# Role of ROCK Isoforms in Regulation of Stiffness Induced Myofibroblast Differentiation in Lung Fibrosis

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Author Contribution

SSH performed experiments and wrote the manuscript. BHC and KY performed experiments. AK, AJK and AMG contributed to the study design and revised the manuscript. AMG supervised the project, contributed to the study design and wrote the manuscript. All authors approved the final manuscript.

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# Abstract

Fibrosis is a major cause of progressive organ dysfunction in several chronic pulmonary diseases. Rho associated coiled-coil forming kinase (ROCK) has shown to be involved in myofibroblast differentiation driven by altered matrix stiffness in fibrotic state. There are two known ROCK isoforms in human, ROCK1 (ROKβ) and ROCK2 (ROKα), but specific role of each isoform in myofibroblast differentiation in lung fibrosis remains unknown. To study this, we developed a Gelatin methacryloyl (GelMA) hydrogel based culture system with different stiffness levels relevant to healthy and fibrotic lungs. We have shown that stiff matrix and not soft matrix, can induce myofibroblast differentiation with high αSMA expression. Furthermore, our data confirm that the inhibition of ROCK signalling by a pharmacological inhibitor (i.e. Y27632) attenuates stiffness induced αSMA expression and fibre assembly in myofibroblasts. To assess the role of ROCK isoforms in this process we used siRNA to knock down the expression of each isoform. Our data showed that knocking down either ROCK1 or ROCK2 did not result in a reduction in αSMA expression in myofibroblasts on stiff matrix as opposed to soft matrix where αSMA expression was reduced significantly. Paradoxically, on stiff matrix, the absence of one isoform (particularly ROCK2) exaggerated αSMA expression and led to thick fibre assembly. Moreover complete loss of αSMA fibre assembly was seen only in the absence of both ROCK isoforms suggesting that both isoforms are implicated in this process. Overall our results indicate the differential role of ROCK isoforms in myofibroblast differentiation on soft and stiff matrices.

# Introduction

Pulmonary fibrosis is a major cause for progressive organ dysfunction in several pulmonary diseases like idiopathic pulmonary fibrosis (IPF), asbestosis and chronic granulomatous diseases such as sarcoidosis. Fibrosis is multifactorial and characterised by (1) fatal accumulation of extracellular matrix (ECM) proteins, (2) hyperproliferation of fibroblasts and (3) their differentiation into myofibroblasts in clusters, termed as fibrotic foci that collectively lead to disruption of normal lung architecture.

In lung fibrosis, myofibroblasts are the most predominant cell type found in fibrotic foci. Functionally, they share the contractile features of smooth muscle cell with the expression and polymerisation of smooth muscle actin isoform (α-SMA) (1, 2). Unlike in normal wound healing, in which myofibroblasts contract the wound and undergo apoptosis after the healing process, in fibrosis myofibroblasts become resistance to apoptosis and produce excessive extracellular matrix (ECM) like collagen. Together with excessive collagen deposition from myofibroblasts, increased cross linking of the collagen makes extracellular matrix more rigid in lung fibrosis (3, 4). In the bleomycin induced lung fibrosis mouse model, ECM rigidity of lung is about 6 times higher than normal lung (5). Moreover decellularised IPF lungs showed significantly higher matrix stiffness, nearly 9 times higher than that of normal lungs (2 kPa vs 17 kPa)(6). Fibroblasts grown on the stiff matrix also trigger further increase in expression of α-SMA independent of TGF-β (7). Rather than simply being a consequence of fibrosis, the increase in matrix stiffness has been recognised as active participant in promoting fibroblast activation and myofibroblast transformation by establishing a feedback loop for ongoing fibrosis (5, 8-11).

Recently Rho associated coiled-coil forming kinase (ROCK) has shown to be involved in lung fibrosis (12-14) and also in stiffness driven myofibroblast differentiation by intrinsic mechanotransduction mechanism (15). The ROCK belongs to the family of serine/threonine kinase and a major downstream effector of the small GTPase RhoA. They are involved in normal cell physiology function such as contractibility of smooth muscle cells through myosin II activity and formation of focal adhesion and stress fibre in non-muscle cells through myosin phosphatase (16, 17). In response to matrix stiffness, through ROCK activation, lung fibroblasts demonstrate increased F actin polymerisation, increased MRTF-A (myocardin

related transcription factor A) nuclear localisation, increased αSMA protein expression and stress fibre assembly through MRTFA-SRF (serum response factor) dependent pathway (7, 9, 18).

There are two known isoforms of ROCK in human, ROCK1 (ROKβ) and ROCK2 (ROKα). These two isoforms have high amino acid sequence homology (65%) and identity (92%) in their kinase domains (19). Pharmacological ROCK inhibitors such as Y27632 or Fasudil (20, 21) were shown to be efficient in preventing myofibroblast differentiation in both *in vitro* (7) and *in vivo* fibrosis models (15). This clearly exhibits ROCK's role in sensing extracellular matrix stiffness changes and subsequent activation of mechanotransduction pathways in fibrosis. However, given the high degree of homology within the kinase domain and the downstream pathway redundancy between two ROCK isoforms, it is not possible to determine the role of each isoform in stiffness induced myofibroblast differentiation through using pharmacological inhibitors only (22). Although ROCK inhibitors have shown promising effect in controlling fibrosis, their off target effect when used in high dose and non-selectivity against other kinases like protein kinase C related kinase (PRK) at the same concentration necessary for ROCK inhibition, limits their efficacy(21). Moreover, advances in gene silencing using short interfering RNA (SiRNA) as well as in knockout animal models, have the potential to provide additional insight into ROCK isoform selectivity. Recent examples include studies focused on the differential role of ROCK isoforms in contractile function of vascular smooth muscle cells (19, 23) and cancer cell migration (24).

