Modulation of the Expression of Connective Tissue Growth Factor by Alterations of the Cytoskeleton*

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Modulation of the cytoskeletal architecture was shown to regulate the expression of CTGF (connective tissue growth factor, CCN2). The microtubule disrupting agents nocodazole and colchicine strongly up-regulated CTGF expression, which was prevented upon stabilization of the microtubules by paclitaxel. As a consequence of microtubule disruption, RhoA was activated and the actin stress fibers were stabilized. Both effects were related to CTGF induction. Overexpression of constitutively active RhoA induced CTGF synthesis. Interference with RhoA signaling by simvastatin, toxin B, C3 toxin, and Y27632 prevented up-regulation of CTGF. Likewise, direct disintegration of the actin cytoskeleton by latrunculin B interfered with nocodazole-mediated up-regulation of CTGF expression. Disassembly of actin fibers by cytochalasin D, however, unexpectedly increased CTGF expression, indicating that the content of F-actin per se was not the major determinant for CTGF gene expression. Given the fact that cytochalasin D sequesters G-actin, a decrease in G-actin increased CTGF, while increased levels of G-actin corresponded to reduced CTGF expression. These data link alterations in the microtubule and actin cytoskeleton to the expression of CTGF and provide a molecular basis for the observation that CTGF is up-regulated in cells exposed to mechanical stress.

In closing wounds or developing fibrotic tissue fibroblasts are exposed to massive mechanical stress, which leads to alterations in cell morphology and reorganization of the cytoskeleton. The impact of morphological changes on gene expression has been analyzed in model systems such as the one described by Grinnell et al. (1), where fibroblasts are cultured in collagen gels under stressed or relaxed conditions. Connective tissue growth factor (CTGF) was among those genes that showed the most striking up-regulation by mechanical stress when cDNA arrays were used to compare gene expression in fibroblasts cultured under mechanically stressed conditions and cells cultured in relaxed collagen I gels (2, 3).

CTGF is a cysteine-rich secreted protein, which functions as modulator of complex cellular processes such as growth, differentiation, adhesion, and migration (4). Because high levels of CTGF are observed during wound healing and in fibrotic lesions, and CTGF induces the synthesis of extracellular matrix proteins, it is believed to play a role in the pathogenesis of fibrosis (5). The fibrogenic growth factor transforming growth factor β (TGFβ) is one of the major inducers of CTGF expression, but other mediators implicated in organ injury and wound healing such as lysophosphatidic acid (LPA), serotonin, or angiotensin II can also directly increase CTGF levels (e.g. see Refs. 6–8). Similar to the up-regulation of CTGF in stressed fibroblasts, deformation of renal mesangial cells by cyclic stretch (9) or by static pressure (10) likewise increased CTGF expression, pointing to changes in the cell architecture as modulator of CTGF gene expression.

An intact actin cytoskeleton proved to be necessary for the induction of CTGF by exogenous stimuli as shown by cytochalasin D treatment, which directly interrupts filamentous actin. Pretreatment of mesangial cells or renal fibroblasts with cytochalasin D reduced the inducibility of CTGF by LPA or TGFβ (6, 7). Members of the Rho family, including RhoA, Rac1, and Cdc42, are key regulators of the actin cytoskeleton (11, 12). Inhibition of RhoA signaling rapidly disassembles actin stress fibers, leaving cortical actin fibers intact. Furthermore, inhibition of the activity of RhoA by C3 exotransferase or inhibition of the downstream Rho-associated kinase (ROCK) by the compound Y27632 prevented induction of CTGF by TGFβ or LPA (7). These data support a role of the actin cytoskeleton in the regulation of CTGF expression not excluding other signaling pathways.

