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Tenascin-C Modulates Matrix Contraction via Focal Adhesion Kinase– and Rho-mediated Signaling Pathways

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A provisional matrix consisting of fibrin and fibronectin (FN) is deposited at sites of tissue damage and repair. This matrix serves as a scaffold for fibroblast migration into the wound where these cells deposit new matrix to replace lost or damaged tissue and eventually contract the matrix to bring the margins of the wound together. Tenascin-C is expressed transiently during wound repair in tissue adjacent to areas of injury and contacts the provisional matrix in vivo. Using a synthetic model of the provisional matrix, we have found that tenascin-C regulates cell responses to a fibrin-FN matrix through modulation of focal adhesion kinase (FAK) and RhoA activation. Cells on fibrin-FN+tenascin-C redistribute their actin to the cell cortex, downregulate focal adhesion formation, and do not assemble a FN matrix. Cells surrounded by a fibrin-FN+tenascin-C matrix are unable to induce matrix contraction. The inhibitory effect of tenascin-C is circumvented by downstream activation of RhoA. FAK is also required for matrix contraction and the absence of FAK cannot be overcome by activation of RhoA. These observations show dual requirements for both FAK and RhoA activities during contraction of a fibrin-FN matrix. The effects of tenascin-C combined with its location around the wound bed suggest that this protein regulates fundamental processes of tissue repair by limiting the extent of matrix deposition and contraction to fibrin-FN-rich matrix in the primary wound area.

INTRODUCTION

Wound repair is a dynamic chain of events involving both soluble factors, blood proteins, and cells in the synthesis of a provisional matrix that is deposited at sites of tissue injury. The provisional matrix is a covalently cross-linked network consisting predominantly of fibrin and plasma fibronectin (pFN) that is formed during the terminal steps of blood coagulation (Clark *et al.*, 1982). At areas of tissue damage, fibrin-FN deposits fill the wound to prevent further blood loss. This matrix also supports cell adhesion and migration into the site of injury and is subsequently remodeled to regain normal tissue structure and function. The synthesis of the fibrin-FN provisional matrix can be recapitulated in vitro using purified components (Wilson and Schwarzbauer, 1992; Corbett *et al.*, 1996). The resulting matrix allows examination of the effects of individual extracellular matrix proteins and dissection of molecular signaling pathways in an environment with physiological relevance. Using a synthetic fibrin-FN provisional matrix, we have previously shown that FN is required for fibroblast attachment and spreading on this matrix and have characterized the molecular requirements for maximal fibrin-FN cross-linking (Corbett *et al.*, 1996, 1997).

Fibroblasts carry out several functions once they have migrated into the provisional matrix. The first major task is the deposition of granulation tissue, which is composed of newly synthesized FN and other matrix proteins (Grinnell *et al.*, 1981; Welch *et al.*, 1990). Fibroblasts then differentiate into myofibroblasts, which express increased amounts of contractile proteins such as alpha smooth muscle actin (Masur *et al.*, 1996). These cells exert mechanical force on the matrix. This contraction is essential for bringing the margins of the wound together to minimize the wound area and the extent of scarring (Clark, 1996).

The fibrin-FN matrix also contacts uninjured tissue adjacent to the wound bed, thus allowing resident extracellular matrix proteins to affect provisional matrix functions. Tenascin-C is an extracellular matrix protein that exhibits a restricted pattern of expression in vivo. It is limited to developing tissues and sites of active remodeling, such as tumors and wounds (Mackie *et al.*, 1988; Erickson and Bourdon,

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Abbreviations used: FAK, focal adhesion kinase; FN, fibronectin; pFN, plasma fibronectin.

1989; Crossin, 1996). Tenascin-C is significantly but transiently upregulated in tissues adjacent to injury sites, suggesting that it may act to regulate cellular response to the provisional matrix. In tenascin-C knockout mice, wound healing is defective and FN matrix deposition at wound sites is decreased (Forsberg *et al.*, 1996; Mackie and Tucker, 1999). A major role for tenascin-C in adhesion modulation is suggested by its adhesive and antiadhesive properties. This protein can antagonize the adhesive effects of matrix proteins such as FN and can cause changes in actin cytoskeleton organization and focal adhesion formation (Chiquet-Ehrismann *et al.*, 1988; Spring *et al.*, 1989; Murphy-Ullrich *et al.*, 1991; Sage and Bornstein, 1991; Orend and Chiquet-Ehrismann, 2000; Huang *et al.*, 2001).

We have shown that tenascin-C dramatically alters fibroblast spreading on a three-dimensional fibrin-FN matrix. In our fibrin-FN matrix model, both fibroblast morphology and actin organization are modulated by tenascin-C via regulation of RhoA GTPase activity (Wenk *et al.*, 2000). The Rho family of GTPases controls actin cytoskeletal organization. In particular, RhoA mediates the formation of actin stress fibers and regulates cellular functions including adhesion, contractility, cell cycle progression, and gene transcription (Hall, 1998).

Based on the RhoA-mediated effects of tenascin-C on fibroblast interactions with a fibrin-FN matrix, we hypothesized that the presence of this protein may regulate wound contraction. A role for FN in fibrin-FN matrix contraction has been previously demonstrated (Corbett and Schwarzbauer, 1999). Here we show that tenascin-C inhibits matrix contraction and that this inhibition can be reversed by treatments that activate RhoA. We also show that tenascin-C downregulates FAK phosphorylation and that expression and phosphorylation of FAK is required for matrix contraction. Deposition of a FN-rich matrix is also blocked by tenascin-C. Our results suggest a model whereby tenascin-C serves a major role in determining the boundaries of the wound. The presence of this protein limits both the deposition of new extracellular matrix and the extent of wound contraction to within the boundaries of the provisional matrix.

MATERIALS AND METHODS

Protein Production

Rat pFN was purified by gelatin-Sepharose (Pharmacia Biotech, Piscataway, NJ) affinity chromatography from freshly drawn plasma (Wilson and Schwarzbauer, 1992). The production of recombinant proteins 70-kDa, 70Ten, and 70TenS has previously been described (Schwarzbauer, 1991; Luczak *et al.*, 1998; Wenk *et al.*, 2000). Native human tenascin-C from U251 glioma cells, consisting of >90% large splice variant, was purchased from Life Technologies (Rockville, MD).

Cell Culture

NIH 3T3 fibroblasts were maintained in DMEM and 10% calf serum (Hyclone Laboratories, Logan, UT). Rat-1 fibroblasts stably transfected with activated RhoA-V14 or control vector cDNA (Qiu *et al.*, 1995; gifts from Dr. Marc Symons, Picower Institute, New York, NY) were maintained in DMEM containing 10% fetal calf serum and 400 μg/ml G418 (GIBCO, Rockville, MD). Because RhoA-V14 is driven by a tetracycline-repressible promoter, medium also contained 2.5 μg/ml puromycin and 2 μg/ml tetracycline. Tetracycline was withdrawn from the medium 48 h before the start of each experiment. Fibroblasts derived from wild-type or FAK-deficient mouse embryos (Ilic *et al.*, 1995; gifts from Dr. Dusko Ilic, UCSF) were cultured in DMEM plus 10% fetal bovine serum (Hyclone Laboratories).

