Pericellular fibronectin is required for RhoA-dependent responses to cyclic strain in fibroblasts

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Summary

To test the hypothesis that the pericellular fibronectin matrix is involved in mechanotransduction, we compared the response of normal and fibronectin-deficient mouse fibroblasts to cyclic substrate strain. Normal fibroblasts seeded on vitronectin in fibronectin-depleted medium deposited their own fibronectin matrix. In cultures exposed to cyclic strain, RhoA was activated, actin-stress fibers became more prominent, MAL/MKL1 shuttled to the nucleus, and mRNA encoding tenascin-C was induced. By contrast, these RhoA-dependent responses to cyclic strain were suppressed in fibronectin knockdown or knockout fibroblasts grown under identical conditions. On vitronectin substrate, fibronectin-deficient cells lacked fibrillar adhesions containing α5 integrin. However, when fibronectin-deficient fibroblasts were plated on exogenous fibronectin, their defects in adhesions and mechanotransduction were restored. Studies with fragments indicated that both the RGD-synergy site and the adjacent heparin-binding region of fibronectin were required for full activity in mechanotransduction, but not its ability to self-assemble. In contrast to RhoA-mediated responses, activation of Erk1/2 and PKB/Akt by cyclic strain was not affected in fibronectin-deficient cells. Our results indicate that pericellular fibronectin secreted by normal fibroblasts is a necessary component of the strain-sensing machinery. Supporting this hypothesis, induction of cellular tenascin-C by cyclic strain was suppressed by addition of exogenous tenascin-C, which interferes with fibronectin-mediated cell spreading.

Key words: Fibronectin, Mechanotransduction, Cyclic strain, RhoA, Actin, MAL/MKL1/MRTF-A, Tenascin-C

Introduction

Cellular responses to mechanical cues are essential for tissue homeostasis, and mechanical stress is linked to the development and progression of several diseases, such as fibrosis, arteriosclerosis and cancer (Butcher et al., 2009; Hahn and Schwartz, 2009; Wipff et al., 2007). Cells constantly integrate mechanical signals into information about the physical properties of their environment (Chen, 2008; Geiger et al., 2009). To study mechanotransduction and signaling pathways triggered by mechanical stress, cells can be cultured on extracellular matrix (ECM)-coated elastic membranes and cyclically strained (Chiquet et al., 2004; Hsieh et al., 2000). This is a physiologically relevant model to analyze mechanotransduction because cellular responses can be measured after mechanical strain of amplitude and frequency within the ranges found in many tissues (e.g. arterial vessels, lung, heart or tendons) (Lehoux et al., 2006; Magnusson et al., 2008). Among other responses, tissues adapt to mechanical load by remodeling their extracellular matrix (ECM). For example, the ECM protein tenascin-C is strongly expressed in tissues bearing high tensile stress, and is induced by dynamic tensile strain both in vivo (Fluck et al., 2000) and in vitro (Chiquet et al., 2004).

Several types of cell-ECM adhesion structures are crucial for mechanotransduction via integrin-dependent signaling (Geiger et al., 2009). Focal complexes are formed during initial contact of fibroblasts with an ECM substrate. Some mature into focal adhesions that contain mainly αvβ3 integrin, which binds to vitronectin and other ECM components with RGD peptide motifs (Horton, 1997; Zamir et al., 2000). During cell spreading, fibroblasts rapidly develop fibrillar adhesions characterized by the presence of mainly α5β1 integrin, which are involved in the assembly of a pericellular fibronectin matrix (Zamir et al., 2000). Activation of major intracellular signaling pathways by mechanical stress, such as NFκB, MAPK and RhoA/ROCK, is both adhesion- and integrin-dependent (Inoh et al., 2002; Orr et al., 2006; Stupack and Cheresh, 2002). The induction of tenascin-C by cyclic strain requires β1 integrins (Chiquet et al., 2007), but does not depend on MAPK signaling (Chiquet et al., 2004). Instead, induction is abolished after actin disassembly or inhibition of RhoA-dependent kinase (ROCK) (Sarasa-Renedo et al., 2006). Upregulation of tenascin-C by cyclic strain also correlates with an increase in actin-stress fibers and the translocation of MAL/MKL1/MRTF-A to the nucleus (Maier et al., 2008). MAL is a transcriptional co-activator of serum response factor that links RhoA activation and actin dynamics to gene expression (Miralles et al., 2003). Thus, induction of tenascin-C by mechanical stress appears to be controlled primarily by the RhoA-ROCK pathway.

In a previous study, we showed that in fibroblasts deficient for integrin-linked kinase (ILK), activation of RhoA, nuclear translocation of MAL and tenascin-C induction by cyclic strain were all abolished (Maier et al., 2008). The defect was specific for RhoA-dependent responses, because the MAPK Erk1/2 was activated normally and the *Fos* gene was induced by mechanical stress in ILK-knockout cells. ILK is an adaptor protein in cell-matrix adhesion sites that forms a complex with PINCH and parvin, joining the cytosolic part of β1 and β3 integrins to the actin cytoskeleton (Legate et al., 2006). $ILK^{-/-}$ fibroblasts do not

assemble fibronectin into fibrils, and classical fibrillar adhesions are absent, indicating an important function for ILK in the formation of these structures (Legate et al., 2006). We speculated that the missing RhoA-ROCK activation upon mechanical stress in ILK$^{-/-}$ cells was due to lack of a fibronectin matrix and of fibrillar adhesions, rather than to a direct involvement of ILK in the activation of this pathway. In this case, the pericellular fibronectin matrix (in conjunction with fibrillar adhesions) would itself be part of the machinery that transduces cyclic (i.e. dynamic) mechanical stress. To test this hypothesis, we prepared stable fibronectin-knockdown fibroblast lines and monitored their RhoA-ROCK-dependent responses to cyclic strain in the presence or absence of exogenous fibronectin. Indeed, when plated on vitronectin, such cells had defects in mechanotransduction that were very similar to those in ILK-deficient fibroblasts. In contrast to ILK$^{-/-}$ cells, however, fibronectin-knockdown cells could be rescued by adding exogenous fibronectin to the culture. Based on our results, we propose a model for mechanotransduction in which cyclic strain activates the RhoA-ROCK pathway mainly via attachment of cells to their own pericellular fibronectin matrix.

Results

Fibronectin-deficient fibroblasts can assemble exogenous fibronectin

To test whether the fibronectin matrix is important for mechanotransduction, we isolated stable fibronectin-knockdown cell lines and tested them for their ability to activate canonical signaling pathways in response to mechanical stress. Fibronectin knockdown was carried out in immortalized wild-type mouse embryo fibroblasts (MEFs) by stable transfection with shRNA. Two clones (1.2 and 8.3) were analyzed further. Clone 1.2 was transfected with one type of shRNA to knockdown fibronectin; however, this clone expressed and secreted normal amounts of fibronectin and was therefore used as a control (Fig. 1A,B). For clone 8.3, another type of shRNA targeting mRNA encoding fibronectin was used, that effectively reduced fibronectin expression to 10% of the original level in wild type MEFs (Fig. 1B). We also tested fibronectin-knockout MEFs (FN$^{-/-}$) and the matched fibronectin-expressing control cells (FN$^{f/f}$). Unlike in control cells, staining for fibronectin was not detected in either fibronectin-knockdown clone 8.3 or the fibronectin-knockout MEFs grown for 2 days on tissue culture plastic (Fig. 1A) or vitronectin (supplementary material Fig. S1) in fibronectin-depleted medium. However, both cell lines showed distinct fibronectin fibrils upon addition of 100 μg/ml soluble fibronectin to the plating medium (Fig. 1A), indicating that the machinery for fibronectin fibrillogenesis was still intact. This assumption was confirmed by the fact that expression levels of integrin αv, α5 and β1 were similar in all cell lines tested (Fig. 1C).

Cyclic-strain-induced activation of RhoA requires pericellular fibronectin

Amongst many other stimuli, mechanical stress is known to increase intracellular levels of active RhoA (Sarasa-Renedo et al., 2006; Smith et al., 2003). To test whether a pericellular fibronectin matrix is required for activation of RhoA by cyclic strain, we plated fibronectin-expressing (FN$^{f/f}$) or fibronectin-deficient (FN$^{-/-}$) MEFs on silicone membranes coated with either vitronectin or fibronectin. Cells were cultured for 24 hours and then subjected to biaxial cyclic strain (10%, 0.3 Hz) for 5 minutes. Levels of active (GTP-bound) RhoA were detected by Rhotekin-RBD binding assays as described in the Materials and Methods. On vitronectin, a clear increase in the amount of active RhoA could be detected in the fibronectin-expressing control cells after 5 minutes of cyclic strain (Fig. 2A,B). However, this activation was absent in fibronectin-knockout cells grown on vitronectin. Interestingly, after plating on fibronectin, a robust activation of RhoA could be detected for both cell lines, indicating that fibronectin as extracellular substrate is necessary for this effect.

