Elsevier Editorial System(tm) for Journal of Molecular and Cellular Cardiology
Manuscript Draft

Manuscript Number: JMCC12086R2

Title: Mechanical strain induces a pro-fibrotic phenotype in human mitral valvular interstitial cells through RhoC/ROCK/MRTF-A and Erk1/2 signaling pathways.

Article Type: Full length article

Keywords: heart valve disease; myxomatous mitral valve; mechanical strain; RhoGTPases; MRTF-A; CTGF/CCN2.

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Abstract: The mitral valve is a complex multilayered structure populated by fibroblast-like cells, valvular interstitial cells (VIC) which are embedded in an extracellular matrix (ECM) scaffold and are submitted to the mechanical deformations affecting valve at each heartbeat, for an average of 40 million times per year. Myxomatous mitral valve (MMV) is the most frequent heart valve disease characterized by disruption of several valvular structures due to alterations of their ECM preventing the complete closure of the valve resulting in symptoms of prolapse and regurgitation. VIC and their ECM exhibit reciprocal dynamic processes between the mechanical signals issued from the ECM and the modulation of VIC phenotype responsible for ECM homeostasis of the valve. Abnormal perception and responsiveness of VIC to mechanical stress may induce an inappropriate adaptative remodeling of the valve progressively leading to MMV. To investigate the response of human VIC to mechanical strain and identify the molecular mechanisms of mechano-transduction in these cells, a cyclic equibiaxial elongation of 14% at the cardiac frequency of 1.16 Hz was applied to VIC by using a Flexercell-4000T™ apparatus for increasing time (from 1h to 8h). We showed that cyclic stretch induces an early (1h) and transient over-expression of TGFβ2 and αSMA. CTGF, a profibrotic growth factor promoting the synthesis of ECM components, was strongly induced after 1 and 2 h of stretching and still upregulated at 8 h. The mechanical stress-induced CTGF up-regulation was dependent on RhoC, but not RhoA, as demonstrated by siRNA-mediated silencing approaches, and further supported by evidencing RhoC activation upon cell stretching and suppression of cell response by pharmacological inhibition of the effector ROCK1/2. It was also dependent on the MEK/Erk1/2 pathway which was activated by mechanical stress independently of RhoC and ROCK. Finally, mechanical stretching induced the nuclear translocation of myocardin related family factor MRTF-A which forms a transcriptional complex with SRF to promote the expression of target genes, notably CTGF. Treatment of stretched cultures with inhibitors of the identified pathways (ROCK1/2, MEK/Erk1/2, MRTF-A translocation) blocked CTGF

overexpression and abrogated the increased MRTF-A nuclear translocation. CTGF is up-regulated in many pathological processes involving mechanically challenged organs, promotes ECM accumulation and is considered as a hallmark of fibrotic diseases. Pharmacological targeting of MRTF-A by newly developed inhibitors may represent a relevant therapy for MMV.

Covering letter to JMCC 12086R1

Dear Editor,

We are most pleased to submit to JMCC our manuscript # 12086R1 "Mechanical strain induces a pro-fibrotic phenotype in human mitral valvular interstitial cells through RhoC/ROCK/MRTF-A and Erk1/2 signaling pathways" revised according to the comments of the reviewer 3. The main requirement of the reviewer was to consolidate the hypothesis of the independence of two signaling pathways regulating CTGF expression upon mechanical stretching. We had no other way than to carry out the experiment to satisfy this request, although you mentioned that performing new experiments was not necessary. These results do not lengthen much the manuscript but strengthen it. Therefore, we took the liberty to include these data in the form of two small panels in figure 3 and to briefly describe them. We can however remove these two small panels and information, as per your decision.

We hope to have adequately addressed the last concerns of the reviewer and we thank you for your consideration.

Looking forward to the editorial decision,

Sincerely yours

Prof. A. Colige
Laboratory of Connective Tissues Biology
University of Liège, Sart Tilman, Belgium

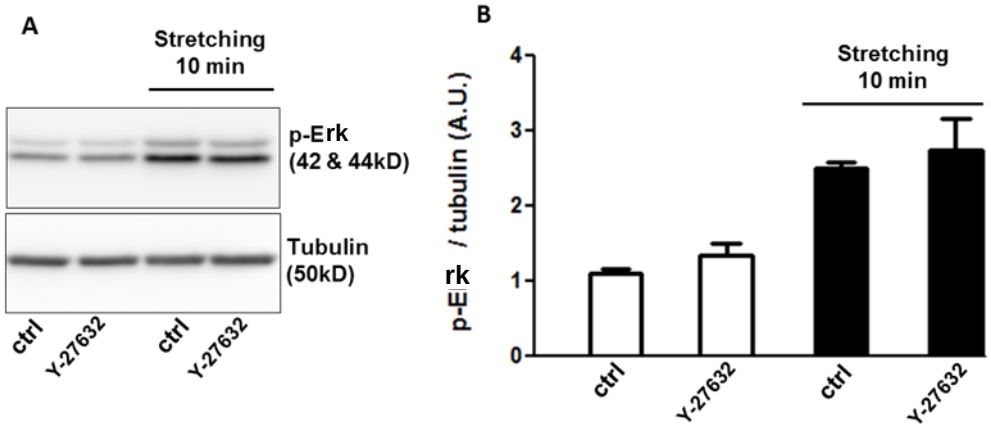
Response to the reviewer JMCC 12086 R1

We thank the reviewer for the positive recommendation for publication of our manuscript and the relevant suggestion to consolidate one of our hypotheses.

Reviewer #3: The present study investigated how the mitral valvular interstitial cells (VIC) respond to mechanical stress and subsequently initiate pathways for extracellular matrix synthesis. Human VIC were subjected to cyclic stretching and CTGF, the key ECM mediator, was found upregulated. Mechanism study revealed that RhoC/ROCK and MEK/Erk pathways independently contribute to MRTF-A nuclear translocation, which works together with SRF to promote transcription of ECM genes. In summary, the use of human VIC and in vitro mechanical strain provide a new approach for the study of valvular diseases. The manuscript is suitable for publication if the following concerns are solved.

1. In fig.3, the authors demonstrated that activation of MEK/ERK pathway by cyclic stretching is independent of RhoC/ROCK pathway. The RhoC knockdown experiment is not sufficient to exclude the involvement of ROCK in this MEK/ERK activation process since ROCK can be turned on by many other factors. Therefore, application of ROCK inhibitor (Y27632) may consolidate the hypothesis that there is a cross-talk between ROCK and MEK/ERK pathways during mechanical stress response in VIC.

To answer the reviewer's concern regarding the involvement of ROCK in the MEK/ERK activation process, we complied with the suggestion to use the ROCK inhibitor Y27632. As shown in the figure below, the tratmpent by Y27632 did not impair the MEK/ERK activation pathway by mechanical stretching in concordance with the RhoC silencing experiment. The efficiency of Y27632 was assessed by checking the inhibition of phosphorylation of myosin light chain, a known ROCK substrate (data not shown).



*VIC were submitted or not to mechanical stretching for 10 min using the experimental procedure described in the manuscript, in control conditions or after a 1 h pre-treatment with Y27632 (5µM). **A**: western-blot analysis of phospho-Erk (p-Erk) in static and stretched cells in absence (ctrl) and presence of Y27632. **B**: quantification of p-Erk relative to α-tubulin used as protein load control (mean±SEM, n=3 experiments).*

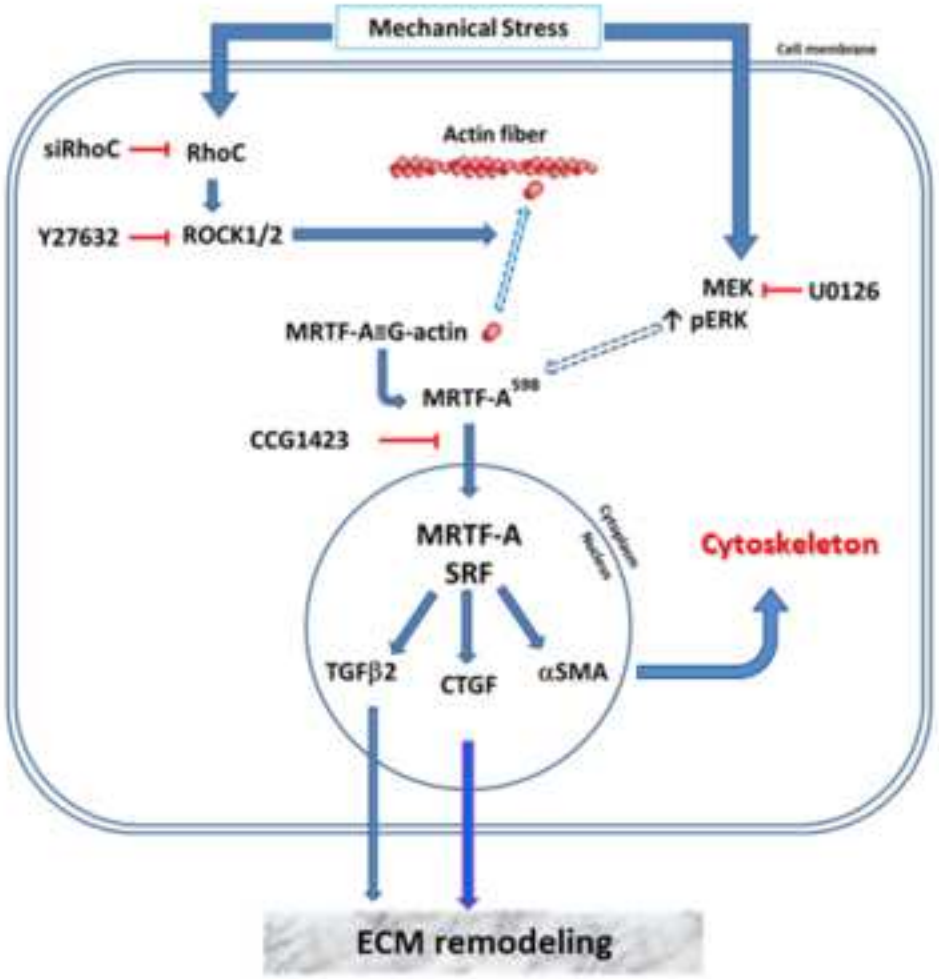
These results have been added in the figure 3 (G & H) of the revised manuscript p 13-14 and briefly described in the text p 12 line 26-27.

2. The authors used recombinant CTGF to demonstrate its specific role in sensing mechanical stress and in the regulation of ECM genes expression (fig. 6). However, the expression levels of ECM genes were not changed when endogenous TCGF were increased after stress stretching (table S3). Why the effects of extra versus endogenous TCGF are different? The controversial findings should be discussed.

Our aim by using recombinant CTGF on VIC was to verify if these cells are able to respond to it, what had never been investigated before, to our best knowledge. We found that they indeed respond to CTGF very similarly to what was previously observed with skin fibroblasts, by an increased expression of type I collagen, fibromodulin, CTGF itself and CYR61. In this experimental setting, the commercially available recombinant CTGF is an 11 kDa peptide, of undisclosed sequence and location in the native CTGF and with a biological activity similar to that of CTGF. It was administered in one shot at 30ng/ml to VIC in culture. By contrast, during the stretching experiments, CTGF is progressively produced at a likely lower concentration and is released as a full size protein of 38kD, as shown in our western-blots. It has been further shown that CTGF can be proteolytically modified to generate polypeptides with different affinity for receptors and biological properties. The discrepancies that we observed in the two experimental settings can be therefore explained by different concentrations and biological properties of CTGF. In these conditions, further comparisons of the relative activity of recombinant and native secreted CTGF was not considered relevant to this experiment aiming only at evaluating whether VIC are able to respond to CTGF, which we proved to be the case.

