FAK, PIP5KIγ and gelsolin cooperatively mediate force-induced expression of α-smooth muscle actin

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Summary

During the development of pressure-induced cardiac hypertrophy, fibroblasts are activated to become myofibroblasts, which exhibit actin-cytoskeletal remodeling and express α-smooth muscle actin (SMA; encoded by *ACTA2*). Currently, the mechanosensing signaling pathways that regulate SMA expression are not defined. Because focal-adhesion complexes are putative mechanosensing organelles, we examined the role of focal adhesion kinase (FAK) and its interaction with gelsolin in the regulation of SMA expression. We subjected NIH3T3 cells to tensile forces (0.65 pN/μm^2) by using collagen-coated magnetite beads attached to integrins. After stimulation by mechanical force, FAK and gelsolin were recruited to magnetite beads and there was increased phosphorylation of Tyr397FAK. Mechanical force enhanced *SMA* promoter activity by twofold; this increased activity was blocked by *FAK* knockdown using siRNA and by deletion of gelsolin. Force-induced nuclear translocation of MRTF-A, a transcriptional co-activator of SMA that is regulated by actin filaments, was also reduced by FAK knockdown. Phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], which uncaps gelsolin from actin filaments, was enriched at sites of force application. Type-I phosphatidylinositol 4-phosphate 5 kinase-γ (PIP5KIγ), which generates PtdIns(4,5)P_2, associated with FAK and was required for force-mediated SMA-promoter activity and actin assembly. Catalytically inactive PIP5KIγ inhibited force-induced phosphorylation of FAK at Tyr397. These data suggest a novel pathway in which mechanosensing by FAK regulates actin assembly via gelsolin and the activity of PIP5KIγ; actin assembly in turn controls SMA expression via MRTF-A.

Key words: Myofibroblasts, FAK, PIP5KIγ, Gelsolin, SMA

Introduction

In cardiac hypertrophy, excessive pressure or volume overload leads to the differentiation of myofibroblasts, which are of crucial importance in the stiffening of the myocardium caused by the synthesis and secretion of fibrillar collagens (MacKenna et al., 2000). Mechanical force has been shown to upregulate the expression of α-smooth muscle actin (SMA), a marker for myofibroblast differentiation (Hinz et al., 2001). Fibroblasts adhere to extracellular matrices via integrins, cell-surface receptors that provide sites for force transfer to the actin cytoskeleton (MacKenna et al., 1998; Gullberg et al., 1992). In the myocardium, the formation of myofibroblasts in response to pressure overload is important for the development of cardiac fibrosis (Leslie et al., 1991). Although the Rho-dependent pathway of myofibroblast differentiation in response to mechanical stimulation, which involves nuclear translocation of a SMA transcriptional co-activator, MRTF-A, has been described (Zhao et al., 2007), the proximal mechanosensory processes in fibroblasts responding to mechanical force are not well defined.

Mechanosensory signaling in anchorage-dependent cells might be initiated in specialized adhesive contacts (e.g. focal adhesions) that are formed at the junctions between extracellular-matrix proteins and integrin in fibroblasts and other mesenchymal cell types (Ingber, 1997). These junctions contain large, complex assemblies of cytoskeletal and signaling proteins, consisting of more than 50 different proteins that play crucial roles in cell adhesion, spreading and migration. One of the crucial signaling proteins in focal complexes and focal adhesions is focal adhesion kinase (FAK). Tyrosine phosphorylation of FAK is an early, transient event that occurs during the formation of focal contacts (Parsons, 2003). In cell adhesion, clustering of integrin receptors triggers the recruitment of FAK to focal adhesions; FAK is subsequently auto-phosphorylated at residue Tyr397 (Calalb et al., 1995). This process in turn increases the kinase activity of FAK and enhances interactions of FAK at its C-terminal domain with other focal-adhesion-associated proteins such as Src, paxillin and proteins with SH2 and/or SH3 domains. Integrin-dependent activation of FAK induces signaling pathways that regulate MAP kinases, as well as the Rho and Arf families of small GTPases (Parsons, 2003). Although a large body of literature on the role of FAK in signal transduction and cytoskeletal organization suggests that FAK might critically regulate and integrate multiple adhesion-related signaling inputs (Schober et al., 2007; Albinsson and Hellstrand, 2007), the proteins that associate with FAK to mediate mechanically induced signals are not defined.

Type-I phosphatidylinositol 4-phosphate 5 kinase (PIP5KI), specifically PIP5KIγ, associates with FAK in focal adhesions in migrating cells (Brakebusch and Fassler, 2003). PIP5KIγ converts phosphatidylinositol 4-phosphate [PtdIns(4)*P*] to phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] (Anderson et al., 1999), which acts as a second messenger in many signaling events that link the cortical actin cytoskeleton to the plasma membrane (Raucher et al., 2000). The regulation of PtdIns(4,5)P_2 synthesis might be crucial in mechanotransduction in view of the ability of PtdIns(4,5)P_2 to modulate the activity of a large number of actin-binding proteins (Yin and Janmey, 2003). Indeed, actin-binding proteins are important regulators of actin polymerization at focal adhesions (Zhang et al., 2007) and are crucial not only for

cell migration (Brakebusch and Fassler, 2003), but also for mechanosensing (Riveline et al., 2001; Kopecki et al., 2009). Notably, gelsolin, which modifies actin-filament length by severing pre-existing filaments and by capping the fast-growing barbed ends, is controlled by Ca^{2+} and by PtdIns(4,5)P_2 (Janmey et al., 1985), and therefore might be a crucial protein in the transduction of mechanical signals at focal adhesions.

Here, we tested the hypothesis that FAK is a mechanosensory element in the cellular response to stretching and the activation of the *SMA* gene. Our results demonstrate that phosphorylation of FAK at Tyr397 is required for actin assembly at sites of mechanical force by a mechanism that involves the interaction of PIP5KIγ with FAK, the generation of PtdIns(4)P_2 and the activation of gelsolin at focal-adhesion complexes. These processes in turn lead to actin assembly and the regulation of *SMA*-promoter activity through the actin-filament-dependent transcriptional co-activator MRTF-A.

Results

FAK activation by mechanical force

We examined whether application of tensile force (0.6 pN/μm^2) through integrins would recruit and activate FAK at force sites. To assess FAK and β-actin at the force-application sites, collagen-bead-associated proteins were analyzed by immunoblotting. Within 20 minutes of force application, there was increased FAK recruitment to collagen beads in force-treated cells compared with untreated cells (Fig. 1A). β-actin was detected in collagen-bead-associated proteins in samples of both force-treated and untreated cells but, in the treated cells, there was enhanced β-actin accumulation over time, indicating increased actin assembly at the force-application sites. We established whether the number of beads separated from cells were equivalent under these conditions. Beads were isolated at 60 minutes from force-treated and untreated cells, and the number of beads were counted (2049±22 beads for force-treated cells; 2135±17 beads for untreated cells; $P>0.2$). β1-integrin immunoblots were used as loading controls for each lane and these showed equal amounts of β1 integrin over the time course for all samples. Cells incubated with BSA-coated beads were used as bead-binding controls and cells were loaded with force for 60 minutes. Immunoblots of these samples showed no differences in the amount of FAK and β-actin between the force-treated and untreated groups. Immunoblots were processed to evaluate the amount of β1 integrin associated with BSA-coated beads, but these blots showed minimal association of β1 integrin. Because of the possibility that the collagen beads had become internalized during the experiment, we examined fluorescence quenching of fluorescein isothiocyanate (FITC)-collagen-coated beads with Trypan Blue, but there was no difference of quenching between 10 and 60 minutes (data not shown), indicating no significant bead internalization during this time interval.

By immunostaining and confocal microscopy we evaluated the spatial localization of FAK at collagen beads following force application. The intensity of fluorescence signals around the beads was quantified with ImageJ software. There was enhanced localization of FAK at collagen-coated beads after force application for 30 and 60 minutes (Fig. 1B), whereas, in cells without force treatment, there was little change of fluorescence intensity. In cells with BSA beads, there was no force-induced enrichment of FAK. In cells treated with *FAK* siRNA, there was very little accumulation of β-actin around beads in both control and experimental groups, and the force-induced increase of β-actin around beads was also very limited (Fig. 1C). Similarly, Alexa-Fluor-488–phalloidin staining around beads in FAK-knockdown cells showed lower fluorescence intensity than in control cells (Fig. 1D).

The FAK signaling pathway is initiated by autophosphorylation of FAK (Carlucci et al., 2008). To study the force-dependent activation of FAK, we examined its phosphorylation at Tyr397; phosphorylation at this residue is required for full activation of FAK and for productive interactions with downstream signaling proteins (Sakurai et al., 2002; Zhao et al., 1998). Compared with untreated controls, immunoblots of force-treated cells showed time-dependent increases of Tyr397 phosphorylation above baseline within 20 minutes of force application, which increased up to 60 minutes (Fig. 2A). Pre-incubation of cells with the β1-integrin-blocking antibody Lia1/2 prevented phosphorylation of Tyr397FAK at 60 minutes of force application. Fibronectin-coated and BSA-coated beads were used as controls and incubated for 60 minutes, but there was no phosphorylation of FAK at Tyr397.

To examine the abundance of other proteins that might be recruited into focal adhesions by force, we immunoblotted collagen-bead-associated proteins (Fig. 2A). We found an enrichment for talin and the Arp2/3 complex following force application. Furthermore, force-induced activation of Src, as indicated by phosphorylation at Tyr416 and dephosphorylation of Tyr527, was also evident. However, pre-incubation of cells with the Src inhibitor SU6656 had no effect on force-induced phosphorylation of FAK at Tyr397. These data suggested that activation of FAK by force is independent of Src activation.

We found enhanced localization of phosphorylated Tyr397FAK (Tyr397FAK-*P*) at collagen-coated beads after force application for 30 and 60 minutes (Fig. 2B), whereas, in cells without force treatment, there was little change of fluorescence intensity. In cells transfected with a dominant-negative (DN) construct (CD2-Tyr397PheFAK), there was a reduction of force-induced Tyr397 phosphorylation, and force-induced β-actin accumulation around collagen beads at 60 minutes was not detected (Fig. 2C). We also found that DN FAK did not change talin recruitment to beads but decreased Arp2 recruitment and inhibited Src activation (data not shown). When we examined the effect of force application on Tyr397FAK phosphorylation after knockdown of FAK with siRNA, we found a twofold decrease in the ratio of Tyr397FAK-*P*:FAK in samples treated with *FAK* siRNA compared with irrelevant siRNA. These data indicated that, in response to force application, the relative abundance of FAK recruited to force-application sites has an impact on the phosphorylation of FAK.