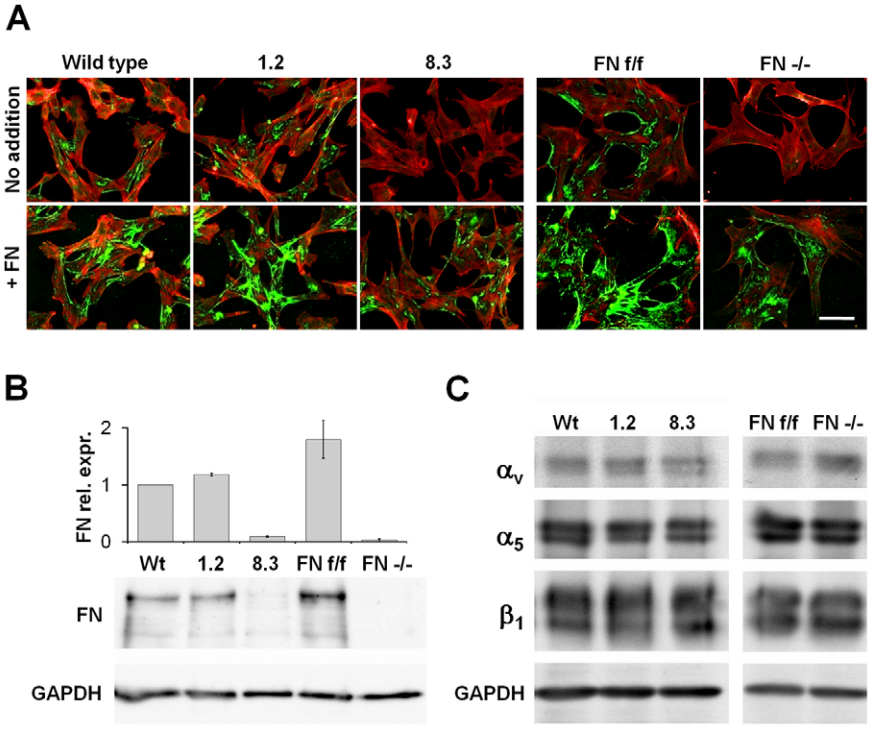

Fig. 1. Fibronectin expression levels and integrin profiles of fibronectin-knockdown, fibronectin knockout and control MEFs. (**A**) Cells were plated on cell culture plastic and grown for 48 hours in DMEM containing 3% fibronectin-depleted FCS, either without (top) or with fibronectin added at 100 μg/ml (bottom). Cells were subsequently fixed and double-stained with phalloidin (red) and anti-fibronectin (green). Scale bar: 50 μm. Note that clone 8.3 and FN$^{-/-}$ cells assembled pericellular fibronectin fibrils only in the presence of exogenous fibronectin. (**B**) Cells were grown for 48 hours on tissue culture plastic and subsequently detached from the matrix by detergent. Matrices were scraped into Laemmli buffer and analyzed by immunoblotting against fibronectin. Cell lysates were blotted for GAPDH as a loading control. Graph shows the average relative expression (mean ± s.e.m.) of fibronectin to GAPDH quantified from independent experiments. (**C**) Cell extracts were immunoblotted for αv, α5 and β1 integrins to check their expression levels; GAPDH was detected as a loading control.

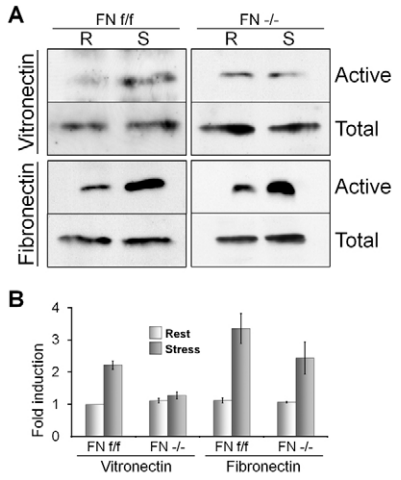

Fig. 2. Cyclic strain activates RhoA only in the presence of pericellular fibronectin. (**A**) Fibronectin-knockout ($FN^{-/-}$) and control MEFs ($FN^{f/f}$) were cultured on either vitronectin (top) or fibronectin (bottom) for 48 hours. Cells were then cyclically strained (10%, 0.3 Hz) for 5 minutes and active RhoA levels were determined by Rho pull-down experiments. (**B**) Graph showing average levels of active RhoA from independent experiments. Values (mean ± s.e.m.) are normalized to levels in $FN^{f/f}$ cells at rest on vitronectin.

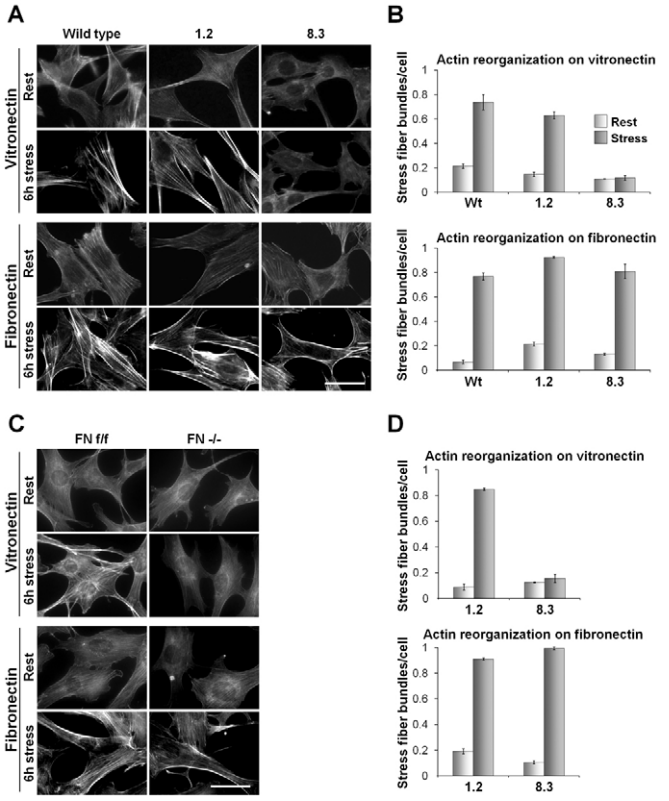

Fig. 3. Cyclic-strain-induced actin reorganization requires a pericellular fibronectin matrix. (**A**) Wild-type MEFs, the fibronectin-expressing clone 1.2 and the fibronectin-knockdown clone 8.3 were seeded on vitronectin-coated (top) or fibronectin-coated (bottom) silicone membranes. Two days after plating in DMEM plus 0.3% fibronectin-depleted FCS, cells were cyclically strained (10%, 0.3 Hz) for 6 hours and subsequently stained for the actin cytoskeleton. (**B**) Statistical analysis of different independent experiments as depicted in A. For every experiment, 100 cells per condition were analyzed and number of peripheral actin-stress-fiber bundles per cell was determined (mean ± s.e.m.). Peripheral actin bundles were defined as locations where single visible stress fibers merged in the cell periphery into larger and thicker, bright foci. (**C**) Fibronectin-knockout MEFs ($FN^{-/-}$) and the matched fibronectin-expressing control cells ($FN^{f/f}$). (**D**) The statistical analysis of different independent experiments shown in C. Note the missing actin reorganization in the fibronectin-deficient clones 8.3 and $FN^{-/-}$ on the vitronectin, but not on the fibronectin substrate. Scale bars: 50 µm.