This is now discussed p19 line 8-18 of the revised manuscript.

Graphical Abstract



Highlights

1. Mechanical stress activates RhoC promoting, via ROCK, pro-fibrotic genes expression.

2. Mechanical stress also activates MEK/Erk1/2, independently of RhoC and ROCK.

3. MRTFA translocates to the nucleus, a process dependent on RhoC and Erk1/2 activation.

4. Nuclear MRTFA associates with SRF to activate the transcription of pro-fibrotic genes.

Mechanical strain induces a pro-fibrotic phenotype in human mitral valvular interstitial cells through RhoC/ROCK/MRTF-A and Erk1/2 signaling pathways.

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Abstract

The mitral valve is a complex multilayered structure populated by fibroblast-like cells, valvular interstitial cells (VIC) which are embedded in an extracellular matrix (ECM) scaffold and are submitted to the mechanical deformations affecting valve at each heartbeat, for an average of 40 million times per year. Myxomatous mitral valve (MMV) is the most frequent heart valve disease characterized by disruption of several valvular structures due to alterations of their ECM preventing the complete closure of the valve resulting in symptoms of prolapse and regurgitation. VIC and their ECM exhibit reciprocal dynamic processes between the mechanical signals issued from the ECM and the modulation of VIC phenotype responsible for ECM homeostasis of the valve. Abnormal perception and responsiveness of VIC to mechanical stress may induce an inappropriate adaptative remodeling of the valve progressively leading to MMV. To investigate the response of human VIC to mechanical strain and identify the molecular mechanisms of mechano-transduction in these cells, a cyclic equibiaxial elongation of 14% at the cardiac frequency of 1.16 Hz was applied to VIC by using a Flexercell-4000T™ apparatus for increasing time (from 1h to 8h). We showed that cyclic stretch induces an early (1h) and transient over-expression of TGFβ2 and αSMA. CTGF, a profibrotic growth factor promoting the synthesis of ECM components, was strongly induced after 1 and 2 h of stretching and still upregulated at 8 h. The mechanical stress-induced CTGF up-regulation was dependent on RhoC, but not RhoA, as demonstrated by siRNA-mediated silencing approaches, and further supported by evidencing RhoC activation upon cell stretching and suppression of cell response by pharmacological inhibition of the effector ROCK1/2. It was also dependent on the MEK/Erk1/2 pathway which was activated by mechanical stress independently of RhoC and ROCK. Finally, mechanical stretching induced the nuclear translocation of myocardin related family factor MRTF-A which forms a transcriptional complex with SRF to promote the expression of target genes, notably CTGF. Treatment of stretched cultures with inhibitors of the identified pathways (ROCK1/2, MEK/Erk1/2, MRTF-A translocation) blocked CTGF overexpression and abrogated the increased MRTF-A nuclear translocation. CTGF is up-regulated in many pathological processes involving mechanically challenged organs, promotes ECM accumulation and is considered as a hallmark of fibrotic diseases. Pharmacological targeting of MRTF-A by newly developed inhibitors may represent a relevant therapy for MMV.

Key words: heart valve disease, MMV, mechanical strain, RhoGTPases, MRTF-A, CTGF/CCN2.

1. INTRODUCTION

The role of the heart valves is to ensure the unidirectional blood flow during the cardiac cycle by opening and closing motion. They possess the remarkable ability to withstand the mechanical forces they experience at each cardiac cycle: tension when closed to prevent retrograde flow, flexion during closing and opening, and shear stress due to blood flow [1]. Like the other cardiac valves, the atrio-ventricular mitral valve is a complex multilayered structure, each layer containing specific extracellular matrix (ECM[1]) components. The fibrosa, the thickest layer of the valve on the ventricular side, is mostly composed of dense and aligned collagen fibers that provide most of the tensile strength to the leaflet. The spongiosa, a glycosaminoglycans and proteoglycans enriched layer, provides mitral valve with its resistance to compression while the atrialis, a layer rich in elastin, confers its elasticity. Mitral valve is populated by fibroblast-like cells, named valvular interstitial cells (VIC), which are in close contact with the ECM and are therefore submitted to the mechanical deformations affecting valve at each heartbeat, for an average of 40 million times per year. The phenotype of VIC is regulated by the various signals issued from their environment and, in turn, VIC are responsible for the homeostasis and remodeling of the valvular ECM, forming intermingled interactive loops between the ECM properties, the mechanical constraints affecting valves and the phenotype of VIC [2].

Myxomatous mitral valve (MMV) is the most frequent heart valve disease, affecting 2-3% of the population[3]. Its prevalence increases markedly after the age of 65 years and it is becoming a health burden due to the current extending life expectancy in the industrialized countries [4]. MMV is characterized by disruption of several valvular structures (enlarged leaflet, annular dilatation, elongated/ruptured chordae), an increased density of Ki-67 positive proliferating cells and alterations of the ECM, notably an accumulation and disorganization of collagen fibers in the fibrosa, a fragmentation of elastic fibers, an expansion of the spongiosa by a loose ECM enriched in proteoglycans [5].

Modifications of the physiological valvular geometry linked to a progressive inappropriate ECM remodeling result in abnormal perception and responsiveness of VIC to mechanical stress

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[1] ***Abbreviations:*** *COL1A1 : α 1 chain of type I collagen; CTGF/CCN2: connective tissue growth factor; ECM: extracellular matrix; F-actin: fibrillary-actin; GAGs: glycosaminoglycans; G-actin: globular actin; GAP: GTPases activating proteins; GDI: guanine nucleotide dissociation inhibitors; GEF; guanine nucleotide exchange factors; GTP: guanosine tri-phosphate; MMP: matrix metalloproteinase; MMV: myxomatous mitral valve; MRTF-A: Myocardin related Transcription Factor A; PAI-1: plasminogen activator inhibitor 1; PGs: proteoglycans; α-SMA : α smooth muscle actin ; SMC: smooth muscle cells; SOD 1: superoxide dismutase 1; TIMP1: tissue inhibitor 1 of MMP; VIC: valvular interstitial cells.*

which, in turn, may lead to a vicious circle and contribute to perpetuation of the disease [2].These structural defects prevent the complete closure of the valve causing symptoms of prolapse and regurgitation which ultimately lead to heart failure and to a significant morbidity and mortality [6, 7]. Besides surgery for the severe forms of MMV, the medical therapies used in some patients aim at limiting the left ventricular remodeling but do not address the primary mechanism of mitral valve dysfunction.

Although MMV has been largely described at clinical, functional and histological levels, the cellular and molecular mechanisms underlying the pathological ECM remodeling are still elusive. Pathogenesis of MMV has been related to biological mediators such as serotonin [8, 9], angiotensin II [10] and activation of the TGFβ pathway [11, 12]. Mechanistic knowledge was also gained from studies of the syndromic forms of MMV, such as Marfan and Loeys-Dietz syndromes [13, 14] characterized by an activated TGFβ signaling. It is currently increasingly recognized that mechanical stress is one major etiological factor underlying soft connective tissue remodeling, including the pathological remodeling observed in MMV. Various in vitro and ex vivo studies have investigated the phenotypic response to mechanical loading of human and animal VIC. Altogether, they show an activation of VIC towards a SMC phenotype, an increased synthesis of PGs, GAGs and collagen, as well as increased expression and activity of proteolytic enzymes [15-19]. Most of these studies were however performed on cells and tissues from aortic valve. Extrapolating results from aortic to mitral valve seems only partly relevant since dysregulated aortic VIC tend to express an osteogenic-like phenotype while MMV are weakened due to a massive deposition of PGs and are characterized by the expression of cartilage markers [20] and a loss of mechano-competent layered structure.

Although these studies clearly demonstrated that mechanical stress plays a fundamental role in valvular ECM remodeling, the mechanisms of transduction of mechanical cues into biochemical signals that drive tissue remodeling have been little investigated in human mitral VIC. Our work aimed at identifying genes modulated by cyclic mechanical deformations and the downstream signaling pathways involved in their regulation. These data should provide a better understanding of the mechanisms driving MMV progression and potentially uncover new targets for pharmacological therapy.

2. MATERIAL AND METHODS

2.1 Cells and culture reagents

Isolation of interstitial cells (VIC) was performed by outgrowth from explants of posterior segment (P2) of human healthy mitral valves from 3 donors as previously described [11]. The study was approved by the Ethics Committee of Liege University Hospital (B70720071262). VIC cultures were amplified in EBM-2 medium containing 5% FBS (Lonza) at 37°C in 95% air, 5% CO2. Cells were used at passage 3–8 and displayed the typical elongated morphology with expanding thin cytoplasmic processes (Fig S1).

2.2 Reagents and chemicals:

Western-blot and immunohistology were performed with antibodies against CTGF (Santa Cruz, sc-14939), RhoA (Santa Cruz, sc-418), RhoC (Santa Cruz, sc-26480 and Cell Signaling Technology #3430), Rac1 (BD Biosciences 610651), MRTF-A (Santa Cruz, sc-398675), p-Erk1/2 (Sigma, M8159), Erk1/2 (Sigma, M-5670), α-tubulin (Sigma, T6199) and YAP (Santa Cruz H125). Secondary antibody Alexa Fluor 555 goat anti-mouse IgG (Invitrogen, A21424) was used for immunofluorescence and secondary horseradish peroxydase-conjugated rabbit anti-mouse (Dako, P0260), rabbit anti-goat (Dako, P0449) and swine anti-rabbit (Dako, P0217) were used for western-blot. Phalloïdin-FITC (Sigma, P-5282) was used for staining fibrillar actin and DAPI (Life Technologies, D1306) for nuclear staining. Recombinant human CTGF was from Gibco (PHG0286). Primers used for qRT-PCR are listed in supplemental material (Table S1). The following pharmacologic inhibitors were used: Y27632 (Selleckchem, S1049), a potent and selective inhibitor of ROCK1/2 competing with ATP for binding to the catalytic site [21]; U0126 (Calbiochem, 662005), a specific inhibitor of MEK1 and MEK2 antagonizing the AP-1 transcriptional activity [22]; CCG-1423 (Calbiochem, 555558), an inhibitor preventing the binding of importin $\alpha/\beta 1$ to MRTFA and its nuclear translocation [23].

2.3 Cyclic stretching of VIC

150 000 cells were seeded in EBM-2 medium supplemented with 2% FBS in a 2 cm Ø glass ring placed in the center of the well (to ensure a homogeneous deformation) of a 6-well Bioflex plate with flexible silicone rubber bottoms coated with type I collagen (Flexcell International Corp). After 24 hours of attachment, cells were starved for 24 hours in EBM-2 medium supplemented with 0.1% FBS. They were then exposed to continuous cycles of equibiaxial stretch (14%) /relaxation for indicated times at the cardiac frequency of 70 cycles/minute by a computer-assisted vacuum pump (Flexercell 4000™, Flexcell International Corp.). When indicated, pharmacologic inhibitors were added 1 hour before stretching.

2.4 CTGF-treated VIC

Subconfluent monolayers of VIC on plastic were treated with 30 ng/ml recombinant human CTGF for 48h and then lyzed for qRT-PCR.

