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SHORT COMMUNICATION

Substrate rigidity-dependent positive feedback regulation between YAP and ROCK2

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ABSTRACT

Extracellular matrix (ECM) stiffness influences gene expression, leading to modulation of various cellular functions. While ROCK2 regulates actomyosin activity as well as cell migration and proliferation, expression of ROCK2 is increased in response to stiffening ECM. However, the mechanism underlying rigidity-dependent *ROCK2* expression remains elusive. Here, we show that YAP, a mechanically regulated transcription coactivator, upregulates *ROCK2* expression in an ECM rigidity-dependent manner. YAP interacted with the *ROCK2* promoter region in an actomyosin activity-dependent manner. Knockdown of *YAP* decreased *ROCK2* expression while activity of the *ROCK2* promoter was upregulated by expressing constitutively active YAP. Furthermore, we found that *ROCK2* expression promotes transcriptional activation by YAP. Our results reveal a novel positive feedback loop between YAP and ROCK2, which is modulated by ECM stiffness.

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Introduction

Actin filaments (F-actin), the polymerized form of globular actin (G-actin), are major components of the cytoskeleton. The actin cytoskeleton adopts various architectures—including stress fibers, filopodia, and lamellipodia—that underlie cellular morphogenesis and migration.[1,2]

Cells bind to the ECM through integrin receptors. This binding initiates the recruitment of focal adhesion (FA) proteins to the cell-ECM adhesion sites. Subsequent activation of Rho GTPases including Rho, Rac, and Cdc42 promotes actin polymerization.[3,4] Rho then increases actin-myosin contractile force generation via Rho-associated coiled coil-containing protein kinase (ROCK)-mediated myosin activation, which leads to further activation of integrin signaling.[5]

The mechanical environment surrounding the cell, such as ECM stiffness and cell density, has a significant impact on cell properties and behaviors.[6,7] Stiffening of the ECM promotes cellular migration and invasion via integrin signal-dependent alteration of actin cytoskeletal architectures.[8,9] Furthermore, ECM stiffness affects the differentiation and proliferation of cells by modulating gene expression, in which remodeling of the actin cytoskeleton plays a significant role.[10,11,12] Thus, the actin cytoskeleton works as a key mediator in mechanical cue-induced signal transduction.

Yes-associated protein (YAP) is a "mechanotransducer" and a major downstream effector of the Hippo pathway.[12] YAP translocates into the nucleus and binds to TEA domain (TEAD) transcription factors, thereby inducing the expression of target genes, such as *CTGF*.[13] Upon cell-cell adhesion mediated by adherens junctions and tight junctions, YAP is phosphorylated by large tumor suppressor kinases (LATS) and subsequently binds to 14–3–3 proteins, resulting in the sequestration of YAP in the cytoplasm. On the other hand, inhibition of actin polymerization causes the inactivation of YAP by both LATS-dependent and -independent mechanisms.[12] The YAP binding protein angiomotin (AMOT) is involved in the LATS-independent regulation of YAP localization. While the interaction of AMOT with YAP sequesters YAP in the cytoplasm, F-actin binding of AMOT attenuates this interaction, leading to nuclear translocation of YAP.[12,14,15]

While ROCK2 is a key regulator of actomyosin, it also regulates cell proliferation, invasion, and metastasis.[16,17]

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We have recently reported that, in human breast cancer MCF-7 cells, stiffened ECM increases ROCK2 expression, which causes an increase in cellular sensitivity against anti-tumor drugs.[18] Thus, ROCK2 is likely to be a mechanical cue-dependent molecule that might affect cancer therapy outcomes. However, the mechanism by which ECM stiffness alters *ROCK2* expression remains unknown.

In the present study, we examine the molecular mechanisms underlying the regulation of *ROCK2* expression by ECM stiffness. Our results suggest that, in response to ECM rigidity, YAP induces *ROCK2* expression in an actomyosin-dependent manner, and ROCK2, in turn, enhances the activation of YAP.