Cyclic-strain-induced actin reorganization requires a pericellular fibronectin matrix

In embryonic fibroblasts cultured on fibronectin-coated elastomer membranes, cyclic strain induces a characteristic change in cell shape and a RhoA-ROCK-dependent increase and relocation of actin-stress fibers (Maier et al., 2008; Sarasa-Renedo et al., 2006). To analyze whether the fibronectin matrix is required for this actin reorganization, we plated fibronectin-expressing or fibronectin-knockdown clone 8.3 on vitronectin-coated silicone membranes. After 2 days in culture, cells were subjected to 6 hours of cyclic strain and subsequently stained with fluorescently labelled phalloidin. After mechanical stress, fibronectin-deficient cells remained triangular or multipolar, without prominent stress fibers (Fig. 3A,B). Conversely, fibronectin-expressing and secreting control cells (wild type and clone 1.2) both assumed a more bipolar shape and formed distinct bundles of stress fibers and actin foci within 6 hours of cyclic strain on a vitronectin substrate. Strong stress-fiber bundles formed predominantly along the cell edges, whereas thinner actin fibers in the central area of the cell disappeared (Fig. 3A,B). Similarly to fibronectin-knockdown clone 8.3, fibronectin-knockout fibroblasts did not respond to cyclic strain by actin rearrangement when cyclically strained for 6 hours on vitronectin (Fig. 3C,D). By contrast, their fibronectin-expressing parental cells ($FN^{f/f}$) formed distinct stress fibers under these conditions. Interestingly, the missing response to strain of either of the fibronectin-deficient cell lines (clone 8.3 or $FN^{-/-}$) was restored when these cells were plated on exogenous fibronectin and cyclically strained. Similarly to fibronectin-expressing control cells, central thinner actin structures in clone 8.3 and $FN^{-/-}$ fibroblasts were replaced by peripheral thick actin-stress fibers after 6 hours of cyclic strain on this substrate. These peripheral actin-stress fiber bundles are easily distinguishable from other actin structures, and were therefore used to quantify our observations (Fig. 3B,D).

Pericellular fibronectin is required for cyclic-strain-induced MAL translocation to the nucleus

MAL (MKL-1; MRTF-A) is a transcriptional co-activator of serum response factor (SRF) that cycles between the cytoplasm and the nucleus under the control of RhoA-dependent actin dynamics (Asparuhova et al., 2009; Miralles et al., 2003). Mechanical strain stimulates actin reorganization and was shown to promote a shift of MAL from the cytoplasm to the nucleus in serum-starved cells (Maier et al., 2008; Zhao et al., 2007). Nuclear translocation of MAL after cyclic strain depends on activation of RhoA, because it was abolished in the presence of the specific inhibitor C3 transferase (supplementary material Fig. S2). Since actin dynamics in response to cyclic strain were clearly affected in fibronectin-deficient fibroblasts plated on vitronectin, we expected to find a similar effect on MAL shuttling. Cyclic strain for 1 hour stimulated

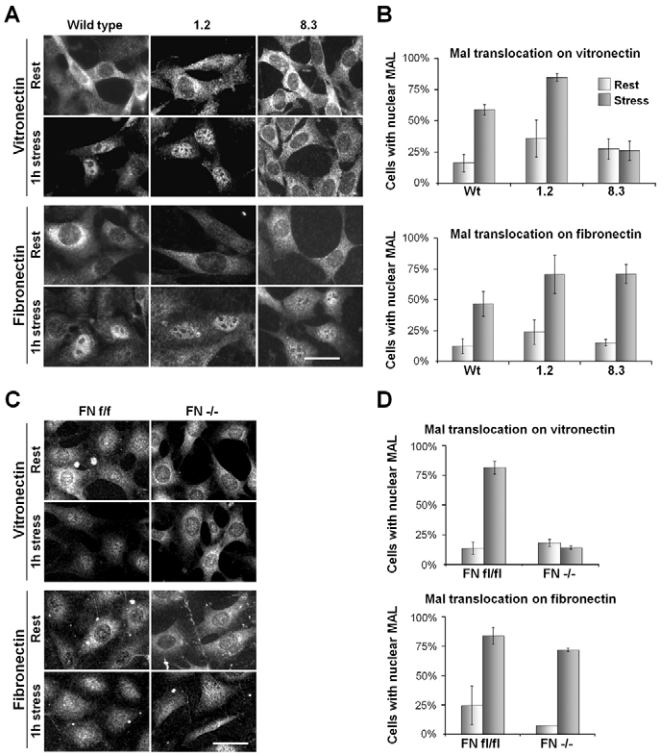

Fig. 4. Pericellular fibronectin is necessary for the cyclic-strain-induced translocation of MAL to the nucleus. (**A**) Wild-type MEFs, the fibronectin-expressing clone 1.2 and the fibronectin-knockdown clone 8.3 were seeded on either vitronectin (top) or fibronectin (bottom). Two days after plating, cells were cyclically strained for 1 hour and subsequently fixed and stained for MAL. Scale bar: 50 μm. (**B**) Statistical analysis of three independent experiments as depicted in A. For every experiment, 100 cells per condition were analyzed and MAL staining was scored as either nuclear or cytoplasmic. The graph shows the mean percentage of cells with nuclear MAL (± s.e.m.). Light columns represent values before and dark columns values after 1 hour of cyclic strain. (**C**) Fibronectin-knockout MEFs ($FN^{-/-}$) and the matched fibronectin-expressing control cells ($FN^{f/f}$). Scale bar: 50 μm. (**D**) Statistical analysis of the results from three independent experiments shown in C. Note the absence of MAL translocation in response to cyclic strain in the fibronectin-deficient clones 8.3 and $FN^{-/-}$, when plated on vitronectin, but not on fibronectin.

translocation of MAL in wild-type MEFs and in fibronectin-expressing clone 1.2; the shift of MAL to the nucleus in these cells was observed not only on fibronectin, but also on vitronectin substrate (Fig. 4A,B). Conversely, in fibronectin-knockdown clone 8.3 cells grown on vitronectin, MAL did not translocate to the nucleus after 1 hour of cyclic strain. However, plating these cells on fibronectin and straining them for 1 hour clearly raised the percentage of cells with nuclear MAL from 15% at rest to 71% after strain (Fig. 4A,B).

To confirm these results, we also analyzed the localization of MAL in fibronectin-knockout cells. Fibronectin-knockout fibroblasts plated on vitronectin showed no increase in nuclear MAL upon cyclic strain, in contrast to the matched wild-type cells (Fig. 4C,D). However, for both knockout and wild-type cells, MAL translocation was observed after strain when fibronectin was used as a substrate. Our findings indicate that on vitronectin substrate, the fibronectin matrix deposited by wild-type cells themselves is required for translocation of MAL after cyclic strain, because fibronectin-deficient cells depended on exogenous fibronectin for this process.

mRNA and protein levels of tenascin-C are induced by cyclic strain only in the presence of pericellular fibronectin

Tenascin-C is a glycoprotein of the extracellular matrix whose expression is induced by cyclic strain (Chiquet et al., 2004). We have shown that this response depends on RhoA-ROCK-induced actin assembly and correlates with translocation of MAL to the nucleus (Maier et al., 2008; Sarasa-Renedo et al., 2006). We therefore investigated tenascin-C expression levels in response to cyclic strain in fibronectin-expressing and non-expressing fibroblasts. Cells were cultured with fibronectin-depleted serum on either vitronectin or fibronectin. Fibroblasts were then cyclically strained for 6 hours and cultured for an additional 18 hours, during which time tenascin-C was deposited. Immunoblotting of cell lysates showed higher amounts of tenascin-C protein in both wild-type MEFs and fibronectin-expressing clone 1.2 after straining, than in the resting control (Fig. 5A,B). This effect did not depend on the respective ECM substrates. However, a response to mechanical stress by the fibronectin-knockdown clone 8.3 was again only observed when cells were plated on fibronectin. On vitronectin, tenascin-C accumulation by clone 8.3 fibroblasts did not change after strain, indicating that the absence of the fibronectin matrix prevented this cellular response.

By quantitative PCR, the induction of tenascin-C by cyclic strain was also analyzed at the mRNA level. After only 1 hour of cyclic strain (10%, 0.3 Hz), there was a significant increase in levels of mRNA encoding tenascin-C ($P<0.05$) in cell lines plated on fibronectin (Fig. 5C,D). However, tenascin-C (*Tnc*) mRNA remained at resting levels when fibronectin-deficient cells (clone 8.3 and $FN^{-/-}$) were plated and strained on vitronectin. By contrast, *Tnc* mRNA was upregulated in fibronectin-expressing control cells (wild type, clone 1.2, $FN^{f/f}$) after cyclic strain, also on a vitronectin substrate (Fig. 5C,D). Thus, as in previous experiments, responses were consistent between fibronectin-knockdown and fibronectin-knockout fibroblasts. Induction of *Tnc* mRNA by cyclic strain appears to depend on pericellular fibronectin, either provided exogenously or deposited by the fibroblasts themselves.