Immunofluorescence

Matrices were prepared as described previously (Corbett and Schwarzbauer, 1999; Wenk *et al.*, 2000). A mass ratio of 20:1 fibrinogen/FN was used, which gives identical results to matrices prepared at a physiological ratio of 10:1 (Wenk *et al.*, 2000). The ratio of FN/tenascin-C was 1:3, a 1:1 M ratio of dimeric FN to hexameric tenascin-C. 600 μg/ml fibrinogen (America Diagnostica Inc., Greenwich, CT), 30 μg/ml FN and 120 μg/ml tenascin-C or 70Ten, and 15 μg/ml coagulation factor XIIIa (Calbiochem, La Jolla, CA) were mixed with 1 mg/ml aprotinin in 150 mM NaCl, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4. Immediately after the addition of thrombin at 2 U/ml, the mixture was pipetted onto a glass coverslip (Fisher Scientific, Pittsburgh, PA). After overnight incubation at 4°C, the clots were carefully aspirated from the coverslip leaving a matrix attached to the surface (Corbett *et al.*, 1996), and the substrate was blocked with 1% BSA in PBS. Fibrinogen and thrombin were reconstituted and contaminating FN removed from fibrinogen as previously described (Wilson and Schwarzbauer, 1992; Corbett *et al.*, 1996). Covalent cross-linking of matrices was monitored by SDS-PAGE (Wilson and Schwarzbauer, 1992).

Cells were released from tissue culture dishes using 0.2 mg/ml EDTA in PBS, washed with PBS, resuspended in serum-free DMEM at 4×10^4/ml, and added to coverslips. Cells were allowed to spread on substrate-coated glass coverslips for 1 h, after which time cells were washed with PBS, fixed for 15 min with 3.7% formaldehyde in PBS, and permeabilized for 15 min with 0.5% NP-40 (Calbiochem) in PBS. Cells were incubated with primary or secondary antibody or phalloidin in 2% ovalbumin (Sigma Chemical Co., St. Louis, MO) in PBS at 37°C for 30 min. Antibodies were used at the following dilutions: anticortactin monoclonal (Upstate Biotechnology, Lake Placid, NY) at 1:100, PT66 antiphosphotyrosine monoclonal (Sigma Chemical Co.) at 1:300, antivinculin monoclonal (Sigma Chemical Co.) at 1:300 and fluorescein-conjugated goat anti-mouse secondary antibody (Molecular Probes Inc., Eugene, OR) at 1:500. For staining actin filaments, rhodamine-conjugated phalloidin (Molecular Probes Inc.) was used at 1:1000 as described (Corbett *et al.*, 1996). Coverslips were mounted with SlowFade Light Antifade Kit (Molecular Probes Inc.). Cells were visualized with a Nikon Optiphot-2 microscope (Garden City, NY), and images were captured using a Photometrics Coolsnap camera (Tucson, AZ) and analyzed using Coolsnap and IP labs software.

FN matrix assembly was assessed by immunofluorescence staining (Sechler *et al.*, 2001). Cells were plated on fibrin-FN matrix on glass coverslips in 24-well dish (Nunc Inc., Napierville, IL) and allowed to spread. Cells were then incubated with 25 μg/ml human pFN for 2 h, after which time cells were washed with PBS + 0.5 mM $MgCl_2$ and then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. FN matrix was detected with culture supernatant from hybridoma cells producing anti-human FN antibody 7.1 at 1:200 (Brenner *et al.*, 2000) and fluorescein-conjugated goat anti-mouse secondary antibody (Molecular Probes Inc.) at 1:500. Coverslips were mounted with SlowFade Light Antifade Kit (Molecular Probes Inc.).

Immunoprecipitation

Matrices and cells were prepared as for immunofluorescence studies. Cells were plated in serum-free DMEM at a density of 1.5×10^6 per 35-mm dish and allowed to spread on matrices for 15, 30, or 60 min. At the end of the incubation period cells were washed with PBS and then lysed in 200 μl RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM $NaVO_4$, 1 mM EDTA, 50 mg/ml leupeptin, 0.5% aprotinin) on

ice for 15 min (Kanner *et al.*, 1989). The cells were scraped with a rubber policeman, and lysate was collected and centrifuged for 10 min at 4°C. The pellet was discarded, and the protein concentration of the supernatant was determined using the BCA Protein Assay (Pierce, Rockford, IL). Four micrograms of anti-FAK mAb (Upstate Biotechnology) was added to 250 μg of total cell lysate and incubated at 4°C overnight while rotating. Fifty microliters of washed and packed protein-G agarose beads (Calbiochem) was added to the lysates. After an additional 2-h incubation the beads were washed three times with cold RIPA buffer. Protein was eluted from the beads by boiling in electrophoresis sample buffer (2% SDS, 80 mM Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue, 100 mM DTT) for 5 min.

Samples were run on a 6% polyacrylamide-SDS gel and transferred to nitrocellulose. Proteins were detected using anti-FAK mAb (Transduction Laboratories, Lexington, KY) at 1:1000, or PT66 antiphosphotyrosine mAb (Sigma Chemical Co.) at 1:3333. Primary antibodies were detected using horseradish peroxidase–conjugated goat anti-mouse secondary antibody (Pierce) diluted 1:50,000 and Supersignal chemiluminescent detection reagent (Pierce).

Contraction Assays

Matrices were prepared as for immunofluorescence except 1×10^6 cells/ml were added to the matrix components. Immediately after the addition of thrombin, the mixture was pipetted into 48-well plates that had been coated with 1% BSA overnight at 4°C. The cell-matrix mixture was allowed to polymerize for 30 min at 37°C. The clots were then carefully detached from the dishes, and matrix contraction was visualized. The area of the matrix was measured over time using a ruler, subtracted from the starting area, and expressed as a percentage of the starting area.

Before the treatment with activators or inhibitors, cells were serum starved for 24 h. After release with EDTA cells were incubated with 5 μM LPA (Sigma Chemical Co.) or 10 μM Y27632 (BIOMOL, Plymouth Meeting, PA) for 30 min at 37°C in suspension before addition to matrix proteins. Cells were serum starved for 24 h with 25 μg/ml C3 transferase (Cytoskeleton, Denver, CO) or 10 μM simvastatin (Calbiochem) added to the medium before being released from tissue culture dishes as described above and added to matrix proteins. Simvastatin was activated before use as described previously (Laufs *et al.*, 1998). In some experiments, serum-starved cells were treated with 5 μM phenylarsine oxide (Sigma Chemical Co.) or 0.1 mM pervanadate as described (Miao *et al.*, 2000). The pretreatments had no effect on the cross-linking of the matrices as determined by SDS-PAGE.

Membrane Fractionation

Cells plated in serum-free DMEM at a density of 1.5×10^6 per 35-mm dish were allowed to spread on matrices for 1 h. At the end of the incubation period cells were washed with PBS and then lysed in 200 μl hypotonic lysis buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 10 mM PMSF, 20 mM β-mercaptoethanol) on ice for 15 min (Gohla *et al.*, 1998). The cells were scraped with a rubber policeman, and lysate was collected and centrifuged for 5 min at 4°C. The supernatant was centrifuged at $50,000 \times g$ for 30 min at 4°C. The pellet was resuspended in lysis buffer without NaCl containing 20 μg/ml leupeptin, and the protein concentration of both the pellet and supernatant was determined using the BCA Protein Assay (Pierce). Equal amounts of total protein were run under reducing conditions on a 13% polyacrylamide-SDS gel and transferred to nitrocellulose. Proteins were detected using anti-Rho mAb (Transduction Laboratories) at 1:250 dilution. Primary antibodies were detected using horseradish peroxidase–conjugated goat anti-mouse secondary antibody (Pierce) diluted 1:50,000 and Supersignal chemiluminescent detection reagent (Pierce).

Analysis of DOC-insoluble Material

Cells were plated at 1.5×10^5/ml in fibrin-FN–coated 24-well dishes and incubated with 25 μg/ml human pFN for 2 h. Cells were then washed with cold PBS and lysed with 200 μl of deoxycholate (DOC) lysis buffer (2% DOC, 0.02 M Tris-HCl, pH 8.8, 2 mM PMSF, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM *N*-ethylmaleimide; Sechler *et al.*, 2001). DOC-insoluble material was isolated by centrifugation at 14,000 rpm for 15 min at 4°C, and then solubilized in 25 μl of 1% SDS, 25 mM Tris-HCl, pH 8.0, plus protease inhibitors. Total protein concentration was determined using BCA Protein Assay (Pierce). Equal amounts of DOC-insoluble material were electrophoresed under reducing conditions on a 5% polyacrylamide SDS gel. Separated proteins were transferred to nitrocellulose (Sartorius Corp., Long Island, NY), and FN was detected using culture supernatant from hybridoma cells producing anti-human FN antibody 7.1 at 1:2000. Primary antibodies were detected using horseradish peroxidase–conjugated goat anti-mouse secondary antibody (Pierce) diluted 1:50,000 and Supersignal chemiluminescent detection reagent (Pierce).

