Cell Contact–dependent Regulation of Epithelial–Myofibroblast Transition via the Rho-Rho Kinase-Phospho-Myosin Pathway

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Epithelial-mesenchymal-myofibroblast transition (EMT), a key feature in organ fibrosis, is regulated by the state of intercellular contacts. Our recent studies have shown that an initial injury of cell–cell junctions is a prerequisite for transforming growth factor- β 1 (TGF- β 1)-induced transdifferentiation of kidney tubular cells into α -smooth muscle actin (SMA)-expressing myofibroblasts. Here we analyzed the underlying contact-dependent mechanisms. Ca^{2+} removal-induced disruption of intercellular junctions provoked Rho/Rho kinase (ROK)-mediated myosin light chain (MLC) phosphorylation and Rho/ROK-dependent SMA promoter activation. Importantly, myosin-based contractility itself played a causal role, because the myosin ATPase inhibitor blebbistatin or a nonphosphorylatable, dominant negative MLC (DN-MLC) abolished the contact disruption-triggered SMA promoter activation, eliminated the synergy between contact injury and TGF- β 1, and suppressed SMA expression. To explore the responsible mechanisms, we investigated the localization of the main SMA-inducing transcription factors, serum response factor (SRF), and its coactivator myocardin-related transcription factor (MRTF). Contact injury enhanced nuclear accumulation of SRF and MRTF. These processes were inhibited by DN-Rho or DN-MLC. TGF- β 1 strongly facilitated nuclear accumulation of MRTF in cells with reduced contacts but not in intact epithelia. DN-myocardin abrogated the Ca^{2+} -removal- $\pm$ TGF- β 1-induced promoter activation. These studies define a new mechanism whereby cell contacts regulate epithelial-myofibroblast transition via Rho-ROK-phospho-MLC-dependent nuclear accumulation of MRTF.

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a key process in tissue development, carcinogenesis, and organ fibrosis (Lee *et al.*, 2006). Recently EMT has emerged as a central mechanism underlying tubulointerstitial fibrosis (TIF), a progressive pathology common to a variety of chronic kidney diseases (Strutz *et al.*, 1995; Liu, 2004). In a transgenic mouse model of TIF, nearly 40% of fibroblasts have been shown to originate from the tubular epithelium that underwent EMT (Iwano *et al.*, 2002). During this process tubular cells lose their polygonal shape and epithelial markers (e.g., E-cadherin), acquire fibroblast-specific proteins (e.g., FSP1), increasingly synthesize extracellular matrix (e.g., fibronectin), and ultimately differentiate into α -smooth muscle actin (SMA)-positive myofibroblasts (for a review see Kalluri and Neilson, 2003). Myofibroblasts represent a highly contractile

cell type that is thought to be critical for wound contraction, tissue repair, and the pathogenesis of fibrocontractive diseases (Gabbiani, 2003; Chaponnier and Gabbiani, 2004; Desmouliere *et al.*, 2005).

Several laboratories including our own have established tubular cell models to study EMT and the development of myofibroblasts (Fan *et al.*, 1999; Yang and Liu, 2001; Masszi *et al.*, 2003). Both in vivo and in vitro, transforming growth factor- β 1 (TGF- β 1) is the main inducer of EMT and fibrogenesis (Bottinger and Bitzer, 2002). However, our previous studies revealed that in intact, confluent monolayers of tubular (LLC-PK1) cells, TGF- β 1 alone is insufficient to induce SMA synthesis and thus myofibroblast formation. The additional prerequisite is a partial loss or injury of intercellular contacts, which can be modeled by subconfluence, mechanical wounding or disassembly of adherens junctions (AJ) via Ca^{2+} removal (Masszi *et al.*, 2004). These studies have defined a two-hit (TGF- β 1 and contact injury) model, in which intercellular junctions are not only targets but also active regulators of EMT. Indeed TGF- β 1 and contact disassembly exert strong synergy in the stimulation of the SMA promoter.

While searching for mechanisms responsible for the cell contact–dependent regulation of SMA expression, we have previously found that β -catenin contributes to this phenom-

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enon. β -catenin, when liberated from the AJs upon contact injury and rescued from proteolysis in a TGF- β 1-dependent manner, exerts a potentiating effect on the activation of the SMA promoter. However, the SMA promoter does not harbor a β -catenin-responsive *cis*-element, and overexpression of β -catenin alone does not activate SMA expression, indicating that the effect is indirect and other contact-dependent factors must also be involved. These considerations prompted us to investigate the possible relationship between contact injury and the main direct regulators of the SMA promoter.

In muscle cells and fibroblasts, the expression of smooth muscle-specific genes, including SMA, is primarily controlled by serum response factor (SRF; Hill *et al.*, 1995; Mack *et al.*, 2000) and its recently discovered coactivators, myocardin and the myocardin-related transcription factors (MRTFs) also called MAL or MKL (Wang *et al.*, 2001, 2002; Cen *et al.*, 2003; Du *et al.*, 2003; Selvaraj and Prywes, 2003). Rho GTPase-mediated actin cytoskeleton reorganization has been long recognized as a key activator of SRF (Sotiropoulos *et al.*, 1999; Mack *et al.*, 2001), but until recently the underlying mechanisms remained undefined. Novel studies suggest that SRF is activated by the Rho-dependent nuclear translocation of MRTF (Miralles *et al.*, 2003; Du *et al.*, 2004). According to the current view, in quiescent cells MRTF is associated with monomeric (G) actin and this complex cannot enter the nucleus. Stimulus-induced Rho activation causes enhanced incorporation of G-actin into actin filaments, which then leads to dissociation of MRTF followed by its nuclear translocation (Sotiropoulos *et al.*, 1999; Miralles *et al.*, 2003). Two Rho effector pathways have been implicated in the mediation of this effect: increased actin polymerization via formin proteins (Copeland and Treisman, 2002) and reduced F-actin severing by the LIM kinase-cofilin pathway (Geneste *et al.*, 2002). Rho might also regulate the localization of SRF; however, this aspect is controversial and the underlying mechanisms are not known (Camoretti-Mercado *et al.*, 2000; Liu *et al.*, 2003a; Cen *et al.*, 2004).

Importantly, contact injury also leads to characteristic changes in the cytoskeleton. Previous work by us and others has shown that disassembly of epithelial junctions leads to robust myosin light chain (MLC) phosphorylation (Frixione *et al.*, 2001; Ivanov *et al.*, 2004; Di Ciano-Oliveira *et al.*, 2005), a process mediated by the downstream Rho effector, Rho kinase (ROK) (Szasz *et al.*, 2005). Epithelial wounding-induced MLC phosphorylation and acto-myosin ring formation is believed to be critical for wound closure (Darenfed and Mandato, 2005). This scenario then raises a number of intriguing questions about the potential connection between cell contacts and the regulation of SMA expression. Specifically we sought to determine whether contact disassembly impacts on the localization of MRTF and/or SRF in epithelial cells, and whether such an effect might be mediated by the Rho-ROK pathway. We also asked whether myosin phosphorylation *per se* is required for the contact-dependent regulation of the SMA promoter. Our results indicate that contact injury-induced Rho-ROK-mediated MLC phosphorylation regulates MRTF distribution, which in turn plays a central role in epithelial-myofibroblast transformation.

MATERIALS AND METHODS

Antibodies and Reagents

Anti-SRF, anti-Myc (9E-10), and fluorescein isothiocyanate (FITC)-conjugated anti-Myc were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- α -SMA, anti- β -actin, and anti-FLAG antibodies were purchased from Sigma (St. Louis, MO), anti-monophospho-MLC from Cell Signaling Technology (Danvers,

MA), and anti-histones from Chemicon (Temecula, CA). The polyclonal anti- α -BSAC antibody raised against the mouse MKL1 protein was described previously (Sasazuki *et al.*, 2002). FITC- and Cy3-labeled, as well as peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). DAPI used for nuclear staining was purchased from Invitrogen (Burlington, ON, Canada). Y-27632 and blebbistatin was from Calbiochem (La Jolla, CA), rhodamine-labeled phalloidin from Cytoskeleton (Denver, CO), and human recombinant TGF- β 1 from Sigma.