In this study, we focus on the role of FAK in mediating force-induced SMA expression. Previous data have shown that force-induced activation of the *SMA* promoter might involve Rho (Zhao et al., 2007). Accordingly, we determined whether force-induced activation of FAK requires Rho. Applying force to cells loaded with collagen-coated beads strongly increased phosphorylation of Tyr397FAK (Fig. 2D). In cells that were pre-treated with the Rho-kinase inhibitor Y27632 (10 μM, a concentration that is known to inhibit Rho kinase in these cells), there was little effect on force-induced FAK activation, indicating that force-induced Rho activation is downstream or independent of FAK. We examined this process in more detail by knocking down FAK with siRNA and then examining Rho activation in response to force. Rho activation was evaluated with rhotekin beads and immunoblotting bead-bound material for Rho. These data showed that force-induced RhoA activation was not dependent on FAK and that the RhoA and FAK signaling pathways might be working in parallel (Fig. 2E).

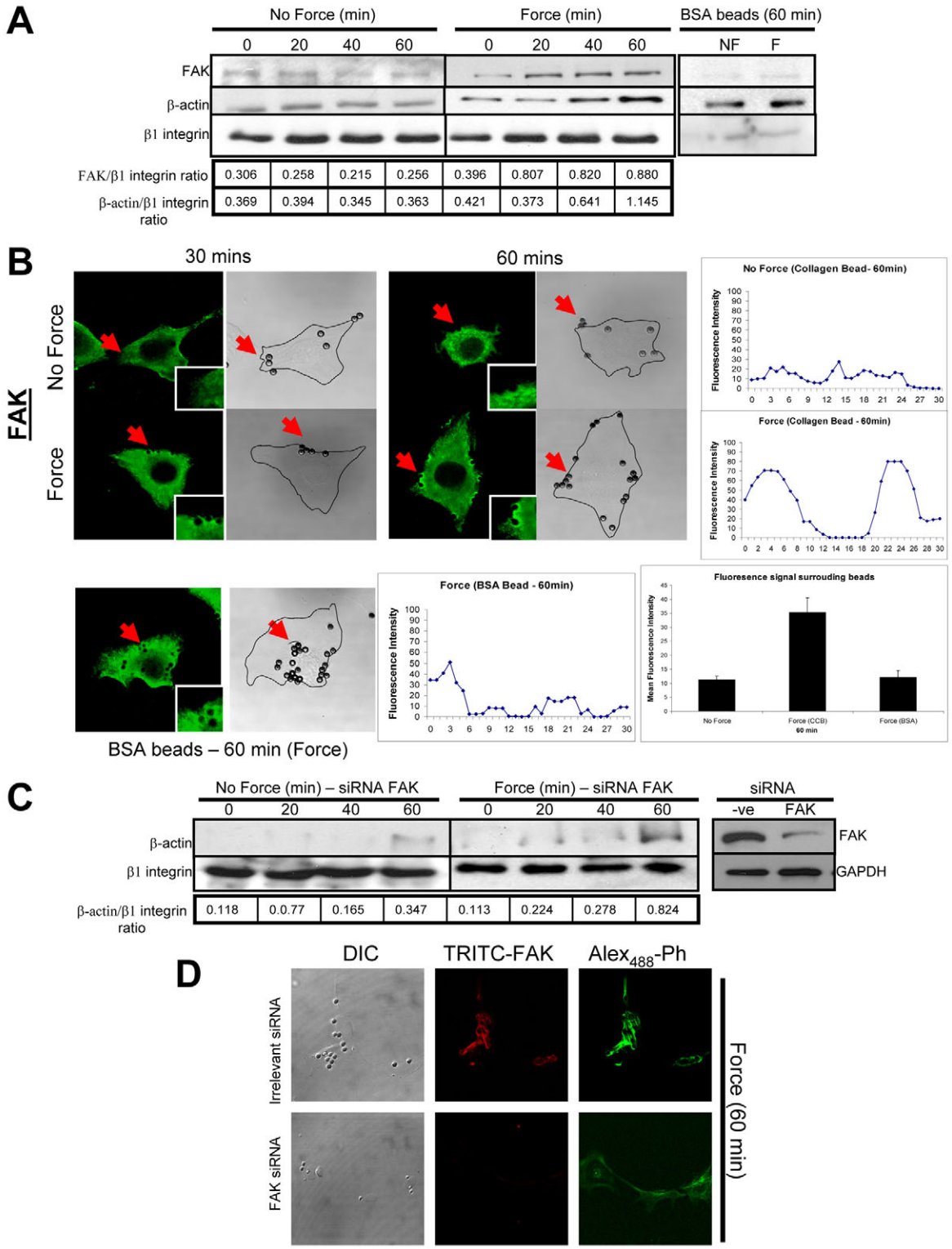

Fig. 1. FAK activation is required for mechanical-force-triggered actin association at focal adhesions. (A) Immunoblot analysis of collagen-coated-bead-associated proteins over a 60-minute time course in the presence of force or no force. An equal number of beads were attached to cells as indicated by the β1-integrin blot of bead-associated proteins and by actual bead counts (see text). BSA-coated beads were used as non-specific controls. (B) Confocal microscopy after collagen-bead incubation with cells showed FAK around beads. DIC images of the same cell show the location of beads. BSA-coated beads with force application were used as non-specific controls. Arrows show the area shown at higher magnification in the insets. Line graphs of fluorescence intensity are shown for insets. Data in histogram (mean fluorescence intensity ± s.e.m.) are representative of 25 beads. (C) Cells with FAK knockdown by siRNA were incubated with collagen-coated magnetite beads and subjected to force or no force. Immunoblots of bead-associated protein show β-actin recruitment. β1-integrin immunoblots show loading controls. Right panel shows efficacy of siRNA knockdown of FAK. (D) Confocal images show actin-filament staining with Alexa-Fluor-488–phalloidin around single collagen-coated beads after 60 minutes of force in cells treated with irrelevant siRNA or siRNA for *FAK*. Cells treated as indicated were stained for Alexa-Fluor-488–phalloidin and with TRITC-conjugated antibody (for FAK). Note that cell treated with siRNA for *FAK* shows no detectable FAK.