RESULTS

Tenascin-C Prevents Focal Contact Formation and Induces Reorganization of Cytoskeleton-associated Proteins

Tenascin-C induces changes in actin organization by suppressing RhoA activation in cells adherent to fibrin-FN matrices (Wenk *et al.*, 2000). To examine the effect of tenascin-C on the distribution of actin filaments, we followed the association of actin with cortactin. Cortactin is a cytoskeletal protein that binds specifically to cortical actin (Wu and Parsons, 1993). It is involved in the signaling pathways of adhesion molecules mediating cytoskeletal reorganization and has been shown to influence cell migration and invasion (Patel *et al.*, 1998). NIH 3T3 fibroblasts plated on fibrin-FN matrices displayed extensive actin stress fibers. Very little actin was associated with cortactin (Figure 1, A–C). In contrast, the addition of tenascin-C to the matrix resulted in a redistribution of actin; no stress fibers were seen and much of the actin became cortical, as indicated by its colocalization with cortactin (Figure 1, D–F).

We have previously described the production of a highly purified recombinant form of tenascin-C, 70Ten. 70Ten contains the amino-terminal 70-kDa region of FN, including the fibrin cross-linking site, connected to all the type III repeats and the terminal knob of tenascin-C, containing adhesive and antiadhesive domains. 70Ten behaves identically to full-length tenascin-C when included in a fibrin-FN matrix (Wenk *et al.*, 2000). The addition of 70Ten to a fibrin-FN matrix resulted in the same reorganization of the cytoskeleton to cortical actin as tenascin-C. This effect, therefore, is not due to other proteins present in preparations of native tenascin-C.

Focal adhesion assembly, cytoskeleton-associated protein distribution, and intracellular signaling were also altered upon cell interaction with a fibrin-FN + 70Ten matrix. Immunofluorescence staining of cells on fibrin-FN matrices revealed typical focal adhesions, which are rich in vinculin, a protein involved in connections to the actin cytoskeleton (Figure 2, A and B). These focal adhesions were not formed by fibroblasts on fibrin-FN + 70Ten matrices (Figure 2, C and D). Staining patterns similar to vinculin were observed

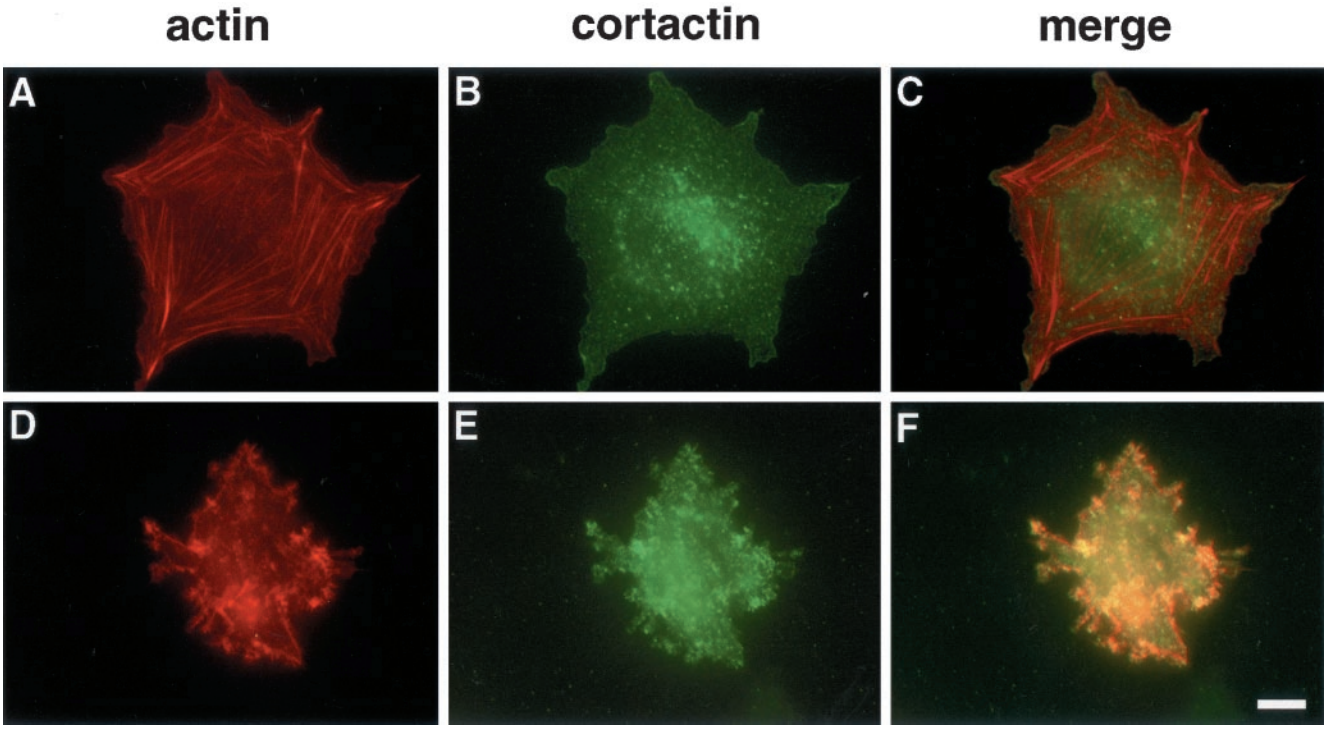

Figure 1. Tenascin-C induces a distinct cytoskeletal organization. NIH 3T3 fibroblasts were allowed to interact with fibrin-FN (A–C) or fibrin-FN+tenascin-C (D–F) matrices for 1 h before fluorescent staining for actin with rhodamine-labeled phalloidin (A and D) and for cortactin with an anticortactin mAb (B and E). Sites of colocalization are shown in yellow (C and F). Bar, 10 μm.

with antiphosphotyrosine antibodies that detect signaling proteins such as FAK.

Biochemical data show that although total FAK remains the same in all cell lysates, FAK phosphorylation was sustained on fibrin-FN substrates but was transient in fibrin-FN matrices including 70Ten (Figure 3). Together these results show that focal adhesion assembly is impaired by the addition of tenascin-C, which has a dramatic effect on cytoskeletal organization and subsequent signaling events.

Tenascin-C Inhibits Matrix Contraction

Fibroblast interactions with fibrin-FN–based matrices are important in the generation of force required to contract the provisional matrix found at sites of tissue injury in vivo (Clark, 1996). To determine the effects of matrix composition on cell contractility and to examine cell phenotype in a more quantitative manner, we used a well-characterized matrix contraction assay (Corbett *et al.*, 1997; Corbett and Schwarzbauer, 1999). NIH 3T3 fibroblasts were incorporated into three-dimensional matrices consisting of fibrin-FN or fibrin-FN+tenascin-C. This cell-matrix mixture was allowed to polymerize in 48-well dishes and then released from the sides of the dish. The ability of cells to contract the matrix was assessed by measuring the area of the matrix over time. This value was subtracted from the starting area, and contraction was expressed as a percentage of the starting area. Fibroblasts rapidly contracted matrices consisting of fibrin-FN. In contrast, fibrin-FN matrix contraction was completely inhibited by the inclusion of tenascin-C or 70Ten (Figure 4). 70TenS is a small splice variant of tenascin-C, which lacks five alternatively spliced type III repeats and has been shown to have the same effects on cell spreading as 70Ten (Wenk *et al.*, 2000). The addition of 70TenS to a fibrin-FN matrix also inhibited the ability of fibroblasts to contract the matrix to the same extent as full-length tenascin-C and 70Ten (Figure 4). The addition of equal molar ratios of BSA or the recombinant protein 70-kDa, which consists of the 70-kDa amino terminal of FN, instead of tenascin-C had no effect on the ability of cells to contract a fibrin-FN matrix (Figure 4). These results show that inhibition of contraction is due to the specific inclusion of tenascin-C in the matrix and demonstrate an important modulatory role for tenascin-C in cell contractility.