The actin cytoskeleton is closely linked to the microtubule network, regulating cell motility and maintenance of cell shape (13). Formation of actin stress fibers was observed upon disruption of microtubuli by drugs like colcemid or vinblastine (14, 15). In the presence of inhibitors of RhoA, stress fiber formation was prevented establishing RhoA as a link between microtubules and actin fibers. Direct evidence for RhoA activation by microtubule destabilizing agents was obtained in Swiss 3T3 cells (16), and this activation was possibly mediated by p190RhoGEF, which binds to microtubules (17). These results lead to the hypothesis that alterations in the microtubule and actin cytoskeleton might be molecular mechanisms underlying the up-regulation of CTGF observed in cells under pressure (2, 3, 9, 10). In the present study, we show that disruption of microtubules by low concentrations of colchicine or nocodazole strongly up-regulates CTGF expression. This enhanced up-regulation was dependent on RhoA activity and
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**Materials**—Nocodazole, colchicine, paclitaxel (taxol), jasplakinolide, PD98059, cytochalasin D, latrunculin B, and DRB (5,6-dichlorobenzimidazole riboside) were obtained from Merck Biosciences, Bad Soden, Germany. Acutax<sup>®</sup> was from PAA Laboratories, Linz, Austria. Y27632 was kindly provided by Yoshitomi Pharmaceutical Industries, Osaka, Japan, simvastatin by Merck Sharp and Dohme, München, Germany. Simvastatin was dissolved in ethanol and activated as described by Jakobisiak et al. (18). Toxin B from *Clostridium difficile* and C3 toxin from *Clostridium limosum* were kindly provided by Drs. F. Hofmann and K. Aktories, Freiburg, Germany (19). For delivery of C3 toxin into cells, the C2IN-C3 fusion toxin was used together with the C2I binding component of *Clostridium botulinum* C2 toxin (20).

**Cell Culture**—Immortalized human renal fibroblasts (21) were cultured as described previously (7, 22). The cells were characterized in detail and shown to have many characteristics of primary fibroblasts (23). Prior to the experiments, cells at subconfluence were serum-starved in Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum for 1 day.

**Northern Blot Analysis**—Northern blot analysis was performed by standard techniques as described previously (23). Hybridization was performed with cDNA probes labeled with [*32*P]dCTP using the Non- aPrimer kit from Appligene, Heidelberg, Germany. A cDNA specific for CTGF was kindly provided by N. Abdel-Wahab, London, UK (24). The blots were analyzed by phosphorimager or autoradiography. Quantitative analysis of the autoradiographs was performed by densitometric scanning. All values were corrected for differences in RNA loading by calculating the ratio of CTGF to 18 or 28 S rRNA expression.

**Western Blot Analysis**—Western blot analyses were performed as described previously (25). CTGF was detected in cellular homogenates. In brief, cells were rinsed twice with ice-cold phosphate-buffered saline and then scraped in 90 μl of lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μM sodium orthovanadate, 25 μM sodium fluoride). Lysates were incubated for 30 min on ice and then centrifuged at 15,000 *g* for 10 min at 4 °C. The supernatant was removed and protein concentrations were determined by the bicinchoninic acid assay, Pierce (Sankt Augustin, Germany). Aliquots of the cell lysates (1 mg of protein) in 50 μl of Rho-IPA buffer were mixed with 20 μl of Rho-RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) and lysed in 500 μl of Rho-IPA buffer (50 mM Tris/HCl, pH 7.2, 500 mM NaCl, 10 mM MgCl<sub>2</sub>, 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mg/ml phenylmethylsulfonyl fluoride). The homogenized cell lysates were cleared by centrifugation at 15,800 × *g* for 15 min at 4 °C, and the protein concentration was determined by the bicinchoninic acid assay, Pierce (Sankt Augustin, Germany).

**RESULTS**

**Morphological Changes Induced by Nocodazole or Colchicine**—To detect the influence of microtubule disrupting agents on the cytoskeletal architecture of immortalized human renal fibroblasts, the cells were incubated with low concentrations of colchicine (1 μM) or nocodazole (1 μM). The actin cytoskeleton was visualized by rhodamine phalloidin staining, and the microtubules were detected by immunocytochemistry. Treatment for 1 h disrupted microtubular structures and increased cell spanning actin stress fibers (Fig. 1). Immunofluorescence images showed that tubulin was no longer detected in fine elongated fibers but appeared as patches distributed within the cytosol. Actin stress fibers, which were sparsely visible in resting fibroblasts, were markedly intensified after disruption of the microtubules, pointing to an interaction of the microtubule system and the actin cytoskeleton in these cells.