Cell Culture and Treatments

LLC-PK1 (CL4) proximal tubular cells were cultured in DMEM (Invitrogen) and Chinese hamster ovary (CHO) cells in α -minimal essential medium (α -MEM, Invitrogen), supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin at 37°C under humidified atmosphere of air/CO₂ (19:1). Cells were grown on 6- or 12-well plates, on glass coverslips, or on 10-cm dishes to either 100% confluence or subconfluence as indicated in the legend of the corresponding figures, and then subjected to various treatments. For acute Ca²⁺ removal, cells were preincubated in an isotonic NaCl-based medium (140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 20 mM HEPES, pH 7.4) for 10 min, and then the medium was replaced with the same basic solution lacking CaCl₂ and supplemented with 1 mM EGTA. For chronic Ca²⁺ deprivation, the cells were washed four times with phosphate-buffered saline (PBS, Invitrogen), and once with serum- and Ca²⁺-free DMEM followed by incubation in the latter solution. Control samples were incubated with serum-free DMEM containing Ca²⁺. Where applied, TGF- β 1 (10 ng/ml or vehicle for controls) was added to the cells for times specified at the individual experiments. For inhibitor studies, cells were preincubated with 10 μ M Y-27632 or 50–100 μ M blebbistatin for various times as described in the figure legends. Wounding of confluent monolayers grown on glass coverslips was achieved by scraping a 1–3-mm gap using a rubber policeman. Cells were fixed 6 h after wounding.

Plasmids

The PA3-Luc vector containing a 765-bp fragment of the rat SMA promoter (pSMA-Luc) was a kind gift from Dr. R. A. Nemenoff (Department of Medicine, University of Colorado), and was used as in our previous studies (Masszi *et al.*, 2003). In certain experiments we used pGL3-SMA-Luc plasmid (provided by Dr. S. H. Phan, University of Michigan Medical School, Ann Arbor; Hu *et al.*, 2003), which harbors the same promoter region inserted into pGL3 luciferase vector. As internal control for transfection efficiency, thymidine kinase-driven Renilla luciferase vector (pRL-TK, Promega, Madison, WI) was used. Plasmids (pcDNA3.1) encoding for the C-terminally His- and Myc-tagged wild-type (WT) myosin regulatory light chain-2 (WT-MLC) and its dominant negative version in which T18 and S19 were replaced with alanine (DN-MLC), were kind gifts from Dr. H. Hosoya (Department of Biological Sciences, Hiroshima University; Iwasaki *et al.*, 2001; Di Ciano-Oliveira *et al.*, 2005). FLAG-tagged MRTF-A, MRTF-B, and the dominant negative truncation mutant (Δ C585) of myocardin were kindly provided by Dr. E. N. Olson (Department of Molecular Biology, University of Texas), and were described previously (Wang *et al.*, 2001). Vectors encoding for Myc-tagged constitutive active RhoA (Q63L, CA-Rho), dominant negative RhoA (T19N, DN-Rho), and GFP-p190RhoGAP were described and used in our previous studies (Masszi *et al.*, 2003). The SBE₄-Luc reporter plasmid, which contains four tandem repeats of the SMAD-binding element, was a kind gift of Dr. A. B. Roberts (National Institutes of Health, Bethesda; Felici *et al.*, 2003).

Transient Transfection and Luciferase Promoter Activity Assays

If not otherwise stated, cells were grown on six-well plates and transfected at a 100% confluence using 2.5 μ l FuGene6 (Roche, Laval, QC, Canada) reagent/1 μ g DNA. For promoter activity measurements, cells were cotransfected with 0.5 μ g pSMA-Luc (or pGL3-SMA-Luc), 0.05 μ g pRL-TK, and 2 μ g of either empty vector (pcDNA3.1) or the specific construct to be tested. After a 24-h incubation period, cells were washed and placed in a serum-free medium, either containing or lacking Ca²⁺. TGF- β 1 (10 ng/ml) or its vehicle was added to the cells after 4 h, and the incubation was continued for an additional 16 h. Cells were then lysed in 500 μ l passive lysis buffer (Promega), and the samples were subjected to a cycle of freezing/thawing, and then clarified by centrifugation (12,000 rpm, 5 min at 4°C). Firefly and Renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay Kit (Promega) using a Berthold Lumat LB 9507 luminometer (Bad Wildbad, Germany) according to the manufacturer's instructions. Results were normalized by dividing the Firefly luciferase activity with the Renilla luciferase activity of the same sample. For each condition duplicate or triplicate measurements were performed, and experiments were repeated at least three times. For immunofluorescence analysis typically 1–2 μ g plasmid DNA was transfected *per* coverslip.

Rho Activity Assay

Rho activation was assessed by an affinity pulldown assay as in our previous studies (Di Ciano-Oliveira *et al.*, 2003). Briefly, after the indicated treatment,

cells grown in 10-cm dishes were lysed in 800 μ l of ice-cold Rho lysis buffer (100 mM NaCl, 50 mM Tris-Base, pH 7.6, 20 mM NaF, 10 mM MgCl₂, and 1% Triton X-100) supplemented with 0.5% deoxycholic acid, 0.1% SDS, 20 μ l/ml protease inhibitor cocktail, 1 mM Na₂VO₄, and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 12,000 rpm for 1 min at 4°C. Glutathione-Sepharose beads (10–15 μ g/sample) covered with GST-Rho-binding domain (RBD) fusion protein were then added to the supernatants and incubated at 4°C for 45 min. The GST-RBD beads were washed three times with Rho lysis buffer, and the captured proteins were diluted with 25 μ l of Laemmli buffer, and subjected to electrophoresis on 15% SDS-polyacrylamide gels followed by Western blotting using an anti-Rho antibody.

Western Blotting

After treatments cells were scraped into Triton lysis buffer (30 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EGTA, 20 mM NaF, 1% Triton X-100, 1 mM Na₂VO₄, 1 mM phenylmethylsulfonyl fluoride, 20 μ l/ml protease inhibitor cocktail (BD Biosciences, Mississauga, Ontario, Canada), the protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA), and the samples were mixed in a 1:1 ratio with 2 $\times$ Laemmli buffer and boiled for 5 min. For pMLC blots, the cells were lysed in ice-cold acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol, followed by centrifugation for 10 min at 12,500 rpm at 4°C. The resulting pellet was washed with pure acetone, allowed to air dry, and dissolved in 60 μ l of Laemmli sample buffer. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Blots were blocked with Tris-buffered saline (TBS), containing 0.1% Tween 20 and 5% albumin for an hour. Membranes were incubated for an additional hour (or overnight for pMLC) with the primary antibody (in TBS-Tween plus 0.5% albumin), extensively washed, and incubated with the corresponding peroxidase-conjugated secondary antibody. After final washes immunoreactive bands were visualized with the enhanced chemiluminescence reaction.

Nuclear Extraction

Nuclear extracts were prepared from confluent layers of LLC-PK1 cells grown on 10-cm dishes, using NE-PER Nuclear Extraction Kit from Pierce Biotechnology (Rockford, IL) according to the manufacturer's recommendation. The nuclear extracts were collected, their protein concentration was determined, and samples of equal protein content were analyzed by Western blotting. Anti-histone antibody was used to check for equal loading of nuclear proteins.

Quantification of Cellular F-Actin Content

F-actin was measured by the rhodamine phalloidin extraction method, essentially as described (Pedersen and Hoffmann, 2002). This technique allows reliable determination of a few percent change in F-actin. Briefly, confluent LLC-PK1 cells grown on six-well plates were serum-deprived, treated with various inhibitors, and then fixed in Tris-buffered saline containing 2% paraformaldehyde. After repeated washes the cells were permeabilized with 0.1% saponin buffer and incubated in 250 μ l of a 0.33 μ M rhodamine phalloidin solution for an hour. The cells were then thoroughly washed, and the bound phalloidin was extracted by incubating the cells for 30 min with 2 ml of pure methanol per well. Rhodamine phalloidin fluorescence in the samples was determined by a Photon Technology cuvette fluorimeter (Lawrenceville, NJ) using 537 nm for excitation and 576 nm for emission.

Immunofluorescence Microscopy

Cells grown on coverslips were fixed with 4% paraformaldehyde for 30 min, washed with PBS, and incubated with 100 mmol/l glycine in PBS for 10 min. Cells were then permeabilized in PBS containing 0.1% Triton X-100, blocked for an hour with 3% albumin, and incubated with the primary antibody or antibodies (in case of costaining) for 1 h. After extensive washes, fluorescently labeled secondary antibodies were added for another hour. The coverslips were washed and then mounted on slides using Fluorescence Mounting Medium (DAKO, Carpinteria, CA). When directly labeled, FITC-conjugated mouse anti-Myc antibody was used together with another mouse primary antibody, and the cells were initially processed for staining with the unlabeled primary and corresponding secondary antibodies, blocked again with mouse serum (1:100), and then incubated with the directly labeled primary antibody for an hour. Samples were analyzed by an Olympus IX81 microscope (60 $\times$ or 100 $\times$ objectives, Melville, NY) coupled to an Evolution QEi Monochrome camera controlled by the QED InVivo Imaging software (Media Cybernetics, Silver Spring, MD). Images were processed by the ImagePro Plus 3DS 5.1 software (Media Cybernetics). Bars on the microscopic images correspond to 20 μ m.