Materials and methods

Cell culture

MCF-7 human breast cancer cells, 293T human embryonic kidney cells, C2C12 mouse myoblasts were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. N-acryloyl-6-aminocaproic acid (ACA)-copolymerized acrylamide gels for polyacrylamide culture substrates were prepared as described previously.[19,20]

Retroviral vectors and retroviral infection

Retroviral infection was performed as described previously.[21] Briefly, pSuper-YAP puro was cotransfected with the pAmpho into 293T cells using the Polyethylenimine as a transfection reagent (Polysciences, Inc.). At 48 h after transfection, the supernatant was collected and then used to infect MCF-7 cells in the presence of 8 μg/mL Polybrene. At 24 h after infection, the cells were selected using puromycin (1.5 μg/mL) for 3 d.

Antibodies and materials

Anti-ROCK2 mouse monoclonal (D-11; Santa Cruz) and anti-α-tubulin mouse monoclonal (DM1A; Sigma-Aldrich) antibodies were used for immunoblot analysis. Anti-YAP rabbit monoclonal antibody (D8H1X; Cell Signaling Technology) was used for immunoblot and immunofluorescence analyses. Latrunculin A was purchased from Sigma-Aldrich. Blebbistatin and Y-27632 were purchased from Merck Millipore.

Fluorescence microscopy

To immunostain for YAP, cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, and blocked with 2% BSA in PBS. The cells were then incubated with the anti-YAP rabbit polyclonal antibody. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) was used as a secondary antibody. DAPI (Vector Laboratories) was used to stain nuclei. Images were acquired using a confocal microscope (LSM700; Zeiss) and then analyzed with ImageJ software (NIH).

Plasmids

To generate retroviruses encoding small hairpin RNAs (shRNAs) against human YAP and human ROCK2, the YAP target sequences #1: 5′- GCCACCAAGCTAGATAAAGAA −3′, #2: 5′-GACATCTTCTGGTCAGAGA-3′ and ROCK2 target sequences #1: 5′-GGTTTATGCTATGAAGCTT-3′, #2: 5′-GGATAAACATGGACATCTA-3′ were cloned into the pSuper retro hygro vector and pSuper retro puro vector (Oligoengine, Seattle, WA), respectively. pCS2-YAP 5SA gift from Dr. Hiroshi Nishina contains the insert phosphorylation-defective YAP 5SA mutant.

Luciferase assay

The reporter construct ROCK2-WT-luc was generated by subcloning the 0.8 kb fragment encompassing the promoter region (−815 to +20) of the human *ROCK2* gene into the pGL3-basic vector. The control plasmid phRL-TK (*Renilla* luciferase reporter) was obtained from Toyobo (Osaka, Japan). Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

Quantitative real-time PCR

Total RNA was purified using NucleoSpin RNA kit (Takara). cDNA was prepared using PrimeScript 1st strand cDNA Synthesis kit (Takara). Quantitative real-time PCR analysis was performed with Thunderbird SYBR qPCR Mix (Toyobo) under the following conditions: 1 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 55°C for 1 min using StepOne Plus Real-Time PCR system (Applied Biosystems). The following primers were used: human *YAP* forward 5′- AGGAGAGAC TGCGGTTGAAA-3′ and reverse 5′-CCCAGGAGAAGACACTGCAT −3′; human *CTGF* forward 5′- ACCGA CTGGAAGACACGTTTG-3′ and reverse 5′- CCAGGTCAGCTTCGCAAGG-3′; human *ROCK1* forward 5′- GA CCTGTAACCCAAGGAGAT-3′ and reverse 5′- GGAA AGTGGTAGAGTGTAGG-3′; human *ROCK2* forward 5′- CAACTGTGAGGCTTGTATGAAG-3′ and reverse 5′- TGCAAGGTGCTATAATCTCCTC-3′; and human *ubiquitin* forward 5′- TGACTACAACATCCAGAA-3′ and reverse 5′- ATCTTTGCCTTGACATTC −3′.