2.5 siRNA transfection

21-nucleotides long siRNAs (desalted, deprotected and PAGE purified) were from Eurogentec (Liège, Belgium). The sequences of the siRNAs targeting RhoA (siRhoA), RhoC (siRhoC), Cdc42 (siCdc42) and Rac1 (siRac1) and that of the control siRNA (siScr) were designed in our laboratory and validated for their specificity and efficacy [24, 25]. They are listed in supplemental material (Table S2). Each pair of oligoribonucleotides was annealed at a concentration of 20 µM in 50 mM NaCl, 1mM EDTA, 10 mM TrisHCl pH 7.5. Calcium phosphate mediated transfection of siRNAs was performed overnight (14-16 h) on subconfluent cells at a final concentration of 20 nM. Cells were washed twice with PBS and maintained in EBM-2 medium containing 5% FBS. After 24 hours, cells were trypsinized and seeded on the Bioflex plates, this step being defined as time 0 post-transfection.

2.6 RNA extraction and purification

Total RNA was purified from VIC cultures using the High Pure RNA isolation kit (Roche Molecular Biochemical) following the manufacturer's instructions and quantified by a Nanodrop 1000 spectrophotometer (Isogen Life Science).

2.7 Real time quantitative PCR

1 µg of total RNA was reversed transcribed using SuperScript III Reverse Transcriptase (Invitrogen) and oligodT. Real time qPCR was performed in a final volume of 25 µl containing 2.5 µl of cDNA (corresponding to 10 ng of total RNA), 300 nM of each primer and 12.5 µl of the qPCR MasterMix Plus for SYBR® green (Eurogentec) in the StepOne™ Real-Time PCR system (Applied Biosystems). The results were analyzed with the StepOneTM Software and normalized to the GAPDH transcript using the $2^{\Delta\Delta Ct}$ method. Results are expressed relative to the values measured in control static samples taken as 1.

2.8 Proteins extraction and western-blot

Cells were lysed in Laemmli buffer and the extracts were denatured at 90°C. Proteins were separated by SDS-PAGE and transferred to a PVDF Transfer Membrane (NEN Life Science Products). Membranes were then blocked for 1 hour with 3 % dry milk in PBS-0.05 % Tween 20 and incubated overnight with the primary antibody. Membranes were then washed three times, incubated with the secondary horseradish peroxydase-conjugated antibody for 1 h, and revealed by using the ECL kit (Amersham Biosciences). Western blots were quantified with

ImageQuant™ LAS 4000 and reported to αTubulin used as control protein loading. Results are expressed relative to the values measured in static non-treated VIC taken as 1.

2.9 RhoC activity assay

The assay was carried out as previously described [26]. Briefly, cells were chilled on ice and lysed in ice-cold buffer containing 0.5% Triton X-100, 25 mM HEPES pH 7.3, 150 mM NaCl, 5mM MgCl2, 0.5 mM EGTA, 4% glycerol, 0.1 mM AEBSF, 4 µg/ml aprotinin. Lysates were centrifuged for 6 minutes at 20000 g. For each time point, the lysates from 6 Flexercell wells were pooled to securely measure the active RhoC. A small aliquot of each supernatant was immediately denatured in SDS-PAGE lysis buffer to measure the total RhoC content by Western blotting and the supernatants were immediately frozen in liquid nitrogen and stored at -80°C until use for pull-down assays. Supernatants were incubated for 30 minutes with 30 µg of GST-RBD protein containing the RhoC binding region of Rhotekin affinity linked to glutathione-Sepharose beads. The beads were washed 4 times in lysis buffer and boiled in 60µl SDS-PAGE lysis buffer. One third of the lysate was separated on SDS-PAGE gel and the RhoC content, corresponding to the active RhoC, was measured by Western blotting.

2.10 MRTF-A nuclear translocation

After cyclic stretching for 30 min, cells were washed with PBS and fixed in the Bioflex wells for 10 min in 4% paraformaldehyde at room temperature. After washing with PBS, cells were permeabilized for 5 min at room temperature with 0.01% Triton X-100 in PBS. After blocking non-specific sites with 5% BSA in PBS, cells were incubated with an anti-MRTF-A monoclonal antibody (1/50) in 1% BSA/PBS overnight at 4°C. Cells were then incubated with Phalloïdin-FITC (2 µg/ml) in 1% BSA/PBS for 1 hour and then with a secondary antibody Alexa Fluor 555 goat anti-mouse IgG (1/1000) in 1% BSA/PBS for 1 hour. Finally, cells were incubated with DAPI (2.5 µg/ml) for 5 min at room temperature to stain nuclei and mounted with Aqua-Poly/Mount Coverslip Medium (18600; PolySciences). Cells were analyzed by using Nikon Eclipse Ti microscope and the NIS-Elements Imaging Software. The total fluorescence was measured in each cell delimited by the FITC-phalloïdin staining of the cortical actin and the nuclear fluorescence (nf) measured in the nucleus delimited by the DAPI staining. Between 29 and 58 cells were analyzed per condition. The nuclear translocation of MRTF-A was expressed as the percentage of fluorescence associated with the nucleus and cells were distributed into 3 categories: nf$\leq$10%; 20%$\geq$nf>10%; nf>20%.

2.11 Statistics:

Data were analyzed by using GraphPad Prism 5.0 and were presented as the mean $\pm$ SEM. Statistical analysis was performed by using the Student's t-Test for unpaired samples and the one sample t-Test when indicated.

3. RESULTS

3.1 Cyclic stretch increases the expression of TGFβ2, αSMA and CTGF/CCN2

A cyclic mechanical strain was applied to subconfluent VIC plated onto flexible silicone rubber membranes coated with type I collagen (Bioflex) by using a Flexercell-4000T™ apparatus. Preliminary experiments using various conditions of cell density seeding, culture medium composition, pre-treatment, stretching amplitude, frequency and duration were performed. After seeding, spreading and starvation as detailed in Materials and Methods, VIC were submitted to a cyclic equibiaxial elongation of 14%, a stretching amplitude remaining in the physiological range, at the cardiac frequency of 1.16 Hz for 1, 2, 4 or 8 h in medium supplemented with 0.1% FBS. Control cultures were handled in parallel except for the mechanical stretching that was omitted (static cultures). Based on previous results from our laboratory [11] and data from the literature [for review, see 27], the expression of a panel of genes relevant to MMV and mechanobiology was investigated by qRT-PCR.

While the expression of TGFβ1 was not modified by stretching at any time point (Fig 1A), the mRNA level of TGFβ2 and αSMA was significantly increased after 1 hour of stretching and then returned to values measured in static cultures (Fig 1B and C).

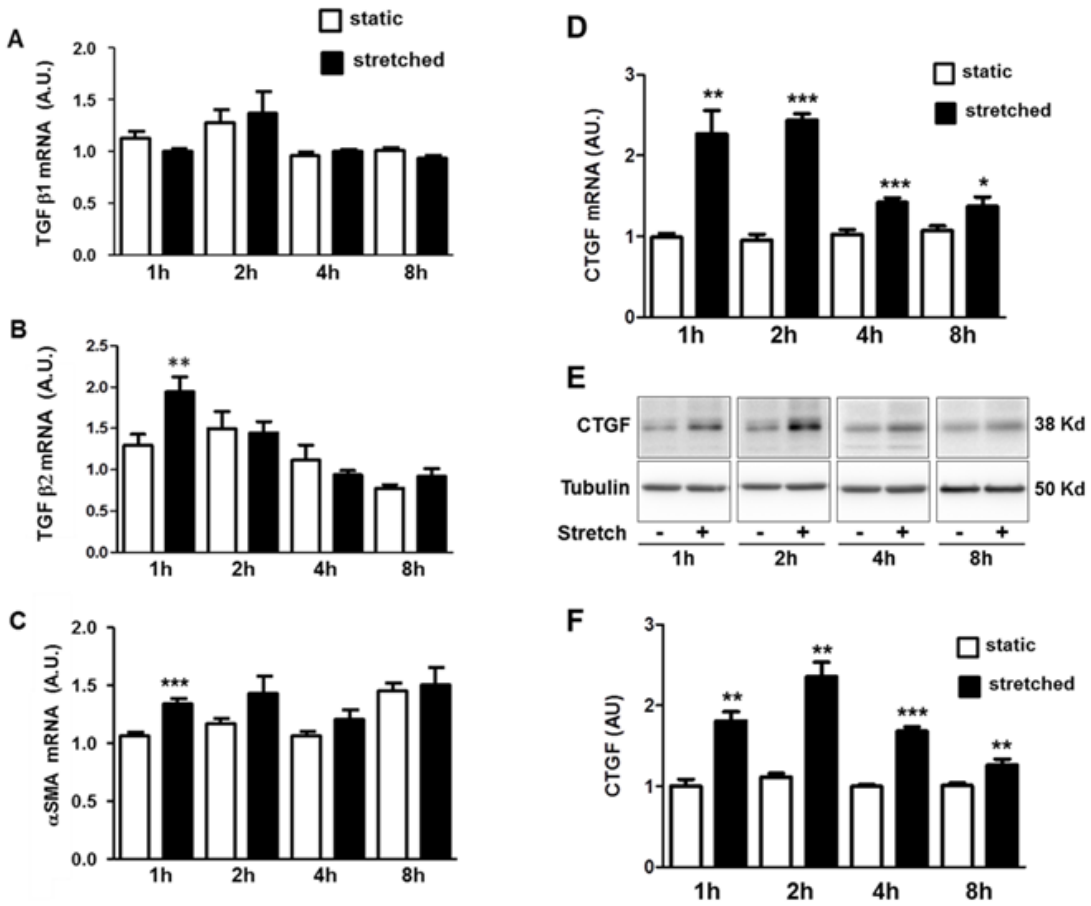

Fig 1: Mechanical stretching induces TGFβ2, αSMA and CTGF overexpression*: Expression of TGFβ1 (A), TGFβ2 (B), αSMA (C) and CTGF (D) was measured by qRT-PCR in VIC submitted to mechanical stretching or kept in control static conditions for increasing time. Results are expressed in arbitrary units (A.U.) relative to GAPDH taking static cultures at 1h as 1 (n=5 to 11). Representative western-blot of the production of CTGF in static (-) and stretched (+) VIC for increasing time using tubulin as protein load control (E). Amount of CTGF produced by VIC submitted to mechanical stretching compared to control static cultures for increasing time. Results are expressed in arbitrary units (A.U.) relative to tubulin taking static cultures at 1h as 1 (F) (n=3 to 6). * p<0.05; ** p< 0.005, ***p<0.0005.*

CTGF/CCN2 is a matricellular protein. Acting as a growth factor, it promotes ECM accumulation and is involved in many fibrotic diseases. In our experimental model, a very significant stimulation of CTGF expression was already observed at the mRNA level after 1 and 2 h of stretching (Fig 1 C) and persisted until 4 and 8 h with a progressive decline. The protein production paralleled the mRNA level with a slight delay as shown by western-blot (Fig 1E & 1F). These data clearly identify CTGF as a specific mechano-responsive marker useful to investigate the molecular mechanisms driving the response of VIC to mechanical deformations.

Among the other tested genes, TGFβ3, COL1A1, SOD1 and MMP1, none was found significantly modulated in our experimental conditions (Table S3).

3.2 The small RhoGTPase RhoC, but not RhoA, is involved in the mechanical stress-induced CTGF up-regulation

The small GTPases of the RhoA family are crucial molecular switches involved in the transduction of mechanical signals issued from the extracellular environment and the cytoskeleton dynamics. We investigated the involvement of RhoA, RhoC, Rac1 and Cdc42 in the mechanical stress-induced up-regulation of CTGF by silencing their expression with siRNAs [21, 22]. SiRNA for RhoA, RhoC and Rac1 efficiently and specifically silenced their target (Fig 2A). Cells transfected with siCdc42 stopped proliferating, detached from their support and were not further used.