A growing body of evidence indicates the importance of ROCK isoforms in different pathologies. Therefore, detail understanding of how each of the ROCK isoforms are involved in myofibroblast differentiation in lung fibrosis pathophysiology could pave the way for the development of more efficient therapeutic approaches to control lung fibrosis in an isoform specific manner (13, 22). In this study, we investigated the role of each ROCK isoform in stiffness induced myofibroblast differentiation in pulmonary fibrosis by using physiologically relevant ECM based hydrogel scaffolds with tuneable stiffness.

**Materials and methods**

**Preparation of different percentages of GelMA hydrogels**

Gelatin methacryloyl (GelMA) macromer as a white porous foam (kindly provided by Khademhosseini Lab, Harvard) was prepared as previously described (25). Prior to fabrication of GelMA hydrogels, 1cm$^2$ glass chips was prepared from TMSPMA treated glass slide and followed by PolyHEMA coating as previously described (26). Briefly, different percentages of GelMA (5%, 10% and 15%) was prepared by dissolving in 0.25% (w/v) of photoinitiator [Sigma-410896] in DPBS at 60°C and then UV crosslinked (Figure S1). The mechanical properties of hydrogel was characterised according to established protocol (27).

**Si RNA Gene silencing of ROCK isoform**

Human lung fibroblast cell line (MRC-5) obtained from ATCC, was routinely cultured as previously described (28). Both SiRNA targeting human ROCK1 and ROCK2 (SiGENOME SMART pool) and non-targeting SiRNA control were purchased from Dharmacon (GE Healthcare). Cells were transfected by DharmaFECT1 transfection agent (GE healthcare) according to manufacturer's instruction. Lung fibroblasts (5x10$^4$) were seeded on the 5% and 15% GelMA hydrogel in non-tissue cultured treated 24 well plate overnight and changed into new plate before transfection. The efficiency and specificity of gene knock down was checked by RT-PCR and western blotting. Effective gene knock down was achieved at 50 nM of SiRNA of ROCK1 and ROCK2 in 1:625 dilution of transfection agent after 48 hour transfection.

**Real time (qRT-PCR) analysis**

Total RNA was harvested using pure Link$^{TM}$ RNA mini kit (Ambion) following Trizol extraction. 100ng cDNA was synthesized using Superscript III first Strand Synthesis SuperMix (Invitrogen) according to manufacturer's protocol. Real time PCR was performed with Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent) and analysed with Agilent Mx3000P QPCR system.

## Western Blot analysis

Before lysis, cells cultured on hydrogel were washed with cold PBS twice for 15 minutes on shaker at 4˚C to remove protein absorbed in hydrogel and then lysed using RIPA buffer with 1x protease inhibitor (Sigma) for 30 min on ice. Protein samples (8 μg) were then separated by SDS-PAGE electrophoresis and followed by protein transfer onto a nitrocellulose membrane. Membranes were blocked in 5% BSA in TBS with 0.1% Tween at RT for 1 hr and then incubated with primary antibodies overnight at 4°C [anti αSMA (Sigma, A5228), anti-alpha tubulin (Abcam); anti ROCK1 (C8F7) and ROCK2 (D1B1) (Cell Signalling)] followed by relevant HRP conjugated secondary antibody for an hour at RT.

## Immunofluorescence Confocal Imaging

Immunofluorescent staining and confocal microscopy were performed as described in our previous study (29). All immunofluorescent images comparing 5% and 15% GelMA conditions are from the same experiment and also analysed together using same microscopy settings on the same day.

## Statistical Analysis

Statistical analysis was performed using Graph Prism 6. Data are expressed as means ± Standard deviation (SD) and analysed by one way ANOVA and two way ANOVA where appropriate with Bonferroni post hoc test for multiple comparisons. Significance level was set at $p < 0.05$.

Additional details on methods used are available in online supplement.