Immunoblot analysis

Immunoblot analysis was performed as described previously.[19] Briefly, cells were solubilized with the lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 10 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, and protease inhibitor cocktail [PIC; Nacalai Tesque]) and then centrifuged at 20,000 × *g* for 20 min after sonication. The supernatants were used as total cell extracts and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using a SimpleChIP Plus Enzymatic Chromatin IP Kit following the manufacturer's protocol provided by Cell Signaling Technology. Cross-linked chromatin was immunoprecipitated with anti-YAP rabbit polyclonal antibody (D8H1X; Cell Signaling Technology) or normal rabbit IgG (Cell Signaling Technology) antibodies. Precipitated DNA was analyzed by quantitative real-time PCR. The following primers were used: human *ROCK2* promoter forward 5′-TGGGTTTACTGGGTCAAAGG −3′ and reverse 5′-AAGAGAACGGGAGAGCAC −3′; and human *CTGF* promoter forward 5′- GCCAATGAGCTGAATGGAGT −3′ and reverse 5′- CAATCCGGTGTGAGTTGATG −3′ as a positive control.

Statistical analysis

Statistical analysis of data was performed using the unpaired Student's 2-sided t-test.

Results and discussion

To elucidate the mechanism underlying rigidity-dependent *ROCK2* expression, we focused on YAP as it is known to be a mechanically regulated transcription

