-1625.

Wang, H. B., Dembo, M., Hanks, S. K. and Wang, Y. (2001). Focal adhesion kinase is involved in mechanosensing during fibroblast migration. Proc. Natl. Acad. Sci. USA 98, 11295-11300.

Weaver, A. M., Karginov, A. V., Kinley, A. W., Weed, S. A., Li, Y., Parsons, J. T. and Cooper, J. A. (2001). Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr. Biol. 11, 370-374.

Webb, D. J., Parsons, J. T. and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. Nat. Cell. Biol. 4, E97-E100.

Weed, S. A., Du, Y. and Parsons, J. T. (1998). Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. J. Cell Sci. 111, 2433-2443.

Weed, S. A., Karginov, A. V., Schaffer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A. and Parsons, J. T. (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. J. Cell Biol. 151, 29-40.

Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R. and Parsons, J. T. (1991). Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol. Cell. Biol. 11, 5113-5124.

Yano, H., Mazaki, Y., Kurokawa, K., Hanks, S. K., Matsuda, M. and Sabe, H. (2004). Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. J. Cell Biol. 166, 283-295.