Quantification of Nuclear/Cytoplasmic Distribution of Proteins

Staining was quantified using the ImagePro Plus software: fluorescence intensities were determined at three random nuclear and cytoplasmic points along a line, or in three equal rectangular areas within the nucleus or the

cytoplasm. An average of three determinations per cell was used, and the nuclear/cytoplasmic ratio was calculated. Ratios measured along lines or within rectangular areas were identical. Nuclei were independently visualized by DAPI staining. MRTF distribution was categorized as cytosolic or nuclear when the nucleus was clearly demarcated either by exclusion or accumulation of the label. Otherwise the distribution was regarded as even (or pancellular). To make these categories exact, distribution data were verified using the nuclear/cytoplasmic ratios as <0.75 (cytosolic), 0.75–1.25 (even), and >1.25 (nuclear). In the vast majority of cells within the nuclear category the ratio was >2.

Statistical Analysis

Data are presented as blots or images from at least three similar experiments or as the means $\pm$ SE for the number of experiments (n) indicated. Statistical significance was determined by Student's *t* test or one-way ANOVA using the GraphPad InStat software (San Diego, CA).

RESULTS

Contact Disassembly Induces Rho/ROK-dependent Myosin Phosphorylation and SMA Promoter Activation

To assess whether the disassembly of intracellular contacts affects Rho signaling in LLC-PK1 cells, we tested the effect of Ca²⁺ removal, a classic maneuver that dismantles Ca²⁺-dependent intercellular junctions. Figure 1A shows that replacement of the normal medium with a Ca²⁺-free solution caused rapid and robust (approx. threefold) Rho activation, as detected by an affinity pulldown assay that precipitates active (GTP-bound) Rho from the cell lysates. Concomitant with this response, the cells exhibited a large increase in their staining for the monophosphorylated myosin light chain (pMLC; Figure 1B, a and b), which occurred predominantly at the cell periphery. This observation together with our earlier finding that Ca²⁺ removal caused sizable rise in peripheral diphospho-MLC staining as well (Di Ciano-Oliveira *et al.*, 2005) indicates that contact disruption enhances contractility via both mono- and diphosphorylation of MLC. Importantly, MLC phosphorylation is a sustained response, because under Ca²⁺-free conditions peripheral pMLC levels remained high in $\approx$ 60% of the cells for days (Figure 1, Bc and C), i.e., through the time course of our transfection and promoter studies (see below). To test whether Rho activity was required for increased monophosphorylation of MLC, 2 d before Ca²⁺ removal, cells were transfected with a Myc epitope-tagged dominant negative (T19N) Rho construct. Double staining for Myc and pMLC revealed that DN-Rho prevented the contact injury-triggered increase in pMLC (Figure 1B, e and e', and C). Moreover, the Rho kinase inhibitor Y-27632 also abolished the enhanced MLC phosphorylation (Figure 1Bd), suggesting that Rho-mediated ROK activation is indispensable for this process.

Next we addressed whether the Rho/ROK pathway might contribute to the Ca²⁺ removal-induced stimulation of the SMA promoter. Cells were transfected with the SMA-Luc reporter plasmid along with empty pcDNA vector or DN-Rho, and subsequently the culture medium was exchanged for serum-free DMEM, either containing or lacking Ca²⁺. Ca²⁺ deprivation caused a 6–10-fold increase in the activity of the SMA promoter (Figure 1D). Importantly, DN Rho, although exerting no significant effect on the basal promoter activity, entirely prevented the Ca²⁺ depletion-induced stimulation (Figure 1D). Similar results were obtained when Y-27632 was used to inhibit ROK: this treatment also abolished the contact-dependent activation of the SMA promoter (Figure 1E). Taken together these data imply that the disruption of intercellular contacts leads to Rho activation and ROK-dependent myosin phosphorylation. Moreover, the Rho/ROK pathway is a key mediator

Figure 1. Contact disassembly induces Rho/Rho kinase-dependent myosin phosphorylation and SMA-promoter activation. (A) Confluent LLC-PK1 cell cultures were serum-starved for 3 h and then preincubated with a Ca^{2+} -containing NaCl-based medium for 10 min. Subsequently the medium was aspirated and either replaced with the same solution (control) or with a Ca^{2+} -free solution containing 1 mM EGTA (no Ca) to rapidly disrupt the intercellular contacts. Five minutes later the cells were lysed, and samples of equal protein content were subjected to the Rho activity assay as described in *Materials and Methods*. Total Rho was determined from the same lysates. One representative blot of three separate experiments is shown. Densitometry (bars) was performed for each experiment, and Rho activation was expressed as fold increase compared with the control. (B) LLC-PK1 cells were grown on coverslips to confluence, and after various treatments were stained with anti-monophospho-MLC antibody: (a) No treatment; (b) cells were exposed to acute Ca^{2+} removal for 5 min using EGTA as in A; (c and d) for chronic Ca^{2+} removal, the normal, serum-free DMEM was replaced with a nominally Ca^{2+} -free DMEM for 24 h. Thirty minutes before Ca^{2+} removal cells were preincubated with vehicle (c) or 10 μM Y-27632 (d), which remained present throughout the whole experiment. To visualize cells, nuclei were stained with DAPI; and (e and e') cells grown to confluence were transfected with Myc-tagged DN-Rho for 24 h, exposed to nominally Ca^{2+} -free conditions for an additional 24 h, and then doubly stained for monophospho-MLC (red) and for the Myc epitope (green). (C) The frequency of peripheral phospho-MLC staining was quantified in control and DN-Rho expressing cells after 24-h incubation in nominally Ca^{2+} -free DMEM. Note that more than 60% of controls cells showed peripheral myosin phosphorylation, whereas this response was negligible in DN-Rho expressing cells. ($n = 3$, in each experiment >60 cells were counted in each cell population). (D) Confluent cells were transfected with pSMA-Luc plus pRL-TK along with either empty vector (pcDNA3.1) or with DN-Rho (see *Materials and Methods*). After 24 h the cells were incubated in serum-free (Cont) or serum- and Ca^{2+} -free DMEM (no Ca) for an additional 24 h, followed by determination of luciferase activity ($n = 3$). (E) The same conditions as in D, except cells were treated for 30 min before Ca^{2+} depletion with vehicle or 10 μM Y-27632 ($n = 3$).

of the contact injury-provoked activation of the SMA promoter.

Myosin Phosphorylation Plays a Critical Role in the Ca^{2+} Removal-triggered Activation of the SMA Promoter

Although the activation of Rho is known to participate in the regulation of SRF-dependent gene expression, the downstream pathways mediating this effect have not been entirely elucidated. Particularly, the potential role of myosin activity or phosphorylation has not been addressed. Given the robust pMLC response upon contact disassembly, we sought to determine whether this event contributes to the activation of the SMA promoter. Initially we applied blebbistatin, a specific inhibitor of myosin ATPase (Straight *et al.*, 2003). Figure 2A shows that pretreatment of the cells with blebbistatin did not affect the basal promoter activity but entirely prevented its activation by Ca^{2+} removal. Next we tested whether the drug also impacts the TGF- β 1-triggered stimulation and its contact-dependent potentiation. In agreement with our previous results, TGF- β 1 added to confluent layers caused only a modest increase in SMA promoter activity (Figure 2A). This effect was significantly reduced but not entirely abolished by blebbistatin. The combined treatment (Ca^{2+} removal + TGF- β 1) led to a robust rise in

promoter activity, and this synergism was fully eliminated by blebbistatin. Although our previous results (Masszi *et al.*, 2004) have implied that contact disruption does not act simply via increasing receptor availability for TGF- β 1, we further substantiated this point by testing the effect of Ca^{2+} removal on another TGF- β -induced effect, the activation of the Smad-binding element (SBE; Felici *et al.*, 2003; Figure 2B). TGF- β 1 exerted a similar stimulation of SBE in the presence or absence of calcium, indicating that altered receptor accessibility does not play a key role in the observed effects, and that the Ca^{2+} -removal-induced potentiation is specific for the SMA promoter.