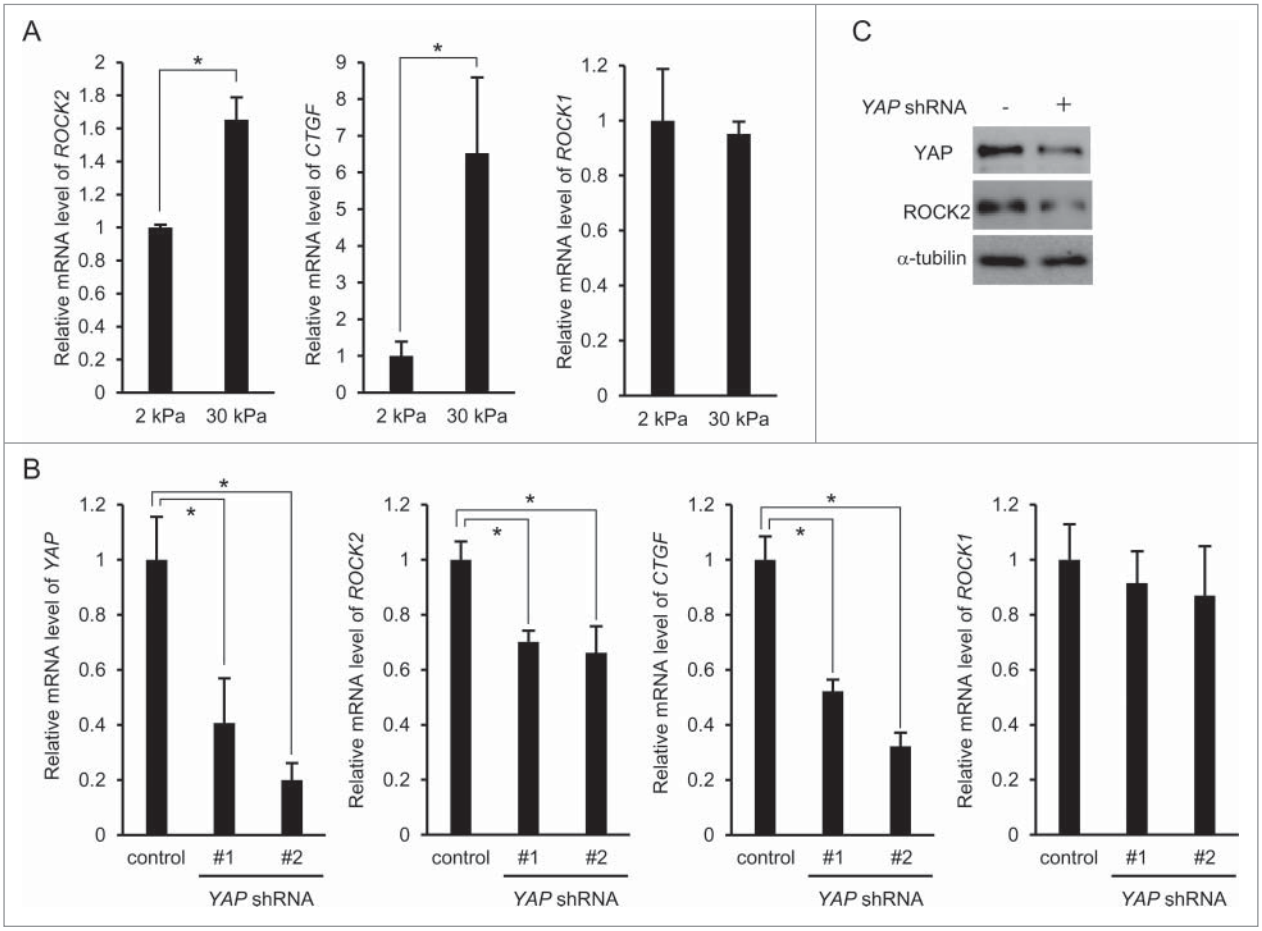

Figure 1. *YAP* is involved in *ROCK2* expression. (A) MCF-7 cells were cultured on substrates with elasticities of 2 and 30 kPa. The expression of ROCK2, CTGF, and ROCK1 were evaluated by quantitative real-time PCR. Each bar represents the mean ± SD; n = 3. Asterisks, $p < 0.05$. (B, C) Cells were infected with a control, *YAP* shRNA-#1, or -#2 -expressing retrovirus. (B) The expression of *YAP*, *ROCK2*, *CTGF*, and *ROCK1* were evaluated by quantitative real-time PCR. Each bar represents the mean ± SD; n = 3. Asterisks, $p < 0.01$. (C) Extracts from control and *YAP* shRNA-#1-expressing cells cultured on plates were subjected to immunoblot analysis with antibodies against *YAP* ROCK2 and α-tubulin as a loading control.

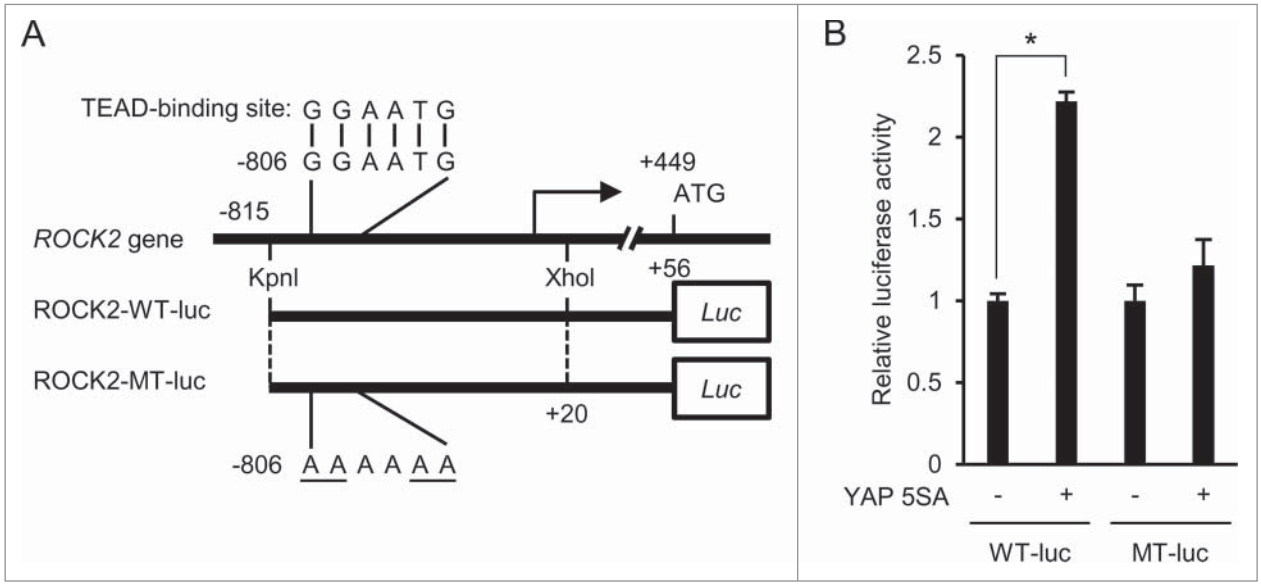

Figure 2. YAP induces activation of the *ROCK2* promoter. (A) The *ROCK2* promoter and luciferase reporter gene constructs. The TEAD-recognition sequence in the *ROCK2* promoter is shown. The following reporter plasmids used in this assay are also indicated: ROCK2-WT-luc containing *ROCK2* promoter and putative TEAD-recognition sequence, and ROCK2-MT-luc in which 4 critical nucleotide residues for TEAD binding were altered (indicated by the underlined letters). (B) The pCS2-YAP 5SA expression vector was transfected into C2C12 cells with each reporter plasmid. The luciferase activity was measured 24 hours after transfection. The activity was normalized with the average value of the cells transfected with control vector. Each bar represents the mean ± SD; n = 3. Asterisks, $p < 0.01$.