Rho Activation Relieves Inhibitory Effects of Tenascin-C

Tenascin-C modulates cell morphology and actin organization on fibrin-FN matrices by regulating RhoA GTPase activity (Wenk *et al.*, 2000). RhoA activation is also essential for contraction of a fibrin-FN matrix. Inhibition of RhoA activity by treatment of cells with C3 transferase or inhibition of RhoA translocation to the membrane by treating cells with simvastatin inhibited fibroblast contraction of a fibrin-FN matrix (Figure 5). Stimulation of the activity of RhoA by preincubation of cells with LPA (Figure 5) or by expression of constitutively active RhoA-stimulated contraction of a

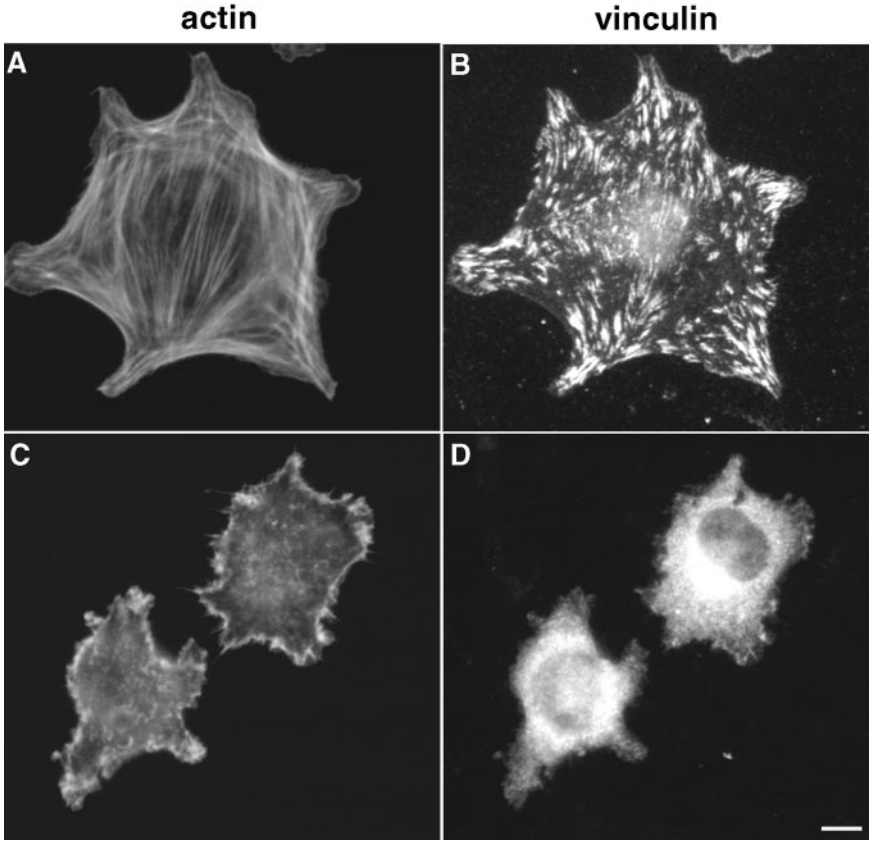

Figure 2. Tenascin-C inhibits the formation of focal adhesions. NIH 3T3 fibroblasts were allowed to interact with fibrin-FN (A and B) or fibrin-FN + 70Ten (C and D) matrices for 1 h before staining for actin (A and C) and for vinculin with an antivinculin mAb (B and D). Bar, 10 μm.

fibrin-FN matrix over untreated or vector control cells. The LPA effect was dose dependent. Treatment of cells with Y27632, an inhibitor of ROCK, a downstream target of RhoA involved in promoting stress fiber and focal adhesion formation (Amano *et al.*, 1997), also inhibited matrix contraction (Figure 5), suggesting that RhoA mediation of this process is via a ROCK-dependent pathway. These results show an important role for RhoA in cell contraction of fibrin-FN matrices.

To determine whether RhoA acts downstream of tenascin-C during contraction, the effect of stimulating RhoA activity on the contraction of fibrin-FN + 70Ten matrices was examined. Treatment of NIH 3T3 cells with LPA or expression of constitutively active RhoA in Rat-1 fibroblasts restored normal levels of contraction of a fibrin-FN + 70Ten matrix (Figure 6). Therefore tenascin-C inhibition of cell contractility can be circumvented by downstream activation of RhoA.

The fact that prevention of RhoA movement to the membrane by simvastatin inhibits matrix contraction suggests that RhoA must be both in its active GTP-bound form and localized properly to mediate contraction. To determine whether tenascin-C regulates RhoA function by affecting its subcellular distribution, we examined the effect of tenascin-C on the localization of RhoA. In cells plated on a fibrin-FN matrix, RhoA was predominantly localized in the membrane fraction of cell lysates (Figure 7). On the addition of 70Ten to the matrix, the majority of RhoA was localized in the cytoplasmic fraction. These data indicate that tenascin-C inhibits the recruitment of RhoA to the cell membrane.

A major role for fibroblasts within the provisional matrix is to deposit a FN-rich matrix as a framework for tissue repair (Grinnell *et al.*, 1981). In addition to regulating matrix contraction, tenascin-C in the surrounding tissue may act to prevent inappropriate FN matrix deposition. To test this idea, NIH 3T3 fibroblasts on a fibrin-FN matrix were exam-

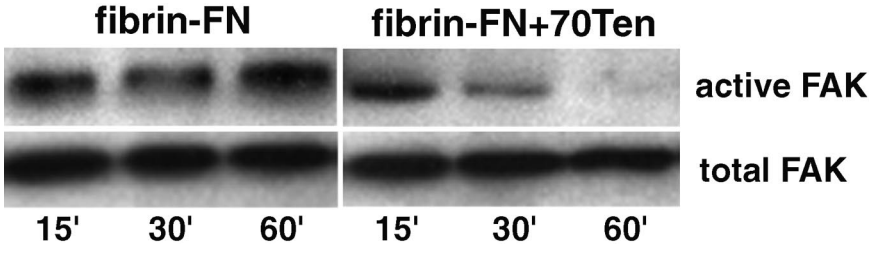

Figure 3. Transient FAK phosphorylation in the presence of tenascin-C. NIH 3T3 fibroblasts were allowed to adhere to fibrin-FN+/−70Ten matrices for 15, 30, and 60 min before lysis and immunoprecipitation with anti-FAK antibodies. Proteins were separated by SDS-PAGE, and immunoblots were probed with an anti-FAK mAb to detect total cellular FAK and an antiphosphotyrosine mAb to detect active FAK.