In our subsequent experiments we chose an alternative and more specific way to interfere with myosin function. We used transfection with a construct encoding for a Myc epitope-tagged, nonphosphorylatable myosin mutant in which the critical target residues T18 and S19 were exchanged with phenylalanine (AA-MLC). This approach offers the advantage over blebbistatin in that it prevents myosin phosphorylation and activation without interfering with basal myosin ATPase activity (Di Ciano-Oliveira *et al.*, 2005). AA-MLC effectively prevented the Ca^{2+} -removal-induced rise in peripheral pMLC (Figure 2C), implying that this mutant acts as a dominant negative (DN-MLC). This conclusion is sup-

Figure 2. Inhibition of myosin ATPase activity or myosin phosphorylation strongly suppresses the contact disruption-induced activation of the SMA promoter and its potentiation by TGF- β 1. (A) Confluent monolayers were transfected with p-SMA-Luc and pRL-TK, and after 24 h were treated with vehicle or 100 μ M blebbistatin for 2.5 h. Subsequently the cells were incubated in serum-free, Ca^{2+} -containing or Ca^{2+} -free DMEM, in the presence or absence of blebbistatin. After 4 h, 10 ng/ml TGF- β 1 was added to the samples where indicated. Sixteen hours later the cells were lysed, and their luciferase activity was determined ($n = 3$). (B) Ca^{2+} removal does not act through increasing receptor availability for TGF- β 1. Cells were transfected with the TGF- β 1-responsive SBE reporter (p-SBE-Luc) and left untreated or challenged with Ca^{2+} removal, TGF- β 1, or the combination of these stimuli as in A. (C) DN-MLC inhibits the Ca^{2+} removal-induced MLC phosphorylation. Cells grown on coverslips in 6-well plates were transfected with Myc-tagged DN-MLC for 24 h, incubated in serum and Ca^{2+} -free DMEM for another 24 h, and then fixed and doubly stained for the Myc epitope (green) and phospho-MLC (red). Note the absence of pMLC staining in the clusters of transfected cells. (D) The effect of DN-MLC on the activation of the SMA promoter. Confluent cells were transfected with empty vector (pcDNA3) or DN-MLC, and after 24 h were subjected to Ca^{2+} removal where indicated. Four hours later, 10 ng/ml TGF- β 1 was added for 20 h to the indicated samples, followed by lysis and determination of luciferase activity. (E) Cells were transfected with pGL3-SMA-Luc, an alternative vector harboring the same 765-base pairs SMA promoter region as PA3-SMA-Luc. Other conditions were identical as in D. (F) Cells were transfected with wild-type (WT) MLC. Other conditions were identical with D.

ported also by our previous observation using other stimuli, including osmotic stress and depolarization. To assess the effect of MLC phosphorylation per se on the activation of the promoter, we cotransfected the cells with DN-MLC along with SMA-Luc and subjected them to various stimuli. DN-MLC nearly abolished the Ca^{2+} deprivation-induced increase in promoter activity, and strongly abrogated the synergistic effect induced by Ca^{2+} removal and TGF- β 1 (Figure 2D). To substantiate these results, we performed two kinds of control experiments. First, to verify that the type of the expression vector was not critical, and that the observed effect was indeed exerted on the promoter, we repeated these experiments using an alternative (pGL3) plasmid harboring the same 765-base pair promoter sequence. DN-MLC effectively inhibited the Ca^{2+} depletion-induced luciferase response in this system as well (Figure 2E). Second, to verify that the mutation is indeed the determining factor for the inhibitory effect, cells were transfected with the Myc-tagged WT (T18, S19) MLC as well (Figure 2F). Overexpression of WT myosin had no effect on the basal promoter activity and did not alter its activation by Ca^{2+} removal. Together these data imply that myosin activity as well as myosin phosphorylation are important contributors to the contact-dependent regulation of the SMA promoter. Together these experi-

ments strongly argued for the participation of myosin activity and activation in the regulation of the SMA promoter.

Because the level of actin polymerization is known to regulate CARG-dependent genes, we considered that the effect of myosin inhibition might be, at least partially, due to an impact on F-actin. Phalloidin staining revealed that in confluent cultures LLC-PK1 cells contained relatively few and fine stress fibers near their ventral surface, and punctate F-actin distribution corresponding to microvilli at their apical surface. Blebbistatin induced substantial stress fiber disassembly in accord with our previous findings (Di Ciano-Oliveira *et al.*, 2005) and a decrease in microvillar F-actin labeling (Figure 3A, top and bottom). These observations suggest that basal myosin activity is necessary for normal F-actin arrangement, but they do not provide quantitative information about any potential change in the size of the F-actin pool. To address this issue, we compared the F-actin levels in control and blebbistatin-treated cells using a phalloidin extraction assay. As shown on Figure 3C, blebbistatin did not alter the total F-actin level, in sharp contrast with the effect of the actin monomer-sequestering drug, Latrunculin B, which was applied as a positive control for the assay. Next we assessed the effect of DN-MLC on the actin skeleton. As opposed to blebbistatin treatment, we were not able

Figure 3. The effect of myosin inhibition on F-actin organization and content. (A) LLC-PK1 cells were either left untreated or exposed to blebbistatin as in Figure 2A, and then fixed and stained with rhodamine phalloidin. F-actin arrangement was visualized near the apical surface (top, microvilli) and the ventral surface (bottom, level of stress fibers). (B) Cells were transfected with Myc-tagged DN-MLC as in Figure 2C, and then doubly stained using an anti-Myc antibody (green) and rhodamine phalloidin (red). No obvious differences were observed in the organization of F-actin between control and DN-MLC-expressing cells. (C) Quantification of the cellular F-actin content. Cells were treated with blebbistatin (blebbs) as in Figure 2A or with 10 μ M latrunculin B (LB) for 2 h (as a positive control), and their total F-actin content was determined with the phalloidin extraction assay as described in *Materials and Methods*. To assess the effect of DN-MLC, transiently transfected cells were exposed to a selection procedure using G-418, until >85% transfection efficiency was achieved. The total F-actin in these cells was then measured as above. (D) Total actin was assessed by Western blotting under the same conditions as in C.

to detect any obvious change in the F-actin arrangement of DN-MLC-expressing (Myc-positive) cells compared with their nontransfected neighbors (Figure 3B). This finding is consistent with the preservation of basal myosin activity in these cells. To assess the impact on F-actin, we had to generate a cell population, in which the majority of cells expressed DN-MLC. Using a selection protocol, we obtained a population with >85% expressors. In these cells there was a slight ($\approx$ 9%) decrease in the total F-actin compared with nonexpressors. Neither blebbistatin nor DN-MLC altered total actin expression (Figure 3D). Taken together, these data show that alteration in myosin activity markedly suppressed the activation promoter without (blebbistatin) or with a small (DN-MLC) change in the total F-actin. Although these experiments do not rule out that myosin might impact on specific actin pools or on stimulus-induced F-actin polymerization,

they suggest that other mechanisms are likely to contribute to the overall effect of myosin inhibition (see *Discussion*).

Cell Contact Disassembly Promotes Nuclear Accumulation of Serum Response Factor

Serum response factor (SRF) is the key *cis*-element driving SMA expression, whose activity could be regulated by nucleocytoplasmic shuttling (Camoretti-Mercado *et al.*, 2000), although both this possibility and the involvement of the Rho pathway in this process remain controversial (Cen *et al.*, 2004). Therefore we asked whether contact disruption affects SRF localization. Figure 4A shows that SRF exhibited nuclear accumulation even in resting, nonstimulated cells. Interestingly, nuclear distribution was more pronounced in subconfluent cultures (not shown), whereas upon reaching confluence, the cytosolic staining increased concomitant