coactivator. We found that the expression of both *ROCK2* and *CTGF*, a well-recognized transcriptional target of the YAP-TEAD complex, was considerably higher on the 30 kPa substrate than on the 2 kPa substrate (Fig. 1A). We then tested whether YAP-TEAD regulated *ROCK2* expression by examining the effect of shRNA-mediated depletion of *YAP* on *ROCK2* expression. Knockdown of *YAP* decreased mRNA expressions of both *ROCK2* and *CTGF* (Fig. 1B). The ROCK2 expression on a protein level was also reduced upon the *YAP* knockdown (Fig. 1C).

The ROCK family consists of ROCK1 and ROCK2, which are functionally redundant in part.[22, 23] We previously reported that, in fibroblasts, *ROCK2* expression is downregulated when cells are cultured on soft (<4 kPa) substrates, whereas *ROCK1* expression remains unaffected.[24] Consistently, *ROCK1* expression in MCF-7 cells on the 2 kPa substrate was comparable to that on the

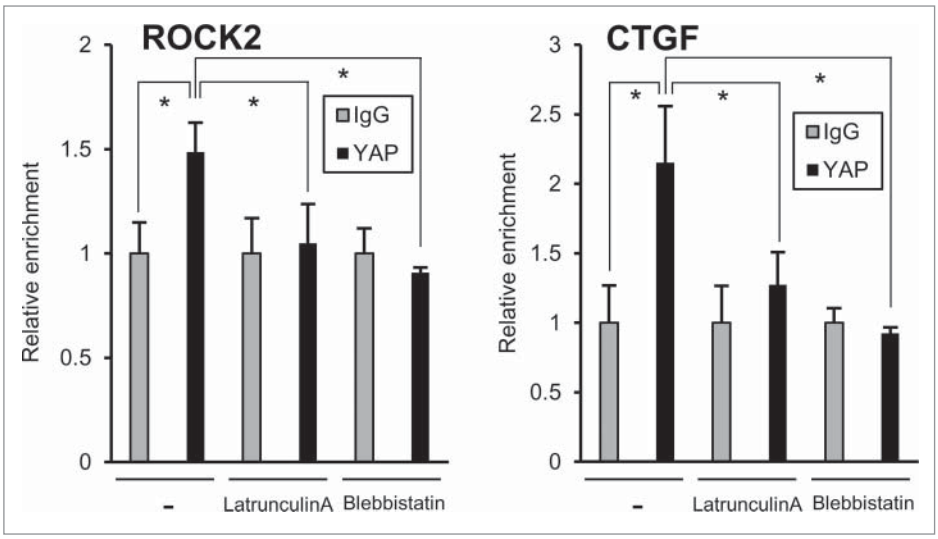

Figure 3. Binding of YAP to the *ROCK2* promoter is decreased by treatment with latrunculin A or blebbistatin. MCF-7 cells were treated with latrunculin A (200 nM) or blebbistatin (50 μM) for 40 min. The binding of YAP to the *ROCK2* and *CTGF* promoters was evaluated by chromatin immunoprecipitation assay and quantitative real-time PCR. Each bar represents the mean ± SD; n = 3. Asterisks, $p < 0.05$.

30 kPa substrate (Fig. 1A). Furthermore, *ROCK1* expression was not decreased by *YAP* knockdown (Fig. 1B). These results indicate that, in contrast to the case of *ROCK2*, expression of *ROCK1* is regulated by neither substrate rigidity nor YAP.