# Results

**Fabrication of GelMA based Hydrogel Scaffolds with Different stiffness**

Mechanical properties of the extracellular matrix environment have shown to be important in lung fibrosis progression (8). GelMA is a well-established ECM based hydrogel system for different types of cell culture and has easily tuneable mechanical properties (27, 30, 31). It is particularly relevant to studying lung fibrosis in which increased stiffness of ECM is mainly induced by collagen deposition. We first fabricated 5%, 10% and 15% GelMA hydrogels with 5mm$^2$ diameter and 100$\mu$m thickness (Figure S1). We then measured the mechanical properties of different percentage GelMA hydrogels using both compressive (Figure 1) and Young's modulus (Figure S2). As shown in Figure 1, different percentages of GelMA hydrogel showed varying degree of stiffness with 3.650 $\pm$ 0.1 kPa (5% GelMA), 15.15 $\pm$ 0.9 (10% GelMA) and 27.73 $\pm$ 0.8 kPa (15% GelMA). This range of stiffness in both compressive and Young's modulus corresponds to the range of normal and fibrotic lung stiffness with 5% GelMA representing the normal lung stiffness while 10 and 15% being closer to fibrotic lung matrix (6).
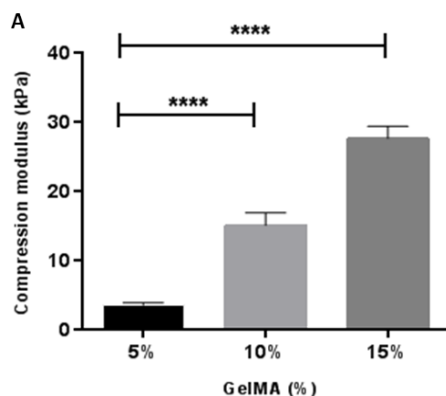


**Figure 1. Mechanical properties of GelMA based Hydrogel platform with different percentages of GelMA.** The stiffness of hydrogel [5%, 10% and 15% (w/v) GelMA] was measured in compressive modulus. Three independent sets of experiments for each condition were performed in triplicate. 10% and 15% (w/v) GelMA were significantly different (****p < 0.001) compared to that of 5% (w/v) GelMA. Mean $\pm$ SD.

**Increased substrate stiffness promote myofibroblast differentiation as evidenced by increased expression of αSMA protein in lung fibroblast**

As seen in Figure 2, there is a significant increase in αSMA mRNA and protein expression levels in lung fibroblasts with increasing substrate stiffness from normal lung (5% GelMA) to fibrotic lung (10% and 15% GelMA) values. In addition, confocal immunostaining also confirms the assembly of αSMA stress fibre in lung fibroblast cultured on higher stiffness hydrogels. Lung fibroblasts cultured on 10% GelMA hydrogels exhibit αSMA as short cortical stress fibres compared to the 15% GelMA where cells expressed long αSMA stress fibres across the whole cytoskeleton. Together these findings suggest that increased stiffness of substrate promotes the differentiation of human lung fibroblasts to myofibroblast.
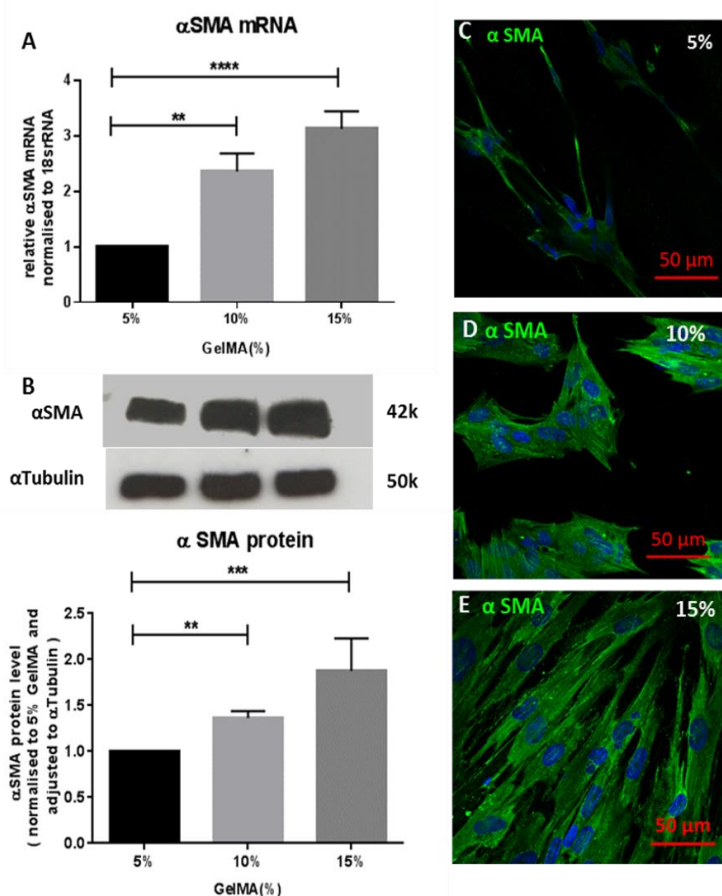
**Figure 2. Increased substrate stiffness promotes αSMA gene and protein expression in lung fibroblast.** (A) The level of αSMA mRNA level was examined in lung fibroblasts by the real time RCR after culturing on 5%, 10% and 15% of GelMA for 24 hours. 18s rRNA was used as reference gene for loading control. The level on 5% GelMA was set at 1 for comparative analysis of RT-PCR. (B) The level of αSMA protein from lung fibroblasts cultured on 5%, 10% and 15% of GelMA for 3 days were determined by Western blot. α tubulin was used as protein loading control. Representative Western blot from 3 independent experiments are shown. Relative levels of αSMA protein expression were determined by densitometry of the blots from 3 independent experiments and normalised to α tubulin expression. (C,D,E) Confocal analysis of αSMA stress fibre formation by immunofluorescence staining at 4 days after gene silencing. Scale bar: 50μm, green= αSMA and blue= DAPI nuclei. Results are Mean ± SEM, n=3, **p<0.01, ***p<0.001, ****p<0.0001