In static cells, the basal level of CTGF was reduced by silencing Rac1 and RhoA, while repressing RhoC had no effect (Fig 2B, open bars). We then compared the effect of cyclic stretching (black bars) to static cultures in each condition. In control cells (siScr), the expression of CTGF was increased upon cyclic stretching, as expected (Fig 2 B). Although the basal CTGF expression was reduced in siRac1 and siRhoA transfected VIC, its fold induction by stretching was similar to siScr cells, or even slightly higher, indicating that Rac1 and RhoA are not involved in the stretch-induced up-regulation of CTGF. By contrast, cells transfected with siRhoC did not respond suggesting a specific implication of RhoC in the regulations triggered by mechanical stretching (Fig 2 B).

To further support the role of RhoC in the transduction of the mechanical signals, we performed a pull-down assay which allows measuring the GTP-linked active RhoC. As shown in figure 2C and D, an increased proportion of active RhoC was observed after 10 min of stretching. Y27632 is a specific and potent inhibitor of ROCK1/2 [21], a main effector of RhoA and RhoC acting on downstream pathways involved in actin polymerization. It did not modify the basal expression of CTGF in static conditions but suppressed the stress-induced CTGF up-regulation, both at the mRNA (Fig 2 E) and protein level (Fig 2 F&G). Since RhoA is dispensable in this process (Fig 2B), this further confirms the participation of RhoC in the response of VIC to mechanical signaling.

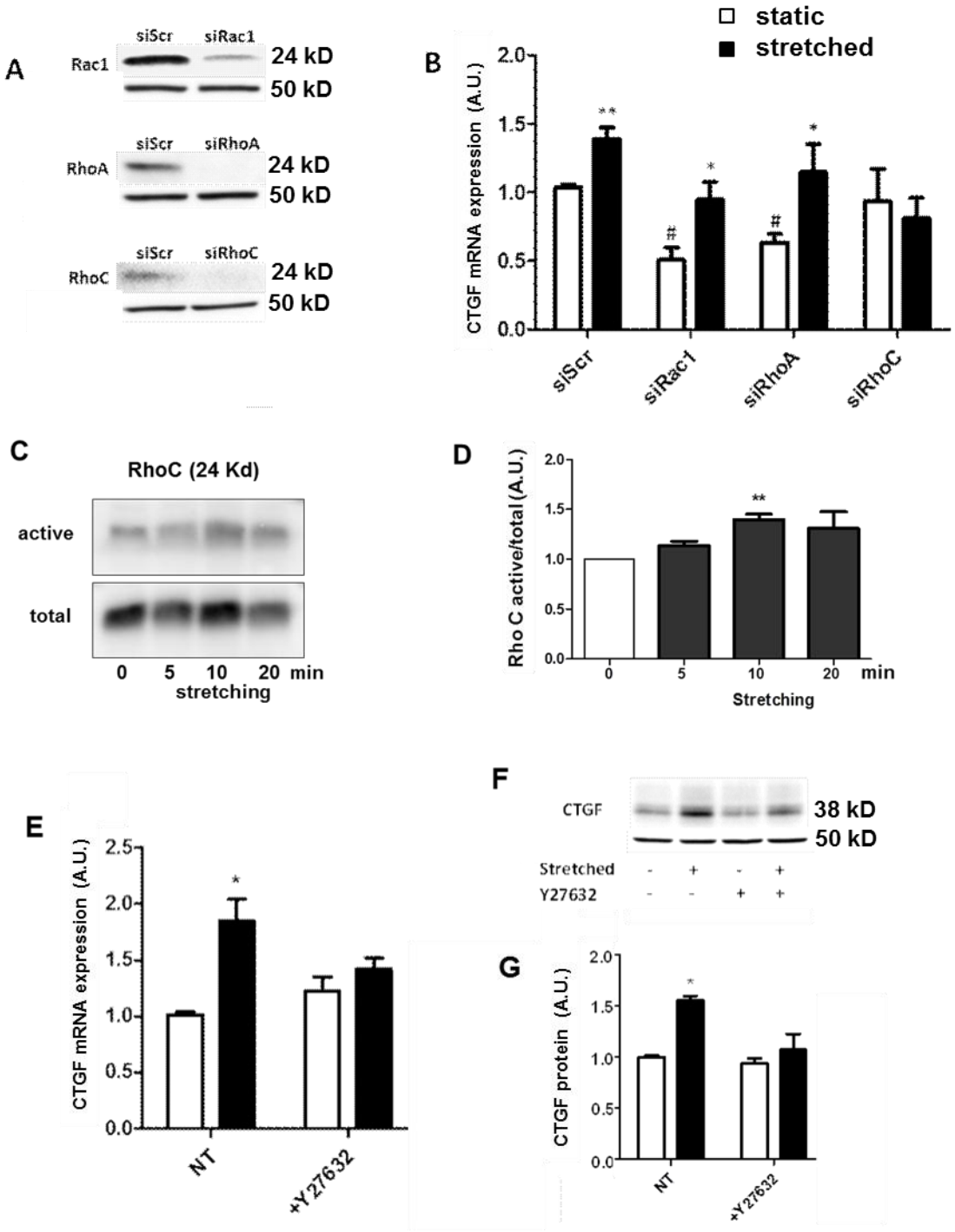

Fig 2: **RhoC regulates mechanical stress-induced CTGF expression through ROCK1/2.** *VIC were transfected with siRNAs targeting Rac1 (siRac1), RhoA (siRhoA) or RhoC (siRhoC) or with an irrelevant scramble siRNA (siScr) used as a negative control. The efficacy of silencing was evaluated by western-blot using antibodies specific to each GTPase. Alpha-tubulin (Tub) was used as protein loading control (A). CTGF expression was measured by qRT-PCR in transfected cells submitted to mechanical stretching for 4h or in static cultures. Results are expressed in*

*arbitrary units (A.U.) relative to GAPDH taking Siscr-static cultures as 1 (n= 3 to 5 separate experiments, each performed in triplicate; # p< 0.01, siRhoA or siRac1 vs siScr; ** p< 0.01, * p< 0.05, stretched vs static) (B). The activation of RhoC was measured by pull-down assay in VIC stretched for 5, 10 and 20 min (C). Active RhoC is expressed in arbitrary units (A.U.) relative to total RhoC in each condition (n=3) **p<0.01 (D). CTGF expression was measured by qRT-PCR in VIC non-treated (NT) or pretreated with Y27632 (5 µM), an inhibitor of of the effector of RhoC, ROCK1/2, and stretched for 4h (E). Representative CTGF western-blot of VIC static or stretched and treated or not by Y27632. Alpha-tubulin (Tub) was probed as control for protein loading (F). Quantification of the CTGF western-blots expressed in arbitrary units (A.U.) relative to alpha-tubulin taking static non-treated (NT) cultures as 1 (n=3 separate experiments performed in duplicate, * p= 0.02) (G).*

3.3 The MEK/Erk pathway is activated by mechanical stress, independently of RhoC and ROCK, and participates in the CTGF up-regulation.

Extracellular signal–regulated kinases (Erk1/2) were evaluated in our experimental model since they are key secondary messengers for many signals issued from the extracellular environment. A rapid and transient phosphorylation of Erk1/2 was observed already after 5 minutes of mechanical stretching (Fig 3A) and returned to basal values after 15 min (not shown). Inhibition of MEK, an upstream activator of Erk1/2, by U0126 [22] efficiently suppressed the phosphorylation of Erk1/2 in VIC. While this inhibitor did not modify the basal level of CTGF in static conditions, it completely abolished its mRNA and protein overexpression upon cyclic stretching (Fig 3B-D).

To evaluate whether the activation of the MEK/Erk pathway was depending on the RhoC/ROCK pathway, the phosphorylation of Erk1/2 was measured in VIC transfected with siRhoC and submitted to mechanical stretching for 10 min. As shown in fig 3E & F, the level of Erk1/2 phosphorylation was similar in siRhoC and siScramble transfected cells under stretching conditions. In accordance, treatment of stretched VIC with Y27632, a ROCK inhibitor, did not impair the activation of Erk 1/2 (Fig 3G and H). These results demonstrate that Erk1/2 pathway activation by mechanical stress is Independent of the RhoC/ROCK axis.

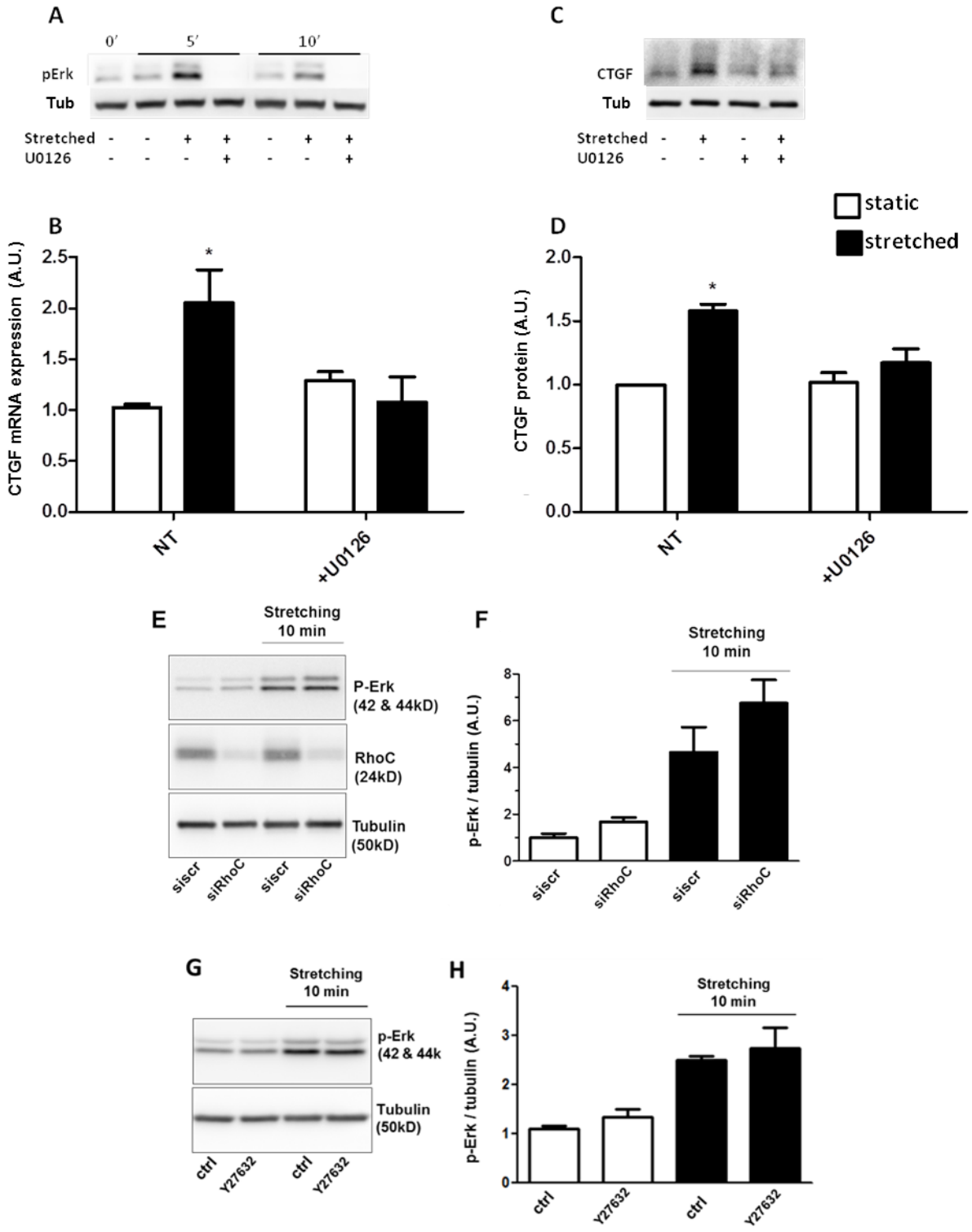

Fig 3: MEK/Erk1/2 signaling is activated by mechanical stress, independently of RhoC and ROCK, and participates to the stress-induced CTGF expression. *Representative western blot of phospho-Erk1/2 (pErk) in VIC submitted (+) or not (-) to cyclic stretching for 5 and 10 min in presence (+) or absence (-) of U0126 (10 µM), a MEK inhibitor (A). CTGF mRNA expression in*