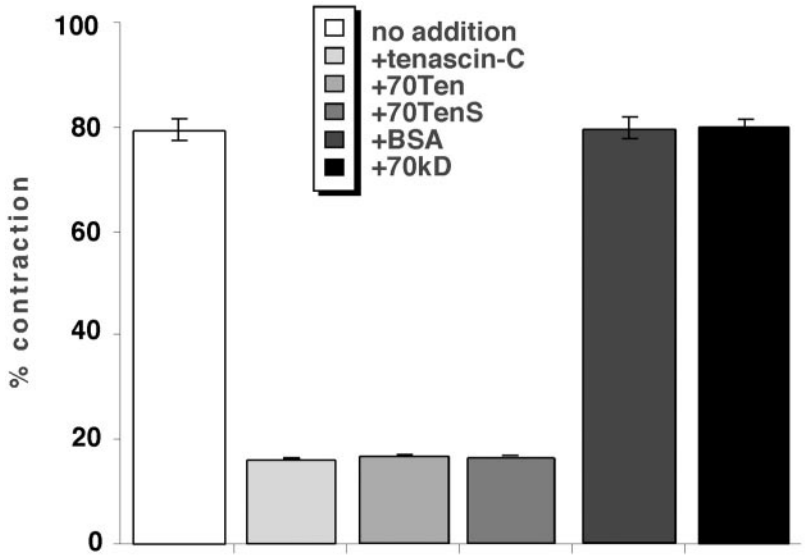

Figure 4. Tenascin-C inhibits cell contractility. NIH 3T3 fibroblasts were mixed together with fibrin-FN and fibrin-FN+tenascin-C, 70Ten, 70TenS, BSA, or 70 kDa, and the matrices were allowed to polymerize in a 48-well dish. The matrix was detached from the dish, and the area of the matrix was measured after 4 h, subtracted from the starting area, and expressed as a percentage of the starting area. The data are expressed as the mean ± SEM of triplicate experiments.

ined for the ability to assemble FN into a matrix. In the absence of 70Ten, cells on a fibrin-FN matrix assembled extensive FN fibrils (Figure 8A). In contrast, cells treated with 70Ten showed a marked decrease in fibril formation (Figure 8B). However, LPA stimulation was able to reverse the effects of 70Ten (Figure 8C). The assembly of FN was also monitored by conversion into DOC-insoluble material, and these results confirmed the immunofluorescence data (Figure 8D). FN matrix assembly has previously been shown to be dependent on Rho GTPase signaling (Zhang *et al.*, 1997). Our data indicate that tenascin-C modulates cell-mediated formation of FN fibrils by suppressing RhoA activity.

FAK Is Required for Matrix Contraction

In addition to effects on Rho signaling, tenascin-C inhibits sustained activation of FAK in fibroblasts adherent to fibrin-FN matrices. To determine whether there is a connection between the suppression of FAK activity by tenascin-C and cell contractility, we examined matrix contraction by cells that do not express FAK. Our results show that expression of FAK is required for maximal contraction of a fibrin-FN matrix. FAK-null fibroblasts showed a 52% reduction in matrix contraction compared with wild-type fibroblasts (Figure 9A). Tenascin-C caused a further decrease in contraction by FAK-null cells (Figure 9B). Stimulation of RhoA activity can only partially compensate for the absence of FAK. Treatment of FAK-null cells with LPA stimulated contraction of a fibrin-FN and of a fibrin-FN+tenascin-C matrix over untreated cells (Figure 9, A and B). However, levels of contraction did not reach those of LPA-treated wild-type cells in either matrix, indicating that activation of RhoA cannot replace the loss of FAK expression.

Reduced matrix contraction by FAK-null cells was not due to an inability to organize the actin cytoskeleton. FAK-null fibroblasts formed robust stress fibers and focal adhesions on fibrin-FN matrices (Figure 10, A and B) and, unlike NIH 3T3 cells, stress fibers also formed in the presence of tenascin-C (Figure 10, C and D). In contrast, wild-type fibroblasts

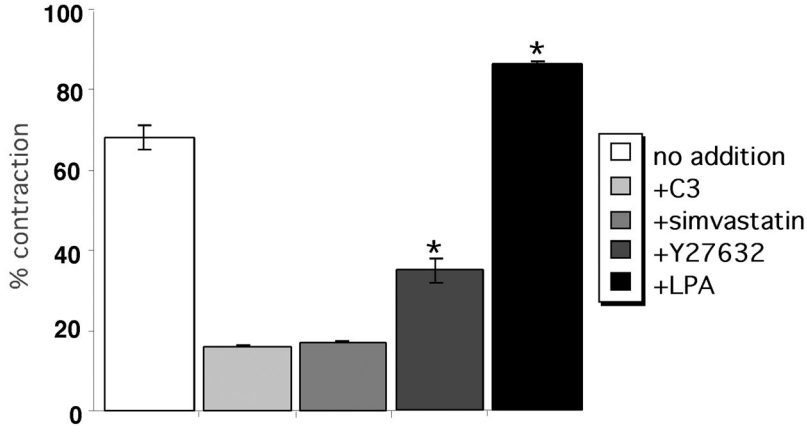

Figure 5. The role of RhoA in the contraction of fibrin-FN matrices. NIH 3T3 fibroblasts treated with LPA, simvastatin, C3 transferase (C3), or Y27632 were mixed together with fibrin-FN and allowed to polymerize in a 48-well dish. Matrix contraction was measured as described in the legend to Figure 4. The data are expressed as the mean ± SEM of duplicate experiments. Contraction of matrices by cells treated with LPA and Y27632 was significantly different from that of untreated cells (*$p < 0.05$, Student's paired *t* test).

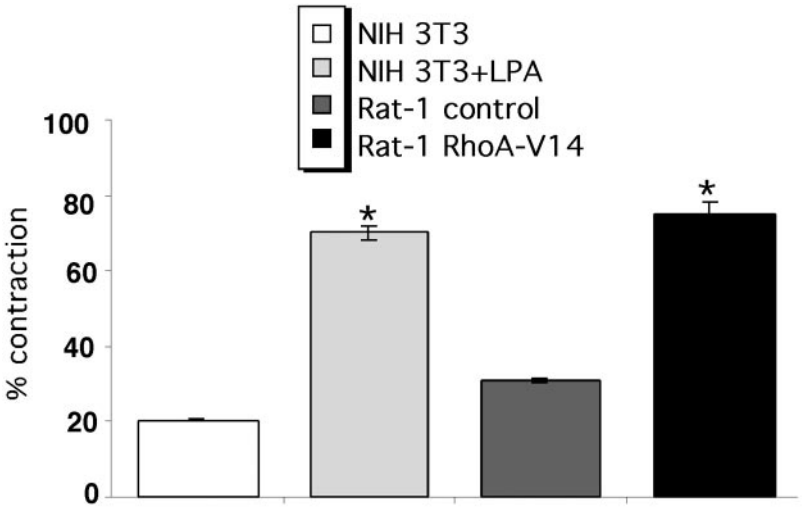

Figure 6. RhoA activation reverses inhibition by 70Ten. NIH 3T3 fibroblasts treated with LPA and Rat-1 fibroblasts expressing constitutively active RhoA or control DNA were incorporated into a fibrin-FN + 70Ten matrix. Contraction data are expressed as the mean ± SEM of duplicate experiments. Compared with untreated cells, the addition of LPA or expression of active RhoA significantly increased contraction of matrices (*$p < 0.05$; Student's paired *t* test).

derived from mouse embryos expressing FAK behaved identically to NIH 3T3 fibroblasts. They were well spread with actin stress fibers and focal adhesions on a fibrin-FN matrix and were rounded with no stress fibers or focal adhesions on a fibrin-FN matrix containing tenascin-C.

Inclusion of tenascin-C did impact spread cell areas, which were significantly less than without tenascin-C. Areas of cells plated on fibrin-FN and fibrin-FN + 70Ten matrices both in the presence and absence of LPA were measured. Both wild-type and FAK-null cells treated with LPA were smaller than untreated cells and yet when included within matrices showed increased levels of contraction of both types of matrix (Table 1). Taken together, these results indicate that activation of both FAK and RhoA is required for maximal matrix contraction.