**Inhibition of ROCK signalling by Y27632 attenuates stiffness induced αSMA expression and fibre assembly in myofibroblast**

Y27632 is one of the commonly used pharmacological ROCK inhibitors. To investigate the role of ROCK activation in stiffness induced myofibroblast transformation, lung fibroblasts cultured on 5% and 15% GelMA were treated with Y27632 (10uM) for 24 hr. On the stiff matrix (15% GelMA), Y27632 treatment caused a significant reduction in αSMA protein level and a complete loss of αSMA fibre assembly compared to untreated controls (Figure 3 C and D). In addition, Y27632 treatment decreased the Filamentous (F actin) assembly induced by stiff matrix. However on the soft matrix (5% GelMA), we did not observe any significance changes in αSMA fibre assembly and αSMA protein level and F actin assembly with or without Y27632 treatment (Figure 3 A and B).
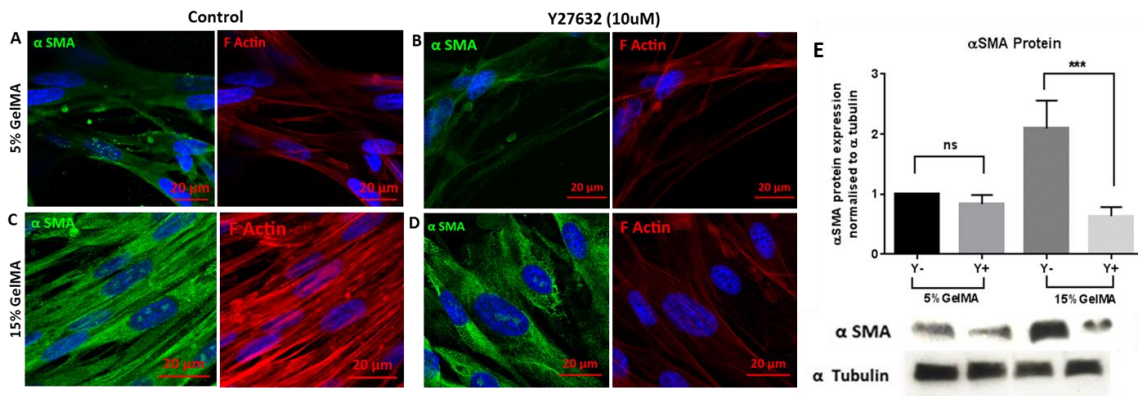
**Figure 3. Y27632 inhibits stiffness induced myofibroblast differentiation.** Lung fibroblasts are cultured on 5% (A,B) and 15% GelMA (C,D). The cells were treated with or without Y27632 (10μM) for 24hrs at 2 days after cells was cultured on different percentages of GelMA. The cells were stained for αSMA (Alexa Flour 488 - green) and F actin (Alexa Flour 647 red and examined by confocal microscopy. Nuclei were stained with DAPI. Scale bar =20μm. (E) The level of αSMA protein with or without treatment with Y27632 (10μM) on both 5% and 15% GelMA for 24hr were determined by Western blot. α tubulin was used as protein loading control. Relative levels of αSMA protein expression were determined by densitometry of the blots from 3 independent experiments and normalised to α tubulin protein. Results are Mean ± SEM, n=3, ***p<0.001.

**Differential role of ROCK isoforms in αSMA expression and stress fibre assembly in fibroblasts grown on soft and stiff matrix**

To further establish whether ROCK1 and ROCK2 have distinct function in regulation of stiffness induced αSMA expression, SiRNA gene silencing was used to knock down ROCK1 and ROCK2 isoforms specifically. As shown in Figure 4, ROCK1 and ROCK2 isoforms could be suppressed individually with high specificity without affecting the other isoform confirmed by RT-PCR and Western Blotting. We then analysed αSMA expression at gene and protein levels on lung fibroblasts cultured on 5% (soft) and 15% (stiff) matrix after ROCK 1 and ROCK2 knockdown (Figure 5). RT-PCR analysis showed that silencing of ROCK isoforms individually or both isoforms simultaneously in fibroblasts cultured on soft matrix reduced αSMA mRNA expression by approximately 40% and 60% respectively without a significant change in αSMA protein level. However, paradoxically silencing individual ROCK isoforms, particularly ROCK 2, in fibroblasts cultured on stiff substrate led to a significant increase in αSMA mRNA expression whereas silencing both isoforms returned αSMA mRNA expression back to control levels. Western blot analysis confirmed a similar pattern for protein expression in lung fibroblasts up to 4 days after transfection on stiff matrix. These data were in line with confocal microscopy showing the formation of thick αSMA stress fibre assembly in ROCK1 or ROCK2 deficient fibroblasts, on stiff matrix compared to ROCK1 or ROCK 2 deficient cells cultured on soft matrix where we did not observe the change in the αSMA fibre assembly compared to control ROCK expressed cells. Importantly the knockdown of both ROCK isoforms simultaneously was associated with complete inhibition of αSMA stress fibre assembly in cells cultured on stiff matrix (Figure 5) indicating the primary function of ROCK isoforms in the αSMA fibre assembly rather than synthesis and breakdown of αSMA protein. In order