*static and stretched cultures for 4 h in absence (NT) and presence of U0126. Results are expressed in arbitrary units (A.U.) relative to GAPDH taking non-treated (NT) static cultures as 1 (B). Representative western blot of CTGF in static (-) and stretched VIC for 4 h (+) in absence (-) and presence (+) of U0126 (C). Quantification of the Western-blots for CTGF expressed in arbitrary units (A.U.) relative to alpha-tubulin (Tub) taking static non-treated (NT) cultures as 1 (D). Representative Western blot of phospho-Erk1/2 (p-Erk) in VIC transfected by a siRNA control (siScr) or a siRNA targeting RhoC (siRhoc), static or submitted to cyclic stretching for 10 min. The silencing of RhoC is shown in the second row (E). Quantification of p-Erk expressed in arbitrary units (A.U.) relative to alpha-tubulin taking the static-si Scr transfected VIC as 1 (F). (n= 3 separate experiments performed in duplicate, * p< 0.05). Representative Western-blot of phospho-Erk1/2 (p-Erk) in VIC static or submitted to cyclic stretching for 10 min in absence (ctrl) or presence of Y27632, a ROCK inhibitor (G). Quantification of p-Erk expressed in arbitrary units (A.U.) relative to alpha-tubulin taking the static non-treated VIC as 1 (H) (n=3 experiments).*

3.4 Cyclic stretch induces the nuclear translocation of MRTF-A

As RhoGTPases/ROCK1/2 are crucial regulators of the dynamics of actin-based cytoskeleton, we hypothesized that mechanical stretching might stimulate the formation of fibrillar actin by recruiting globular actin molecules, which would result in the release of MRTF-A (myocardin-related transcription factor A) from its complex with globular actin. Once free in the cytoplasm, MRTF-A is translocated into the nucleus where it associates with SRF to activate the transcription of target genes, notably CTGF. This process was evaluated by immunostaining of MRTF-A in static and stretched cells (Fig 4A) and quantification of the nuclear labeling relative to the total fluorescence of the cell (Fig 4B).

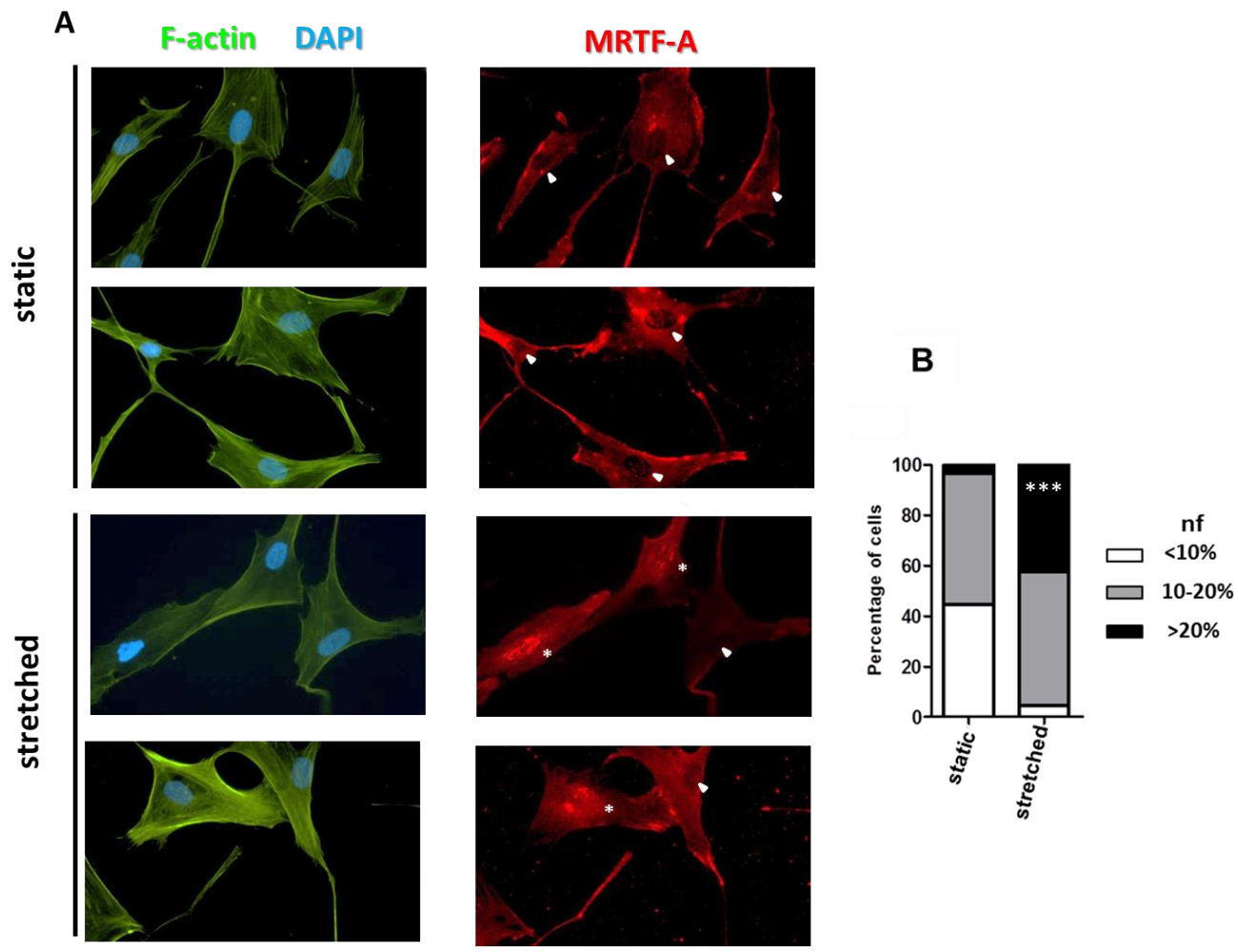

Fig 4: Cyclic-stretch induces MRTF-A nuclear translocation. *Representative staining of actin fibers by FITC-phalloïdin (green), nuclei by DAPI (blue) (left panel), and immune staining of MRTF-A (red, right panel) in static and stretched VIC for 30 min. Nuclei with a low MRTF-A fluorescence are pointed by an arrowhead and those with a strong MRTF-A fluorescence by an asterisk (A). Distribution of cells (in %) according to the nuclear fluorescence (nf) staining for MRTF-A relative to the total fluorescence of the cell (%) measured on 58 (static) and 45 (stretched) cells in 4 separate experiments (***p<0.001) (B).*

In static cultures, more than 40 % of cells had a nucleus containing very low amount of MRTF-A (<10 % of the total fluorescence detected per cell) and only 3 % of cells were found to have stronger nuclear labeling (>20%). Upon mechanical stretching, the percentages were completely reversed evidencing the nuclear translocation of MRTF-A.

In line with our hypothesis, CCG1423, an inhibitor of the MRTF-A nuclear translocation [23], suppressed the CTGF up-regulation in stretched cells (Fig 5A, B, C) without affecting its basal expression in static cultures.

Treatment of stretched cultures with the 3 inhibitors shown to block CTGF expression (Y-27632, CCG1423, U0126) effectively abrogated the increased MRTF-A nuclear translocation (Fig 5D & E).

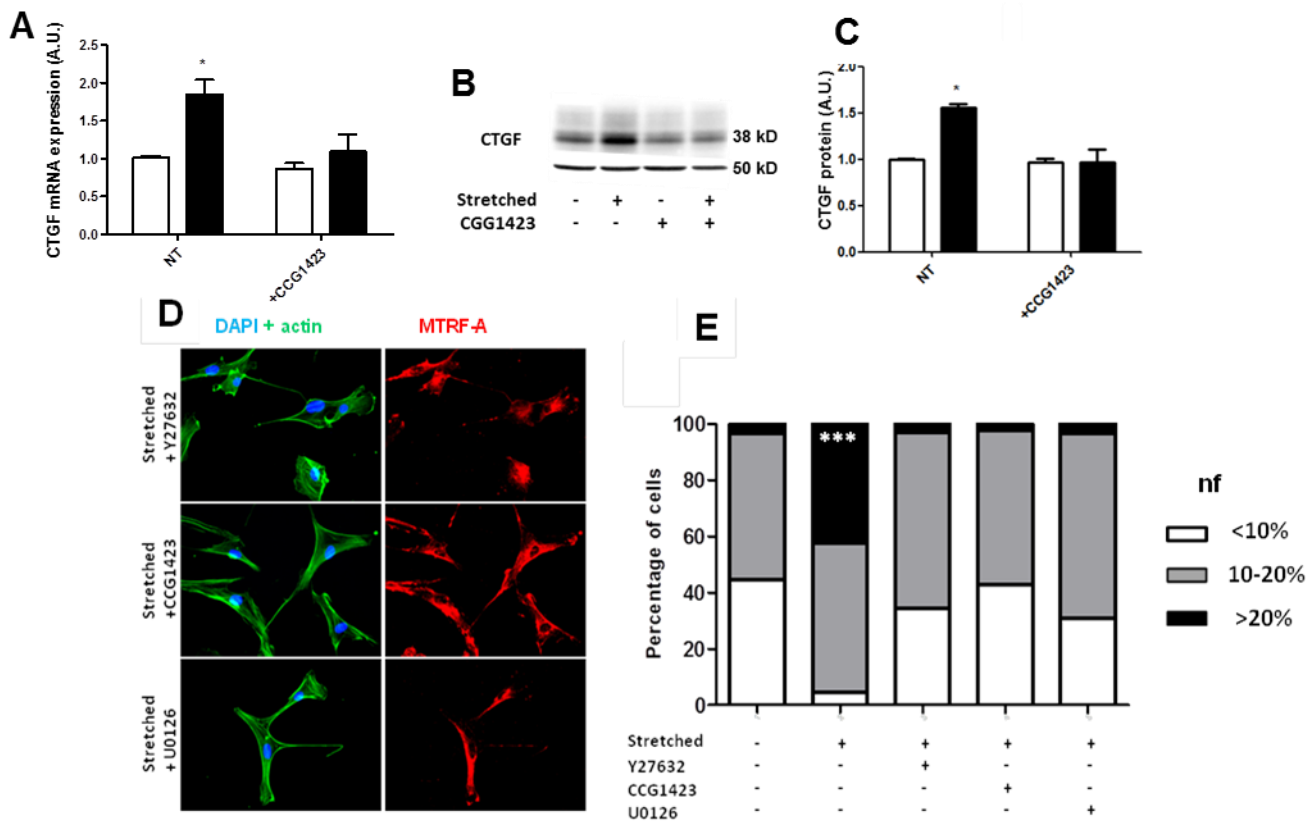

Fig 5. Inhibition of MRTF-A translocation abrogates stretching-induced CTGF overexpression. *CTGF expression was measured by RT-qPCR in VIC kept static or stretched for 4h after pretreatment or not with CCG1423 (2 µM), an inhibitor of MRTF-A nuclear translocation. Results are expressed in arbitrary units relative to GAPDH taking the non-treated (NT) static VIC as 1 (A). Representative western blot of CTGF in the same conditions (B) and its quantification relative to alpha-tubulin (Tub) expressed in arbitrary units taking the static non-treated (NT) VIC as 1(C) (n=3 separate experiments performed in duplicate, * p≤ 0.05). Representative staining by FITC-phalloïdin to label actin fibers (green), DAPI to label nuclei (blue) (left panel), and MRTF-A (red, right panel) in VIC stretched for 30 min after pretreatment with the ROCK1/2 inhibitor Y27632 (5 µM), with CCG1423 (2 µM) or U0126 (10µM), an inhibitor of MEK/Erk1/2 (D). Distribution of cells*

(in %) according to the nuclear fluorescence (nf) staining for MRTF-A relative to the total fluorescence of the cell (%) measured on 32 (Y27632), 42 (CCG1423) and 29 (U0126) cells in 4 separate experiments. The two first bars on the left showing static and stretched VIC without any treatment are reproduced from figure 4B (E).