Figure 4. Contact disassembly facilitates the nuclear accumulation of serum response factor (SRF) in a Rho and MLC phosphorylation-dependent manner. (A) Confluent monolayers were serum-starved for 3 h and then bathed in Ca^{2+} -free DMEM for the indicated times. Cells were then fixed and stained for SRF. (B) Nuclear extracts were prepared from Control or Ca^{2+} -deprived (3 h) cells followed by Western blotting for SRF and H3 histones as a nuclear marker. (C) Confluent cells were transfected with constitutive active Myc-tagged Rho (CA-Rho) for 24 h, and then doubly stained for SRF (red) and Myc (green). Note the robust nuclear accumulation of the transfected cells compared with their non-transfected neighbors. (D) Cells were transfected with Myc-tagged dominant negative Rho (DN-Rho) for 24 h followed by incubation in nominally Ca^{2+} -free DMEM for another 24 h. Cells were then fixed and stained for SRF (red) and Myc (green). To facilitate the identification of the same cells on the two corresponding fluorescent images, successfully transfected cells or clusters of cells are circled with dashed lines. Note the substantial reduction in the nuclear SRF staining of DN-Rho-expressing cells. (E) Conditions were as in D, except the cells were transfected with Myc-tagged DN-MLC. (F) The intracellular distribution of SRF was quantified by measuring the nucleo-cytoplasmic ratio of the fluorescence intensity. For each cell determinations were made along lines drawn across the nucleus (see dashed line in E). The ratios were calculated for control (pcDNA3) and DN-MLC-transfected cells, which were incubated either in Ca^{2+} -containing or nominally Ca^{2+} -free medium for a day. In each category at least 60 cells were analyzed. Ca^{2+} removal significantly enhanced the nuclear accumulation of SRF ($p < 10^{-10}$), and this effect was significantly suppressed ($p < 10^{-6}$) by DN-MLC.

with a drop in nuclear labeling. Nonetheless, the latter still remained significantly higher than the extranuclear signal (Figure 4A). Ca^{2+} depletion of the cultures caused a sizable and time-dependent increase in nuclear SRF staining. To verify that this effect was not an optical artifact due to cell contraction-associated cytosolic shrinkage, we performed Western blots on nuclear extracts obtained from control and Ca^{2+} -deprived cultures. Figure 4B shows that an increase in nuclear SRF upon Ca^{2+} removal was detected also by this technique. Next we tested whether a Myc-tagged constitutively active Rho mutant was able to impact on SRF localization. We observed enhanced nuclear accumulation of SRF in active Rho-transfected cells (Figure 4C). Curiously however, this effect was clearly visible only in cells that showed a modest Rho expression (as visualized by Myc staining), whereas it was not apparent in cells with high level (and possibly longer lasting) expression of active Rho. This finding suggests that the increase in nuclear SRF accumulation may be transient, or various Rho-dependent pathways might be involved both in nuclear import and export processes. Expression of DN-Rho substantially mitigated or completely prevented the increase in nuclear SRF (Figure 4D). Indeed in many DN-Rho-expressing, stimulated cells the level of nuclear SRF was lower than in nonstimulated controls (not shown). Finally we tested whether inhibition of myosin phosphorylation might impact on SRF traffic. To this end cells were cotransfected with DN-MLC, and after Ca^{2+}

removal doubly stained for Myc (DN-MLC) and SRF. DN-MLC expression appeared to mitigate the increase in nuclear SRF accumulation (Figure 4E). To quantify the results, the fluorescence intensity of SRF staining was determined in the nucleus and cytosol of individual (control or DN-MLC expressing) cells and the nucleocytoplasmic ratio was calculated under various conditions (Figure 4F). Under resting conditions in control cells, SRF exhibited a ≈ 1.4 -fold nuclear accumulation over the cytosol, which upon Ca^{2+} removal increased to ≈ 2.1 -fold. The expression of DN-MLC did not have a significant effect on the resting SRF distribution, but it reduced the Ca^{2+} deprivation-induced rise by 60%. These data are consistent with some contribution of myosin phosphorylation in the contact-dependent traffic of SRF. However, given the fact that there is a substantial amount of SRF in the nucleus even under resting conditions, and that the effect of DN-MLC was only partial, we continued to examine the contribution of another Rho-dependent process, namely localization of MRTF.

Localization of MRTF Isoforms and the Effect of the Rho-F-Actin Pathway on MRTF Distribution in Tubular Cells

Because the distribution of MRTF, and its regulation has hitherto not been characterized in epithelial cells, initially we sought to compare the localization of MRTF isoforms in LLC-PK1 cells and fibroblast-like CHO cells. To achieve this, and also to overcome the restricted availability of MRTF

antibodies, we transfected cells with constructs encoding FLAG epitope-tagged MRTF-A and MRTF-B, and followed their localization through staining with an anti-FLAG antibody. As expected, in CHO cells both MRTF-A and -B exhibited predominantly cytosolic staining (in 67 and 82% of the cells, respectively), although the rest of the cells showed even (pancellular) staining (nuclear/cytosolic ratio 0.75–1.25) or nuclear accumulation (nuclear/cytosolic ratio >1.25; Figure 5A). In contrast, in LLC-PK1 cells MRTF-A was mostly nuclear (>70%), whereas MRTF-B was mainly cytosolic (>70%; Figure 5A). This finding indicates that there are significant cell type-specific differences in MRTF distribution between fibroblasts and LLC-PK1 epithelial cells. Next we investigated whether the distribution of MRTF-B, which under resting conditions partitioned mostly in the cytosol, was responsive to Rho signaling and cytoskeletal changes in epithelial cells. Coexpression of constitutively active GFP-Rho resulted in a large (≈ 8 -fold) increase in nuclear MRTF-B accumulation (Figure 5, B and D, a and a'). Overall >80% of Rho-transfected cells showed pancellular or fully nuclear distribution, whereas this fraction was only 15% in the control (GFP expressing) cells. To verify that the changes in actin organization are indeed able to redistribute MRTF-B in kidney epithelial cells, we used jasplakinolide, a potent actin-polymerizing agent. Similar to active Rho, this drug also provoked robust nuclear accumulation of MRTF-B (Figure 5Db).

Next we investigated the effect of contact disassembly on MRTF-B distribution. Ca^{2+} removal caused a more than 2.5-fold increase in nuclear localization of MRTF-B. Moreover, inhibition of Rho signaling by the overexpression of Rho-GAP suppressed the contact disruption-promoted nuclear translocation of MRTF-B (Figure 5C).

Next we studied whether forced F-actin polymerization or MRTF overexpression might result in SMA promoter activation and protein expression in LLC-PK1 epithelial cells. Jasplakinolide (in the absence of any other stimulus) was able to provoke a large increase in SMA promoter activity (Figure 6A) and induced SMA protein expression (Figure 6B), indicating that drastic actin polymerization is sufficient to trigger myofibroblast transformation of normal epithelial cells. Consistent with an important role of MRTF in the regulation of SMA expression, transfection of MRTF isoforms led to a robust increase in the SMA promoter activity (Figure 6C), which—in agreement with the localization data—was stronger in case of MRTF-A than MRTF-B. Further, expression of the MRTF constructs was often accompanied with SMA protein expression, as verified by double immunostaining for SMA and the FLAG epitope (Figure 6E). The expression of SMA was robust considering that transient transfection of a few percent of the cells with MRTF-A or B resulted in SMA synthesis that was readily detectable in total cell lysates by Western blotting (Figure 6D). Control or mock-transfected epithelial cells never expressed SMA (Figure 6, D and E).

Taken together these results indicate that although MRTF isoforms may be differentially regulated in fibroblasts and certain epithelial cells, Rho-dependent cytoskeleton remodeling is a key factor in the control of MRTF distribution also in epithelial cells. Furthermore, MRTF is a potent inducer of SMA expression in an epithelial setting as well.