**Characterization of CTGF Up-regulation by Microtubule Disrupting Agents**—Next, it was investigated whether disruption of the microtubular network modulated CTGF expression. CTGF mRNA was rapidly up-regulated when the fibroblasts were treated with nocodazole or colchicine (1 μM) for 1–5 h (Fig. 2A). Maximal CTGF mRNA levels were obtained after 1 to 2 h, and comparative analysis revealed the nocodazole-mediated up-regulation of CTGF mRNA.
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Fig. 2. **Induction of CTGF by disruption of microtubules.** A, fibroblasts were incubated with nocodazole (Noc, 1 μM) or colchicine (Colch, 1 μM) for the times indicated. CTGF mRNA expression was detected by Northern blot analysis. B, the expression of CTGF was quantified by densitometry. To compare different experiments, stimulation after 2 h was set to 100%. Data are means ± S.D. or half-range of two to four experiments. ■, colchicine; ■, nocodazole. C, fibroblasts were incubated with nocodazole (Noc, 1 μM) or colchicine (Colch, 1 μM) for the times indicated. CTGF protein expression was detected by Western blot analysis. D, fibroblasts were incubated with increasing concentrations of nocodazole for 1 h. CTGF mRNA expression was detected by Northern blot analysis. E, fibroblasts were preincubated with paclitaxel (Pac, 1 μM) for 1 h and then further incubated with nocodazole (1 μM) or colchicine (1 μM) for 2 h. CTGF protein was detected by Western blot analysis.

Increase being more rapid than the colchicine-mediated up-regulation of CTGF mRNA (Fig. 2B). CTGF mRNA levels remained elevated for several hours. Consistently, a long lasting increase of CTGF protein was detectable in the cellular homogenates, which contained intracellular protein and secreted CTGF protein that remained attached to the cells (Fig. 2C). Induction of CTGF expression was dependent on the nocodazole concentration, stimulation still being detectable at 0.1 μM nocodazole (Fig. 2D). Induction of CTGF by colchicine or nocodazole was not restricted to the human fibroblast cell line but also observed in other cell types, such as mouse embryonic fibroblasts, primary cultures of mesangial cells, or endothelial cells (data not shown). In contrast to the microtubule disrupting agents nocodazole and colchicine, there was no significant effect of paclitaxel (taxol), a microtubule stabilizing agent, on CTGF expression measured from 1–5 h (Fig. 2E and data not shown). Furthermore, stabilization of the microtubuli by paclitaxel prevented induction of CTGF by nocodazole or colchicine (Fig. 2E), indicating that disruption of the microtubuli was essential for the induction of CTGF.

The rapid induction of CTGF mRNA within 1 h suggested transcriptional regulation. This was supported by the use of DRB, an inhibitor of transcription. When DRB was added after 1 h during the initial phase of increased synthesis of CTGF mRNA, the further increase was blunted (Fig. 3). DRB added during the phase of mRNA decrease (after 3 h, as shown in Fig. 3) did not affect the stability of the mRNA.

**RhoA as a Mediator of CTGF Expression**—Previous studies had shown that Rho proteins are involved in the regulation of CTGF induction by external stimuli (6). Here we show that pretreatment of the cells with simvastatin, which interferes with protein isoprenylation, or toxinB, which inactivates proteins of the Rho family, prevented the induction of CTGF expression by nocodazole or colchicine (Fig. 4A). Specific inhibition of ROCK by the compound Y27632 similarly reduced CTGF expression, indicating that the RhoA-ROCK pathway serves as a linker between disruption of microtubular structures and CTGF gene expression. CTGF mRNA levels in the presence of 1 μM nocodazole were reduced to 23 ± 11% (means ± S.D., n = 4) by pretreatment with 10 μM Y27632. Specific inhibition of RhoA signaling by C3 toxin decreased colchicine-mediated induction of CTGF (Fig. 4B). In line with data in the literature, C3 toxin proved to be less effective than Y27632, possibly due to its poor penetration of the cells even as C2-C3 complex (27). In accordance with this interpretation, changes in the actin cytoskeleton were less pronounced with C3 exotoxin than with Y27632 (data not shown). Involvement of RhoA in CTGF expression was confirmed by overexpression of constitutively active RhoA (RhoA V14). Two different constructs were used for transfection, resulting in essentially the same results (Fig. 4C). The transfection process per se led to an increase in CTGF expression which was increased 3-fold by the overexpression of active RhoA for 24 h. Essentially the same data were obtained in human embryonic kidney cells (HEK293, data not shown).