3.5 CTGF induces the expression of extracellular matrix genes

To evaluate if VICs can respond to CTGF, monolayer cultures were treated with recombinant human CTGF. After 48h of treatment, the expression of COL1A1, a known CTGF target gene, and of fibromodulin was significantly up-regulated (Fig 2A & B).

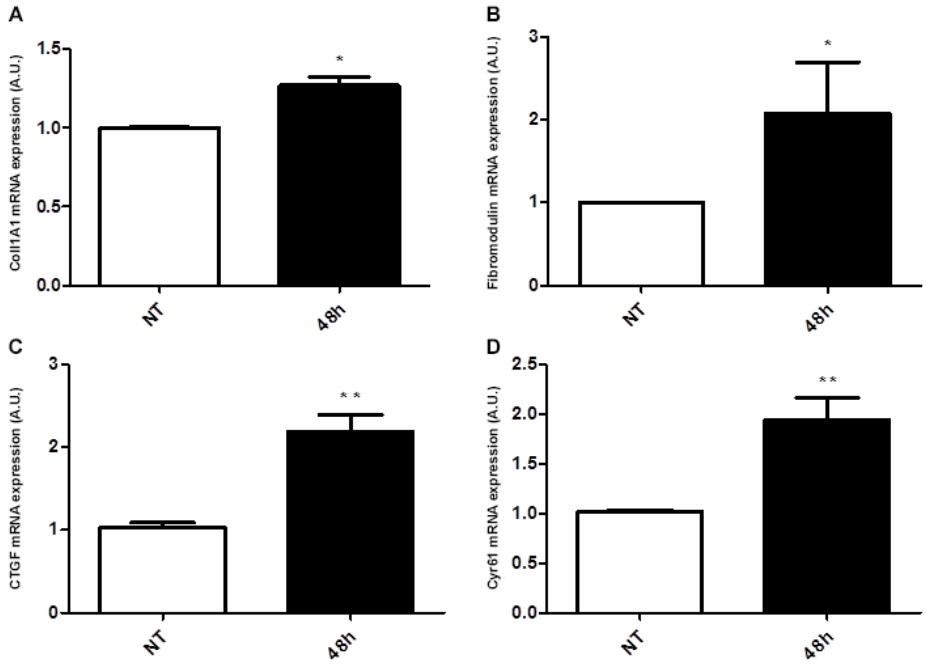

Fig 6: CTGF promotes ECM genes and its own expression. *Expression of COL1A1 (A), fibromodulin (B), CTGF (C) and Cyr61 (D) was measured in VIC treated with human recombinant CTGF (30ng/ml) for 48h. Results are expressed in arbitrary units (A.U.) relative to GAPDH taking the non-treated (NT) VIC as 1. (n=3 independent experiments performed in triplicate) (* p≤ 0.05, ** p≤ 0.01).*

Interestingly, CTGF also induced its own expression and the expression of Cyr61/CCN1, another member of the CCN family (Fig 5 C & D). Other genes potentially modulated by CTGF were tested, such as PAI-1, TIMP1 and proteoglycans such as versican, biglycan, lumican and decorin. None of them was modulated in our experimental setting showing specificity in the response of VIC to CTGF (Table S4).

4. DISCUSSION

In order to investigate the influence of mechanical strain on the VIC phenotype and to relate it to the pathogenesis of MMV, we used a model of cyclic equibiaxial strain. Although this model does not reconstitute the complex 3D mechanical environment of the valve in vivo, it allows deciphering the cascade of signaling events triggered by mechanical deformation. Silicone membranes coated with collagen were used since data from the literature show that mitral VIC adhere to collagen through the integrin $\alpha 2\beta 1$ and that this coupling drives VIC deformation when the valvular tissue is strained [28]. We performed preliminary experiments to define the optimal regimen of deformation (14%) at the cardiac frequency for maximum induction of several genes in human VIC in our experimental setting while remaining below supra-physiological range [29].

Mechanical strain has been shown to activate the TGFβ pathway in many cell types including VIC [30, 19]. Furthermore, myxomatous degeneration of the mitral valve is a constant feature in patients suffering from connective-tissues disorders related to enhanced TGF-β signaling, such as Marfan and Loeyts-Dietz syndromes [31, 14]. Our starting experimental strategy was therefore to check any regulation of the TGFβ pathways and its potential target genes. While TGFβ1 is the most frequently reported up-regulated or activated member of the TGFβ family by mechanical stimuli, we found here that only TGFβ2 was significantly up-regulated in stretched VIC. This up-regulation was rapid and transient. This finding is consistent with our previous in vivo study showing an increased TGFβ2 expression and deposition in human non-syndromic MMV leaflets [11], data confirmed later by Thalji [32]. It is worth noting that the activation of the TGFβ–dependent Smad 2/3 effector was not increased in our stretched VIC (data not shown). α-SMA is one of the target genes of the TGFβ pathway and is considered as a marker of the phenotypic activation and transition of fibroblasts into biosynthetically active myofibroblasts. Its induction by mechanical strain has been reported in several studies [33] mainly performed on aortic valvular cells or tissues [34,35]. In our experimental conditions, the expression of α-SMA was also rapidly and transiently increased similarly to that of TGFβ2.

We showed here that CTGF was significantly up-regulated in VIC upon stretching. CTGF is a member of the CCN genes family. It is a regulatory matricellular protein involved in the control of a large variety of important biological functions, notably ECM macromolecules synthesis, and most often acts as a downstream effector of TGFβ. It is recognized as mechano-responsive gene acting through integrin-mediated signaling [36, 37]. It is up-regulated in many pathological processes involving mechanically challenged organs where it promotes ECM accumulation, explaining why it is considered as a hallmark of fibrotic diseases [38]. In agreement with our findings in this study, CTGF expression was found significantly increased in human MMV tissues and could contribute to the ECM remodeling featuring this disease [39, 40]. The capacity of

CTGF to induce its own expression and to act as an autocrine growth factor has been shown in fibroblasts [41, 42]. When tested by RT-PCR in static monolayer culture, CTGF stimulated its own expression also in VIC. The expressions of type I collagen and fibromodulin, a small rich-leucine proteoglycan regulating collagen fibrillogenesis, were also induced while other proteoglycans, such as decorin, biglycan or versican, were not significantly modified, showing that VIC actually respond to CTGF in a quite specific way that does not involve a general activation of transcription. However, we did not observe overexpression of collagen or fibromodulin upon stretching, although CTGF expression was increased. Several hypotheses can be made to explain such apparent discrepancy. Upon stretching, CTGF being only progressively overerexpressed and secreted in a large volume of culture medium, its concentration does not reach that used for recombinant CTGF. This does not mean, however, that CTGF could not play a significant role in vivo where local concentrations of factors secreted in the extracellular microenvironment are much higher than those obtained in vitro. A second explanation relates to CTGF itself. The only source of recombinant CTGF is a 11 kDa biologically active peptide, of undisclosed sequence and location within the full size protein. By contrast, endogenous CTGF is secreted by VIC as a 38 kDa full size protein that can be further modified or cleaved, processes known to modulate its activity [37].

Small GTPases of the Rho family are known to play a key role in the integrin-mediated transduction of mechanical signals into activation of intracellular signaling pathways and remodeling of the cytoskeleton [43, 44]. By using very specific siRNA tools targeting each of the archetypal Rho family members, we pointed out RhoC as the RhoGTPase involved in the transduction of mechanical stimuli in human VIC. The specific role of RhoC was further supported by evidencing its activation. While RhoA has been shown to contribute to the mechano-transduction and phenotypic regulation in valvular cells, mainly of aortic valve origin, it is to our knowledge the first report of the implication of RhoC and not RhoA, in this process probably because of the very specific molecular tools that we used. Although some studies suggest the redundant roles between RhoA and RhoC, their different modulation by the numerous regulatory factors, GEFs, GAPs and GDI, strongly suggests that they play also context- and localization-dependent distinct roles [45, 46]. ROCK is a downstream effector of RhoA and RhoC. We have demonstrated that its inhibition (using Y27632) abolished the cyclic stretching-dependent upregulation of CTGF expression, similarly to what is observed upon siRhoC depletion. Remarkably, Y27632 does not modify the “basal” expression of CTGF observed in static condition, while siRhoA represses it. These data suggest that RhoC regulates cell response to mechanical deformation through ROCK, while RhoA would participate to the maintenance of a basal level of CTGF expression via another effector [45].

Among the intracellular signaling pathways initiated by mechanical signals, the HIPPO effectors YAP and TAZ are involved in mechano-transduction and translocate to the nucleus in Rho- and actomyosin tension-dependent manner [47]. We did not observe any increase in nuclear labeling of YAP in stretched VIC (data not shown) excluding therefore this pathway in our experimental setting. These data are in line with recent publications reporting a functional link between YAP and RhoA specifically [48]. We then investigated the alternative pathway of the myocardin related family factors, MRTF A/B, which play a critical role as co-factor of SRF-dependent gene transcription, including CTGF [49]. MRTF-A resides mainly in the cytoplasm as a complex with G-actin. Upon RhoGTPases activation, G-actin is recruited to polymerize into F-actin liberating MRTF-A which is transiently translocated to the nucleus [50]. Nuclear MRTF-A forms a transcriptional complex with SRF to promote the expression of target genes, such as CTGF [51]. It is worth noting that CTGF, TGFβ2 and αSMA possess SRF binding sites in their promoter. Two complementary approaches, pharmacological inhibition of MRTF-A and measurement of its nuclear translocation, allowed us to clearly establish that this pathway was triggered in VIC by mechanical strain. In support of its potential involvement in MMV remodeling, MRTF-A loss-of-function mice have been shown to be resistant to cardiac, skin and lung fibrosis [52]. We were however unable to reproducibly evidence an increased ratio of fibrillar/globular actin in stretched VIC. This may be related to the very short and transient timing of actin polymerization that we could not detect in the experimental configuration of the Flexercell.

The MEK/Erk1/2 pathway is another mechano-responsive pathway that we found effectively activated in stretched VIC but independently of the RhoC/ROCK axis. It was previously shown that Erk-mediated phosphorylation of MRTF-A at Ser_{98} inhibits the binding of G-actin on the MRTF-A regulatory RPEL domain, which promotes its nuclear import [48]. This mechanism was not further investigated in our study due to the unavailability of the antibody recognizing p-Ser_{98}. However, the existence of such mechanism in our model is supported by the observation showing that pharmacological inhibition of the MEK/Erk1/2 pathway resulted in the abrogation of stretch-induced MRTF-A nuclear translocation and CTGF up-regulation. It is interesting to note that increased p-Erk 1/2 was reported in mitral leaflets of patients with fibroelastic deficiency [5].