Sustained FAK Phosphorylation Rescues Contraction of Fibrin-FN + 70Ten Matrices

If FAK is required for maximal matrix contraction, then stimulation of FAK activity may modulate contraction of fibrin-FN + 70Ten matrices. NIH 3T3 cells were treated with phosphatase inhibitors pervanadate or phenylarsine oxide. Both inhibitors have been shown to inhibit FAK dephosphorylation and phenylarsine oxide predominantly prevents the dephosphorylation of FAK and paxillin (Miao *et al.*, 2000; Retta *et al.*, 1996). Immunodetection of phosphorylated and total FAK demonstrated that both phosphatase inhibitors prevent FAK dephosphorylation in response to tenascin-C in a fibrin-FN matrix without affecting total FAK levels (Figure 11A). Neither inhibitor affected FAK phosphorylation in cells adherent to a fibrin-FN matrix, suggesting that FAK is already maximally activated by this substrate.

Cells treated with either phosphatase inhibitor contracted a fibrin-FN + 70Ten matrix to an extent equivalent to LPA-treated cells (Figure 11B). Therefore, tenascin-C inhibition of matrix contraction can be partially overcome by downstream activation of FAK. To achieve complete contraction of fibrin-FN + 70Ten matrix required treatment with both a phosphatase inhibitor and LPA (Figure 11B). These results further support the idea that both FAK and RhoA activities are required for maximal matrix contraction.

DISCUSSION

Fibroblasts participate in multiple stages of tissue repair including wound contraction. In this article, we have demonstrated that tenascin-C downregulates matrix contraction. This reduction correlates with transient FAK activation, the absence of focal adhesions, and cortical actin filament distribution. Partial reversion of tenascin's suppressive effects can be obtained by activation of either FAK or RhoA. Maximal matrix contraction, however, requires both FAK and RhoA activities. Thus, the combined effects of these two

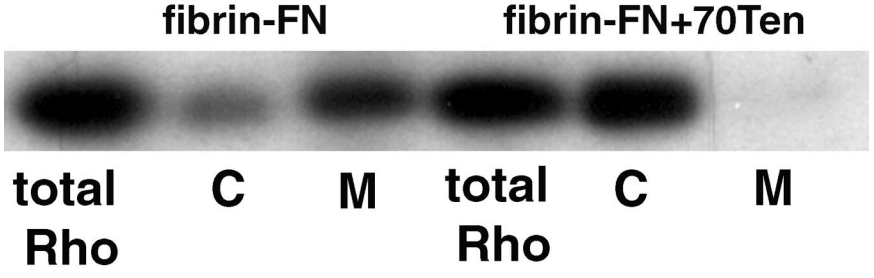

Figure 7. Tenascin-C inhibits RhoA localization to the cell membrane. NIH 3T3 fibroblasts were allowed to adhere to fibrin-FN+/−70Ten matrices for 1 h before lysis and ultracentrifugation to separate cytoplasmic (C) and membrane (M) fractions. Proteins were separated by SDS-PAGE, and immunoblots were probed with an anti-Rho mAb.

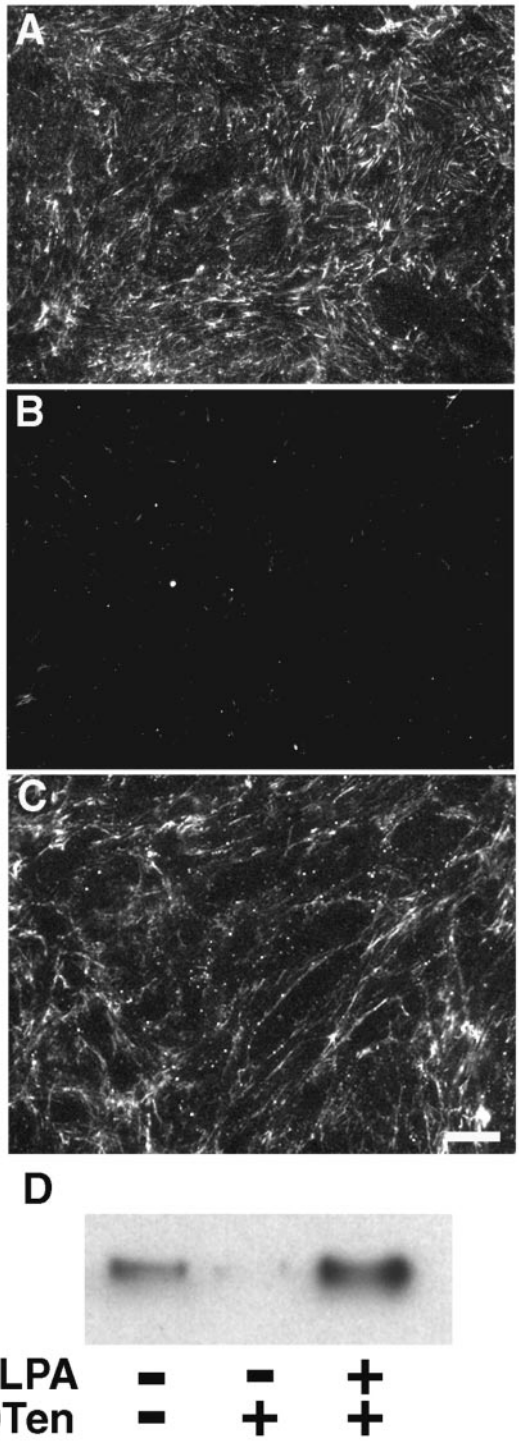

Figure 8. Tenascin-C inhibits matrix assembly. Untreated NIH 3T3 fibroblasts (A) or fibroblasts treated with 70Ten (B) or both 70Ten and LPA (C) were allowed to adhere to fibrin-FN matrices in the absence of serum and in the presence of exogenous human pFN for 2 h, before immunofluorescence staining with an anti-human FN antibody 7.1 (Bar, 50 μm). In parallel experiments, matrix assembly was assessed by isolation of DOC-insoluble material. Proteins were separated by SDS-PAGE, and immunoblots were probed with antihuman FN antibody 7.1 (D).

signaling molecules appear to be critical components of the intracellular machinery that regulates matrix contraction.

The disruption of focal adhesions and modulation of downstream signaling pathways by tenascin-C has many implications for the cell. Of particular interest at sites of tissue repair is the effect on cell contractility. The ability of cells to contract matrices is essential in bringing the margins of a wound together (Clark, 1996). Changes in matrix content have previously been shown to control cell contractility. For example, polymerized FN included in collagen gels stimulates cell spreading and contractility via a RhoA-dependent mechanism, but nonpolymerized FN does not (Hocking *et al.*, 2000). Strong interactions between integrins and FN are also required for maximal fibrin-FN matrix contraction by fibroblasts (Corbett *et al.*, 1997). Focal adhesions serve to connect the actin cytoskeleton to the matrix, and this physical link enables cells to exert force on the surrounding matrix (Burridge and Chrzanowska-Wodnicka, 1996). It follows that cells unable to assemble focal adhesions and stress fibers would be unable to contract surrounding matrices as we have observed with cells in fibrin-FN+tenascin-C matrix.

Activation of the GTPase RhoA induces stress fiber formation via a ROCK-dependent pathway (Amano *et al.*, 1997). We observed that RhoA signaling through ROCK is required for contraction of fibrin-FN matrices. The inhibitory effects of tenascin-C can be reversed by stimulation of RhoA activity with LPA or overexpression of activated RhoA. Tenascin-C also disrupts intracellular localization of RhoA, which is not recruited to the cell membrane under these matrix conditions. The capacity of Rho to cycle on and off membranes is thought to be integral to its biological activity. Vascular smooth muscle proliferation is inhibited by the prevention of the membrane localization of Rho (Laufs *et al.*, 1999), and LPA-induced cytoskeletal contraction of neuronal cells requires translocation of Rho from the cytosol to the plasma membrane (Kranenburg *et al.*, 1997). It appears that RhoA function and targeting to the plasma membrane are also necessary for matrix contraction. Tenascin-C has no apparent effect on the activation of other members of the Rho family GTPases in this assay. Rac and Cdc42 showed similar levels of activity in cells on fibrin-FN matrices both in the presence and absence of tenascin-C (unpublished observations).