to examine whether these findings are reproducible on another hydrogel system, we also repeated same experiment on polyacrylamide hydrogel which is also widely used in stiffness studies (5, 7, 15, 32, 33). Consistent with data obtained from GelMA hydrogels, increased αSMA level with thick fibre assembly was observed on the stiff polyacrylamide hydrogels in the absence of one ROCK isoform (Figure S3) proving that our findings are regardless of type of hydrogel system and mainly due to substrate stiffness.
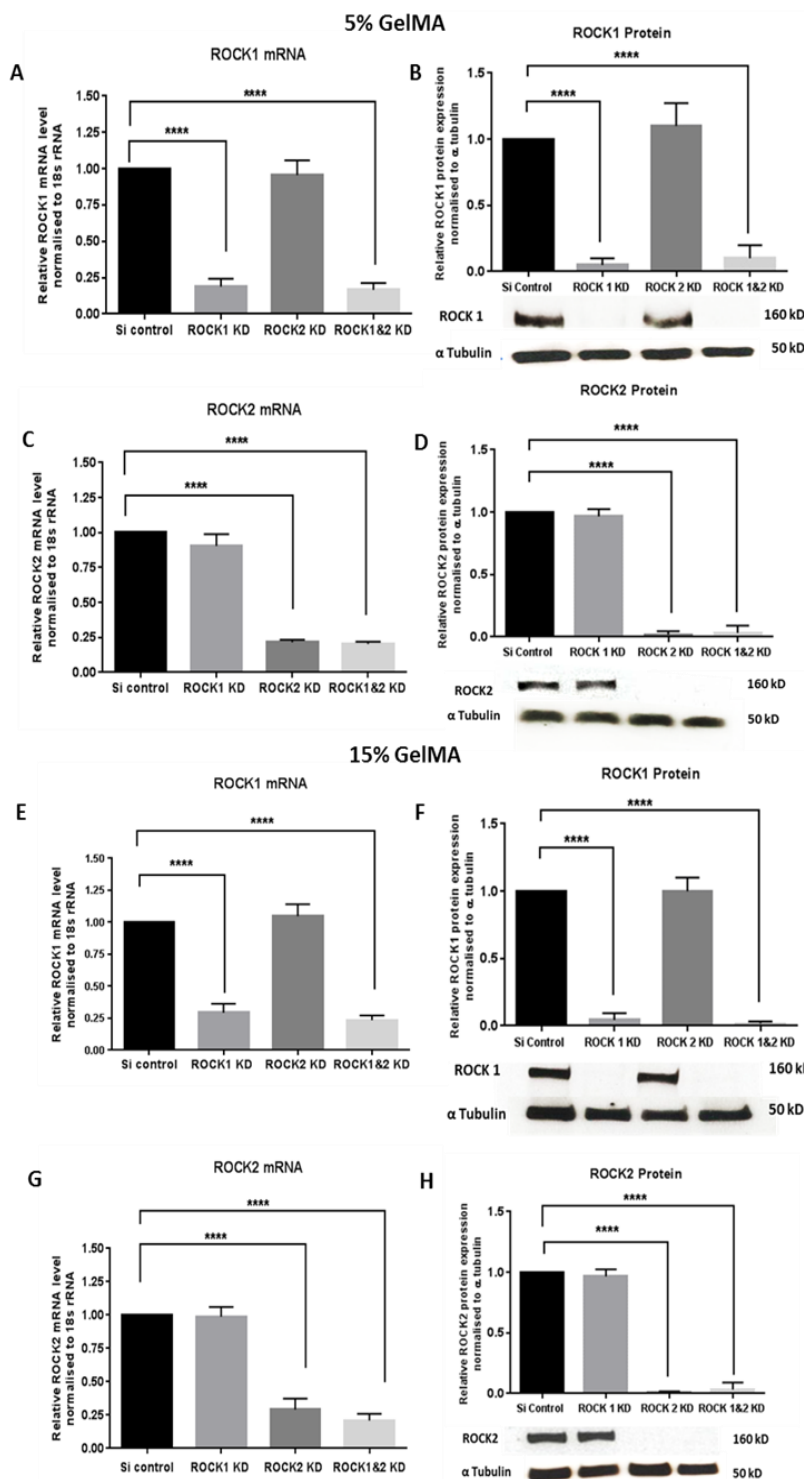