We identified a potential TEAD-binding sequence (GGAATG) located at -806 to -801 in the promoter region of *ROCK2* (Fig. 2A). To examine whether YAP-TEAD directly activates the *ROCK2* promoter, we constructed a luciferase reporter gene expressed under the *ROCK2* promoter (ROCK2-WT-luc in Fig. 2A). *ROCK2* promoter activity was increased by expressing the constitutively active YAP mutant, YAP 5SA (Fig. 2B). In contrast, expression of YAP 5SA did not increase the activity of the reporter gene carrying point mutations in the TEAD binding sequence (ROCK2-MT-luc in Fig. 2A and 2B). Furthermore, the ChIP assay revealed that YAP bound to the *ROCK2* promoter as well as the *CTGF* promoter (Fig. 3). These results reveal that YAP-TEAD induces *ROCK2* expression by directly activating the *ROCK2* promoter.

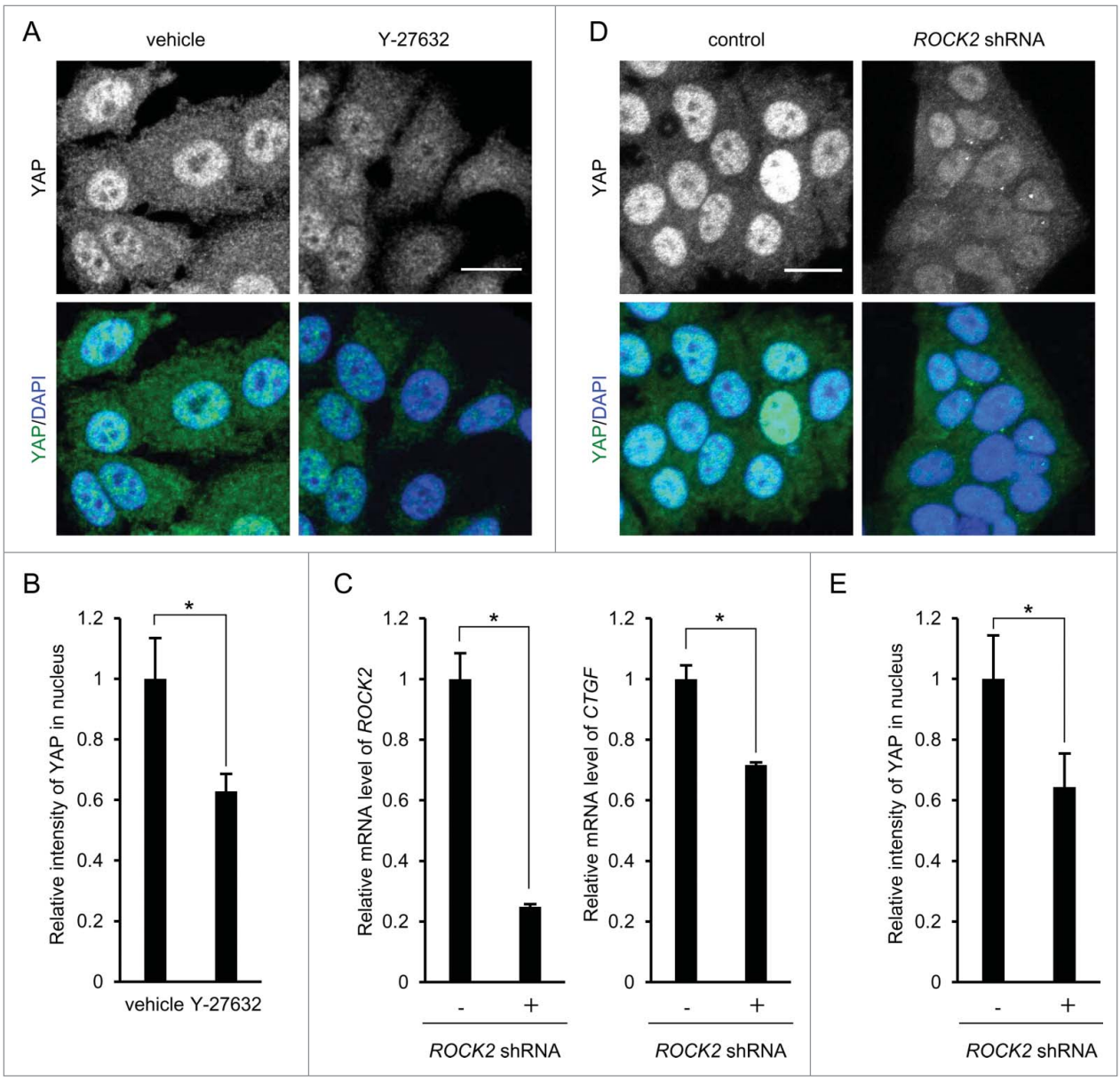

Figure 4. Knockdown of *ROCK2* decreases nuclear accumulation of YAP. (A, B) MCF-7 cells were treated with Y-27632 (10 μM) for 3 h. (C–E) MCF-7 cells were infected with either a control or *ROCK2* shRNA-expressing retrovirus. (A, D) Confocal images of cells stained for YAP (green) and DAPI (blue). Z-stack images with an interval of 1.0 μm were obtained using a confocal microscope, and projected images are shown. Scale bars, 20 μm. (B, E) The mean nuclear fluorescence intensity for the stained YAP was quantified using ImageJ software. The relative fluorescence intensities are shown. Each bar represents the mean ± SD; n = 10 (B) or n = 130 (E). Asterisks, $p < 0.01$. (C) The expression of *ROCK2* and *CTGF* were evaluated by quantitative real-time PCR. Each bar represents the mean ± SD; n = 3. Asterisks, $p < 0.01$.

Stiff ECM increases actin polymerization and myosin activity, which then promotes the nuclear accumulation of YAP.[12,25,26] We therefore tested whether actomyosin was involved in YAP-dependent *ROCK2* expression. Treatment of cells with latrunculin A, an inhibitor of actin polymerization, or blebbistatin, a myosin II inhibitor, diminished YAP binding to the *ROCK2* promoter as well as the *CTGF* promoter (Fig. 3), suggesting that YAP-TEAD-mediated *ROCK2* expression depends on actomyosin activity.