Our data show that contraction of fibrin-FN matrices involves the activation of both FAK and RhoA. FAK plays an important role in the remodeling of focal adhesions and control of cytoskeletal tension (Crowley and Horwitz, 1995; Parsons *et al.*, 2000). FAK expression is also required for smooth muscle cell contractility (Tang and Gunst, 2001). FAK-deficient fibroblasts showed decreased motility and enhanced focal adhesion formation on planar FN substrates, implicating FAK in the regulation of focal adhesion turnover (Ilic *et al.*, 1995). We have shown that, in contrast with wild-type cells, FAK-null cells form robust stress fibers and focal adhesions on fibrin-FN+tenascin-C–based substrates. Despite this level of cytoskeletal organization, FAK-null cells showed a significant reduction in contraction, suggesting that the presence of stress fibers is insufficient to drive matrix contraction. Treatment of wild-type fibroblasts with phosphatase inhibitors prevents the dephosphorylation of FAK that occurs in the presence of tenascin-C and allows

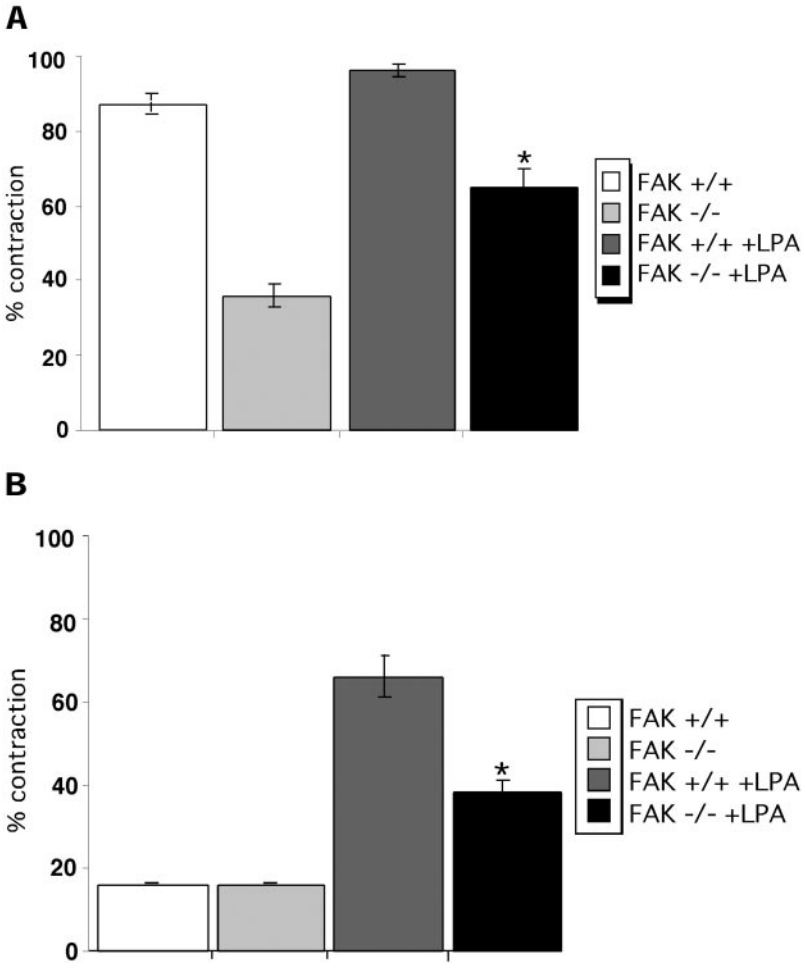

Figure 9. FAK is required for matrix contraction. Untreated fibroblasts derived from wild-type (+/+) or FAK-deficient (−/−) mouse embryos or fibroblasts treated with LPA (+LPA) were mixed together with fibrin-FN (A) or fibrin-FN + 70Ten (B) and allowed to polymerize in a 48-well dish. Matrix contraction data are expressed as the mean ± SEM of duplicate experiments. The addition of LPA significantly stimulated contraction of FAK-deficient cells, compared with untreated cells (*$p < 0.05$; Student's paired *t* test).

these cells to partially contract matrices. Maximal levels of matrix contraction were seen in cells treated with both LPA and phosphatase inhibitors. Because this activation is additive, it appears that FAK and RhoA are acting through different pathways. It seems likely that there may be cross-talk between these two pathways, because the activities of FAK and RhoA appear to be closely linked. FAK regulates focal adhesion turnover via a RhoA-dependent pathway (Ren *et al.*, 2000), and manipulation of the activity of RhoA can influence the activation of FAK (Sinnett-Smith *et al.*, 2001). Our results indicate that both FAK activity in focal adhesions and RhoA GTPase-mediated reorganization of the actin cytoskeleton are required for cells to contract the surrounding matrix. Tenascin-C suppresses the activity of both these signaling molecules, resulting in a complete inhibition of matrix contraction.

Fibroblasts on a fibrin-FN matrix assemble actin stress fibers and focal adhesions. Inclusion of tenascin-C promotes a distinct morphology with cortical actin filaments and actin-filled filopodia. In these cells, actin filaments show significant colocalization with the cytoskeletal protein cortactin. Cortactin as a c-Src substrate is found primarily at sites of dynamic actin assembly and acts to regulate the formation and stabilization of a filamentous actin network (Weaver *et al.*, 2001). Cortactin plays a role in cell migration by mediating localized cross-linking of actin at the leading edge of migrating cells (Bowden *et al.*, 1999; Bourguignon *et al.*, 2001), and overexpression of cortactin in NIH 3T3 fibroblasts increases cell motility in vitro (Patel *et al.*, 1998). Tenascin-C has also been shown to stimulate cell migration (Chung *et al.*, 1996). Increased migration may occur through tenascin-C–mediated reduction in FAK phosphorylation and focal adhesion formation as shown in this report and by suppression of RhoA activation as shown previously (Wenk *et al.*, 2000). The loss of a stationary cell phenotype and intracellular effects that correlate with cell motility in cells on fibrin-FN+tenascin-C matrix may reflect the ability of tenascin-C to promote cell migration at sites of tissue repair.

Tenascin-C expression is tightly regulated in adult tissues. It appears ~2 d after wounding at sites of tissue injury and shows a large increase in expression before wound contraction (Betz *et al.*, 1993; Forsberg *et al.*, 1996). Levels of tenascin-C are highest adjacent to the wound bed (Mackie *et al.*, 1988). This is a region of highly controlled cell movement, because macrophages, fibroblasts, and endothelial cells migrate into the wound bed and epithelial cells migrate across the defect in order to reepi-