Activation of ERK and PKB/Akt by cyclic strain does not depend on the presence of pericellular fibronectin

We showed that the presence of pericellular fibronectin was required for reorganization of the actin cytoskeleton, translocation of MAL to the nucleus, and upregulation of tenascin-C protein and mRNA in response to cyclic strain. As reported previously (Chiquet et al., 2004; Sarasa-Renedo et al., 2006; Zhao et al., 2007), these events all depended on RhoA-ROCK, indicating that the fibronectin matrix is essential for the activation of this signaling pathway by mechanical stress. To test whether mechanotransduction was affected in general in the absence of fibronectin, we analyzed other pathways known to be triggered by mechanical stress. Erk1/2 was phosphorylated upon 5 minutes of cyclic strain, independently of the cell type and the underlying ECM substrate, indicating that activation of the MAPK pathway by mechanical stress does not require the presence of fibronectin (Fig. 6A,B). As with wild-type fibroblasts and clone 1.2, an analysis of the time-course of PKB/Akt phosphorylation showed a robust signal of phosphorylated PKB/Akt in fibronectin-knockdown clone 8.3 after strain, independently of whether cells adhered to vitronectin or fibronectin (Fig. 6C,D).

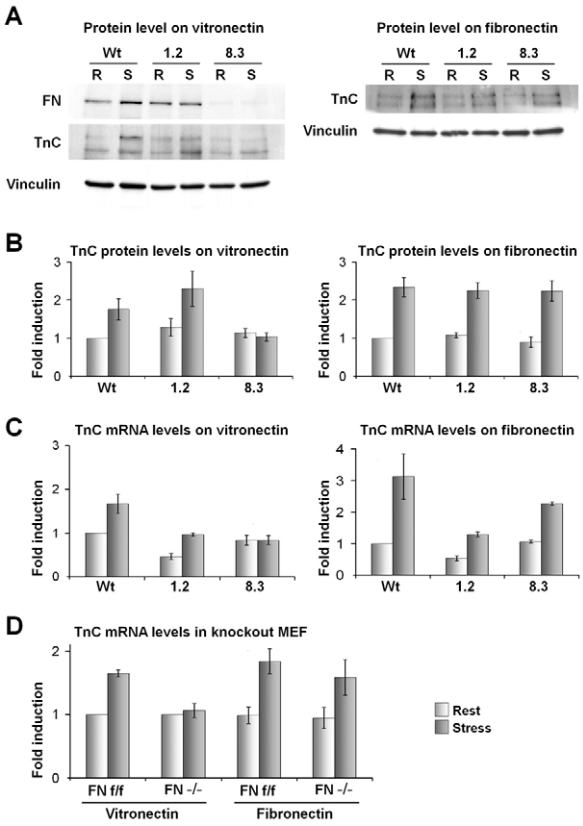

Fig. 5. mRNA and protein levels of tenascin-C are induced by cyclic strain only in the presence of pericellular fibronectin. (**A**,**B**) Wild-type MEFs (Wt), the fibronectin-expressing MEF clone 1.2 and the fibronectin-knockdown MEF clone 8.3 were seeded on (left) vitronectin- or (right) fibronectin-coated silicone membranes and grown for 48 hours. Cells were then left at rest (R) or subjected to cyclic strain (S) for 6 hours followed by a further 18 hours at rest, during which tenascin-C was deposited into the matrix. (A) Lysates were immunoblotted for fibronectin (FN), tenascin-C (TnC) and vinculin as standard. (B) The ratios of tenascin-C to vinculin were calculated from densitometry measurements on immunoblots from three independent experiments. Light bars represent rest and dark bars strained conditions. (**C**) Wt, clone 1.2 and clone 8.3 cells were seeded on vitronectin (left) or fibronectin (right), cultured for 48 hours as above and then subjected to 1 hour of cyclic strain. *Tnc* mRNA levels were measured by quantitative PCR relative to *Gapdh* and normalized to the values of wild-type cells at rest. (**D**) Fibronectin-knockout ($FN^{-/-}$) and control ($FN^{f/f}$) MEFs. Owing to variation in basal tenascin-C levels, $FN^{f/f}$ and $FN^{-/-}$ were normalized individually to their respective rest values on vitronectin.

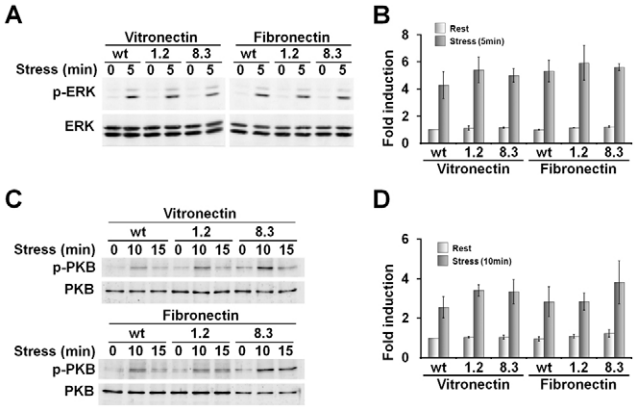

Fig. 6. Activation of ERK and PKB/Akt by cyclic strain does not depend on the presence of pericellular fibronectin. Wild-type and fibronectin-deficient MEFs were cultured on vitronectin- or fibronectin-coated silicone membranes and cyclically strained for the indicated times (minutes). (**A**) Immunoblots of ERK and phosphorylated ERK (p-ERK) at rest (0) and after 5 minutes of cyclic strain. (**B**) The ratios of phosphorylated ERK to total ERK were calculated from densitometry measurements on immunoblots from three independent experiments. Light bars represent rest and dark bars strained conditions (5 minutes). (**C**) Immunoblot of PKB/Akt and phosphorylated PKB/Akt (p-PKB) at rest (0) and after 10 and 15 minutes of cyclic strain. (**D**) The statistical analysis of the results from three independent experiments shown in C. Light bars represent rest and dark bars strained conditions (10 minutes).

Taken together, these results show that activation of the MAPK and the PKB/Akt pathways by mechanical stress does not depend on the presence of fibronectin.

The response of fibroblasts to cyclic strain depends on the amount of deposited fibronectin

Stable shRNA transfection of a rat embryo fibroblast cell line (REFs) resulted in a partial fibronectin-knockdown clone (R2A). These cells built up a fibronectin matrix only slowly over a few days. R2A cultures showed only faint intracellular staining for fibronectin 24 hours after plating (Fig. 7A). However, after 48 hours, intracellular staining was more intense and extracellular fibronectin fibrils appeared. By contrast, normal fibronectin-expressing REFs showed staining of distinct extracellular fibronectin fibrils by 24 hours after plating. The intensity of fibronectin-matrix staining correlated with the responsiveness of the cells to mechanical stress in terms of tenascin-C induction. Upregulation of *Tnc* mRNA after 1 hour of cyclic strain was generally higher in REFs than in MEFs. Both, wild-type and knockdown REFs on fibronectin, showed a six- to eightfold induction compared with the respective cells at rest. The induction levels were similar when cells were cyclically strained for 1 hour, after 24 hours or 48 hours in culture (Fig. 7B). Interestingly, in clone R2A grown on vitronectin, *Tnc* mRNA was induced only ~2.5-fold by cyclic strain 24 hours after plating, correlating with the poor fibronectin deposition at this time point. However, tenascin-C induction after 1 hour of strain increased almost sixfold when cells were allowed a further 24 hours for fibronectin deposition.

The cell-binding site and adjacent heparin-binding region of fibronectin are necessary for full induction of *Tnc* mRNA by cyclic strain

As shown so far, pericellular fibronectin is necessary for cyclic-strain-induced activation of RhoA, reorganization of actin, MAL translocation to the nucleus and induction of tenascin-C. We then asked which domains of fibronectin were required for activation of this pathway. We therefore tested the response of fibronectin-knockout MEFs on different fibronectin fragments. Stable cell- and heparin-binding fragments of fibronectin (160 and 135 kDa) were generated from serum fibronectin by mild digestion with chymotrypsin (Ehrismann et al., 1982) (supplementary material Fig. S3A). These fragments lacked the 60 kDa N-terminal gelatin-binding, self-assembly region and part of the C-terminal domain, including the interchain disulfide bridge; thus, they could not

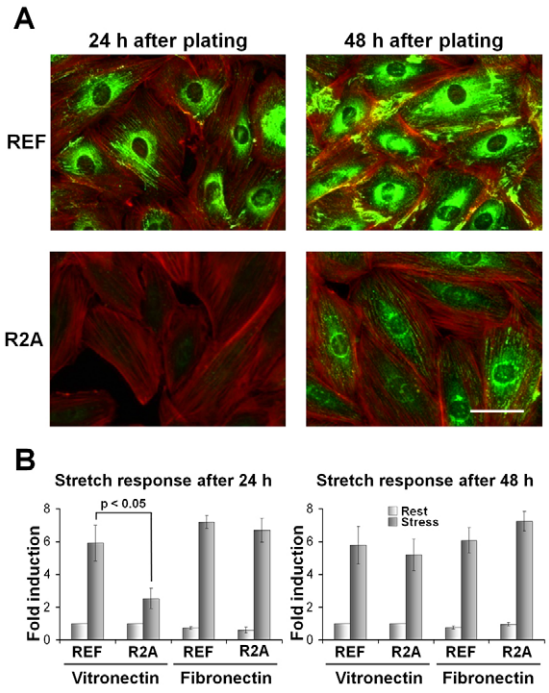

Fig. 7. Response of rat embryo fibroblasts to cyclic strain depends on the time endogenous fibronectin has been allowed to accumulate in the matrix. R2A is a partial fibronectin-knockdown clone derived from REFs. (**A**) Immunofluorescence staining of actin (red) and endogenous fibronectin (green) of detergent-permeabilized REFs and R2A plated on tissue culture plastic. Cells were fixed 24 hours (left) or 48 hours (right) after plating. Scale bar: 50 µm. (**B**) REFs and R2A cells were plated on vitronectin or fibronectin and strained for 1 hour after 24 or 48 hours in culture. Induction of *Tnc* mRNA was measured by qPCR. Light columns represent values before and dark columns values after 1 hour of cyclic strain. Note the correlation between fibronectin matrix accumulation on vitronectin and the level of tenascin-C induction in R2A cells.