In contrast to the RhoA signaling pathway, only a minor contribution of p42/44 MAP kinase signaling was observed. Pretreatment of the cells with the MEK inhibitor PD98059 (10 μM) reduced CTGF induction by about 20% and completely inhibited CTGF protein expression together with Y27632 (Fig. 4D).

To analyze whether disruption of microtubules by nocodazole or colchicine resulted in activation of RhoA, the GTP-bound form of RhoA was detected by an in vitro pull down assay with GST-Rhotekin as binding partner. As a positive control, the cells were stimulated with lysophosphatidic acid for 4 min (Fig. 5A). Treatment of the fibroblasts with nocodazole or colchicine for 10 min resulted in a marked activation of RhoA. To separate the effects of activated RhoA on gene expression and stress fiber organization, the cells were treated with Y27532 for only
expression of CTGF by modulation of the cytoskeleton

The current studies show that regulation of CTGF is modu-
lation of changes in the microtubular as well as actin fiber
network, thereby connecting expression of CTGF to changes in
cell morphology. Up-regulation of CTGF mRNA and protein
was observed within 1 h after treatment of the cells with low
concentrations of nocodazole or colchicine. Kinetics were
consistently faster with nocodazole than with colchicine, but no
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vented up-regulation of CTGF relating the effect of both drugs
to the disruption of the microtubular network. Prolonged in-
cubation with colchicine or nocodazole led to apoptosis of the
fibroblasts, which was not observed during the short period of
time necessary to induce CTGF expression.

There are few examples documented in the literature linking
microtubule disruption to gene expression. Expression of the
early response gene cyclooxygenase-2 (Cox-2) was reported to
be sensitive to chemical disruption of microtubuli but also to
stabilization of the microtubule by paclitaxel (29). In contrast
to these data, CTGF expression was inhibited rather than
stimulated by paclitaxel in our experiments with renal fibro-
blasts (Fig. 2E). Furthermore, p42/44 MAP kinase signaling
was implicated in Cox-2 expression. Depending on the stimulus
used, p42/44 MAP kinase may be involved in CTGF induction
at low concentrations of nocodazole further up-regulated CTGF,
indicating that attachment of the cells was not a prerequisite
for CTGF induction by disruption of microtubuli. At the protein
density of 3-fold was observed after 1 h of treatment
(3.0 ± 0.6, means ± S.D., n = 3 independent experiments). A
partial inhibition was observed when the cells were pretreated
with C3 toxin or Y27632 prior to the detachment, indicative of
additional signaling pathways involved in CTGF up-regulation
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ton—To more directly evaluate the modulation of CTGF gene
expression by alterations of the actin cytoskeleton, cells were
treated with jasplakinolide, which stabilizes actin stress fibers
independent of the activation of RhoA (28). Treatment of cells
with increasing concentrations of jasplakinolide resulted in a
concentration-dependent increase in CTGF mRNA expression
concomitant with a stabilization of the actin filament network
(Fig. 7A). On the other hand, disruption of the actin cytoskel-
et with latrunculin B reduced basal CTGF expression by about
50% (reduction to 52 ± 8%, means ± S.D. in n = 3 independent
experiments) and prevented nocodazole-mediated up-regulation
of CTGF (Fig. 7B). Disruption of the cytoskeleton by cyto-
chalasin D, however, transiently increased CTGF (Fig. 6C).
Maximal expression was observed after 1 h (4.7 ± 1.7-fold
induction by 1 μM cytochalasin D, means ± S.D. in n = 6
independent experiments). Treatment of the cells with 2 μM
cytochalasin D led to a similar stimulation, while lower con-
centrations of cytochalasin D (0.5 μM) were slightly less effec-
tive (data not shown). The different effects of latrunculin B and
cytochalasin D on CTGF expression suggested that structured
F-actin per se was not essential for CTGF expression, because
stress fibers were disintegrated by both compounds as shown
by fluorescence staining (Fig. 7, B and C). Compared with
latrunculin B, cytochalasin D-mediated disruption of stress
fibers was accompanied by an aggregation of actin within the
cytosol.