Contact Integrity Regulates the Nucleocytoplasmic Transfer of Endogenous MRTF in a Rho- and MLC Phosphorylation-dependent Manner

To substantiate the relevance of these findings, we followed the localization of endogenous MRTF using a polyclonal

Figure 5. The impact of the Rho-F-actin pathway and cell contact injury on the localization of MRTF isoforms in epithelial cells. (A) CHO cells or LLC-PK1 cells were transfected with either FLAG-tagged MRTF-A or -B and 2 d later stained with an anti-FLAG antibody. FLAG-expressing CHO cells (145 and 167 cells for MRTF-A and -B, respectively) and LLC-PK1 cells (335 and 3606 for MRTF-A and -B, respectively) were counted for nuclear, even or cytosolic distribution. These categories were objectified as described in *Materials and Methods*. Typical examples of distribution are shown on the right panels. (B) Confluent layers of LLC-PK1 cells were cotransfected with FLAG-MRTF-B and GFP or GFP-CA-Rho, and 48 h later analyzed for intracellular distribution of FLAG staining. Note that CA-Rho induced large nuclear accumulation of MRTF-B. (C) Cells were either transfected with FLAG-MRTF-B alone or along with p190 RhoGAP, and after 48 h, cells were serum-deprived for 3 h. Subsequently, the medium was replaced with Ca^{2+} -free DMEM where indicated, and after 2.5 h incubation the cells were fixed and stained with an anti-FLAG antibody. Data are from 3 to 9 separate experiments, and in each category 300–1800 cells were counted. Ca^{2+} removal raised the percentage of cells with fully nuclear distribution from 10.1 ± 1.8 to $26.2 \pm 5.1\%$ ($p < 0.005$, $n = 9$), and this effect was entirely prevented by Rho-GAP. (D) In a and a' typical images showing the entirely nuclear accumulation of MRTF-B (FLAG-staining, red) in a GFP-CA-Rho-expressing cell (green); (b) jasplakinolide (Jas) treatment (0.5 μM for 12 h) induces strong nuclear accumulation of the transfected MRTF-B.

antibody raised against BSAC, the mouse homologue of MKL1/MRTF-A. In resting LLC-PK1 cells endogenous MRTF showed entirely cytosolic distribution with strong nuclear exclusion (Figure 7, Aa and B). Expression of constitutive active Rho redistributed MRTF into the nucleus (Figure 7A, c and c', and B), verifying the Rho responsiveness of the endogenous protein in this epithelial setting. Importantly, Ca^{2+} removal also caused a marked nuclear

Figure 6. Jasplakinolide or the overexpression of MRTF isoforms is sufficient to induce SMA protein synthesis in tubular cells. (A) Cells were transfected with the pSMA-Luc/pRL-TK system for 24 h and either left untreated for a day, or treated with jasplakinolide (Jas, 0.5 μ M) for 24 h or for the last 3 h of this 24-h period. SMA promoter activity is expressed as fold increase compared with the untreated sample. (B) Cells were left untreated or exposed to Jas for 24 h, lysed, and subjected to Western blotting using an anti-SMA antibody. (C) Tubular cells were cotransfected with the pSMA-Luc/pRL-TK and either MRTF-A or -B. Twenty-four hours later SMA promoter activity was determined as in A. (D) Untransfected controls (none) or cells transiently transfected with MRTF-A or -B for 48 h were lysed, and subjected to SDS-PAGE followed by Western blotting using an anti-SMA antibody. Control cells do not express SMA, whereas both MRTF-A and -B were able to induce SMA expression. The response was stronger in the case of MRTF-A in agreement with the strong nuclear localization and greater SMA promoter-activating capacity of this construct. (E) Cells were transfected with MRTF-A or -B, and after 48 h, fixed and stained for SMA (red), FLAG (green) to visualize successful transfection, and the nuclear dye DAPI to visualize every cell.

shift: after this treatment ~16% of the cells exhibited strong nuclear accumulation, whereas the nuclear exclusion disappeared in the majority of cells (Figure 7, Ab and B). Dominant negative Rho strongly mitigated the contact disruption-induced nuclear transfer (Figure 7, A, d-d", and B). Importantly, expression of DN-MLC significantly suppressed the translocation as well, suggesting the contribution of myosin phosphorylation in the process (Figure 7, A, e-e", and B).

These findings raised the possibility that the contact-dependent regulation of MRTF distribution might play an important role in the differential responsiveness of confluent and nonconfluent cultures to the EMT-inducing effect of TGF- β 1. To test this assumption, we compared MRTF distribution in confluent and nonconfluent cultures exposed to TGF- β 1 for various times. Endogenous MRTF was entirely cytosolic in confluent cultures (Figure 8A, top panels). Treatment of intact confluent layers with TGF- β 1 (0–24 h) did not induce nuclear transloca-

tion of MRTF, and most cells showed no change in MRTF localization at all, whereas some exhibited a punctate, perinuclear labeling. A radically different picture was observed in subconfluent cultures. Under resting condition, ~75% of the cells located at the free edges of cellular islands showed cytosolic MRTF staining, whereas ~17% showed clear nuclear accumulation and 8% had even cytosolic and nuclear distribution (Figure 8A, bottom panels and graph). The extent of the nuclear accumulation of MRTF in subconfluent layers was in good agreement with the values obtained in cells in which the contacts were disassembled by Ca^{2+} removal. In cells located in the intact inner regions of these multicellular islands, MRTF was fully cytosolic. In subconfluent layers (as opposed to the confluent ones), TGF- β 1 exposure induced a dramatic change in MRTF distribution: in cells at the free edges, perinuclear MRTF condensation was apparent after 1 h treatment (not shown), whereas after 6 h, 95% of peripheral cells showed strong nuclear accumulation of MRTF (Figure 8A). Cells in rows adjacent to the peripheral row also showed increased nuclear localization (Figure 8A), whereas in the inner areas MRTF remained cytosolic (not shown). To our surprise nuclear accumulation of MRTF in the peripheral cells was transient: after 24 h of TGF- β 1 treatment, the response significantly decreased: only 25% of the cells showed clear nuclear MRTF localization, whereas even distribution or punctate, perinuclear labeling was visible in 12% of the cells (Figure 8A).

We argued that if myosin phosphorylation indeed plays a permissive role in nuclear translocation of MRTF, then TGF- β 1 might not be able to elicit large or sustained MLC phosphorylation in fully confluent layers. Indeed, TGF- β 1 exposure for 16 h had no discernable effect on phospho-MLC content when the cytokine was added to confluent layers (Figure 8B). In contrast substantial phospho-MLC staining was observed at the free edges of cells located in the periphery of untreated subconfluent islands (Figure 8B), i.e., at the same locus where cells are susceptible to TGF- β 1-induced SMA expression. The phospho-MLC signal at these sites appeared to further increase upon TGF- β 1 treatment. Consistent with these findings, acute Ca^{2+} removal caused robust myosin phosphorylation in confluent layers (as visualized by Western blotting), whereas short-term treatment with TGF- β 1 caused a much smaller response. Nonetheless, TGF- β 1 was able to cause a significant and rapid albeit transient MLC phosphorylation, indicating that the confluent monolayer remained responsive to the cytokine. Ca^{2+} removal combined with TGF- β 1 gave the most robust effect on MLC phosphorylation (Figure 8C).

In addition to Ca^{2+} removal and subconfluence, the third, and from a pathological standpoint possibly the most relevant, model of contact disruption is mechanical wounding of a confluent monolayer. Cells at the wound edge showed marked MLC phosphorylation (Figure 8D). Interestingly, at this location a number of cells exhibited nuclear accumulation of endogenous MRTF as well (Figure 8D).

Finally we tested whether interfering with myosin phosphorylation indeed impacts on SMA protein expression. As shown on Figure 8E DN-MLC reduced the number of SMA-expressing cells in sparse, TGF- β 1-treated cultures. Under these conditions approx. 22% of control cells expressed SMA, whereas this number dropped more than fourfold (approx. 4%) in DN-MLC-expressing cells (Figure 8E).

Taken together, the nuclear accumulation of endogenous MRTF is regulated in a cell contact- and contractility-dependent manner in tubular cells, and impaired contact integrity plays a deterministic role in the regulation of MRTF distribution upon TGF- β 1 treatment.

Figure 7. Contact disassembly induces Rho- and MLC phosphorylation-dependent nuclear translocation of endogenous MRTF in epithelial cells. (A) In a and b cells were serum-deprived for 3 h and then placed into either Ca²⁺-containing or Ca²⁺-free DMEM for 24 h. Cells were then fixed and stained for endogenous MRTF using a polyclonal antibody raised against BSAC, the mouse MKL1 or MRTF-A protein. (c and c') Cells were transfected with Myc-tagged CA-Rho and 24 h later fixed and stained for endogenous MRTF (red) and Myc (green). Note the large nuclear accumulation of MRTF in the CA-Rho-expressing cells. (d–d') Cells were transfected with Myc-tagged DN-Rho and 24 h later subjected to Ca²⁺ removal for 24 h, fixed, and stained for Myc (green), endogenous MRTF (red), and all nuclei were visualized with DAPI (blue). Note the reduced nuclear accumulation of MRTF in the DN-Rho-expressing cells compared with their untransfected neighbors. (e–e') Similar experiments were performed as in d, except the cells were transfected with DN-MLC. Note the substantial nuclear translocation in many nontransfected cells and the preservation of nuclear exclusion in the DN-MLC-expressing cells. (B) Distribution of endogenous MRTF was quantified in each transfected group. The number of evaluated cells was as follows: control, 283; No Ca, 438; CA-Rho, 52; DN-Rho, 52; DN-MLC, 224.