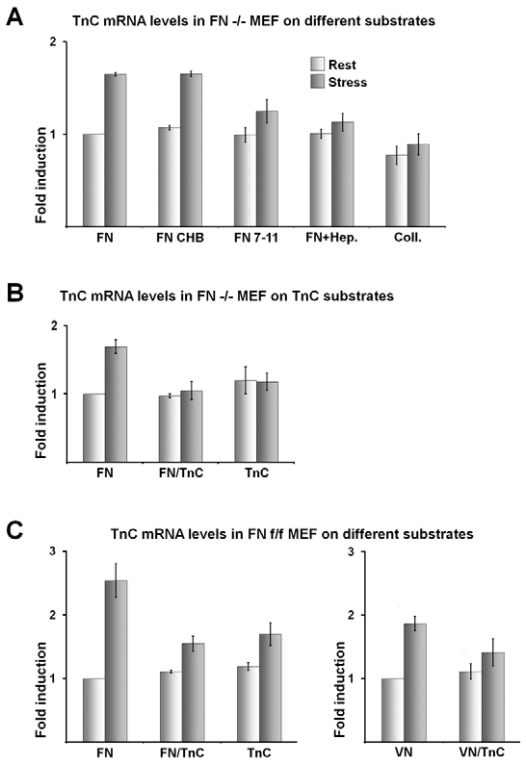

Fig. 8. The cell-binding site and adjacent heparin-binding region of fibronectin are necessary for full induction of *Tnc* mRNA by cyclic strain. (**A**) Fibronectin-knockout MEFs ($FN^{-/-}$) were plated on elastic silicone membranes coated with either fibronectin (FN), fibronectin 160 and 135 kDa chymotrypsin fragments (FN CHB), fibronectin type 3 domain 7-11 (FN 7-11) or collagen I (Coll) and cultured for 48 hours. In FN+Hep cells were plated on fibronectin and soluble heparin was added at 100 µg/ml 24 hours after plating. For all conditions, cells were then kept at rest or subjected to 1 hour of cyclic strain. *Tnc* mRNA levels were measured by quantitative PCR relative to *Gapdh* and normalized to the rest-values on fibronectin. Light columns represent values before and dark columns values after 1 hour of cyclic strain. (**B**) $FN^{-/-}$ MEFs were plated on elastic silicone membranes coated with either fibronectin (50 µg/ml), fibronectin and tenascin-C (50 µg/ml and 50 µg/ml) or tenascin-C (50 µg/ml). (**C**) Fibronectin-expressing MEFs ($FN^{f/f}$). Tenascin-C induction was also measured on vitronectin (20 µg/ml) or vitronectin and tenascin-C (20 µg/ml and 50 µg/ml).

dimerize or form fibrils. However, these large fragments contained the RGD and the synergy site (FNIII 9-10) required for integrin α5β1 binding, as well as a major heparin-binding site (FNIII 13-14) known to be recognized by cellular syndecan-4 (Mao and Schwarzbauer, 2005). A second smaller fragment of fibronectin type III repeats 7-11 (which contains the RGD and the synergy but not the heparin-binding site) was expressed in bacteria (Bloom et al., 1999) and purified on glutathione-Sepharose beads (supplementary material Fig. S3B). To analyze their ability to trigger induction of tenascin-C upon cyclic strain, the fragments were coated on silicone membranes. Fibronectin-knockout MEFs were seeded, cultured for 2 days and subsequently strained for 1 hour. Induction of *Tnc* mRNA on the large cell- and heparin-binding fragment of fibronectin (FN CHB) reached a similar level as seen on full-length fibronectin (FN) (Fig. 8A). Interestingly, even though cells attached and spread normally on the fibronectin type III 7-11 (FN 7-11) fragment (Fig. 9), induction was clearly diminished (Fig. 8A). In agreement with these data, addition of soluble heparin (100 µg/ml) inhibited induction of *Tnc* mRNA on native fibronectin after 1 hour of cyclic strain (Fig. 8A). This supports the notion that the heparin-binding site is important for full induction. On a substrate of fibrillar collagen I, fibronectin-knockout MEFs attached, but did not fully spread (not shown). Induction of *Tnc* mRNA was not observed (Fig. 8A), indicating the specificity of the response for fibronectin.

Tenascin-C was shown to interfere with fibroblast spreading on fibronectin (Chiquet-Ehrismann et al., 1988) by interacting with the 13th type III repeat which is part of the heparin-binding site (Huang et al., 2001). We therefore asked whether substrate-bound tenascin-C also affected its own induction upon cyclic strain. To address this question, silicone membranes were coated with either fibronectin, a 1:1 mixture of fibronectin and tenascin-C, or tenascin-C alone. Indeed, *Tnc* mRNA was not induced after cyclic strain in fibronectin-knockout cells either on tenascin-C alone or on mixed tenascin-C and fibronectin substrates (Fig. 8B). This indicates that substrate-bound tenascin-C could quench the effect of exogenous fibronectin on mechanotransduction. By analyzing fibronectin-expressing MEFs on the same substrates, we found a clearly decreased response on tenascin-C and mixed substrates compared with fibronectin substrates, but no full inhibition (Fig. 8C). This indicates that at least