Our results show for the first time the central role of the axis RhoC/ROCK/MRTF-A which, together with MEK/Erk1/2, regulates the induction of a pro-fibrotic phenotype in mitral valvular interstitial cells by mechanical strain. A series of MRTF-A inhibitors have been recently developed and proved to be potential new therapeutics for treating fibrotic diseases such as scleroderma [54]. In view of our original data, it would be worth investigating if the pharmacological treatment of MMV could be another therapeutic application of these inhibitors.

Aknowledgements
We thank T. Heyeres and L. Vanoorschot for skillful technical assistance.

Funding
This work was supported by J. Grommersch Award and Prodex/Belgian Science Policy. AC is Senior Research Associate and CD is Research Associate of the Belgian FRS-FNRS.

Disclosures
None

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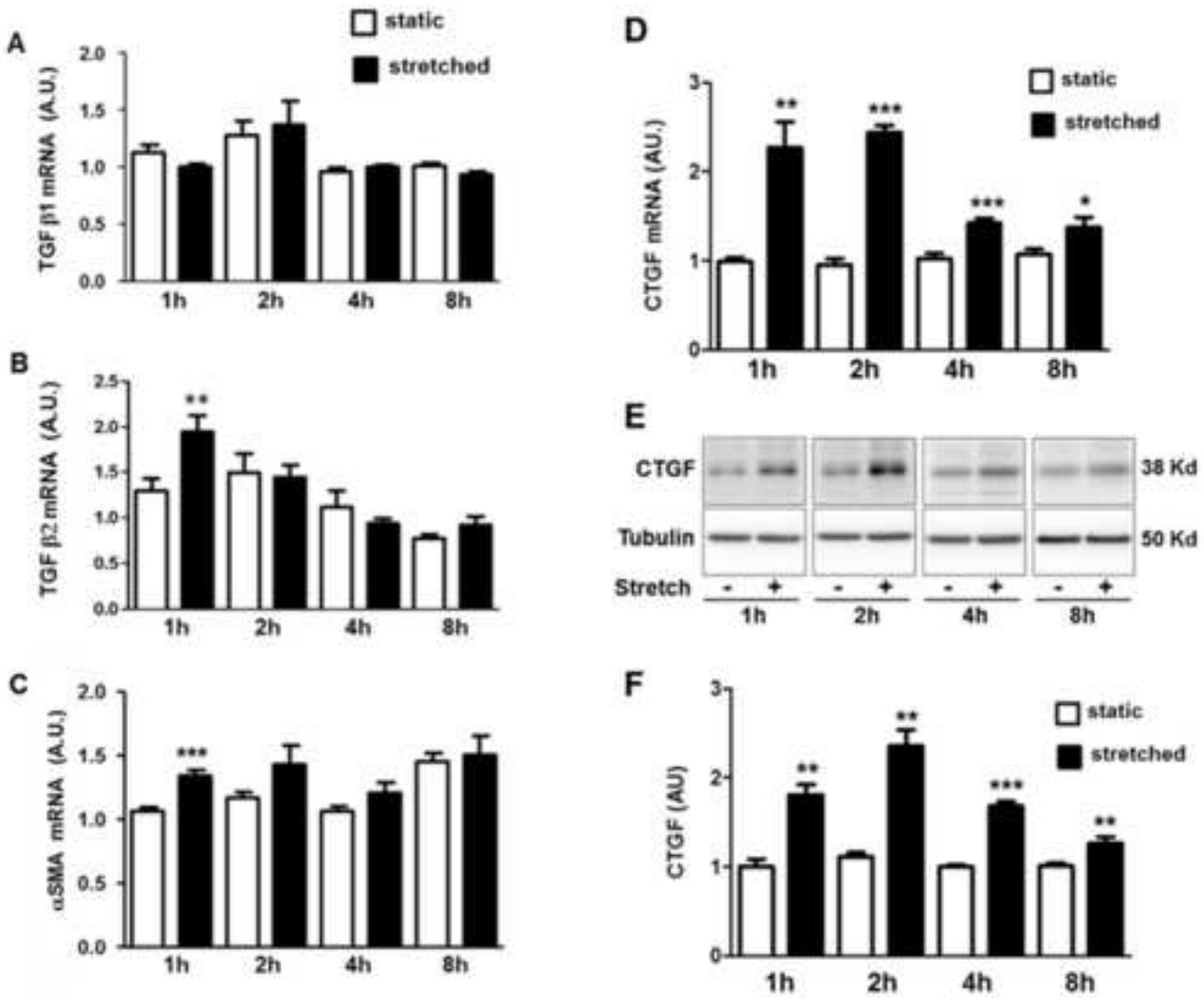

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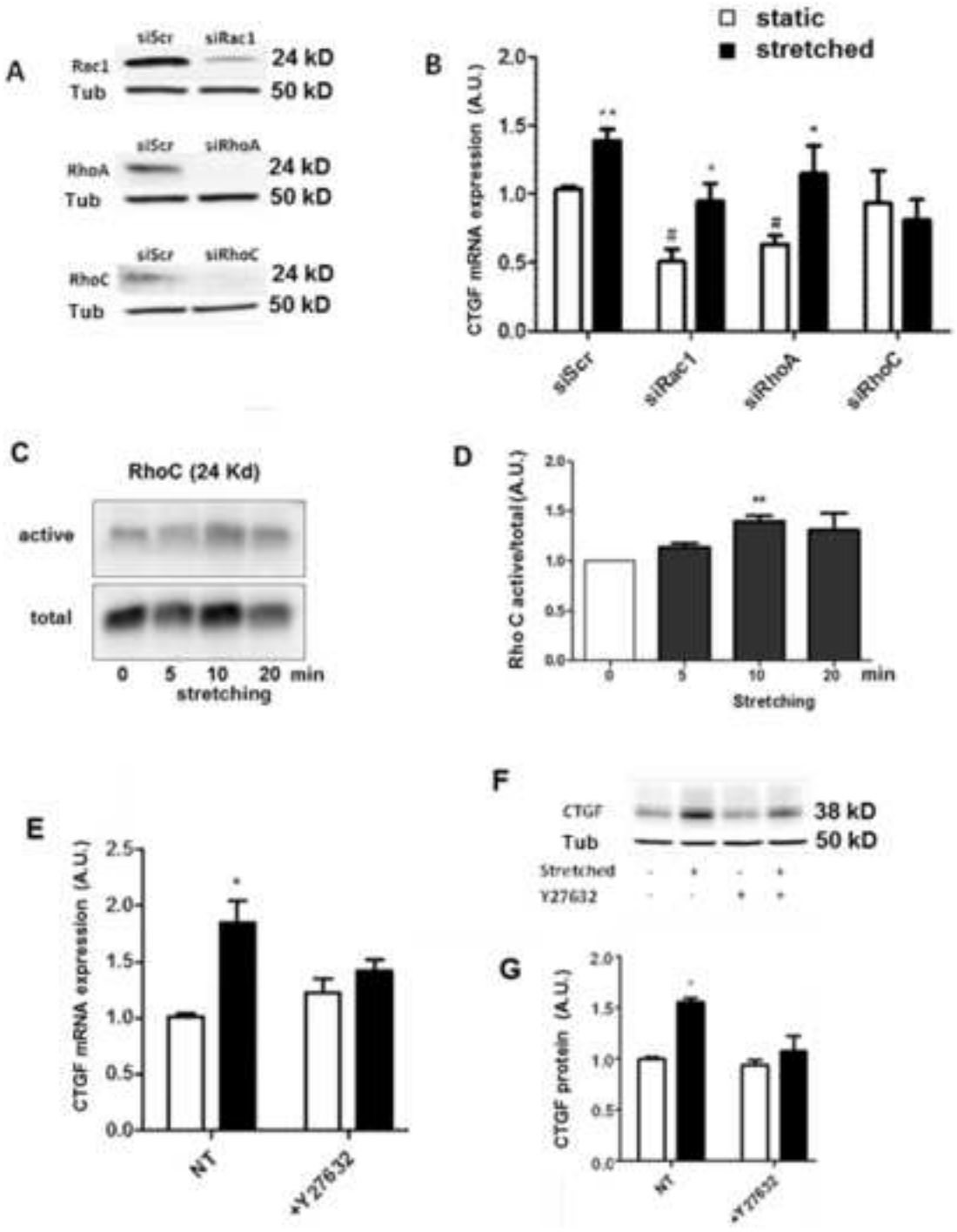

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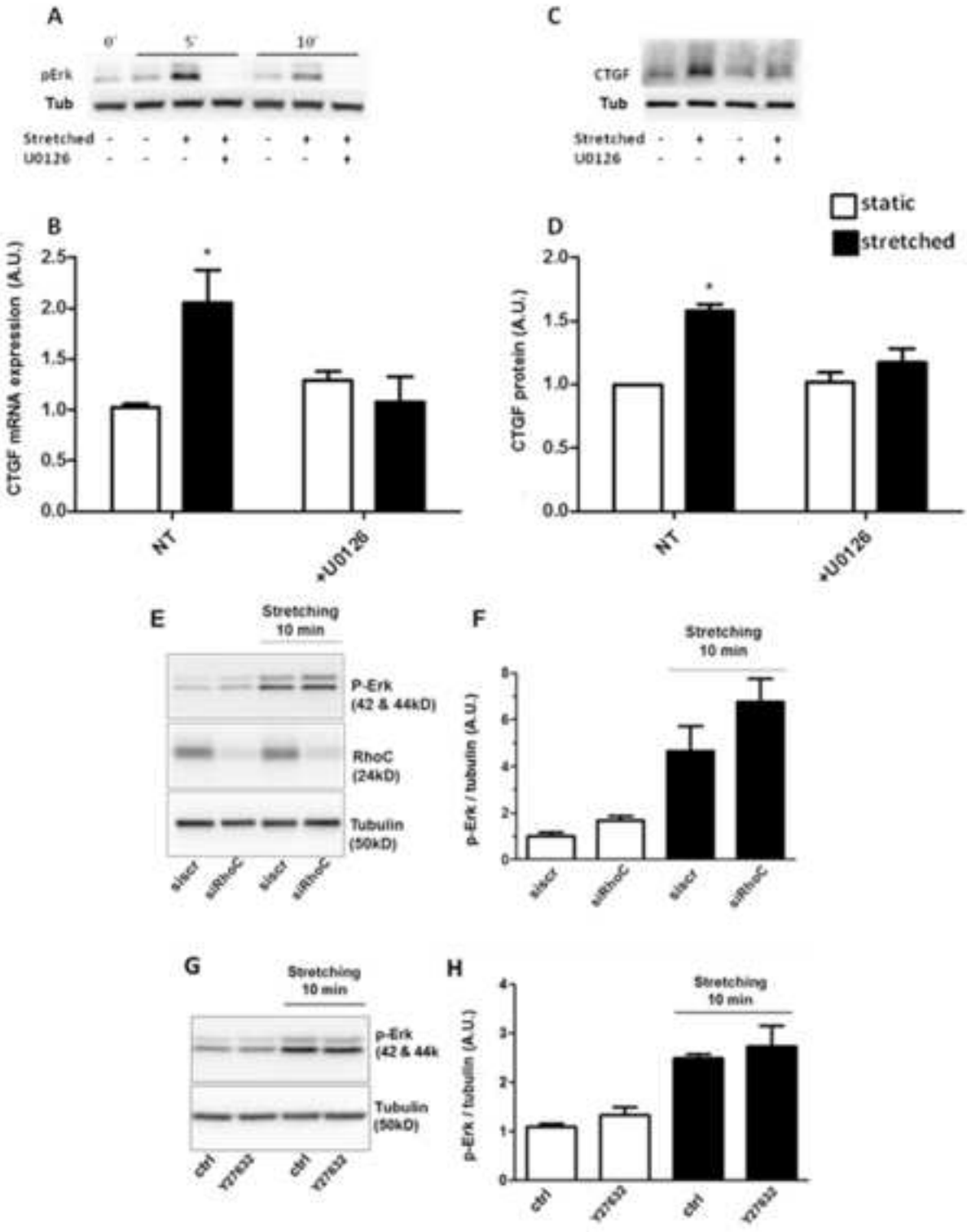