MRTF Is a Central Contributor to the Cell Contact-regulated and TGF- β 1-modulated Activation of the SMA Promoter

To address whether in our system MRTF has a causal role in the contact injury-dependent SMA promoter response, and its potentiation by TGF- β 1, we transfected the cells with a mutant FLAG-tagged myocardin (Δ C585), which lacks the transactivation domain, and has been shown to act as dominant negative against each member of the MRTF family (Wang *et al.*, 2001). This mutant showed spontaneous accumulation in the nucleus and was present in the cytosol too, as revealed by immunostaining with an anti-FLAG antibody (Figure 9A). Importantly, expression of Δ C585 abolished the Ca²⁺ deprivation-triggered increase in promoter activity and strongly suppressed the synergism between contact disassembly and TGF- β 1 (Figure 9B). These observations suggest that endogenous MRTF activity is a central target of the cell contact- and TGF- β 1-dependent regulation of the SMA promoter, and it plays an indispensable role in myofibroblast differentiation of kidney tubular cells.

DISCUSSION

Epithelial-mesenchymal transition is a key process in organ fibrosis (Kalluri and Neilson, 2003). Injury or absence of

intercellular contacts exerts a permissive and potentiating effect on the transdifferentiation of epithelial cells to myofibroblasts (Masszi *et al.*, 2004). This phenomenon may have a key importance from a pathobiologic standpoint: although intact epithelia may be partially resistant to the fibrogenic effect of TGF- β 1, an initial injury may render the wounded region susceptible for this cytokine, thereby generating focally transformed areas. From these foci the process can spread to neighboring regions. Furthermore, the development of myofibroblasts in the wound has been associated with a change in the type of the expressed cadherins (Hinz *et al.*, 2004). With this scenario in mind, the present study aimed to identify contact-dependent mechanisms that can facilitate EMT. We found that contact disassembly causes Rho activation and ROK-mediated MLC phosphorylation. These events then contribute to the nuclear redistribution of SRF and mainly its coactivator, MRTF, which in turn activate the SMA promoter, and strongly synergize with the TGF- β 1-induced SMA protein expression.

Cell Contacts, Rho Activation, and MLC Phosphorylation

Our finding that the Ca²⁺ removal-induced disruption of cell junctions activates Rho is in good accord with the reported converse phenomenon, i.e., that during the Ca²⁺-triggered formation of intercellular junctions Rho activity is

Figure 8. The effect of TGF- β 1 in confluent and subconfluent layers on the intracellular distribution of endogenous MRTF and MLC phosphorylation. (A) Cells were grown to 100% confluence or approx. 30% confluence (subconfluent) and left untreated and fixed or treated with 10 ng/ml TGF- β 1 for the indicated times and then fixed and stained for MRTF. The bar diagram on the right indicates the intracellular distribution of endogenous MRTF in cells at the periphery of cellular islands, under control conditions or after treatment for the indicated times with TGF- β 1. (B) Confluent or subconfluent layers were left untreated or exposed to TGF- β 1 for 16 h and then fixed and stained for pMLC. Nuclei were visualized by DAPI. (C) The acute effect of Ca^{2+} removal and TGF- β 1 on myosin phosphorylation. Cells were treated with normal or EGTA-containing medium in the absence or presence of 10 ng/ml TGF- β 1 for 15 min and then processed for Western blotting with the anti-pMLC antibody as described in *Materials and Methods*. Tubulin was used as loading control. (D) A wound was generated in a confluent monolayer with a rubber policeman, and 6 h later the cells were fixed and stained either for MRTF or for pMLC. (E) Cells were seeded sparsely, transfected with Myc-tagged DN-MLC, and then treated daily with 10 ng/ml TGF- β 1 for 3 d. Cells were then fixed and doubly stained for SMA and for the Myc epitope. Note the robust SMA expression in the control cells and the absence of SMA expression in the DN-MLC-expressing cells. To quantify the effect, three separate experiments were performed, in which 910 randomly selected control (nontransfected) cells and 311 DN-MLC-expressing cells were assessed for SMA expression ($p < 2.0 \times 10^{-5}$).

gradually down-regulated (Noren *et al.*, 2003). The mechanism whereby the destabilization of tight or adherent junctions stimulates Rho remains to be elucidated. It is noteworthy that recently a Rho-specific guanine nucleotide exchange factor, the Dbl family member GEF-H1/Lfc has been found to localize to the tight junction, where it regulates paracellular permeability, a myosin-modulated function (Benaï-Pont *et al.*, 2003). Future studies should address whether GEF-H1 is activated by contact disruption. In tubular cells, contact disassembly led to rapid and long-lasting MLC phosphorylation, which was most prominent at the cell periphery. This response was mediated by the Rho/ROK pathway because it was inhibited by genetic or pharmacological interference with this signaling route. The same maneuvers abolished the Ca^{2+} removal-induced activation of the SMA promoter as well, indicating that the Rho/ROK pathway has a key role in cell contact-dependent regulation of gene expression. In addition to the spatially restricted activation of Rho, junctional ROK and/or myosin localization or accumulation may also contribute to the focal MLC phosphorylation. Indeed, a subpool of ROK was found to be associated with the adherens junctions (Walsh *et al.*, 2001), and a peripheral myosin ring is present in epithelial cells (Ivanov *et al.*, 2004, 2005). Thus, each component of the Rho/ROK/MLC pathways can be junction-associated, facilitating the

preferential activation of this particular downstream Rho pathway at the contacts.

Myosin Phosphorylation and the Regulation of the SMA Promoter

Rho has been shown to increase the transcriptional activity of SRF on those target genes, including SMA, whose promoter harbors CArG boxes (Hill *et al.*, 1995; Mack *et al.*, 2001; Masszi *et al.*, 2003). Elegant studies have revealed that the effect of Rho is mediated by cytoskeletal reorganization, a key component of which is enhanced F-actin polymerization (Miralles *et al.*, 2003). So far two downstream Rho effector pathways have been implicated in SRF-dependent transcription: the activation of the formin protein mDia, which induces net F-actin polymerization (Copeland and Treisman, 2002) and the activation of the Rho/ROK/LIM kinase/cofilin phosphorylation pathway, which stabilizes F-actin due to decreased severing (Geneste *et al.*, 2002). The former mechanism was predominant in fibroblasts, whereas both were critical in neuron-like PC12 cells. Our studies point to the importance of a third Rho effector pathway, namely ROK-dependent MLC phosphorylation. This mechanism, at least in our epithelial cells, seems to be an important contributor, because the myosin inhibitor blebbistatin or a phosphorylation-incompetent DN myosin mutant abolished the contact

Figure 9. Dominant negative MRTF inhibits the contact disassembly-induced activation of the SMA promoter and suppresses the synergism between contact disruption and TGF- β 1. (A) Localization of DN myocardin (DN-MyoC). Cells transfected with FLAG-tagged DN-MyoC for 24 h were serum-starved, incubated in Ca^{2+} -containing or Ca^{2+} -free medium for 24 h, fixed, and stained using an anti-FLAG antibody. Note the predominantly nuclear localization of the construct irrespective of the state of the intercellular contacts. (B) Cells were cotransfected with pSMA-Luc/pRL-TK along with either empty vector (pcDNA3) or DN-MyoC for 24 h, and then exposed to Ca^{2+} removal, 10 ng/ml TGF- β 1, or the combination of these treatment as in Figure 2D.

disruption-provoked SMA promoter expression, eliminated the synergism between contact injury and TGF- β 1 on the promoter, and suppressed SMA protein expression. Peripheral myosin activity (junctional contractility) has been proposed to participate in the regulation of various functions including paracellular permeability (Turner, 2000), junction remodeling (Ivanov *et al.*, 2004, 2005; Shewan *et al.*, 2005), cell scattering (de Rooij *et al.*, 2005), morphogenesis (Bertet *et al.*, 2004), and closure of epithelial wounds (Darenfed and Mandato, 2005). Our data assign yet another critical role for this process: the regulation of SRF-dependent gene expression (Figure 10). This mechanism efficiently couples the mechanical and genetic responses to wounding: Formation of actin-myosin complexes triggers contractile wound closure and at the same time initiates genetic reprogramming, leading to enhanced generation of extracellular matrix proteins and contractile elements.