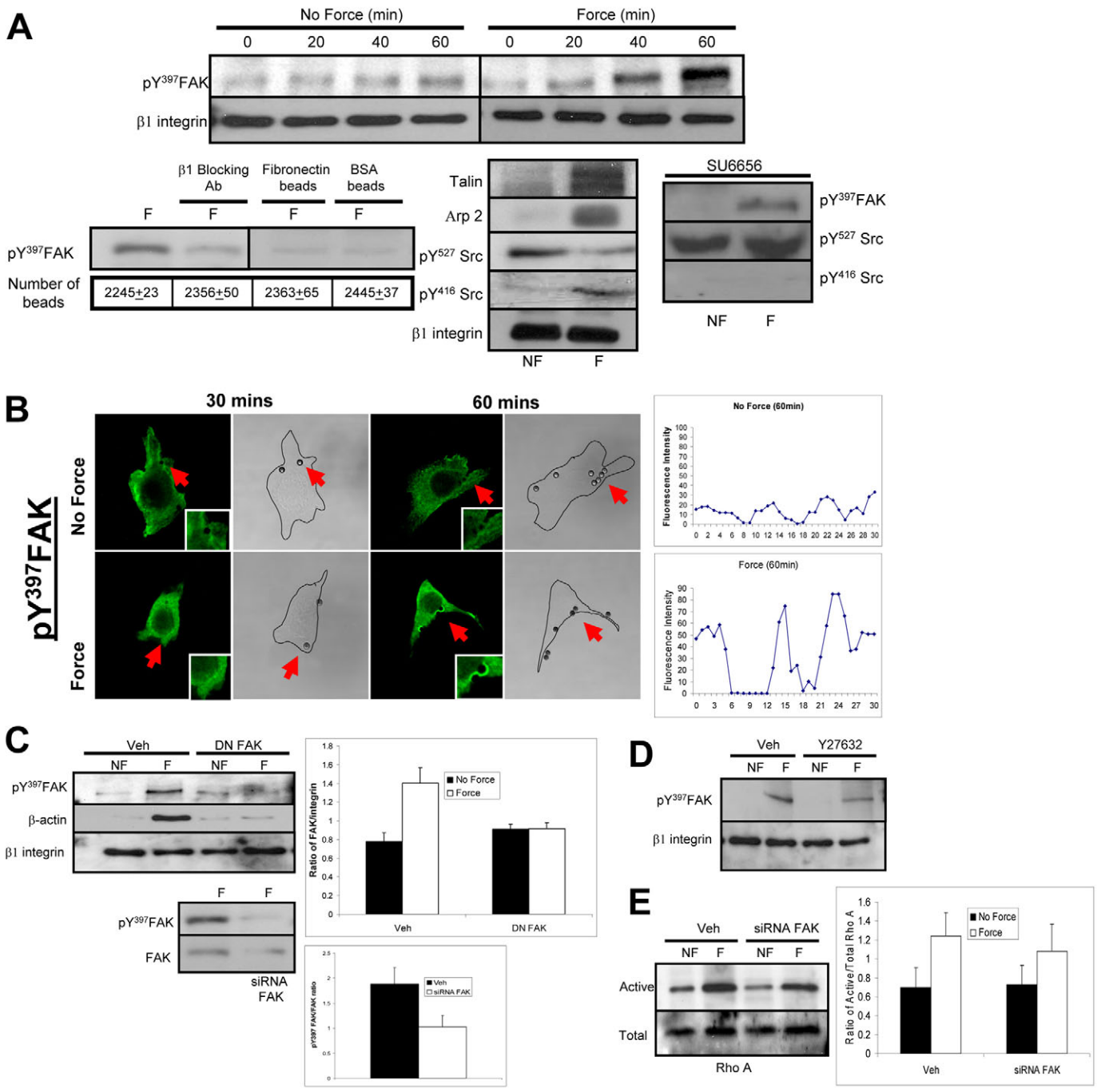

Fig. 2. FAK activation after mechanical force is independent of RhoA. (A) Top panels: immunoblot analysis of Tyr397FAK-*P* (pY^{397}FAK) in collagen-coated-bead-associated proteins after force application for the indicated time intervals. β1-integrin immunoblots show loading for each bead preparation. Bottom left panels: cells were incubated with collagen beads and force was applied for 60 minutes, the optimal time for phosphorylation of Tyr397FAK. Lia1/2 β1-integrin-blocking antibody was co-incubated with collagen beads prior to force application and was used here as a β1-integrin control to test for specificity. Fibronectin and BSA-coated beads were used as alternative controls in cells treated with force for 60 minutes. F, force. Bottom center panels: immunoblots show force-induced collagen-coated-bead association of talin and Arp2 after 60 minutes of force. NF, no force. Bottom right panels: activation of Src by force was determined by immunoblots of Tyr527Src-*P* (pY^{527}-Src) and Tyr416Src (pY^{416}-Src). Note that pre-incubation of cells with the Src inhibitor SU6656 at 1 μM does not inhibit force-induced phosphorylation of Tyr397FAK. (B) Confocal microscopy after force application shows Tyr397FAK-*P* around collagen beads. DIC images of the same cells show the location of beads. BSA-coated beads with force application were used as non-specific controls. Arrows show the area shown at higher magnification in the insets. Line graphs show fluorescence intensity across bound beads. (C) Membrane-anchored, dominant-negative Tyr397Phe FAK (DN FAK) was transfected into cells and protein recruitment to collagen-coated beads was analyzed in bead-associated proteins. After force application, immunoblots show increased levels of Tyr397FAK-*P* and β-actin in bead-associated proteins in vehicle (Veh)-treated cells but not in DN-FAK-treated cells. Ratios of β-actin to integrin were computed from densitometry measurements of immunoblots (mean ± s.e.m.). FAK knockdown by siRNA showed decreased Tyr397FAK-*P* in relation to the amount of FAK present. (D) Cells treated with Rho inhibitor, Y27632, were assessed for phosphorylation of Tyr397FAK. (E) Rho activation was measured in cells with or without siRNA knockdown using rhotekin-binding assay and immunoblotting for RhoA. Ratios of active Rho A to total Rho A were computed from densitometry measurements of immunoblots (mean ± s.e.m.).

Role of PIP5KIγ in force-mediated actin assembly

Our data on force-induced activation of FAK (above) suggested that FAK is involved in early stages of mechanotransduction and consequently might associate with other proteins in focal adhesions. Previous data have shown that PIP5KIγ is localized to focal adhesions (Ling et al., 2002; Sun et al., 2007), and that FAK and PIP5KIγ might interact at focal adhesions to promote cell adhesion to type-I collagen (Ling et al., 2002). Accordingly, we considered that FAK is functionally linked through PIP5KIγ to mediate force-induced actin assembly. Immunoprecipitation of cell lysates following force application showed that, in untreated cells, FAK associated with PIP5KIγ and this association was increased by force (Fig. 3A). In cells treated with *FAK* siRNA, PIP5KIγ was present in bead-associated proteins and this recruitment was increased by force. We next determined the impact of PIP5KIγ on force-induced FAK activation. Lysates were prepared from cells that had been transfected with either an irrelevant construct, a construct encoding wild-type (WT)-PIP5KIγ or one encoding DN-PIP5KIγ. Cells were stimulated with or without force, immunoblotted for Tyr397FAK-*P* and normalized to GAPDH. Immunoblots showed decreased amounts of force-mediated phosphorylation of Tyr397FAK in the presence of the catalytically inactive PIP5KIγ construct compared with the irrelevant construct or the WT-PIP5KIγ construct (Fig. 3B). There was also no change in the amount of total FAK. In cells treated with siRNA to knock down PIP5KIγ, we found no difference in force-induced FAK recruitment but the level of Tyr397FAK-*P* was reduced.

PtdIns(4,5)P_2 is generated by PIP5KIγ at focal adhesions and regulates actin-binding proteins, thereby enabling new actin-filament growth (Janmey and Stossel, 1989). We examined the localization of PtdIns(4,5)P_2 around collagen beads. Cells were co-transfected with PIP5KIγ constructs and with the pleckstrin-homology (PH) domain of phospholipase Cγ fused to GFP in order to show the localization of PtdIns(4,5)P_2. There was bright GFP staining around collagen beads of force-treated cells transfected with WT-PIP5KIγ, but not with the DN-PIP5KIγ construct (Fig. 3C). BSA beads were used as controls and they did not show enhanced localization of GFP staining to beads.

We examined the effect of PIP5KIγ on the incorporation of actin monomers into actin filaments at the junction near the cell surface associated with collagen beads (30 minutes after force). Quantification of rhodamine–actin-monomer incorporation was examined in permeabilized cells; fluorescence intensity was measured around attached collagen beads in confocal images. Whereas force enhanced actin-monomer incorporation around collagen beads in control transfected cells (Fig. 3D; $P<0.05$), cells transfected with a DN-PIP5KIγ construct exhibited no increase of actin-monomer incorporation around collagen beads. There was also no increase of actin-monomer incorporation in cells not treated with force or in cells incubated with BSA-coated beads and treated with force. The localization of Tyr397FAK-*P* and actin around collagen beads was examined by confocal microscopy. Cells were co-transfected with WT- or DN-PIP5KIγ constructs and stained for both Tyr397FAK-*P* and actin after force application. There was bright staining of Tyr397FAK-*P* and actin around collagen beads of force-treated cells transfected with PIP5KIγ WT, but not around those transfected with the DN-PIP5KIγ construct (Fig. 3E).

Actin assembly by gelsolin

The data above on localized generation of PtdIns(4,5)P_2 at sites of tensile-force application suggested a mechanism by which force could promote actin assembly in localized sites. PtdIns(4,5)P_2 uncaps gelsolin from actin barbed ends, thereby enhancing actin-filament assembly (Janmey and Stossel, 1987). Previously, we have shown that PtdIns(4,5)P_2 that is bound to gelsolin can be detected by immunoprecipitation of gelsolin and immunoblotting for PtdIns(4,5)P_2 (Arora et al., 2005). Following force application, there was increased PtdIns(4,5)P_2 immunoprecipitated with anti-gelsolin antibody compared with no-force samples (Fig. 4Ai). When DN-PIP5KIγ was expressed in cells by transfection, the association of PtdIns(4,5)P_2 with gelsolin was reduced compared with cells transfected with WT-PIP5KIγ constructs. We next examined whether gelsolin was required for PIP5KIγ recruitment to focal adhesions. WT or gelsolin-null (Gsn–) fibroblasts were incubated with collagen beads and stimulated with force for 30 minutes. Collagen-bead-associated proteins were isolated from cell lysates and immunoblotted for PIP5KIγ and for β1 integrin. Because there were similar amounts of force-induced recruitment of PIP5KIγ to focal-adhesion complexes in WT and Gsn– fibroblasts (Fig. 4Aii), force-induced recruitment of PIP5KIγ to focal adhesions was evidently independent of gelsolin.

Because our data above showed that gelsolin associated with PtdIns(4,5)P_2 and accumulated at focal adhesions, possibly to regulate actin-filament assembly, we determined whether force would mediate an increase in the relative abundance of gelsolin-actin complexes. Immunoblots showed that force application caused a 50% increase of the relative abundance of gelsolin-actin complexes compared with controls in both bead-associated proteins and ultracentrifuged pellets (Fig. 4B). The ratios of force-induced gelsolin-actin complexes were reduced to control levels when FAK was knocked down with siRNA.

Pyk2 is a focal-adhesion protein with significant protein sequence similarity to FAK (60% in the catalytic domain, and 40% in the N- and C-terminals) (Lev et al., 1995; Sasaki et al., 1995; Avraham et al., 1995) that can associate with gelsolin in regulating actin-cytoskeletal reorganization and that might compensate when FAK is absent (Wang, Q. et al., 2003). Because Pyk2 associates with gelsolin in spreading osteoclasts, we investigated FAK association with gelsolin in mechanotransduction. Immunoprecipitates that were prepared with either anti-FAK or -gelsolin antibodies showed enhanced association of FAK with gelsolin following force application (Fig. 4C). This association was dependent on the presence of phalloidin (1 μM) in the immunoprecipitating buffer to maintain intact actin filaments (Arora and McCulloch, 1996). Although our data showed that FAK associated with gelsolin, it is not known whether gelsolin is a downstream component of the force-induced FAK signaling cascade. To determine the potential sequence of interactions between FAK and gelsolin, WT and Gsn– fibroblasts were incubated with collagen beads, stimulated with force for 60 minutes and proteins were isolated from collagen-coated beads. We found similar amounts of force-induced recruitment of FAK and Tyr397FAK-*P* to bead-associated proteins in both wild-type and Gsn– fibroblasts (Fig. 4D). These data indicated that gelsolin did not regulate force-enhanced FAK recruitment to force-application sites (i.e. presumptive focal adhesions) or regulate FAK activation.

Regulation of SMA expression by FAK-PIP5KIγ-gelsolin

Our objective was to define the molecules in the pathway leading from force to SMA expression. Specifically, because SMA expression in fibroblasts is enhanced by tensile forces (Wang, J. et al., 2003), we determined whether *SMA*-promoter activity is

regulated by FAK. Cells were co-transfected with *SMA*-promoter–luciferase and *lacZ* constructs, and luciferase data were normalized to β-galactosidase levels. Application of force to collagen-coated beads for 4 hours caused a twofold increase of *SMA*-promoter activity ($P<0.001$) in human cardiac fibroblasts (Fig. 5A). More-detailed experiments were conducted using NIH3T3 cells (Fig. 5Bi); these showed a similar force-induced increase of *SMA*-promoter activity. Notably, force application to cells incubated with