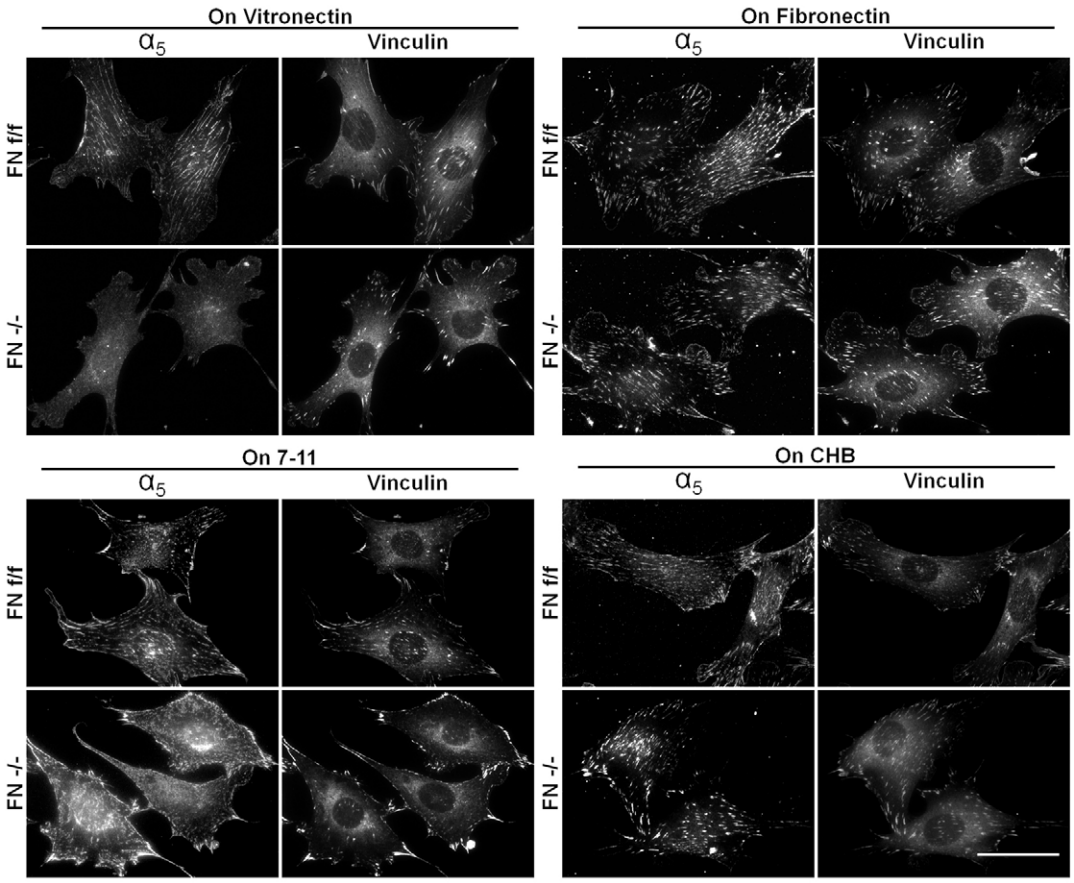

Fig. 9. Fibronectin-deficient MEFs lack integrin-α5-containing fibrillar adhesions when plated on vitronectin. Fibronectin-expressing ($FN^{f/f}$) and deficient ($FN^{-/-}$) MEFs were grown on vitronectin (upper left), fibronectin (upper right), the fibronectin type III repeat 7-11 fragment (7-11, lower left) or the fibronectin cell-binding and adjacent heparin-binding site fragment (CHB, lower right) for 48 hours. Cells were then fixed and subsequently stained for vinculin and α5 integrin. Note that fibronectin-deficient cells do not form α5-integrin-containing fibrillar adhesions on vitronectin. Scale bar: 50 μm.

part of the endogenously expressed fibronectin could not be inhibited by substrate-bound tenascin-C. This assumption was confirmed by straining fibronectin-expressing cells on vitronectin and mixed vitronectin and tenascin-C substrates (Fig. 8C).

Fibronectin-deficient MEFs lack fibrillar adhesions containing α5 integrin and tensin when plated on vitronectin

Since fibronectin-deficient fibroblasts showed clear defects in mechanotransduction in the absence of pericellular fibronectin, we asked whether matrix adhesion sites might also be altered. To address this question we cultured fibronectin-expressing and fibronectin-deficient cells on different substrates for 48 hours. Cells were then co-stained for the cell-matrix adhesion components vinculin and α5 integrin, a subunit of the fibronectin receptor that is enriched in fibrillar adhesions (Zaidel-Bar et al., 2004; Zamir et al., 2000). On fibronectin (Fig. 9, upper right panel) as well as on its large cell- and heparin-binding (CHB) fragment (Fig. 9, lower right panel), both cell lines formed adhesions that were rather homogeneous in shape and generally stained equally well for both vinculin and α5 integrin. On vitronectin, however, two types of cell-matrix adhesions could be found (Fig. 9, upper left). One adhesion type was rather oval in shape, and stained strongly for vinculin but faintly for α5 integrin (‘focal adhesions’). This type could be found in both fibronectin-expressing and fibronectin-deficient cells. The other type of cell matrix adhesion was thin and elongated in shape and strongly stained for α5 integrin (‘fibrillar adhesions’). Interestingly, on vitronectin, these fibrillar adhesions could only be found in fibronectin-expressing cells. In fibronectin-deficient cells on this substrate, α5 integrin localized very faintly to vinculin-positive focal adhesions, but staining was mostly diffuse, and no elongated fibrillar adhesions were observed (Fig. 9, second row left). To further analyze adhesion sites in these cells, we studied the localization of tensin, an adaptor protein that is enriched in fibrillar adhesions (Katz et al., 2000; Zamir et al., 2000). To do so, we transfected fibronectin-expressing and fibronectin-deficient cells with tensin-GFP and grew them on fibronectin or vitronectin substrates for 48 hours. Cells were then fixed and co-stained for vinculin and fibronectin (supplementary material Fig. S4). In wild-type cells plated on vitronectin, tensin was present in elongated adhesions that colocalized with extracellular fibronectin fibrils. By contrast, we could not detect tensin-GFP in cell-matrix adhesions of fibronectin-deficient cells on vitronectin (supplementary material Fig. S4), in accordance with their diffuse staining for α5 integrin. However, tensin clearly localized to adhesion sites in the deficient cells when plated on fibronectin. These results suggest a correlation between the lack of fibrillar adhesions rich in α5 integrin and tensin, and the defective mechanotransduction observed in the absence of fibronectin.

When fibronectin-deficient cells were stretched on substrate coated with fibronectin type III domain 7-11 fragment, tenascin-C induction was diminished (Fig. 8A). Interestingly, cells on this substrate stained for α5 integrin in bright, irregular patches on their lower surface (Fig. 9, lower left) rather than in elongated adhesion structures. Together, these results indicate that α5 integrin has to be organized in fibrillar adhesions for efficient RhoA-dependent mechanotransduction. Experiments with fibronectin-

knockdown clone 8.3 and control cells confirmed this (data not shown).

Discussion

The mechanism by which mechanical stress is transduced into the altered expression of specific genes has received much attention in recent years, but is still not fully understood. Although several signaling pathways are activated by cyclic strain in fibroblasts (Chiquet et al., 2009), reorganization and increase in actin-stress fibers as a result of RhoA activation is a prominent response (Sarasa-Renedo et al., 2006). Recent evidence indicates that mechanical stress might regulate a specific group of genes directly via a change in RhoA-dependent actin dynamics, among them are genes encoding α-smooth muscle actin, CTGF/CCN1, Cyr61/CCN2 and tenascin-C (Chaqour and Goppelt-Struebe, 2006; Chaqour et al., 2007; Schild and Trueb, 2004; Zhao et al., 2007). We have shown previously that induction of tenascin-C by cyclic strain requires the presence of β1 integrin (Chiquet et al., 2006) and ILK (Maier et al., 2008), the activation of RhoA (Sarasa-Renedo et al., 2006), actin reorganization (Sarasa-Renedo et al., 2006), and that it correlates with translocation of MAL to the nucleus (Maier et al., 2008). The results presented here show that the mechanotransduction machinery requires pericellular fibronectin for a complete response to cyclic strain. In the absence of exogenous fibronectin, fibroblasts deficient for this protein have defects in RhoA-dependent mechanotransduction identical to those of ILK-knockout cells: in cells subjected to cyclic strain, RhoA is not activated, the actin cytoskeleton is not reorganized, MAL is not translocated to the nucleus, and tenascin-C protein and mRNA are not induced. Nevertheless, similarly to ILK-knockout cells, Erk1/2 and PKB/Akt are still activated in fibronectin-deficient fibroblasts after cyclic strain, indicating that the defect is specific for RhoA-mediated responses. $ILK^{-/-}$ fibroblasts secrete normal amounts of fibronectin into the medium but fail to assemble it on the cell surface and the substrate, which might explain their similar phenotype.

On any artificial or ECM substrate, a fibronectin matrix is rapidly deposited from normal wild-type fibroblasts (Grinnell and Feld, 1979). There is increasing evidence that fibronectin-specific integrin α5β1 is involved in the activation of RhoA in the process of fibroblast adhesion and spreading. RhoA activation was promoted during spreading of cells with elevated levels of α5 and β1 chains, but not of cells predominantly expressing the vitronectin receptor αvβ3 (Danen et al., 2002). The absence of α5 and β1 integrin chains also affected the RhoA-dependent process of fibronectin fibrillogenesis (Danen et al., 2002; Huveneers et al., 2008). Moreover, fibronectin was the only substrate to stimulate RhoA activity, and thereby cell-cycle progression, upon plating (Danen et al., 2000). These data clearly indicate that activation of RhoA is dependent on fibronectin and α5β1 integrin during interaction of fibroblasts with their substrate. Our data reveal a requirement for fibronectin in other RhoA-dependent responses, namely those induced by cyclic substrate strain in fully spread cells, in which a large integrin pool is already activated and engaged with ligands.

Different types of cell-matrix adhesions can be distinguished in cultured fibroblasts and these are assembled in a hierarchical manner (Zaidel-Bar et al., 2004). Early focal complexes contain mainly integrin αvβ3, talin and paxillin. Later, the adaptor protein vinculin is recruited and helps to stabilize focal complexes (Ziegler et al., 2006). Cytoskeletal force and the recruitment of additional proteins such as zyxin, tensin and α5β1 integrin lead to the formation of early focal adhesions. From these structures, specific proteins such as α5β1 integrin and tensin are pulled out by actomyosin contraction and form fibrillar adhesions (Zamir et al., 2000). The latter are essential for the assembly of the pericellular fibronectin fibrils to which they are attached (Mao and Schwarzbauer, 2005). We observed that in fibronectin-deficient MEFs, α5 integrin and tensin-GFP were integrated in elongated matrix contacts when cells were plated on fibronectin, but not on a vitronectin substrate. This indicates that on vitronectin these cells do not form fibrillar adhesions and suggests that RhoA-mediated responses to cyclic strain depend on the presence of these adhesions (Fig. 10). Fibrillar adhesions are also absent in $ILK^{-/-}$ fibroblasts that fail to assemble fibronectin (Vouret-Craviari et al., 2004) and show a similar defect in mechanotransduction (Maier et al., 2008).