What is the mechanism whereby myosin activity regulates contact-dependent SMA expression? Contact disassembly promoted the nuclear translocation of SRF and its coactivator MRTF, whereas interference with the Rho/ROK pathway or directly with myosin phosphorylation suppressed the nuclear accumulation and/or retention of these factors. The effect of myosin inhibition on the accumulation or retention of SRF was modest, suggesting that myosin-phosphorylation is not the major Rho-mediated pathway regulating this process. In addition, high Rho activity appeared to facilitate the nuclear export of SRF, pointing to a complex regulation. In any case, our results suggest that contractility or tension per se may be critical regulators of MRTF-mediated gene expression. Intriguingly, active (movement-asso-

Figure 10. The synergistic effect of cell contact injury and TGF- β 1 in the complex regulation of the SMA promoter. Contact disassembly activates Rho which, in turn stimulates mDia (1; Copeland and Treisman, 2002) and ROK (2). The former process leads to increased actin polymerization, whereas the latter activates the LIMK/cofilin pathway (2a) and stimulates MLC phosphorylation (2b). The LIMK/cofilin pathway may stabilize F-actin via decreased severing (Geneste *et al.*, 2002). Enhanced MLC phosphorylation may contribute to SMA expression by acting through various nonexclusive mechanisms: it might promote actin polymerization/stabilization (i), may directly participate in the nuclear translocation or retention of MRTF (ii), or might be required for the internalization of cell contact components (iii; Ivanov *et al.*, 2004). Increased nuclear accumulation of MRTF acts in concert with SRF through the CARg boxes. In addition, contact injury triggers the internalization of β -catenin, which—through yet unidentified mechanisms—potentiates the activation of the SMA promoter (Masszi *et al.*, 2004). Finally, TGF- β 1 activates a multitude of signaling pathways, which through various transcription factors impact on the TCE and SBE *cis* elements. TGF- β 1 may also contribute to Rho activation; however, this effect in itself is insufficient to provoke long-lasting MLC phosphorylation, which has a permissive effect on the activation of the SMA promoter. The underlined processes or pathways are addressed in the current study. Question marks denote potential mechanisms. The relative contribution of the primary, permissive, and potentiating mechanisms remains to be defined.

ciated) or passive (traction-transmitted) tension induced the nuclear localization of drosophila MAL (Somogyi and Rorth, 2004) and has been associated with increased SMA expression (Hinz *et al.*, 2001).

The original question then translates into asking how myosin activity impacts on MRTF localization or activity (Figure 10). Several possibilities can be evoked: 1) MRTF localization is regulated by the G/F actin ratio. Binding of monomeric actin (presumably through a yet unidentified protein) to MRTF prevents its translocation to the nucleus whereas actin polymerization removes G-actin from MRTF, thereby exposing its nuclear localization sequence (Miralles *et al.*, 2003; Posern *et al.*, 2004). It is conceivable that myosin activity, which promotes actin filament bundling, can also

“steal away” monomeric actin from MRTF, or the formation of actin-myosin complexes may specifically reduce the MRTF-binding competent pool of actin. Indeed, blebbistatin, which inhibits basal myosin activity as well, promoted the dissociation of stress fibers. However we found no evidence that blebbistatin would significantly decrease the total F-actin pool. Furthermore, using DN-MLC, we did not observe any major change in the actin skeleton organization and only a modest reduction in F-actin. In this regard, it is worth pointing out that both the monomer-sequestering drug latrunculin B and the barbed end capper cytochalasin D induce strong F-actin disassembly, yet the former inhibits while the latter triggers the nuclear translocation of MRTF (Miralles *et al.*, 2003). This observation suggests that the critical factor is not necessarily the content of F-actin or the F/G actin ratio, but rather the interaction of G-actin with inhibitors or proteins, which will determine whether G-actin can bind to MRTF. Taken together our observations suggest that a gross alteration in the G/F actin ratio is unlikely to be required for the inhibition of SMA expression by suppressed myosin phosphorylation. Indeed, several observations suggest that Rho impacts on SRF signaling not exclusively via actin polymerization: Certain Rho mutants stimulate SRF without inducing stress fibers formation (Sahai *et al.*, 1998; Zohar *et al.*, 1998), and a newly described Rho effector hCNK1 activates SRF without promoting stress fiber assembly (Jaffe *et al.*, 2004). Nonetheless our data do not rule out the possibility that inhibition of myosin might interfere with stimulus-induced localized F-actin assembly. Clearly, future studies should directly address whether myosin activity impacts on the formation of the actin/actin-binding protein/MRTF complexes. 2) Another potential mechanism is that myosin, as a force-generating protein, might be required for the efficient nuclear import or retention of MRTF. There is accumulating evidence that both the microtubule and the microfilament cytoskeleton is involved in the nuclear import of certain proteins (Campbell and Hope, 2003). Recently, endothelial MLC kinase has been shown to regulate the nuclear translocation of NF κ B and the consequent reporter gene activation (Wadgaonkar *et al.*, 2005). Although the underlying mechanism remains to be clarified, a similar process might participate in MRTF translocation as well, except in LLC-PK1 cells the predominant enzyme responsible for MLC phosphorylation is ROK and not MLCK (Szasz *et al.*, 2005). 3) Finally, myosin may affect other processes in addition to MRTF translocation. We have previously shown that upon contact injury E-cadherin is degraded allowing the liberation of β -catenin, which potentiates the activation of the SMA promoter (Masszi *et al.*, 2004). Importantly, Ivanov *et al.* (2004) have shown that myosin activity is essential for the contact disassembly-induced internalization of E-cadherin, and blebbistatin maintains E-cadherin at the cell surface. Similarly, the Src-mediated delocalization of E-cadherin from the AJ also requires MLC phosphorylation (Avizienyte *et al.*, 2004). Taken together, junction stabilization by myosin inhibition may contribute to the inhibition of the SMA promoter.

MRTF and EMT

The present work identifies MRTF as a central factor in mesenchymal/myofibroblast transdifferentiation of epithelial cells. This conclusion is supported by the findings that 1) overexpression of MRTF is sufficient to induce SMA promoter activation and protein expression in tubular cells; 2) induction of robust actin polymerization induces nuclear accumulation of MRTF concomitant with SMA expression, and 3) DN-myocardin prevents the Ca²⁺ depletion-induced

promoter activation and the synergism between contact injury and TGF- β 1. In nonstimulated LLC-PK1 cells endogenous MRTF (as visualized by the anti-BSAC antibody) was cytosolic. Interestingly, the heterologously expressed MRTF-A was predominantly nuclear, whereas MRTF-B localized mainly to the cytosol. In contrast, both MRTF-A and -B were cytosolic in fibroblasts, pointing to tissue-specific differences in localization, presumably due to a different set of actin-binding proteins. Indeed, recently a striated muscle-specific actin- and MRTF-binding protein (STARS) has been identified (Kuwahara *et al.*, 2005). Further studies are warranted to investigate the contribution of endogenous MRTF isoforms to EMT.

We observed that TGF- β 1 was unable to induce MRTF translocation in fully confluent layers, but it enhanced nuclear accumulation after contact disassembly. This finding implies that MRTF localization is one of the key target mechanisms that underlies the synergy between TGF- β 1 and contact injury. Presumably, the strong, contact-dependent Rho activation is indispensable for the efficient nuclear accumulation of MRTF. On the other hand, moderate translocation of endogenous MRTF may not be sufficient to induce SMA expression, because cells adjacent to the wound are not transformed in the absence of TGF- β 1. The SMA promoter harbors several transcriptional regulatory elements, including the SRF/MRTF-binding CArG-boxes, the Kruppel factor-binding TGF- β control element (TCE), and the TGF- β -responsive SBE (Hautmann *et al.*, 1997; Hu *et al.*, 2003; Liu *et al.*, 2003b). Accordingly, the promoter can be collectively regulated by contact-dependent (Rho-mediated) and TGF- β 1-dependent (partially Rho-independent) pathways (Figure 10). Interestingly MRTF may have multiple roles: in addition to forming a ternary complex with SRF and CArG boxes, it was found to bind to the SMAD proteins too, and thus it might facilitate transcription through the SBE (Qiu *et al.*, 2005). Moreover SMADs were shown to associate with β -catenin as well (Tian and Phillips, 2002). These multiple inputs then can culminate in robust promoter activation. Finally, we observed that even under the maximally effective two-hit conditions, MRTF accumulation in the nucleus is transient. Future studies should investigate the regulation of the nuclear export of MRTF.

In summary, we propose that the contact injury-induced, Rho-ROK-phospho-myosin-mediated MRTF translocation represents a central signaling pathway in transdifferentiation of epithelial cells, and thereby in the pathogenesis of organ fibrosis.

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