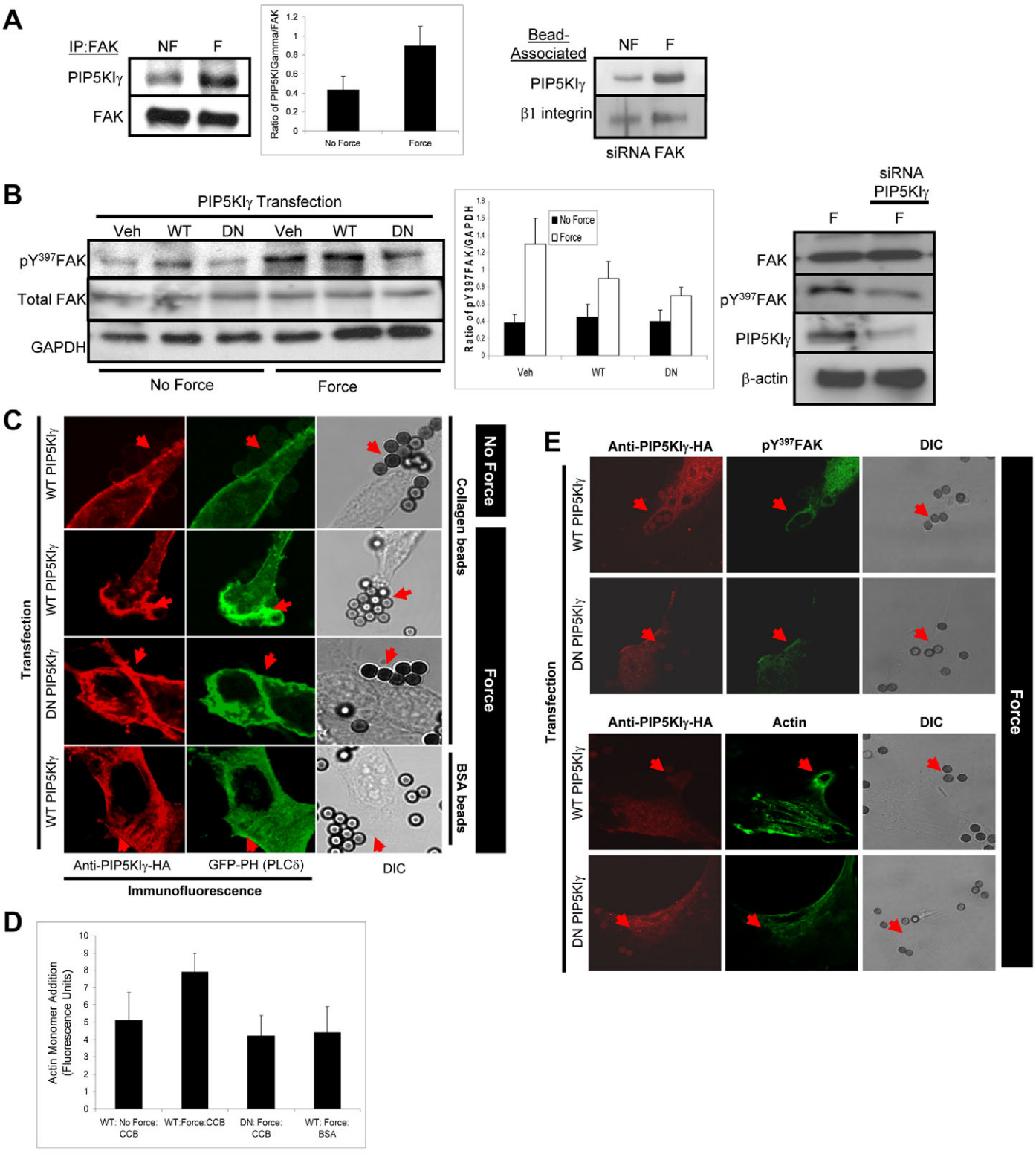

Fig. 3. See next page for legend.

BSA beads caused no change. This lack of difference with BSA beads was not because of differences in bead loading, because there were equivalent numbers of collagen and BSA beads attached to cells in both conditions. In cells that were pre-treated with siRNA to knock down FAK, force-induced *SMA*-promoter activity after force application was substantially reduced ($P<0.01$) compared with force-loaded cells with normal FAK levels. Consistent with the *SMA*-promoter data, after 4 hours of force treatment, immunoblots of cell lysates showed increased expression of SMA protein, but this effect was not observed when FAK was knocked down with siRNA (Fig. 5Bii). We also found that, although Src is not required for FAK activation (Fig. 2A), it is required for force-induced SMA expression (data not shown).

Because application of tensile force to fibroblasts mediates actin-filament-dependent nuclear translocation of MRTF-A, which is required for activation of the *SMA* promoter (Zhao et al., 2007), we investigated whether FAK is required for force-induced translocation of MRTF-A. In fixed cells immunostained for endogenous MRTF-A, immunofluorescence images showed nuclear translocation of MRTF-A following force application. Quantification of these cells showed that force-induced MRTF-A nuclear translocation was inhibited by *FAK* siRNA knockdown (Fig. 5C). In cells transfected with an irrelevant (GFP) siRNA as a negative control, there was a ninefold increase of MRTF-A nuclear translocation after 60 minutes of force application, consistent with previous data (Zhao et al., 2007).

PIP5KIγ is localized to focal adhesions (Ling et al., 2002; Ling et al., 2006); thus, we considered whether FAK and gelsolin are functionally linked through PIP5KIγ to mediate force-induced SMA expression. WT- or catalytically inactive (DN)-PIP5KIγ constructs were co-transfected with the *SMA*-luciferase promoter construct. *SMA*-promoter activity was then assessed in force-treated cells. The expression of catalytically inactive PIP5KIγ reduced force-mediated *SMA*-promoter activity by about 50% compared with cells transfected with WT-PIP5KIγ ($P<0.02$; Fig. 5D). Gsn– fibroblasts showed no significant enhancement ($P>0.2$) of force-induced *SMA*-promoter luciferase activity, but there was a 2.3-fold increase in WT cells (Fig. 5E; $P<0.01$). When Gsn– fibroblasts were reconstituted with gelsolin cDNA, force-activated *SMA*-promoter activity was observed (twofold increase above controls; $P<0.02$). Immunoblots showed the presence of gelsolin in the cell lysates of WT and gelsolin-reconstituted cells but not in Gsn– cells.

Fig. 3. PIP5KIγ is required for FAK-dependent mechanotransduction. (A) FAK immunoprecipitates from cells subjected to force (F) or no force (NF) were immunoblotted for PIP5KIγ. Ratios of PIP5KIγ to FAK were computed from densitometry measurements of immunoblots. Force-induced recruitment of PIP5KIγ to collagen-coated-beads were assessed in FAK-deficient cells. (B) Cells were transfected with vehicle (Veh), wild-type (WT) or catalytically inactive (DN) PIP5KIγ constructs and analyzed for force-induced phosphorylation of Tyr397FAK. From immunoblots of Tyr397FAK-*P* (pY^{397}FAK) and GAPDH loading controls, ratios were computed from densitometry measurements. FAK recruitment and activation to collagen-coated beads following force in cells in which PIP5KIγ was knocked down by siRNA were analyzed by immunoblots. (C) Localization of PtdIns(4,5)P_2 at mechanically stimulated bead junctions following transfection with WT or catalytically inactive (DN) PIP5KIγ constructs. PtdIns(4,5)P_2 accumulation at bead-binding sites was studied by transient transfection of cells with a construct expressing the PH domain of PLCγ fused to GFP. Confocal microscopy at 15 minutes after bead incubation with cells shows the location of PtdIns(4,5)P_2 (green) around collagen beads and PIP5KIγ constructs (red). DIC images show the location of beads. (D) Effect of PIP5KIγ on force-induced rhodamine–actin-monomer incorporation at the collagen-bead–cell interface. Cells were transfected with WT or catalytically inactive (DN) PIP5KIγ constructs and treated with or without force. Rhodamine fluorescence around beads was quantified from 25 cells for each treatment group in defined 4-μm^2 zones. Histogram shows data (mean s.e.m.) of rhodamine-actin fluorescence. CCB, collagen-coated bead. (E) Localization of Tyr397FAK-*P* and actin at mechanically stimulated bead junctions following transfection with WT or catalytically inactive (DN) PIP5KIγ constructs. Confocal microscopy at 60 minutes after bead incubation with cells shows the location of Tyr397FAK-*P* (green, top panels) and actin (green, bottom panels) around collagen beads and PIP5KIγ constructs (red). DIC images show the location of beads.

Discussion

Cardiac cells can respond to increased loading by undergoing phenotypic changes, including the conversion of cardiac fibroblasts to myofibroblasts, cells that promote interstitial fibrosis. Our principal finding is that FAK acts as a proximal sensor for transferring mechanical signals into downstream processes that enhance SMA expression, a classical marker of the myofibroblast. We have identified PIP5KIγ and gelsolin as crucial molecules that functionally connect FAK to actin assembly and, via MRTF-A, to SMA expression. These data provide evidence for a mechanotransduction system that links the application of tensile forces at collagen-binding sites to the regulation of actin assembly (Vadali et al., 2007) and SMA induction in myofibroblast differentiation (Fig. 6).

Focal adhesions have been postulated as mechanosensory organelles that can generate molecular signals in response to applied mechanical forces (Schober et al., 2007; Vadali et al., 2007; Le Clainche and Carlier, 2008). FAK, an important signaling protein in focal adhesions, is a crucial component of integrin-based signaling (Guan and Shalloway, 1992), and has been extensively studied in adhesion, migration and growth-regulation processes that rely on focal-adhesion regulation (Cohen and Guan, 2005). Recent studies have indicated that FAK is activated in the stretching of blood-vessel walls (Albinsson and Hellstrand, 2007) and is required for the regulation of actin assembly in migrating keratinocytes (Schober et al., 2007). When we examined the collagen-bead-associated proteins of cells that had been treated with tensile force, we found an enrichment of β-actin that was dependent on the recruitment and activation of FAK and phosphorylation of the Tyr397 residue of FAK. There is a paucity of data relating mechanical force, FAK-related actin assembly and SMA expression, the hallmark protein of myofibroblast differentiation (Li et al., 2002; Wu et al., 2007). Previous data showed that shear-stress-induced actin reorganization is linked to the RhoA-ROCK-LIMK-cofilin signal-transduction pathway (Wu et al., 2001; Li et al., 1999) and this pathway has been invoked in tensile-force-induced SMA expression in myofibroblast differentiation (Zhao et al., 2007). Because suppression of RhoA activity has been reported to increase FAK activation (Ren et al., 2000) and activation of RhoA is linked to SMA expression (Zhao et al., 2007), we examined whether RhoA is involved in FAK-dependent SMA regulation. Incubation of cells with the Rho-kinase inhibitor Y27632 only slightly reduced the amount of force-induced phosphorylation of Tyr397FAK. Furthermore, FAK knockdown by siRNA did not affect RhoA activation following application of tensile force. Therefore, FAK-dependent upregulation of SMA by mechanical force seems to involve a signaling pathway that is independent of RhoA.