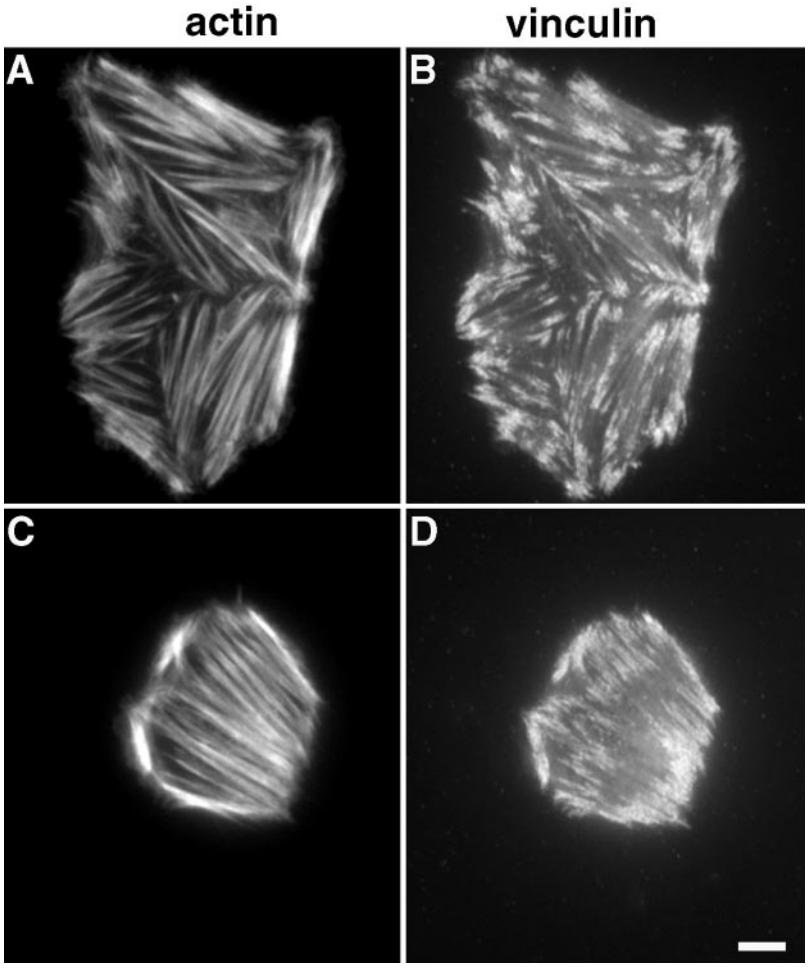

Figure 10. FAK-deficient fibroblasts form stress fibers and focal adhesions. Fibroblasts derived from FAK-deficient mouse embryos were allowed to interact with fibrin-FN (A and B) or fibrin-FN + 70Ten (C and D) matrices for 1 h before staining for actin (A and C) and for vinculin (B and D). Bar, 10 μm.

thelialize the wound (Clark, 1996). This localization of tenascin-C implicates it in the coordination of cell motility and matrix contraction during the healing process. Its presence and clearance may represent an important temporal or spatial switch to prevent and then induce wound contraction. This may help to explain how the differences in the timing of expression of tenascin-C in fetal and adult wounds gives rise to differences in the extent of scar formation. Early expression of tenascin-C in the fetus correlates with optimal contraction and minimal scarring, but delayed expression in adults correlates with delayed contraction and increased scarring (Whitby *et al.*, 1991).

Table 1. Comparison of cell size and matrix contraction

	Fibrin-FN		Fibrin-FN+70Ten	
	Cell area (μm^2)	Matrix contraction (%)	Cell area (μm^2)	Matrix contraction (%)
FAK +/+	812 ± 31	88 ± 2	314 ± 15	16 ± 0
FAK +/+ +LPA	346 ± 17	96 ± 3	174 ± 6	66 ± 0
FAK −/−	226 ± 12	36 ± 2	158 ± 10	16 ± 5
FAK −/− +LPA	126 ± 10	65 ± 5	162 ± 11	38 ± 3

Untreated fibroblasts derived from wild type (+/+) or FAK-deficient (−/−) mouse embryos or fibroblasts treated with LPA (+LPA) were mixed together with fibrin-FN or fibrin-FN+70Ten and allowed to polymerize in a 48-well dish. Matrix contraction data are expressed as the mean ± SEM of duplicate experiments. In parallel experiments, cells were plated on fibrin-FN+/− 70Ten matrices for 1 h, after which time areas for 50–75 cells from random fields were measured using IPlab Software and expressed in μm^2 ± SD.

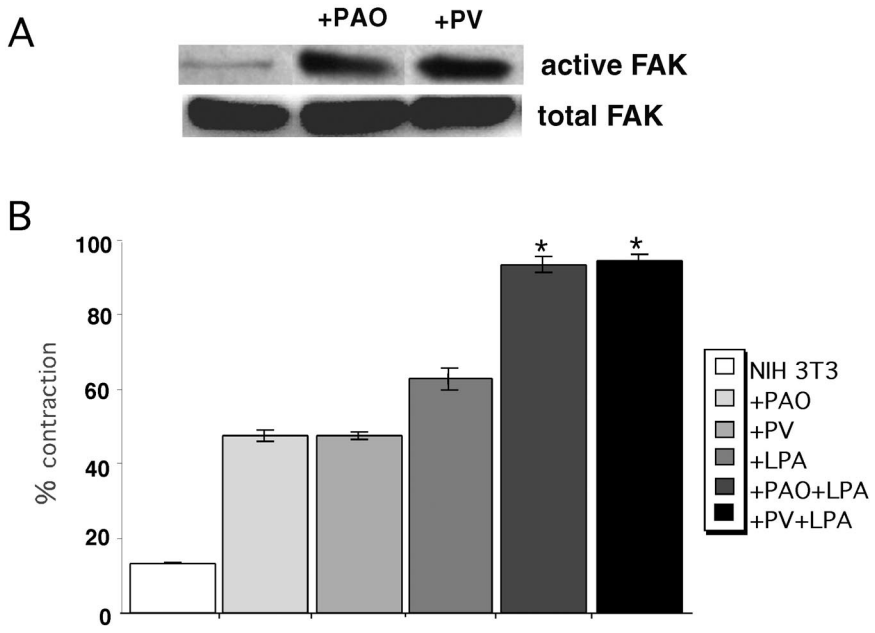

Figure 11. Sustained FAK phosphorylation rescues contraction of fibrin-FN + 70Ten matrices. (A) NIH 3T3 fibroblasts were treated with phenylarsine oxide or pervanadate and then allowed to adhere to fibrin-FN + 70Ten matrices for 60 min before lysis and immunoprecipitation with anti-FAK antibodies. Proteins were separated by SDS-PAGE, and immunoblots were probed with an anti-FAK mAb to detect total cellular FAK and an antiphosphotyrosine mAb to detect active FAK. (B) NIH 3T3 fibroblasts treated with phenylarsine oxide (PAO), pervanadate (PV), LPA, PAO+LPA or PV+LPA were mixed together with fibrin-FN + 70Ten and allowed to polymerize in a 48-well dish. Matrix contraction was measured as described in the legend to Figure 4. The data are expressed as the mean ± SEM of duplicate experiments. The addition of both LPA and PAO or PV significantly stimulated contraction of matrices, compared with cells treated with LPA alone (*$p < 0.05$; Student's paired *t* test).

As part of the process of laying down new tissue, fibroblasts assemble an FN matrix that acts as a framework for wound repair. During wound healing, the FN matrix influences directed cell migration by establishing chemotactic gradients in and around the wound space (Clark *et al.*, 1988). Similarly angiogenesis relies on appropriate matrix-derived cues, and the extent of postwound scarring is determined by the amount of matrix deposited (Welch *et al.*, 1990; Madri and Marx, 1992). It has been shown that Rho-mediated contractility induces matrix assembly by exposing a cryptic site in FN (Zhong *et al.*, 1998). We have found that tenascin-C causes a dramatic reduction in fibroblast assembly of a FN matrix by regulating the activity of RhoA.

Taken together, our results suggest a new function for tenascin-C, to limit the boundaries of the wound bed. Within these boundaries toward the center of the wound, where tenascin-C is absent, the cells primarily contact a fibrin-FN matrix. The fibrin-FN matrix promotes cell adhesion and formation of stress fibers and focal adhesions. These firm attachments allow the cells to exert mechanical force, thus contracting the matrix. Upregulation of tenascin-C at the edges of the injured tissue induces a motile cell phenotype, with loose connections to the matrix. The function of tenascin-C at this site would be to inhibit matrix contraction and prevent extensive deposition of newly synthesized FN matrix. Clearance of tenascin-C as healing progresses would then allow the development of stable cell–matrix interactions and the deposition of new FN matrix. Tenascin-C programs intracellular pathways through modulation of RhoA and FAK activation, leading to downstream effects on the actin cytoskeleton. In this way, tenascin-C plays an important role in regulating key events during tissue repair and regeneration.

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