ROCK increases actomyosin activity through phosphorylation of myosin light chain (MLC) and myosin phosphatase (MYPT).[23] Since actomyosin activity promotes YAP-mediated gene expression,[12,25,26] we next asked whether YAP-dependent regulation of ROCK2 in turn affects YAP activity. Inhibition of ROCK with Y-27632 diminished nuclear localization of YAP in MCF-7 cells (Fig. 4A and B), which is consistent with previous reports using other cell lines.[27] Furthermore, knockdown of *ROCK2* decreased both expression of *CTGF* (Fig. 4C, and Supplementary Fig. 1A) and nuclear accumulation of YAP (Fig. 4D, E, and Supplementary Fig. 1B and C). These results indicate that ROCK2 is involved in activation of YAP.

Shi et al. used MEFs derived from *ROCK1*$^{-/-}$ and *ROCK2*$^{-/-}$ mice to demonstrate that ROCK2, but not ROCK1, regulates MLC2 phosphorylation in cells cultured on stiff substrates (plastic plates; $\sim 10^6$ kPa).[28,29] Thus, ROCK2, but not ROCK1, appears to be crucial for the activation of actomyosin on stiff ECM, which might underlie ROCK2-dependent YAP activation (Fig. 4).

Cell proliferation is largely affected by ECM rigidity.[30-32] While YAP promotes cell cycle progression and cell proliferation through the expression of TEAD-target genes,[13,26,33-35] ROCK is also indispensable for these cellular behaviors.[17,36,37] Our results in this study suggest that YAP and ROCK2 form a positive feedback loop that is modulated by ECM rigidity. This feedback loop may be involved in the rigidity-dependent regulation of cell proliferation.

ECM stiffness, ROCK2, and YAP all affect the susceptibility of cancer cells against chemotherapy, even though their effects are somewhat controversial. We have reported that stiff ECM upregulates ROCK2 expression, which enhances the susceptibility of breast cancer cells against the antitumor drug doxorubicin in a p53-dependent manner.[18] Furthermore, while overexpression of YAP is observed in many cancers,[33,38,39] antitumor drug treatments activate YAP and thereby induce apoptosis through transcriptional activation of p73, a p53 family tumor suppressor.[40] Therefore, although its effect may be dependent on cellular context, the YAP-ROCK2 axis found in this study is likely to be a critical factor that determines the susceptibility of cancer cells against chemotherapy. Further studies on the mechanism underlying the mechanical regulation of YAP and ROCK2 would contribute to the development of effective chemotherapy treatments of cancer.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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