We found enhanced phosphorylation of Tyr397 FAK in collagen-bead-associated protein preparations within 40 minutes of force application to cells, and this response was dependent on the relative abundance of FAK because downregulation of FAK by siRNA reduced the relative amount of force-induced phosphorylation of

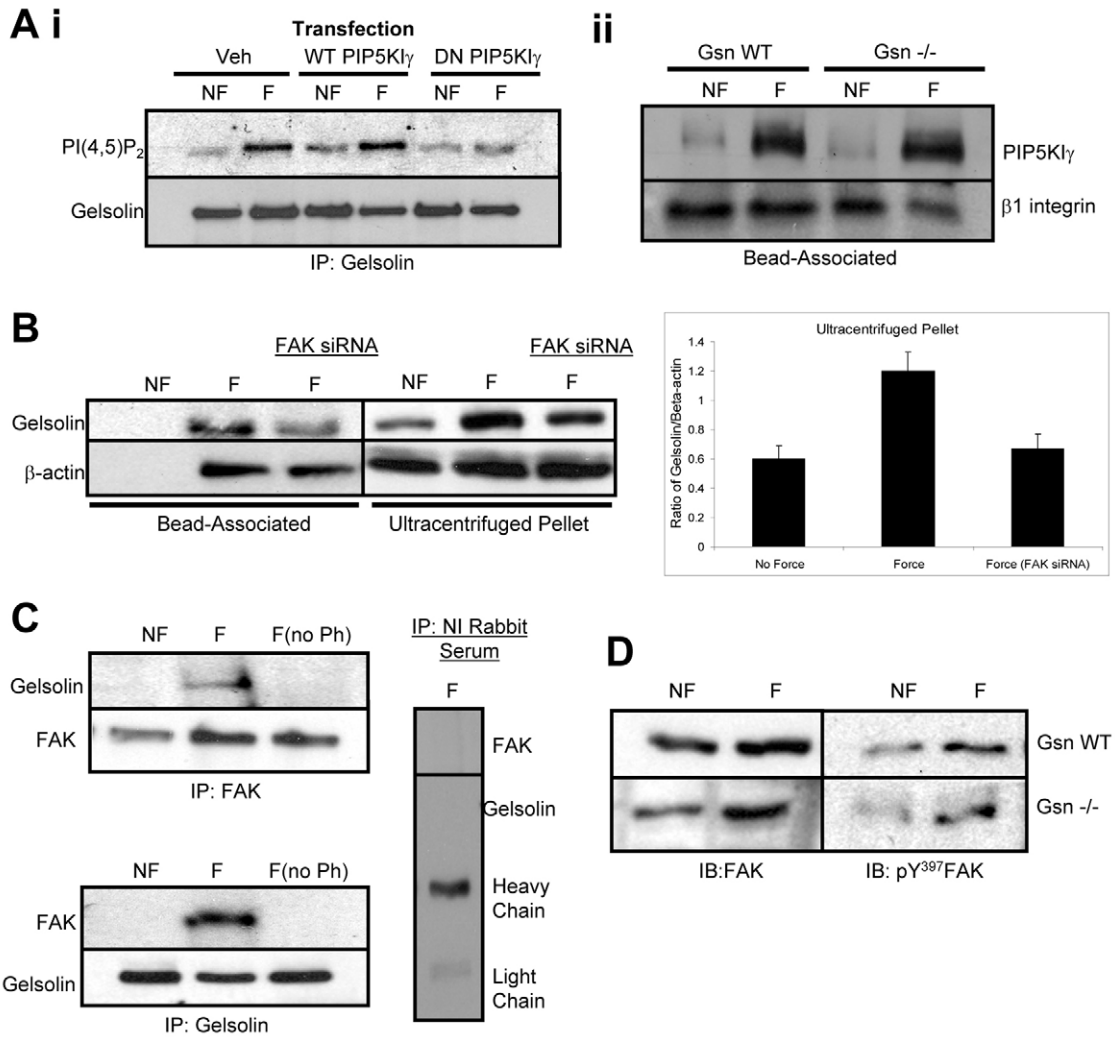

Fig. 4. Gelsolin interacts with FAK and PIP5KIγ after force application. (Ai) Gelsolin-PtdIns(4,5)P_2 complexes measured in gelsolin immunoprecipitates from cells transfected with WT or inactive PIP5Kγ. (Aii) Immunoblots of PIP5KIγ recruitment to force-stimulated collagen-coated magnetite beads in WT and Gsn– cells. F, force; NF, no force; Veh, vehicle. (B) Force-induced gelsolin-β-actin complexes were analyzed in bead-associated fractions and ultracentrifugation fractions. Cells were treated with *FAK* siRNA as indicated and then either subjected to force or no force. Proteins were measured by BCA assay and immunoblots were normalized by loading an equal amount of protein. Densitometry of immunoblot ratios for gelsolin and β-actin are shown as mean ± s.e.m. (C) After 1 hour of force, FAK immunoprecipitates were analyzed for the presence of gelsolin. Gelsolin associates with FAK only in the presence of force and phalloidin (Ph; 1 μm). Gelsolin immunoprecipitates blotted for FAK under similar conditions show FAK-gelsolin association. Immunoprecipitation using pre-immune rabbit serum was used a control. (D) Immunoblots of FAK and Tyr397FAK-*P* recruitment to collagen beads following force stimulation show no difference in WT or Gsn– cells.

Tyr397FAK. Previous studies of migrating cells showed that phosphorylation of FAK can trigger multiple protein-protein interactions in which FAK associates with Src (Xing et al., 1994), Cas (Harte et al., 1996; Owen et al., 1999) and paxillin (Schaller et al., 1999). Apparently, accumulation of Tyr397FAK-*P* in focal adhesions increases the abundance of adaptor sites for downstream signaling proteins and correlates with enhanced anchorage of FAK to actin filaments. Notably, the phosphorylation of Tyr397FAK creates a high-affinity binding site for Src-family kinases that leads to increased phosphorylation of FAK and recruitment of other FAK-binding proteins, including paxillin (Schaller et al., 1999) and PIP5KIγ (Ling et al., 2002). These FAK-binding proteins not only establish more-stable focal adhesions but can also generate downstream signals (Owen et al., 1999).

Because the structure of FAK suggests that it does not contain domains that allow direct interactions with actin filaments (Girault et al., 1999; Sun et al., 2002; Hildebrand et al., 1993; Martin et al., 2002), we considered whether PIP5KIγ is a downstream signal arising from the activation of FAK that leads to β-actin recruitment and the subsequent increased expression of SMA. We determined the relative abundance of PIP5KIγ at force-application sites by immunoprecipitating FAK. We found that there was increased association of PIP5KIγ with FAK following mechanical force. However, FAK knockdown did not prevent force-induced recruitment of PIP5KIγ to focal adhesions. These data suggest that PIP5KIγ recruitment to collagen beads is dependent on mechanical force but not on FAK. The expression and activation of PIP5KIγ are crucial components in focal-adhesion maturation because PIP5KIγ modulates the recruitment of talin (Ling et al., 2002). In turn, talin binds to and activates β1 integrin and serves to anchor PIP5KIγ to FAK. Notably, we found that force application enhanced talin recruitment to collagen beads. Furthermore, we showed here