**Figure 4. Specificity and efficiency of ROCK isoforms gene silencing in lung fibroblasts on 5% and 15% GelMA.** ROCK1 and ROCK2 isoform was knocked down on 5% GelMA (A,B,C,D) and 15% GelMA (E,F,G,H) individually and in combination by SiRNA gene silencing. (A,C,E,G) The level of ROCK1 and ROCK2 isoform mRNA level were examined in lung fibroblasts by RT RCR at 2 days after transfection to assess specificity and efficiency of gene knockdown. 18s rRNA was used as reference control. The level of mRNA in sample transfected with non-targeted SiRNA was set at 1 for comparative analysis of RT-PCR. (B,D,F,H) The ROCK1 and ROCK2 protein level was also checked by western blotting at 4 days after transfection. α Tubulin was used as protein loading control in WB analysis (n=3). Relative levels of αSMA protein expression of 4 days after gene silencing were determined by densitometry (n=3) normalised to α Tubulin. ****p<0.0001, Results are Mean ± SEM, n=3.
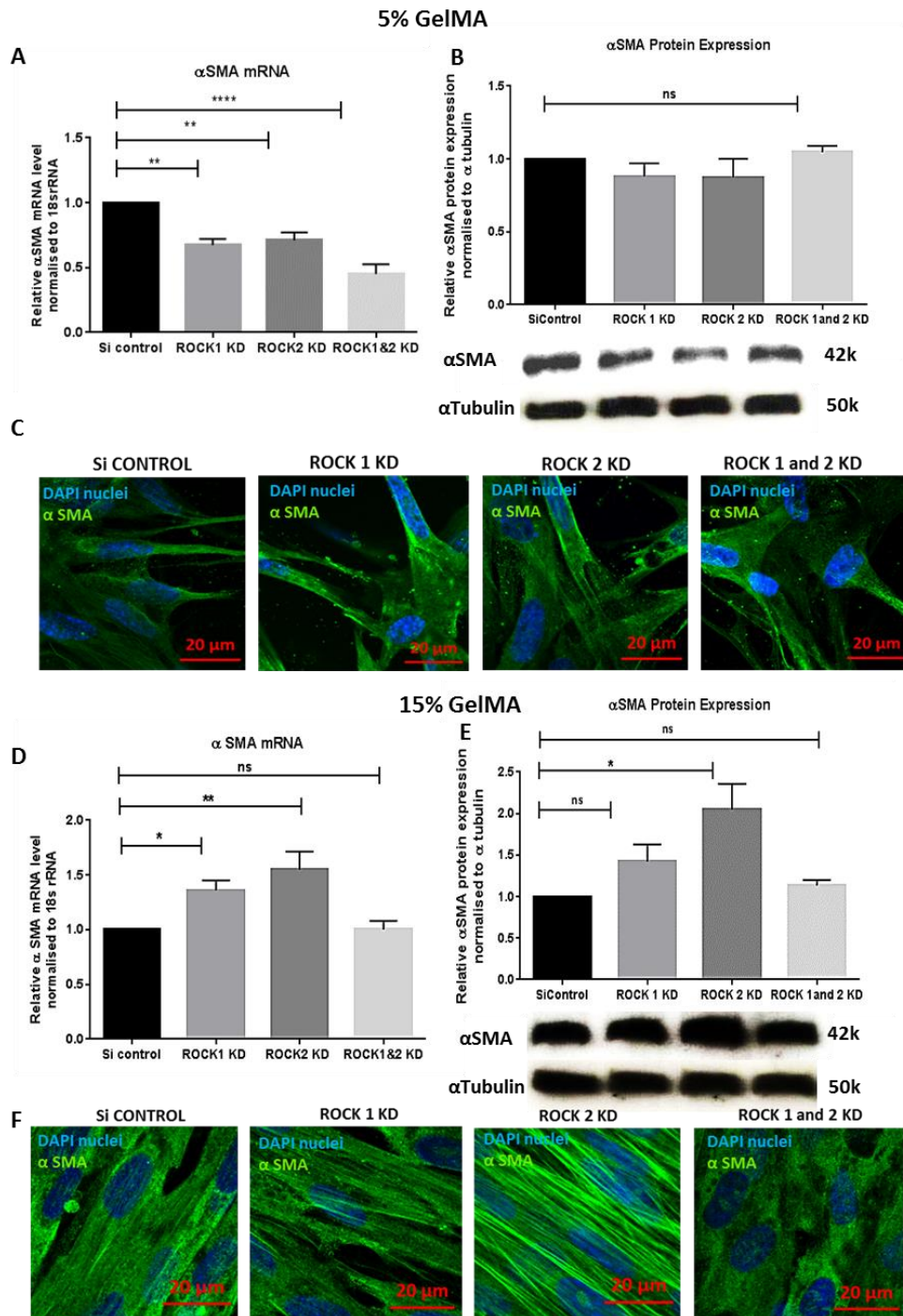
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**Figure 5. ROCK isoform regulation of αSMA (gene and protein expression) and stress fibre formation in lung fibroblasts cultured on 5% and 15% GelMA.** Lung fibroblasts are cultured on 5% (A,B,C) and 15% GelMA (D,E,F) for 2 Days for gene analysis and 4 days for protein level detection (B,E) after ROCK isoform gene silencing. The level of αSMA mRNA level was examined by the RT- RCR. rRNA 18s was used as reference control. The level of αSMA mRNA from Si Control was set at 1 for comparative analysis in RT-PCR. α Tubulin was used as protein loading control in WB analysis (n=3). Relative levels of αSMA protein expression at 4 days after gene silencing were determined by densitometry (n=3) normalised to α Tubulin. Results are Mean ± SEM, n=3, *p<0.05, *p<0.01, ***p<0.001, ****p<0.0001, ns=not significant. (C, F) Confocal analysis of αSMA stress fibre formation by immunofluorescence staining at 4 days after gene silencing. Scale bar: 20μm, green= αSMA and blue= DAPI nuclei staining