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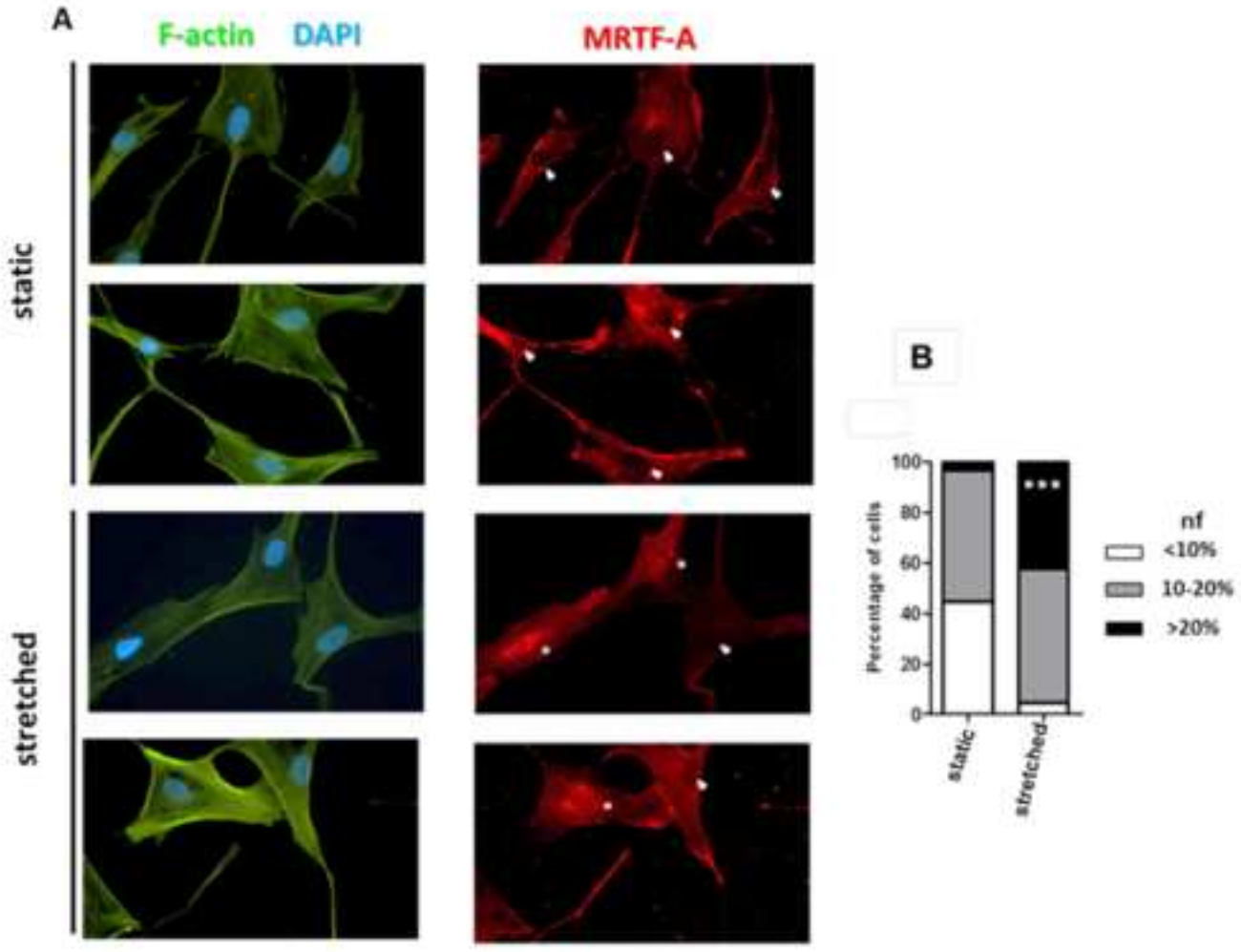

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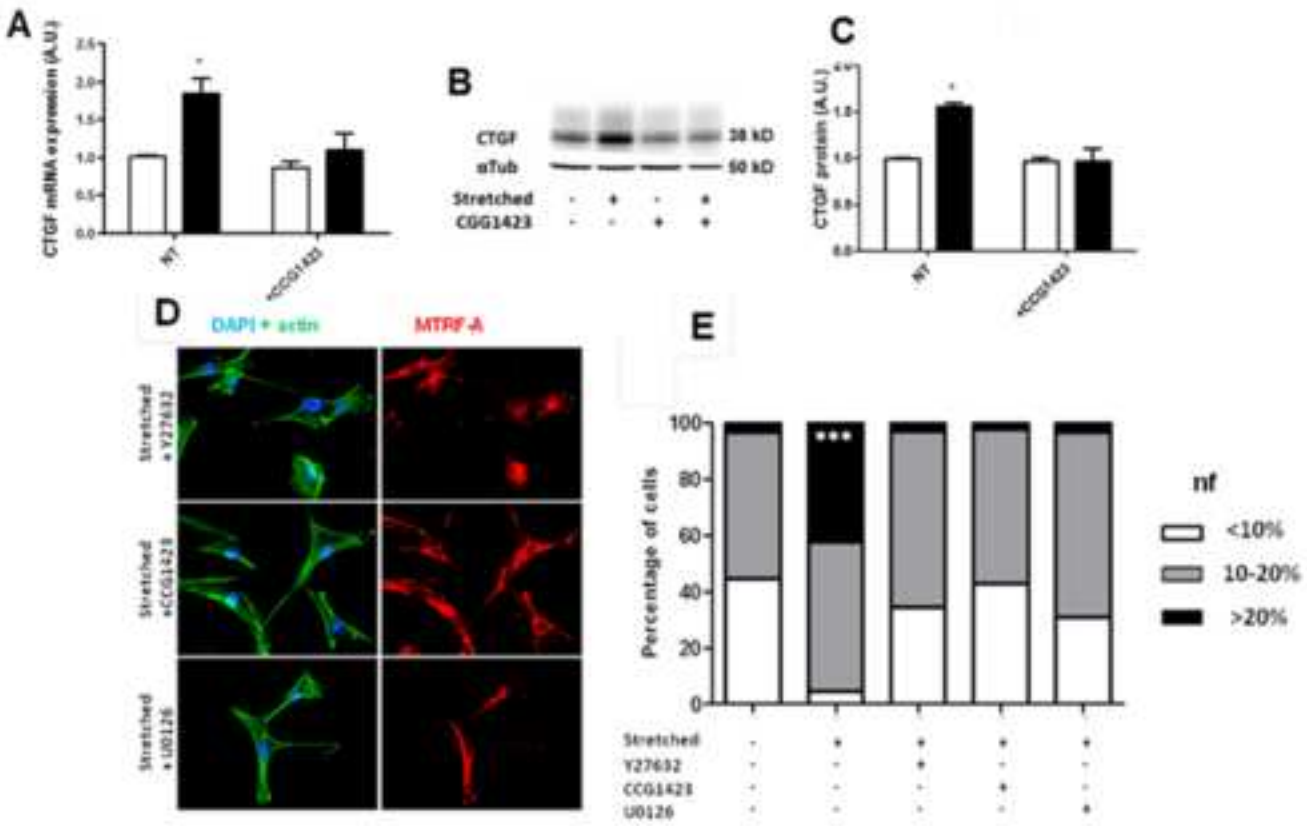

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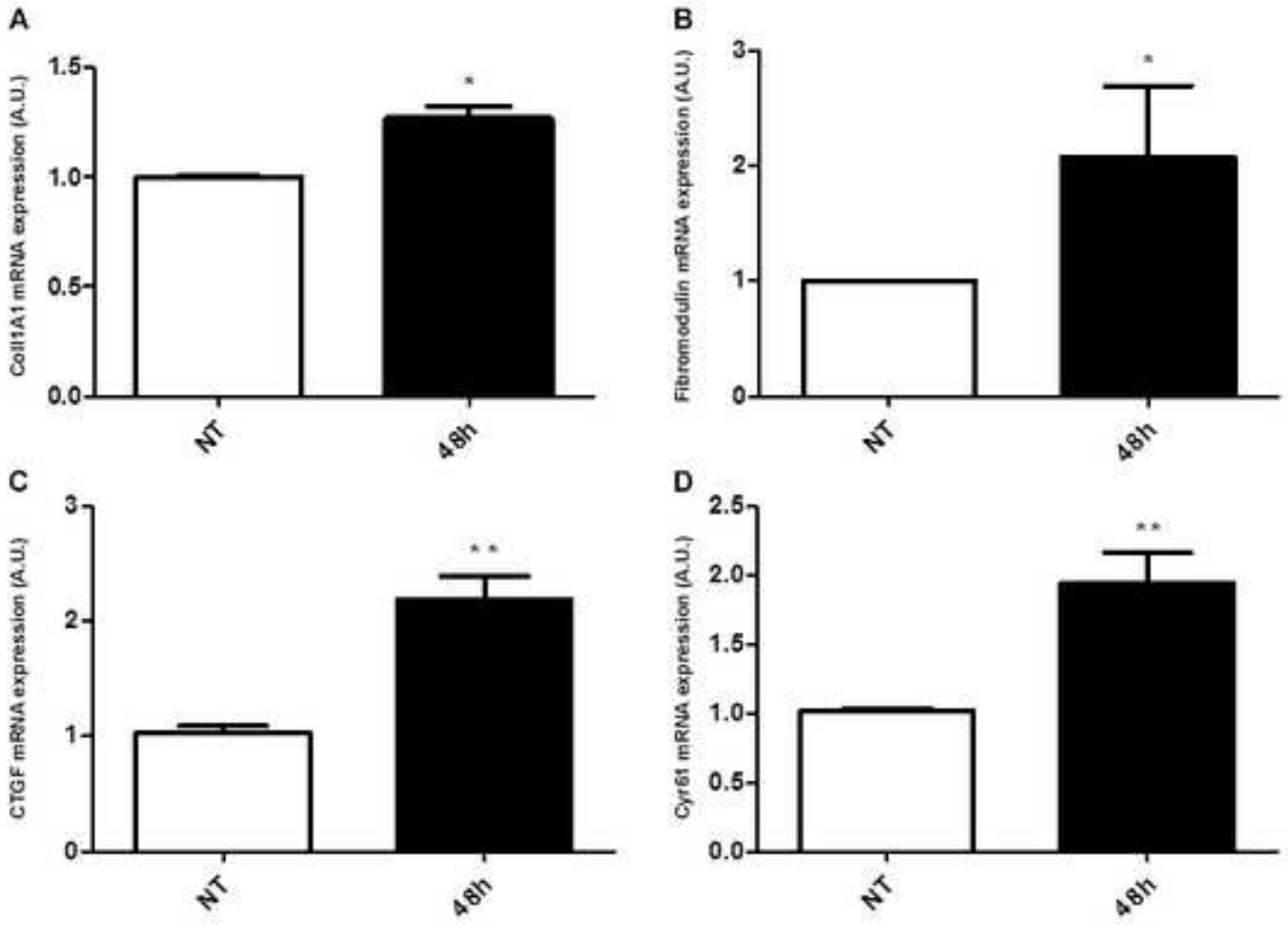

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