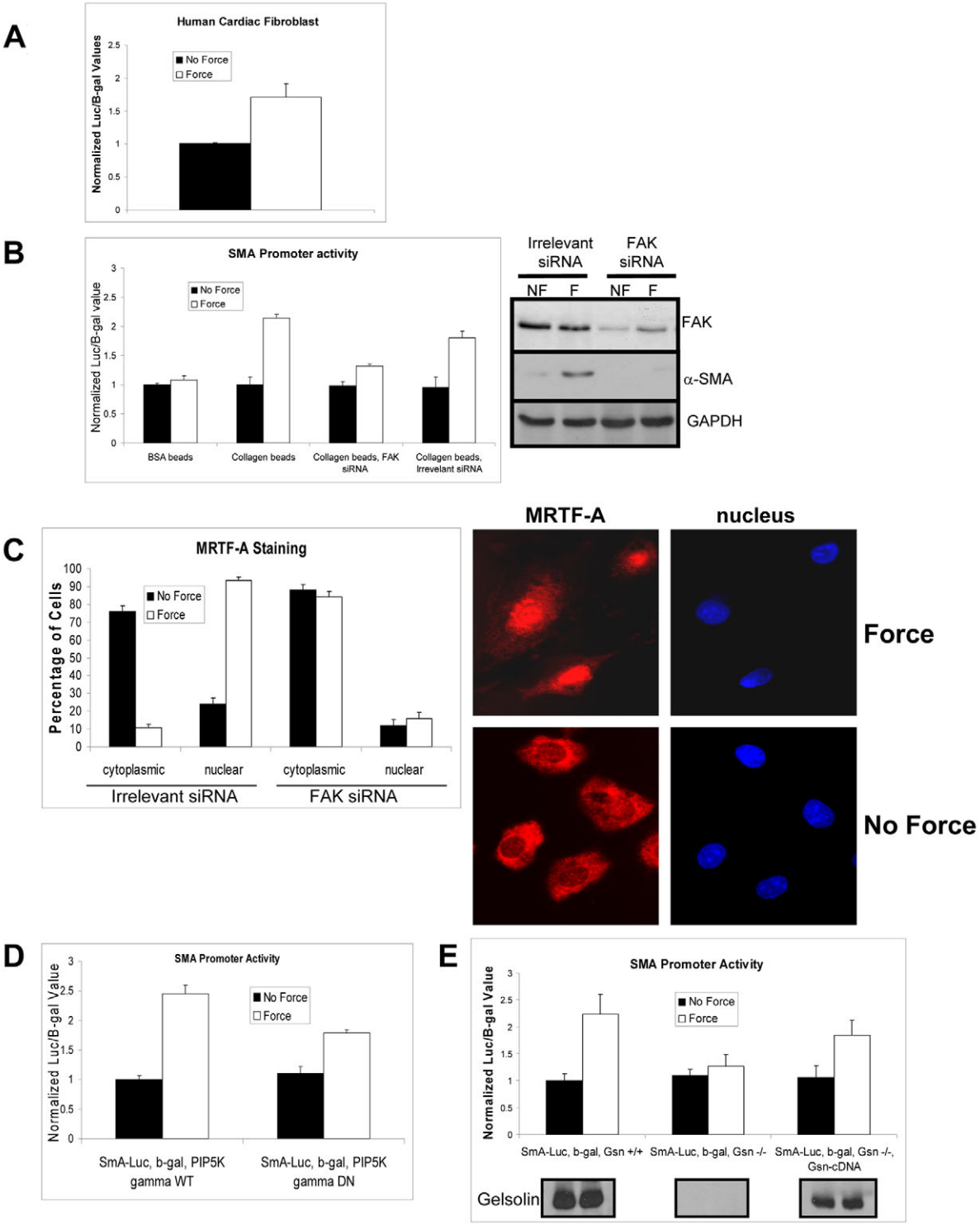

Fig. 5. Force-induced SMA expression by the FAK-PIP5KIγ-gelsolin signaling pathway. (A,B) Force-induced *SMA*-luciferase promoter activity normalized to β-galactosidase was measured in human cardiac fibroblast (A) and NIH3T3 cells (B) with or without knockdown of FAK by siRNA. BSA beads and irrelevant (GFP) siRNA were used as controls. Immunoblots show protein expression of SMA. F, force; NF, no force. (C) MRTF-A nuclear translocation following force stimulation requires the presence of FAK. Nuclear translocation of MRTF-A was measured in cells that had been subjected to force or no force treated with irrelevant or *FAK* siRNA. Immunofluorescence images were quantified after 60 minutes of force. Data are given as mean ± s.e.m. for MRTF-A nuclear translocation, quantified by counting 25 cells from each experimental group. (D) Activation of the *SMA* promoter by PIP5KIγ. Data show *SMA*-promoter activity following force application and transfection of constructs encoding WT- or DN-PIP5KIγ. (E) Gelsolin is required for force-induced activation of the *SMA* promoter. *SMA*-promoter activity was measured after force application in gelsolin WT cells, Gsn– cells and Gsn– cells reconstituted with *Gsn*-WT cDNA. Equivalent amounts of protein were loaded in each lane as measured by the BCA assay.

that catalytically active PIP5KIγ was required to promote force-induced activation of Tyr397FAK. However, when catalytically inactive PIP5KIγ was expressed, there was a 50% reduction in Tyr397FAK-*P*. This finding showed that PIP5KIγ can regulate force-induced activation of FAK, in addition to being recruited to force-induced focal adhesions. Thus, it seems that there is a feedback loop between FAK and PIP5KIγ, in which PIP5KIγ is required for full activation of FAK (Fig. 6).

PIP5KIγ generates PtdIns(4,5)P_2 at focal adhesions by phosphorylating PtdIns(4)*P* (Ling et al., 2002), and PtdIns(4,5)P_2, in turn, is necessary to sustain FAK signaling (Linseman et al., 1999). The accumulation of PtdIns(4,5)P_2 was detected at force-applications sites only when the catalytically active PIP5KIγ was expressed. Similarly, de novo actin assembly at force-application sites only occurred when catalytically active PIP5KIγ was present. In view of these findings, we suggest that PtdIns(4,5)P_2 at focal adhesions might modulate the activity of actin-binding proteins. In this context, PtdIns(4,5)P_2 can regulate the binding of vinculin to the Arp2/3 complex (DeMali et al., 2002), the association of ezrin and F-actin (Janke et al., 2008), and the binding of EFA6 (an Arf6-specific exchange factor) to phospholipid membranes and actin filaments (Macia et al., 2008).

Another abundant actin-binding protein that is also regulated by PtdIns(4,5)P_2 is gelsolin (Janmey and Stossel, 1987): PtdIns(4,5)P_2 can modulate both the actin-severing activity of gelsolin and the uncapping of actin filaments. Gelsolin is a multifunctional, six-domain protein that mediates actin-filament length by severing and capping barbed-end filaments (Janmey et al., 1985). Domain 2 of the six domains of gelsolin contains a PtdIns(4,5)P_2-regulated binding site that mediates the uncapping of gelsolin from actin barbed ends, thereby enabling filament growth (Sun et al., 1999). In experiments using force application to cells, we found increased production of PtdIns(4,5)P_2 at mechanically stimulated focal adhesions, leading to more-abundant association of gelsolin with PtdIns(4,5)P_2. This increased association was found in cells expressing catalytically active PIP5KIγ but not the inactive form. These data are consistent with the notion that the activity of actin-binding proteins that are associated with focal adhesions can be regulated to mediate actin assembly in response to force (Guo and Wang, 2007) and is in agreement with our finding that PIP5KIγ at force-treated collagen-bead-binding sites generates PtdIns(4,5)P_2. In turn, PtdIns(4,5)P_2 regulates gelsolin and leads to increased numbers of actin-free barbed ends (Fig. 6). The importance of this sequence of interactions is supported by experiments in Gsn– cells, in which the recruitment of PIP5KIγ to focal adhesions was no different than in WT cells. This finding indicated that gelsolin is downstream of PIP5KIγ in the signaling sequence that leads to de novo actin assembly at force-application sites. Conceivably, gelsolin must be present initially at focal adhesions to regulate actin assembly (Arora et al., 2000).

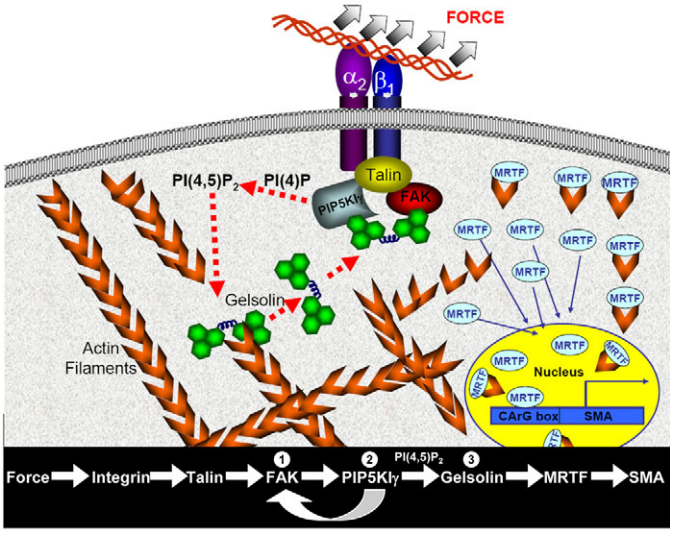

Fig. 6. Proposed model of mechanotransduction. Application of tensile forces to collagen beads triggers the recruitment and phosphorylation of FAK to focal adhesions. FAK recruits PI5KIγ, which generates PtdIns(4,5)P_2. In turn, PtdIns(4,5)P_2 mediates actin assembly by uncapping gelsolin from actin barbed ends. The dissociation of MRTF-A from actin monomers during actin assembly initiates the nuclear translocation of MRTF-A. In the nucleus, MRTF-A acts as transcriptional co-activator for the induction of SMA expression. A flow diagram illustrates the mechanotransduction pathway, in which the DN constructs and/or siRNAs are thought to exert their effects on (1) FAK, (2) PIP5KIγ and (3) gelsolin

In collagen-bead-associated proteins prepared from cells treated with force, we found an enrichment of gelsolin that was associated with β-actin. Force-induced association of gelsolin with β-actin was also associated with enhanced gelsolin and β-actin in the high-speed pellet. These data suggest that there was a detectable increase of gelsolin binding to actin not only in focal adhesions but also in the rest of the cytoskeleton. However, the increased binding of gelsolin to actin was only observed when FAK was present, as demonstrated by the reduced abundance of gelsolin-actin complexes in samples treated with *FAK* siRNA. Therefore, FAK seems to regulate the interaction of gelsolin with actin in response to force.

An association of gelsolin and FAK in actin-filament assembly has been previously postulated (Wang, Q. et al., 2003). In our magnetite-bead model, we found that FAK associated with gelsolin, but only after application of tensile force. This association required intact actin filaments, because immunoprecipitation of FAK-gelsolin complexes only occurred when cell lysates were stabilized with phalloidin. We found that force-induced increases of gelsolin binding to actin and recruitment to focal adhesions was dependent on FAK. However, the reverse was not true because, in Gsn– cells, the amount of force-induced Tyr397FAK-*P* was similar to WT cells. Thus, FAK, acting as a putative mechanosensor, is required for gelsolin-dependent actin assembly.

To determine the effect of the FAK-PIP5KIγ-gelsolin mechanotransduction pathway on SMA expression, we investigated the requirement of each of these proteins in the pathway leading to force-induced upregulation of the *SMA* promoter as well as SMA protein expression. With a *SMA*-luciferase promoter, we found that FAK was required for the force-induced upregulation of *SMA*-promoter activity and SMA protein expression. Furthermore, we demonstrated that catalytically active PIP5KIγ was important for increased *SMA*-promoter activity following force stimulation. The incomplete suppression of force-induced *SMA*-promoter activity by either siRNA knockdown of FAK or PIP5KIγ showed that other signaling pathways, including the RhoA-ROCK-LIMK-cofilin pathway, might be required for complete activation of SMA, as we have shown earlier (Zhao et al., 2007). Similarly, Rho inhibition by Y27632 did not completely suppress force-induced SMA expression (75% reduction; data not shown), indicating that the RhoA-ROCK-LIMK-cofilin pathway is not the only pathway for SMA expression. Nevertheless, our current data indicate that FAK and PIP5KIγ cooperatively act to mediate mechanotransduction and that their binding (Ling et al., 2002) might be important for these functions.

To study the effect of gelsolin on force-induced SMA expression, we used Gsn– fibroblasts and compared their force-induced SMA expression with WT cells. We found that force-induced *SMA*-promoter activity was reduced in Gsn– cells. However, this effect was reversed when WT gelsolin cDNA was reconstituted into Gsn– fibroblast, indicating that gelsolin is a necessary component for FAK-dependent force-induced upregulation of SMA expression. Conceivably, in view of the importance of PtdIns(4,5)P_2 in this force-induced model, the uncapping of gelsolin from actin barbed ends is required for actin assembly at focal adhesions following force. The assembly of actin is an important requirement for liberation of MRTF-A from actin monomers (Zaromytidou et al., 2006). This force-induced actin-filament assembly and activation of the serum-response factor then allows the nuclear translocation of MRTF-A to upregulate SMA expression (Zhao et al., 2007). Consistent with these observations, we found that increased SMA expression was also due to FAK-dependent nuclear translocation of MRTF-A. Together, these data provide a novel mechanism in which force induces the FAK-PIP5KIγ-gelsolin pathway that leads to the upregulation of SMA expression (Fig. 6).