**Loss of αSMA fibre assembly in the absence of ROCK isoforms correlates with diminished F actin fibre on stiff matrix**

F actin polymerisation from G actin monomer in cytoplasm is known to be upstream to the αSMA promoter activation in nucleus after activation by stiff matrix (7). Therefore, in addition to showing αSMA fibre assembly in ROCK isoforms depleted fibroblasts on soft and stiff matrix, we also tried to investigate any potential association between ROCK activation and F actin fibre formation under these conditions. As shown in figure 6A, there was no change in the F actin fibres formation with ROCK isoform depletion on soft matrix. However, on stiff matrix, even though F actin staining did not show any significant increase in fibroblasts with individual silencing of ROCK isoform, it was significantly reduced when both isoforms are absent which also correlates with the loss of αSMA fibre assembly after knocking down of both isoforms (Figure 6B). These data clearly suggest a differential role for ROCK signalling in the control of F actin polymerisation in association with αSMA fibre assembly on soft and stiff matrices.
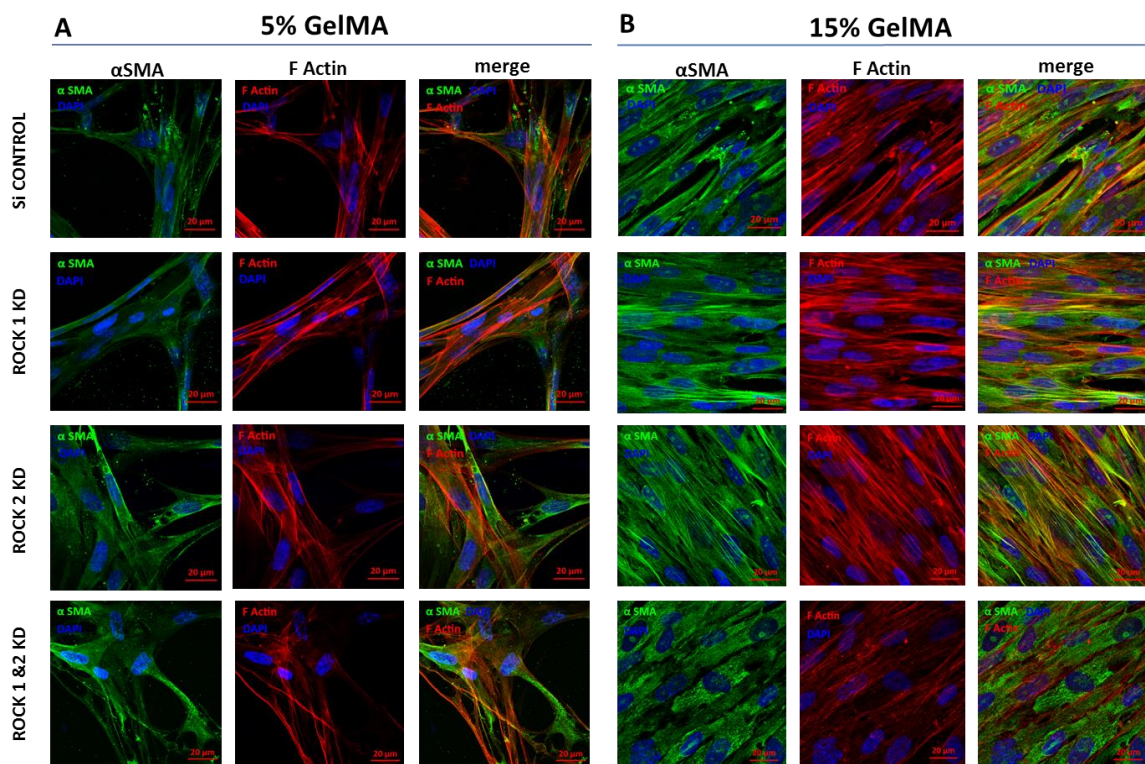


**Figure 6. ROCK isoform regulation of F actin polymerisation in association with αSMA stress fibre formation in lung fibroblasts on soft and stiff matrix.** Lung fibroblasts are cultured on 5% GelMA (A) 15% GelMA (B) for 4days after gene silencing of ROCK isoform. αSMA stress fibre (green) and F actin fibre (red) assembly was examined by confocal microscopy. Nuclei are stained with DAPI. Representative images from the three independent experiments. Scale bar =20μm

**ROCK isoform imbalance promotes nuclear translocation of MRTF-A in a stiffness dependent manner in favour of αSMA stress fibre assembly**