The RhoA-dependent responses to cyclic strain measured on vitronectin depended on the secretion of fibronectin and are therefore likely to be mediated mainly via integrin α5β1 in fibrillar adhesions. By contrast, activation of ERK and PKB/Akt by cyclic strain was also observed in fibronectin-deficient cells in the absence of exogenous fibronectin, indicating that the triggering of these pathways is independent of fibrillar adhesions.

Activity of the fibronectin receptor α5β1 is known to be influenced by force. Externally applied tension switches α5β1 from a relaxed to a tensioned state, which engages the fibronectin synergy site and shows increased bond strength (Friedland et al., 2009; Kong et al., 2009). However, application of external force leads to a lesser activation of α5β1 on soft substrates in comparison to rigid substrates, indicating that a resistant counterforce is needed to trigger the integrin switch (Friedland et al., 2009). Our finding that substrate-bound fibronectin is important for activation of RhoA-dependent responses by cyclic strain is likely to be linked to the fact that actin contraction induced upon RhoA activation counteracts the applied strain. More stress resulting from increased external and internal forces might trigger the catch-bond character of α5β1.

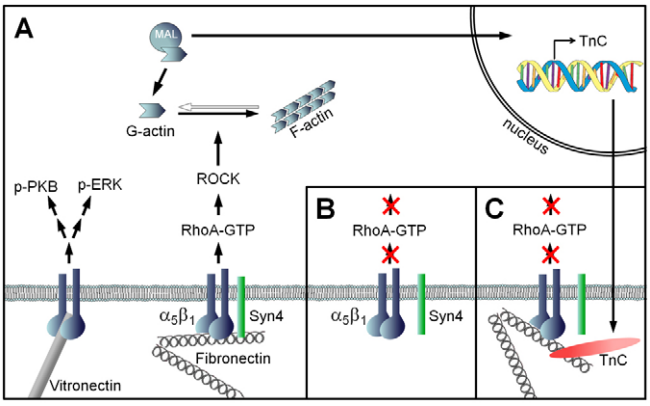

Fig. 10. Schematic model representing activation of RhoA-mediated responses by cyclic strain and induction of a negative-feedback loop. (**A**) Phosphorylation of PKB/Akt and ERK by cyclic strain is not dependent on the presence of fibronectin and might be mediated mainly by αvβ3 integrins that are enriched in focal adhesions. Induction of RhoA-mediated responses such as actin reorganization, MAL translocation and upregulation of tenascin-C by cyclic strain require fibronectin, which is mainly bound to α5β1 in fibrillar adhesions. (**B**) In the absence of fibronectin, these RhoA-mediated responses are not induced. (**C**) In the presence of fibronectin, the tenascin-C synthesized and deposited in response to cyclic strain might suppress RhoA activation on further mechanical stress and negatively regulate its own induction.

Experiments with a small fibronectin fragment (type III domains 7-11) indicated that the RGD and the adjacent synergy site are not sufficient for a full response to cyclic strain in terms of induction of tenascin-C. By contrast, 160 and 135 kDa chymotryptic fibronectin fragments that contained in addition a major heparin-binding site, led to full induction of tenascin-C by cyclic strain in fibronectin-deficient cells; cell-adhesion sites appeared normal on this substrate. However, on intact fibronectin substrates, mechanotransduction was suppressed when soluble heparin was added to the medium. These experiments strongly suggest the involvement of the respective heparin-binding site of fibronectin in RhoA-dependent responses to cyclic strain. This region of fibronectin also contains an interaction site for syndecan-4, a co-receptor of α5β1 integrin that is necessary for full spreading of fibroblasts (Woods and Couchman, 1994). Interestingly, the ECM protein tenascin-C interacts with the 13th type III repeat of fibronectin, which is part of this heparin-binding site, and thereby inhibits fibronectin interaction with syndecan-4 (Huang et al., 2001). This might be the reason why tenascin-C interferes with fibroblast attachment to fibronectin (Chiquet-Ehrismann et al., 1988). Tenascin-C is induced by mechanical stress via the RhoA-ROCK pathway (Chiquet et al., 2007; Maier et al., 2008; Sarasa-Renedo et al., 2006). We show here that the induction of tenascin-C by cyclic strain also depends on the presence of pericellular fibronectin. Tenascin-C was shown to suppress RhoA activation (Wenk et al., 2000) and inhibit cell-mediated contraction of mixed fibronectin and fibrinogen matrices (Midwood and Schwarzbauer, 2002). In addition, several other functions of tenascin-C were reported to depend on the presence of syndecan-4 (Midwood et al., 2004). We now demonstrate that addition of exogenous tenascin-C protein to coated fibronectin interferes with the induction of endogenous *Tnc* mRNA following cyclic strain. Together with our previous results, this further strengthens our hypothesis that pericellular fibronectin is crucial for the activation of the RhoA-ROCK pathway by mechanical stimuli. In addition, tenascin-C expressed upon mechanical stimulation could interfere with fibronectin binding and thereby prevent further activation of the RhoA-ROCK pathway brought about by future bouts of mechanical stress (Fig. 10). This feedback loop, in which tenascin-C protein negatively regulates its own induction, might avoid mechanical overload of the cell that results from excess adhesion to a strained substrate.

Our data indicate that the pericellular fibronectin matrix is fundamental to the activation of RhoA and RhoA-dependent responses to cyclic mechanical stress. Three different mechanically induced events reported to depend on RhoA activation (Chiquet et al., 2004; Maier et al., 2008; Miralles et al., 2003; Sarasa-Renedo et al., 2006; Zhao et al., 2007) were shown to be defective in the absence of fibronectin. All effects occurred in both fibronectin-knockdown and fibronectin-knockout fibroblasts. However, the normal activation of ERK and PKB/Akt following mechanical stress in the absence of fibronectin indicates that mechanotransduction is not affected in general. Experiments made with fibronectin fragments and addition of soluble heparin showed that RhoA-dependent responses to cyclic strain depend on a major heparin-binding site of fibronectin. This fact and the inhibitory effect of tenascin-C protein on its own induction by mechanical stress both seem to indicate the possible involvement of syndecan-4 in the observed responses. The precise role and putative co-operation of fibronectin receptor α5β1 integrin and syndecan-4 (Fig. 10) have to be addressed in future experiments. Nonetheless, our present results implicate pericellular fibronectin in the activation of a specific pathway that changes cytoskeletal structure and gene expression in response to external mechanical stress, and they suggest ways in which this pathway is regulated.

Materials and Methods

Cell culture

A kidney MEF cell line immortalized by stable transfection with SV40 large-T antigen was used to produce stable fibronectin-knockdown mouse embryo fibroblasts (MEFs) (Graness et al., 2006). Cells were transfected with purified plasmid DNA encoding shRNA against mouse fibronectin (MISSION shRNA Bacterial Glycerol Stock from Sigma, Buchs, Switzerland) by means of the MEF nucleofactor kit (Amaxa, Lonza, Basel, Switzerland). Transfected cells were seeded and clones resistant to puromycin were selected. By the same procedure, fibronectin-knockdown REFs were generated from wild-type REFs. A kidney MEF cell line containing a floxed fibronectin gene ($FN^{f/f}$) and a fibronectin-knockout MEF cell line ($FN^{-/-}$) (Fontana et al., 2005; Sakai et al., 2001) were obtained from Michael Leiss and Reinhard Fässler (Max-Planck Institute for Biochemistry, Martinsried, Germany). The original cells were maintained at 37°C with 6% CO_2 in Dulbecco's modified Eagle medium (DMEM; Seromed, Basel, Switzerland) containing 10% fetal calf serum (FCS; Gibco/Invitrogen, Basel, Switzerland). Fibronectin-knockdown clones were maintained in the same medium to which puromycin was added at 2 μg/ml.