Materials and Methods

Reagents

Latex (2-μm diameter) beads were purchased from Polysciences (Warrington, PA). Antibodies to β-actin (clone AC-15), talin (clone 8D4) and rabbit polyclonal IgG conjugated to horseradish peroxidase, as well as FITC-conjugated goat anti-mouse antibody, tetramethylrhodamine B isothiocyanate-phalloidin and Y-27632, were from Sigma-Aldrich (St Louis, MO). Rabbit polyclonal anti-FAK antibody was purchased from Upstate (Lake Placid, NY). Rabbit polyclonal phosphospecific antibody against FAK (Tyr397-*P*) was purchased from BioSource (Camarillo, CA). FITC-goat anti-rabbit and anti-mouse β1-integrin antibodies were purchased from Cedarlane (Hornby, ON). A polyclonal antibody that recognizes MRTF-A was generously donated by H. Nakano (Juntendo University School of Medicine, Tokyo, Japan). The affinity-purified polyclonal antibody to recombinant gelsolin has been described previously (Azuma et al., 1998). PIP5KIγ isoform-specific polyclonal antibody, PIP5KIγ661 WT and inactive kinase constructs were generously donated by Richard A. Anderson (University of Wisconsin-Madison, WI). Antibodies to Arp2, Tyr527Src-*P* and Tyr416Src-*P* were purchased from Cell Signaling Technology (Danvers, MA). DN FAK construct consisting of a transmembrane-anchored chimeric FAK with a point mutation Tyr397Phe, CD2-FAK(Tyr397Phe), was generously donated by Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA (Chan et al., 1994). BCA Protein Assay kit was purchased from Pierce (Rockford, IL).

Cells

NIH3T3 cells or fibroblasts obtained from either WT or Gsn– 12-day mouse fetuses (Witke et al., 1995) were cultured at 37°C in complete DME medium containing 5% fetal bovine serum and a 1:10 dilution of an antibiotic solution (0.17% w/v penicillin V, 0.1% gentamycin sulfate and 0.01% μg/ml amphotericin). Cells were maintained in a humidified incubator gassed with 95% air and 5% CO_2, and were passaged with 0.01% trypsin (Gibco, Burlington, ON). Prior to force experiments, cells were incubated in DME medium containing 1% serum, incubated with beads and force was applied for various time periods.

Force application

A force-generation model was used as described previously (Glogauer et al., 1995; Glogauer et al., 1998). Magnetite 2-μm-diameter beads (Spherotech, Lake Forest, IL) were coated with collagen as described (Glogauer et al., 1995), rinsed in PBS and attached to the dorsal surface of cultured cells. A ceramic permanent magnet (Jobmaster, Mississauga, ON) was used to generate tensile forces (0.6 pN/μm^2) (Glogauer and Ferrier, 1998) perpendicular to the dorsal surface of the cell. To restrict examination of mechanosensory events to periods of time prior to bead internalization (determined by Trypan Blue quenching of FITC-collagen-coated beads), forces were applied to cells only up to 60 minutes.

Collagen bead-associated proteins

Collagen or BSA-coated magnetite 2-μm-diameter beads (Spherotech) were attached to 7×10^6 cells at a 10:1 bead:cell ratio for 30 minutes. Cells and collagen-coated magnetic beads were collected by scraping into ice-cold cytoskeletal extraction buffer (0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, pH 6.8) containing protease inhibitors. BSA-coated beads attach to cells by non-specific interactions and allow comparisons of proteins associated with a broad array of membrane proteins to be contrasted with those associating with β1 integrins (Arora et al., 2000; Arora et al., 2004; Arora et al., 2005). Beads were separated from cell lysates using a side-pull magnet. Bead-associated proteins were removed from beads with sample bugger, quantified (by BCA assay), separated on SDS-PAGE gels and immunoblotted.

Actin-monomer incorporation

Actin-monomer incorporation into actin filaments in permeabilized cells was performed as described (Arora et al., 2004). Briefly, cells were permeabilized for 20 seconds in 0.1 volume of OG buffer (PHEM buffer containing 2% octyl glucoside and 2 μM phalloidin). Permeabilization was stopped by diluting the detergent with buffer without detergent. Immediately thereafter, freshly sedimented rhodamine-actin monomers (0.23 μM) in buffer containing 120 mM KCl, 2 mM $MgCl_2$, 3 mM EGTA, 10 mM PIPES and 0.1 mM ATP were added to samples for 10 seconds followed by fixation with 3.7% formaldehyde. Rhodamine fluorescence in single cells was quantified using PCI Imaging (Compix) and a Nikon TE300 microscope. For estimation of background correction, detergent treatments were omitted, fluorescence was quantified and this background signal was subtracted from experimental samples.

Immunofluorescence and confocal microscopy

Cells plated on beads were allowed to spread and bind to collagen beads for 30 minutes. Cells were fixed with 3% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 and stained with the appropriate primary antibody followed by immunofluorescence-tagged secondary antibody. Spatial distributions of protein staining around beads were determined by confocal microscopy (Leica, Heidelberg, Germany; 40×, 1.4 numerical aperture oil-immersion lens). Transverse optical sections were obtained at 1-μm nominal thickness.

Gelsolin-actin complexes

To examine the relative abundance of gelsolin-actin complexes following mechanical force, we quantified gelsolin that was associated with actin in the ultracentrifuged pellet. Cells were collected and suspended in a non-detergent buffer (1 mM EGTA, 2 mM Tris, pH 7.4, 0.2 mM ATP, 0.2 mM $MgCl_2$, 0.2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride) containing 1 μM phalloidin. Phalloidin at this concentration stabilizes F-actin (Arora and McCulloch, 1996). Freshly prepared cell lysates were pipetted several times with a narrow-gauge pipette tip and sedimented (100,000 ***g*** for 1 hour at 4°C). The pellet, which contained gelsolin complexed with actin filaments, was suspended in 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM ATP, 1 mM $NaHCO_3$ and 1 mM $NaNa_3$. Protein concentrations in the fractions were equilibrated, separated by SDS-PAGE, and immunoblotted for detection of actin and gelsolin.

FAK knockdown

Specific inhibition of mouse FAK was conducted with Silencer siRNAs (Dharmacon) with the following sequences: 5′-GCUAGUGACGUAUGGAUGUUU-3′, 5′-ACAUCCAUACGUCACUAGCUU-3′ (Tilghman et al., 2005). Cells were transfected with siRNA (200 nM) targeting FAK or a negative-control siRNA targeting GFP (Dharmacon) using DharmaFECT transfection reagent 2 for 72 hours. Cells were washed in PBS, lysed with Laemmli buffer and immunoblotted to assess the efficacy of the siRNA-dependent knockdown. After transfection and before force experiments, cells were incubated in DME medium containing 1% serum.

Transfection and *SMA*-promoter studies

Cells were transfected with a 765-bp rat *SMA*-luciferase construct (Raphael Nemenoff, University of Colorado, Denver, CO) and were co-transfected with a *lacZ* construct as a control to normalize for variations of transfection efficiency. Transfections were done with Fugene 6 (Roche Applied Science) according to the manufacturer's instructions. Cells were incubated with normal growth medium (5% serum in DME medium) within the first 36 hours and then cells were cultured in serum-reduced conditions (1% serum in DME medium) overnight. After transfections, cells were loaded with collagen-coated beads and magnetic force was applied for specific time periods. Cells were harvested, and luciferase and β-galactosidase activities were determined as described (Wang et al., 2002). Transfection data were computed as the fold change compared with basal promoter activity normalized to β-galactosidase activity.

Statistical analyses

For continuous variables, means and standard errors of means (s.e.m.) were computed. Differences between groups were evaluated by Student's unpaired *t*-test or analysis of variance for multiple comparisons. Statistical significance was set at $P<0.05$. Post hoc comparisons were performed with Tukey's test. For all experiments, at least three independent experiments were evaluated, each performed in triplicate.

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References

Albinsson, S. and Hellstrand, P. (2007). Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle. *Am. J. Physiol. Cell Physiol.* **293**, C772-C782.

Anderson, R. A., Boronenkov, I. V., Doughman, S. D., Kunz, J. and Loijens, J. C. (1999). Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. *J. Biol. Chem.* **274**, 9907-9910.

Arora, P. D. and McCulloch, C. A. (1996). Dependence of fibroblast migration on actin severing activity of gelsolin. *J. Biol. Chem.* **271**, 20516-20523.

Arora, P. D., Manolson, M. F., Downey, G. P., Sodek, J. and McCulloch, C. A. (2000). A novel model system for characterization of phagosomal maturation, acidification, and intracellular collagen degradation in fibroblasts. *J. Biol. Chem.* **275**, 35432-35441.

Arora, P. D., Glogauer, M., Kapus, A., Kwiatkowski, D. J. and McCulloch, C. A. (2004). Gelsolin mediates collagen phagocytosis through a rac-dependent step. *Mol. Biol. Cell* **15**, 588-599.

Arora, P. D., Chan, M. W., Anderson, R. A., Janmey, P. A. and McCulloch, C. A. (2005). Separate functions of gelsolin mediate sequential steps of collagen phagocytosis. *Mol. Biol. Cell* **16**, 5175-5190.

Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L. M., White, R. A., Groopman, J. E. et al. (1995). Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J. Biol. Chem.* **270**, 27742-27751.

Azuma, T., Witke, W., Stossel, T. P., Hartwig, J. H. and Kwiatkowski, D. J. (1998). Gelsolin is a downstream effector of rac for fibroblast motility. *EMBO J.* **17**, 1362-1370.

Brakebusch, C. and Fassler, R. (2003). The integrin-actin connection, an eternal love affair. *EMBO J.* **22**, 2324-2333.

Calalb, M. B., Polte, T. R. and Hanks, S. K. (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol. Cell. Biol.* **15**, 954-963.

Carlucci, A., Gedressi, C., Lignitto, L., Nezi, L., Villa-Moruzzi, E., Avvedimento, E. V., Gottesman, M., Garbi, C. and Feliciello, A. (2008). Protein-tyrosine phosphatase PTPD1 regulates focal adhesion kinase autophosphorylation and cell migration. *J. Biol. Chem.* **283**, 10919-10929.

Chan, P.-Y., Kanner, S. B., Whitney, G. and Aruffo, A. (1994). A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK*. *J. Biol. Chem.* **269**, 20567-20574.

Cohen, L. A. and Guan, J. L. (2005). Mechanisms of focal adhesion kinase regulation. *Curr. Cancer Drug Targets* **5**, 629-643.

DeMali, K. A., Barlow, C. A. and Burridge, K. (2002). Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. *J. Cell Biol.* **159**, 881-891.

Girault, J. A., Labesse, G., Mornon, J. P. and Callebaut, I. (1999). The N-termini of FAK and JAKs contain divergent band 4.1 domains. *Trends Biochem. Sci.* **24**, 54-57.