DISCUSSION

The current studies show that regulation of CTGF is modu-
lated by changes in the microtubular as well as actin fiber
network, thereby connecting expression of CTGF to changes in
cell morphology. Up-regulation of CTGF mRNA and protein
was observed within 1 h after treatment of the cells with low
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centrations of cytochalasin D (0.5 μM) were slightly less effec-
tive (data not shown). The different effects of latrunculin B and
cytokinase A by forskolin interferes with CTGF induction (7, 8).
Similarly, depolymerization of the microtubules may activate
NF-κB signaling (33), but activation of NF-κB by TNFα has
been shown to inhibit CTGF expression (34). Release of Smads

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unpublished results.
from microtubule was observed upon colchicine treatment and related to TGFβ induction (35), but we found no activation of a plasmid containing a luciferase reporter fused to Smad-binding elements (SBE-Lux) by nocodazole or colchicine for 60 min as indicated. CTGF mRNA was detected by Northern blot analysis. B, fibroblasts were preincubated with C3 toxin (200 ng/ml) for 3 h or Y27632 (10 μM) for 30 min and then further incubated with nocodazole or colchicine for 90 min. The Western blot shown is representative of three similar ones. C, renal fibroblasts were transfected with RhoA V14 or RhoA V14 coupled to GFP for 24 h. CTGF and RhoA were detected by Western blot analysis. As control for equal loading the blot was re-probed with anti-tubulin. D, fibroblasts were preincubated with Y27632 (Y, 5 μM), PD98059 (P, 10 μM), or a combination of both compounds for 30 min and then stimulated with nocodazole for 2 h. CTGF and tubulin were detected by Western blot analysis.

from microtubule was observed upon colchicine treatment and related to TGFβ induction (35), but we found no activation of a plasmid containing a luciferase reporter fused to Smad-binding elements (SBE-Lux) by nocodazole or colchicine in the human fibroblasts used in this study.2 Taken together, these data suggested that other signaling pathways than those described in the literature so far were essential for the induction of CTGF by microtubule disruption in fibroblasts.

Previous studies had been indicative of a role for the small GTPase RhoA in CTGF induction (25), and this was confirmed in the present study by overexpression of constitutively active RhoA. Activation of RhoA was observed within minutes after incubation of the cells with nocodazole or colchicine and confirmed the link established between microtubules and RhoA activation in Swiss 3T3 fibroblasts (16). Activation of RhoA and Rho-associated kinase was of particular functional relevance for the induction of CTGF by disruption of the microtubuli, as shown by C3 toxin and more importantly by the specific Rho-associated kinase inhibitor Y27632. Pretreatment of the cells with Y27632 was sufficient to blunt nocodazole- or colchicine-induced CTGF expression. The concurrent interference with gene expression and the disintegration of the actin stress fibers by Y27632 did not allow us to separate these processes, and cytoskeleton-independent signaling pathways cannot be fully ruled out. On the other hand, the importance of the actin cytoskeleton was further supported by the effect of jasplakinolide, which stabilized actin fibers and increased CTGF expression, whereas latrunculin B, which leads to actin depolymerization, decreased CTGF expression. The stimulating effect of cytochalasin D, which also rapidly disintegrated actin stress fibers, was unexpected. In cardiomyocytes and in Swiss 3T3 fibroblasts, cytochalasin D was shown to activate RhoA (16, 36). Inhibition of RhoA signaling via Rho-associated kinase by 10 μM Y27632 partially inhibited cytochalasin D-mediated CTGF induction, but did not reach...
CTGF was then further treated with or without nocodazole (1 μM) for 25 and 55 min. CTGF mRNA expression was detected by Northern blot analysis. The experiment is representative of three similar ones. B, fibroblasts were preincubated with C3 toxin 200 ng/ml for 3 h or Y27632 (10 μM) for 30 min and then detached for 5 min (Co-5'). They were then further treated with or without nocodazole (1 μM) for 60 min (Co-60', Noco-60'). CTGF expression was detected by Western blot analysis. The blot is representative of two independent experiments with essentially the same result.