Generation of fibronectin fragments

160- and 135-kDa cell-binding fragments of fibronectin were generated as described (Ehrismann et al., 1982) (supplementary material Fig. S3A). Briefly, fibronectin from 50 ml horse serum was adsorbed to gelatin-Sepharose and digested on the column with 20 μg/ml crystalline chymotrypsin (Serva, Heidelberg, Germany) for 5 minutes. Eluted (non-gelatin binding) fragments were collected into tubes containing protease inhibitors, pooled, and adsorbed to heparin-Sepharose to remove chymotrypsin and protease inhibitors. Bound 160 and 135 kDa fragments were eluted from the heparin affinity column with 1 M NaCl, 20 mM sodium phosphate, pH 7.4. A fibronectin fragment comprising type III domains 7-11 was expressed as described (Bloom et al., 1999). Briefly *E. coli* strain DH5a harboring the expression vector (obtained from Gertraud Orend, University of Strasbourg, Strasbourg, France) was grown to late log-phase and induced with 1 mM IPTG for 4 hours. Cells were harvested, lysed by sonification in lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 1 mg/ml lysozyme, protease inhibitors (Roche, Basel, Switzerland), and the GST-tagged recombinant fragment was purified using glutathione-Sepharose beads (Qiagen, Basel, Switzerland).

Matrix coating and plating of cells

For cyclic strain experiments, silicone membranes of Flexercell II six-well plates were coated for 3 hours with purified horse serum fibronectin at 50 μg/ml in Dulbecco's phosphate buffered saline (PBS) (Chiquet et al., 2004). Alternatively, silicone membranes were coated with vitronectin at 20 μg/ml (Abcam, Cambridge, UK) for 90 minutes and then allowed to air dry. Concentrations used for coating were saturating for cell adhesion and roughly adjusted to the molecular mass of the respective proteins. Air drying was used because a more homogeneous coating of the very hydrophobic silicone membrane was achieved, and no loss of adhesive activity was observed compared with coating glass or culture plastic without drying. In some experiments tenascin-C, purified from chick embryo fibroblast conditioned medium (Chiquet-Ehrismann et al., 1988), was mixed with fibronectin or vitronectin during coating. Concentrations used for coating of tenascin-C (TnC)-containing substrates were: FN and TnC (50 μg/ml and 50 μg/ml), TnC (50 μg/ml), VN and TnC (20 μg/ml and 50 μg/ml). Other matrix proteins were used at the following coating concentrations: FN CHB (30 μg/ml), FN 7-11 (20 μg/ml), Collagen (Serva, Heidelberg, Germany; 2 mg/ml). For inhibition experiments, porcine intestinal mucosa heparin (Sigma, Buchs, Switzerland) was added at 100 μg/ml 24 hours before applying strain. For immunofluorescence experiments, 40,000 cells and, for biochemical experiments 90,000 cells were plated in DMEM containing 3% FCS depleted of fibronectin by two passages over gelatin-Sepharose (Amersham, Wädenswil, Switzerland). Cells were allowed to attach, spread and deposit their own matrix for 20 hours. After washing with serum-free medium, cells were starved for a further 20 hours in DMEM containing a low concentration of fibronectin free serum (0.03% to determine tenascin-C protein and mRNA, 0.3% for all other experiments) and cells were subsequently subjected to cyclic strain in this medium. For analyzing tenascin-C deposition into the matrix, cells were kept in culture for a further 18 hours after straining.

Mechanical loading of cells

Culture dishes were mounted on a Flexcell FX-4000 machine (Dunn Labortechnik, Asbach, Germany) and cells were subjected to equibiaxial cyclic strain (10%, 0.3 Hz) at 37°C for the times indicated (5 minutes to 6 hours). After mechanical stimulation, cells were fixed with 4% paraformaldehyde for phalloidin and immunofluorescence staining (see below). Alternatively, cells were lysed in RNA isolation buffer from an RNA purification kit (RNeasy, Qiagen, Basel, Switzerland).

If used, cell-permeable C3-transferase at 0.25 μg/ml (Cytoskeleton, LuBioScience, Luzern, Switzerland) was added 30 minutes before cyclic strain.

Rho-activity assay

Silicone membranes with attached cells were washed with cold TBS, lysed and Rho activity was measured using a previously described method (Ren and Schwartz, 2000). Briefly, cells were lysed in cold lysis buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaC1, 10 mM $MgC1_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF) and GTP-Rho was affinity precipitated using Rhotekin-GST-Sepharose beads (for production of beads and precipitation see Ren and Schwartz (Ren and Schwartz, 2000) and Sarasa-Renedo et al. (Sarasa-Renedo et al., 2006). Affinity precipitated RhoA was quantified in parallel with total cellular RhoA from cell lysates by western blot analysis with an antibody against RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:75 in TBS, 5% BSA, 0.1% Tween.

Immunofluorescence and phalloidin staining

Silicone membranes with attached, fixed cells were permeabilized and incubated at room temperature for 30 minutes in PBS containing 3% BSA and 0.1% Triton X-100. Rabbit polyclonal antiserum to horse serum fibronectin (crossreacting with mouse) (Wehrle and Chiquet, 1990) and monoclonal antibody 65F13 against mouse MAL (Maier et al., 2008) were described previously. Cells were then stained for 1 hour at room temperature with anti-fibronectin antiserum diluted 1:300, anti-MAL mAb 65F13 supernatant diluted 1:10, anti-vinculin 1:1000 (Sigma, Buchs, Switzerland) and/or anti-α5-integrin 1:500 (BD Pharmingen, Basel, Switzerland) in PBS containing 3% BSA and 0.1% Triton X-100. After staining, cells were washed three times with PBS, 0.1% Triton X-100, incubated for 1 hour with Alexa Fluor 546-labeled phalloidin (Sigma) and either Alexa Fluor 488-labeled goat anti-rabbit or Alexa Fluor 488-labeled goat anti-mouse IgG (Sigma). Cells were again washed three times with PBS, 0.1% Triton-X100 and mounted in Prolong Gold antifade reagent (Invitrogen, Basel, Switzerland). Slides were examined with a Zeiss Z1 microscope equipped with a 20×/0.8 NA objective, Zeiss filter cubes no. 38HE for Alexa Fluor 488, no. 43 for Alexa Fluor 546 and a Zeiss MRm camera.

Immunoblotting

Wild-type, clone 1.2 and clone 8.3 cells were left at rest or subjected to cyclic strain for 5, 10 and 15 minutes, respectively. Cells were then immediately washed with ice-cold PBS and subsequently scraped in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate and 1 mM EDTA) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Lysates were cleared by centrifugation, resolved on 10% polyacrylamide-SDS gels and blotted to nitrocellulose. Blots were incubated with one of the following antibodies diluted in TBS (0.1% Tween, 3% BSA): rabbit antibody against Erk1/2 (1:1000), mouse mAb against phosphorylated Erk1/2 (1:1000), rabbit antibody against phosphorylated Akt (S473) (1:1000) (all Cell Signaling; BioConcept, Allschwil, Switzerland) or rabbit antibody Ab10 against PKB/Akt (1:500) (Jones et al., 1991). Tenascin-C in cell or cell-matrix lysates was detected using the rat monoclonal anti-tenascin-C antibody mTn-12 (1:50) (Aufderheide and Ekblom, 1988). For detection of fibronectin, blots were incubated with polyclonal antiserum against fibronectin (see above) diluted 1:300. Non-reducing gels were run to analyze integrin expression levels. After blotting to PVDF membranes, α5 integrin (rat monoclonal, 1:250, BD Bioscience, Allschwil, Switzerland), αv integrin (mouse anti-CD51, 1:250, BD Biosciences) and β1 integrin (rat monoclonal, 1:1500, BD Bioscience) were used for detection. In all cases, blots were washed and subsequently incubated with peroxidase-labeled secondary antibodies (1:2000; Cappel/ICN Biomedicals; EGT Chemie, Switzerland) and developed with ECL reagent (Amersham, Wädenswil, Switzerland).

Quantification of mRNA levels by real-time PCR

RNA was isolated from scraped cell lysates by the RNeasy procedure (Qiagen, Basel, Switzerland). RNA was then reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland). TaqMan real-time PCR primer/probe mixtures for mouse tenascin-C (Mm00495662_ml), and mouse GAPDH (Mm99999915_gl) as well as TaqMan Universal Master Mix were purchased from Applied Biosystems. For each experimental condition, reverse-transcribed cDNA was amplified for each gene on an ABI Prism 7000 real-time PCR cycler (Applied Biosystems). Data were analyzed by the ΔCt method (Livak and Schmittgen, 2001), i.e. values for tenascin-C were normalized to GAPDH for each sample. These data are shown in the graphs rather than data produced by further normalization to the resting controls [ΔΔCt method (Livak and Schmittgen, 2001)]. Data are the means ± s.e.m. of 3-4 independent experiments. Statistical significances between rest and stressed conditions were determined by two-way ANOVA. Differences of $P<0.05$ were considered significant.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/9/1511/DC1

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