Glogauer, M. and Ferrier, J. (1998). A new method for application of force to cells via ferric oxide beads. *Pflugers Arch.* **435**, 320-327.

Glogauer, M., Ferrier, J. and McCulloch, C. A. (1995). Magnetic fields applied to collagen-coated ferric oxide beads induce stretch-activated Ca2+ flux in fibroblasts. *Am. J. Physiol.* **269**, C1093-C1104.

Glogauer, M., Arora, P., Chou, D., Janmey, P. A., Downey, G. P. and McCulloch, C. A. (1998). The role of actin-binding protein 280 in integrin-dependent mechanoprotection. *J. Biol. Chem.* **273**, 1689-1698.

Guan, J. L. and Shalloway, D. (1992). Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* **358**, 690-692.

Gullberg, D., Gehlsen, K. R., Turner, D. C., Ahlen, K., Zijenah, L. S., Barnes, M. J. and Rubin, K. (1992). Analysis of alpha 1 beta 1, alpha 2 beta 1 and alpha 3 beta 1 integrins in cell-collagen interactions: identification of conformation dependent alpha 1 beta 1 binding sites in collagen type I. *EMBO J.* **11**, 3865-3873.

Guo, W. H. and Wang, Y. L. (2007). Retrograde fluxes of focal adhesion proteins in response to cell migration and mechanical signals. *Mol. Biol. Cell* **18**, 4519-4527.

Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H. and Parsons, J. T. (1996). p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *J. Biol. Chem.* **271**, 13649-13655.

Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J. Cell Biol.* **123**, 993-1005.

Hinz, B., Mastrangelo, D., Iselin, C. E., Chaponnier, C. and Gabbiani, G. (2001). Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am. J. Pathol.* **159**, 1009-1020.

Ingber, D. E. (1997). Tensegrity: the architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.* **59**, 575-599.

Janke, M., Herrig, A., Austermann, J., Gerke, V., Steinem, C. and Janshoff, A. (2008). Actin binding of ezrin is activated by specific recognition of PIP2-functionalized lipid bilayers. *Biochemistry* **47**, 3762-3769.

Janmey, P. A. and Stossel, T. P. (1987). Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature* **325**, 362-364.

Janmey, P. A. and Stossel, T. P. (1989). Gelsolin-polyphosphoinositide interaction: full expression of gelsolin-inhibiting function by polyphosphoinositides in vesicular form and inactivation by dilution, aggregation, or masking of the inositol head group. *J. Biol. Chem.* **264**, 4825-4831.

Janmey, P. A., Chaponnier, C., Lind, S. E., Zaner, K. S., Stossel, T. P. and Yin, H. L. (1985). Interactions of gelsolin and gelsolin-actin complexes with actin. Effects of calcium on actin nucleation, filament severing, and end blocking. *Biochemistry* **24**, 3714-3723.

Kopecki, Z., Arkell, R., Powell, B. C. and Cowin, A. J. (2009). Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. *J. Invest. Dermatol.* doi:10.1038/jid.2008.461.

Le Clainche, C. and Carlier, M. F. (2008). Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol. Rev.* **88**, 489-513.

Leslie, K. O., Taatjes, D. J., Schwarz, J., vonTurkovich, M. and Low, R. B. (1991). Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am. J. Pathol.* **139**, 207-216.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B. and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* **376**, 737-745.

Li, S., Chen, B. P., Azuma, N., Hu, Y. L., Wu, S. Z., Sumpio, B. E., Shyy, J. Y. and Chien, S. (1999). Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *J. Clin. Invest.* **103**, 1141-1150.

Li, S., Butler, P., Wang, Y., Hu, Y., Han, D. C., Usami, S., Guan, J. L. and Chien, S. (2002). The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. *Proc. Natl. Acad. Sci. USA* **99**, 3546-3551.

Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W. and Anderson, R. A. (2002). Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. *Nature* **420**, 89-93.

Ling, K., Schill, N. J., Wagoner, M. P., Sun, Y. and Anderson, R. A. (2006). Movin' on up: the role of PtdIns(4,5)P(2) in cell migration. *Trends Cell Biol.* **16**, 276-284.

Linseman, D. A., Sorensen, S. D. and Fisher, S. K. (1999). Attenuation of focal adhesion kinase signaling following depletion of agonist-sensitive pools of phosphatidylinositol 4,5-bisphosphate. *J. Neurochem.* **73**, 1933-1944.

Macia, E., Partisani, M., Favard, C., Mortier, E., Zimmermann, P., Carlier, M. F., Gounon, P., Luton, F. and Franco, M. (2008). The pleckstrin homology domain of the Arf6-specific exchange factor EFA6 localizes to the plasma membrane by interacting with phosphatidylinositol 4,5-bisphosphate and F-actin. *J. Biol. Chem.* **283**, 19836-19844.

MacKenna, D. A., Dolfi, F., Vuori, K. and Ruoslahti, E. (1998). Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J. Clin. Invest.* **101**, 301-310.

MacKenna, D., Summerour, S. R. and Villarreal, F. J. (2000). Role of mechanical factors in modulating cardiac fibroblast function and extracellular matrix synthesis. *Cardiovasc. Res.* **46**, 257-263.

Martin, K. H., Boerner, S. A. and Parsons, J. T. (2002). Regulation of focal adhesion targeting and inhibitory functions of the FAK related protein FRNK using a novel estrogen receptor "switch". *Cell Motil. Cytoskeleton* **51**, 76-88.

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**, 1409-1416.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P. and Meyer, T. (2000). Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* **100**, 221-228.

Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D. and Schwartz, M. A. (2000). Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J. Cell Sci.* **113**, 3673-3678.

Riveline, D., Zamir, E., Balaban, N. Q., Schwarz, U. S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B. and Bershadsky, A. D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J. Cell Biol.* **153**, 1175-1186.

Sakurai, S., Sonoda, Y., Koguchi, E., Shinoura, N., Hamada, H. and Kasahara, T. (2002). Mutated focal adhesion kinase induces apoptosis in a human glioma cell line, T98G. *Biochem. Biophys. Res. Commun.* **293**, 174-181.

Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K. and Sasaki, T. (1995). Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J. Biol. Chem.* **270**, 21206-12119.

Schaller, M. D., Hildebrand, J. D. and Parsons, J. T. (1999). Complex formation with focal adhesion kinase: a mechanism to regulate activity and subcellular localization of Src kinases. *Mol. Biol. Cell* **10**, 3489-3505.

Schober, M., Raghavan, S., Nikolova, M., Polak, L., Pasolli, H. A., Beggs, H. E., Reichardt, L. F. and Fuchs, E. (2007). Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J. Cell Biol.* **176**, 667-680.

Sun, C. X., Robb, V. A. and Gutmann, D. H. (2002). Protein 4.1 tumor suppressors: getting a FERM grip on growth regulation. *J. Cell Sci.* **115**, 3991-4000.

Sun, H. Q., Yamamoto, M., Mejillano, M. and Yin, H. L. (1999). Gelsolin, a multifunctional actin regulatory protein. *J. Biol. Chem.* **274**, 33179-33182.

Sun, Y., Ling, K., Wagoner, M. P. and Anderson, R. A. (2007). Type I gamma phosphatidylinositol phosphate kinase is required for EGF-stimulated directional cell migration. *J. Cell Biol.* **178**, 297-308.

Tilghman, R. W., Slack-Davis, J. K., Sergina, N., Martin, K. H., Iwanicki, M., Hershey, E. D., Beggs, H. E., Reichardt, L. F. and Parsons, J. T. (2005). Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells. *J. Cell Sci.* **118**, 2613-2623.

Vadali, K., Cai, X. and Schaller, M. D. (2007). Focal adhesion kinase: an essential kinase in the regulation of cardiovascular functions. *IUBMB Life* **59**, 709-716.

Wang, J., Su, M., Fan, J., Seth, A. and McCulloch, C. A. (2002). Transcriptional regulation of a contractile gene by mechanical forces applied through integrins in osteoblasts. *J. Biol. Chem.* **277**, 22889-22895.

Wang, J., Chen, H., Seth, A. and McCulloch, C. A. (2003). Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. *Am. J. Physiol. Heart Circ. Physiol.* **285**, H1871-H1881.

Wang, Q., Xie, Y., Du, Q. S., Wu, X. J., Feng, X., Mei, L., McDonald, J. M. and Xiong, W. C. (2003). Regulation of the formation of osteoclastic actin rings by proline-rich tyrosine kinase 2 interacting with gelsolin. *J. Cell Biol.* **160**, 565-575.

Witke, W., Sharpe, A. H., Hartwig, J. H., Azuma, T., Stossel, T. P. and Kwiatkowski, D. J. (1995). Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell* **81**, 41-51.

Wu, C. C., Li, Y. S., Haga, J. H., Kaunas, R., Chiu, J. J., Su, F. C., Usami, S. and Chien, S. (2007). Directional shear flow and Rho activation prevent the endothelial cell apoptosis induced by micropatterned anisotropic geometry. *Proc. Natl. Acad. Sci. USA* **104**, 1254-1259.

Wu, C. H., Lin, C. S., Hung, J. S., Wu, C. J., Lo, P. H., Jin, G., Shyy, Y. J., Mao, S. J. and Chien, S. (2001). Inhibition of neointimal formation in porcine coronary artery by a Ras mutant. *J. Surg. Res.* **99**, 100-106.

Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D. and Guan, J. L. (1994). Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Mol. Biol. Cell* **5**, 413-421.

Yin, H. L. and Janmey, P. A. (2003). Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* **65**, 761-789.

Zaromytidou, A. I., Miralles, F. and Treisman, R. (2006). MAL and ternary complex factor use different mechanisms to contact a common surface on the serum response factor DNA-binding domain. *Mol. Cell. Biol.* **26**, 4134-4148.

Zhang, W., Wu, Y., Wu, C. and Gunst, S. J. (2007). Integrin-linked kinase regulates N-WASp-mediated actin polymerization and tension development in tracheal smooth muscle. *J. Biol. Chem.* **282**, 34568-34580.

Zhao, J. H., Reiske, H. and Guan, J. L. (1998). Regulation of the cell cycle by focal adhesion kinase. *J. Cell Biol.* **143**, 1997-2008.

Zhao, X. H., Laschinger, C., Arora, P., Szaszi, K., Kapus, A. and McCulloch, C. A. (2007). Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. *J. Cell Sci.* **120**, 1801-1809.