Fig. 6. Adhesion-independent induction of CTGF. A, fibroblasts were detached with accutase and then further incubated in medium consisting of 50% accutase solution and 50% medium with 0.5% fetal calf serum for 60 min, conditions that prevented attachment. After 5 min of detachment, the cells were incubated with nocodazole (1 μM) for 25 and 55 min. CTGF mRNA expression was detected by Northern blot analysis. The experiment is representative of three similar ones. B, fibroblasts were preincubated with C3 toxin 200 ng/ml for 3 h or Y27632 (10 μM) for 30 min and then detached for 5 min (Co-5'). They were then further treated with or without nocodazole (1 μM) for 60 min (Co-60', Noco-60'). CTGF expression was detected by Western blot analysis. The blot is representative of two independent experiments with essentially the same result.

statistical significance, arguing against a pronounced role of RhoA in cytochalasin D-mediated induction of CTGF expression. Both cytochalasin D and latrunculin B enhance the cellular content of G-actin (37); however, the availability of G-actin as modulator of gene expression seems to be different upon treatment with both agents; cytochalasin D was shown to sequester and thus reduce the effective level of G-actin, thereby activating a subset of genes regulated by serum response factor (38, 39). The published promoter sequence of CTGF does not contain a serum response element, and thus far, CTGF transcription has not been characterized as being dependent on activation by serum response factor, suggesting a different molecular mechanism of cytochalasin D action. Furthermore, RhoA activation has been linked to serum response factor activation via diaphanous-related forms (mDia1 and mDia2) rather than Rho-associated kinase (40). Although the molecular mechanism responsible for the increased CTGF mRNA levels has not yet been unraveled, sequestration of G-actin by cytochalasin D is in agreement with up-regulation of CTGF being regulated by decreased levels of G-actin.

CTGF has been shown to be up-regulated in fibroblasts embedded in attached collagen gels (3). These cells are under tension and show large cell spanning stress fibers (41). Microtubules have not been investigated under those experimental conditions, but cross-talk between microtubules and the actin cytoskeleton is observed in locomotion of cells with microtubules exerting a relaxation response opposing actin contraction (13). Under physiologic conditions, microtubules act in concert with signals from extracellular matrix to modulate actin fibers. As a model system, chemical depolymerization of microtubules is sufficient to elude diverse pleiotropic effects on cells, including the stabilization of actin fibers as summarized in Ref. 42. High concentrations of colchicine or nocodazole (10 μM) were shown to induce the development of focal adhesions and to activate adhesion-dependent signal transduction in fibroblasts (31). Interestingly, up-regulation of CTGF was also observed in detached cells (Fig. 6), indicative of CTGF induction being at least in part independent of cell adhesion. In line with our results Ren et al. (16) showed activation of RhoA by colchicine in the absence of adhesion. However, the reduced effect of the inhibitor of the Rho-associated kinase suggests that additional signaling pathways play a role in CTGF induction under these experimental conditions.

Taken together, our data are in agreement with an inverse correlation between the availability of G-actin and CTGF expression. Interference with the microtubular network or treatment of the cells with jasplakinolide, and CTGF mRNA expression was detected by Northern blot analysis. Right panel, actin stress fibers were stained with rhodamine phalloidin after 1-h treatment with 100 nM jasplakinolide. B, fibroblasts were treated with latrunculin B (LatB, 1 μg/ml) for the times indicated. After 1-h treatment, the cells were stimulated with nocodazole (Noc, 1 μM) for 1 h, and CTGF mRNA expression was detected by Northern blot analysis. Right panel, actin stress fibers were stained with rhodamine phalloidin after 1-h treatment with 100 nM latrunculin B. C, fibroblasts were incubated with cytochalasin D (CytoD, 1 μg/ml) for the times indicated and CTGF mRNA expression was detected by Northern blot analysis. Right panel, actin stress fibers were stained with rhodamine phalloidin after 1-h treatment with cytochalasin D.

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