Myocardin related transcription factor A (MRTF-A) has been shown to be involved in stiffness induced myofibroblast differentiation (7) by increasing F actin to G actin protein ratio. Generally actin monomers are bound to MTRF-A in the cytoplasm. When the ROCK signalling is activated, G actin monomers are released from MRTF-A molecules and polymerise to form F actin fibre assembly and MRTF-A is translocated into the nucleus where it promotes the fibrotic gene program. To determine the role of MRTFA activation in ROCK isoform dependent changes in stiffness induced myofibroblast differentiation, we examined the MRTF-A nuclear localisation in ROCK isoform depleted fibroblasts on 5% and 15% GelMA. Interestingly, immunofluorescent analysis demonstrated increased nuclear localisation of MRTF-A (30 to 40%) in lung fibroblasts with single ROCK isoform depletion on 15% GelMA compared to control (transfected with scrambled siRNA) and cells where both ROCK isoforms were knockdown (Figure 7D). This was opposite to cells cultured on the 5% GelMA where the increased nuclear localisation of MRTF-A was not detected in all conditions (Figure 7B).  Taken together, these data support the important role of MRTF-A transcriptional factor activation in induction of αSMA gene expression and protein synthesis when one ROCK isoform was absent in lung fibroblast cultured on a stiff matrix.
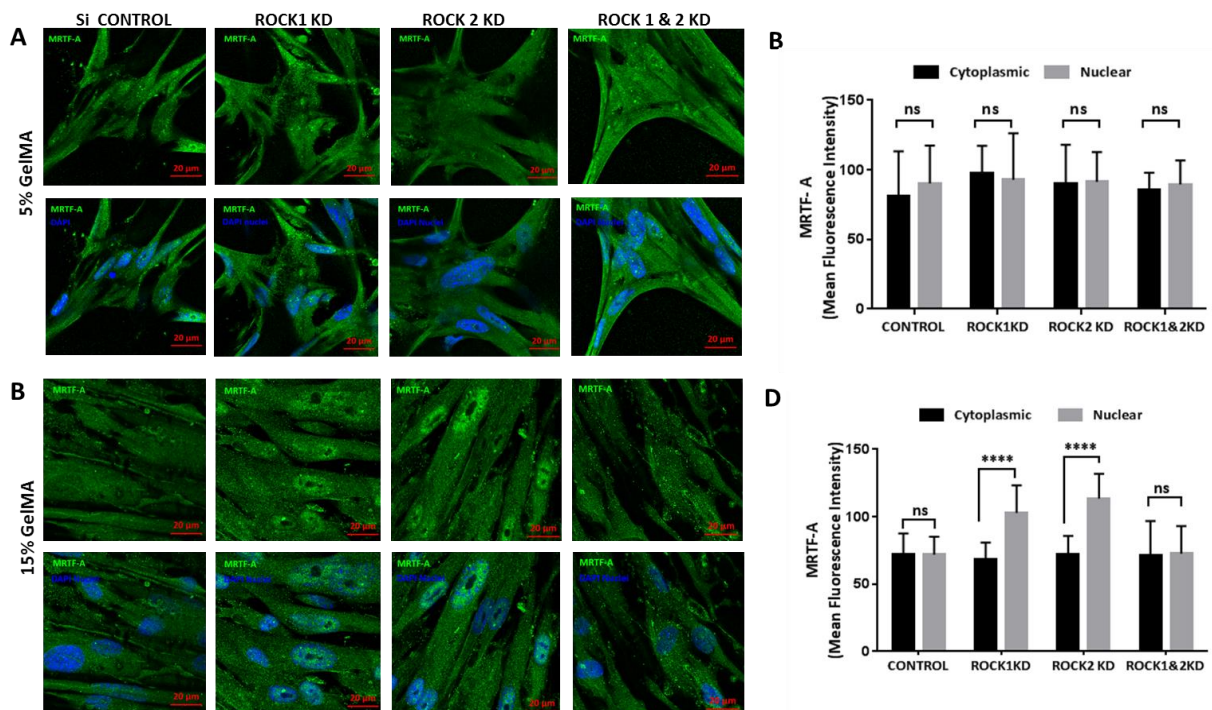


**Figure 7. ROCK isoform regulation of matrix stiffness induced nuclear localisation of MRTF-A in lung fibroblasts.** Lung fibroblasts are cultured on the 5% (A, B) and 15% GelMA (C, D). The cells were fixed at 3 Days after gene silencing of ROCK isoform and stained for MRTF-A (Alexa Flour 488 – green colour). Nuclear

localisation of MRTF-A was examined by confocal imaging and overlaid with DAPI nuclei (blue colour) to confirm nuclear localisation of MRTF-A. (B, D) The mean Fluorescence intensity (MFI) of subcellular localisation of MRTFA was quantified using ImageJ software. For each condition, MFI of nuclear and cytoplasmic MRTF-A was measured in 60 randomly selected cells from three independent experiments. Scale bar =20µm, n=3.

## Discussion

Experiments using pharmacological inhibitors such as Y27632 and Fasudil have shown ROCK activation as a critical step in the stiffness induced myofibroblast transformation (5, 7). In addition to showing high efficiency in *in vitro* experiments these pharmacological inhibitors have also shown good efficacy in preventing development of lung fibrosis as well as reversing the established fibrosis in the bleomycin induced lung fibrosis mouse models (15). However, given the non-selective nature of the ROCK inhibitors used in these studies, the relative contribution of ROCK 1 versus ROCK2 isoforms in the development of lung fibrosis is yet to be elucidated. Such information could not only enhance our understanding of lung fibrosis at the molecular level but could also pave the way for development of more effective intervention strategies for treatment of lung fibrosis.

The main aim of this study was to evaluate the importance of the balance between ROCK 1 and 2 isoforms and their relative contribution in stiffness induced myofibroblast transformation in lung fibroblasts. Using SiRNA gene silencing, we achieved highly specific knock down of ROCK1 and ROCK2 isoforms in spite of the high homology between them. This enabled us to investigate the effect of substrate stiffness on αSMA expression and assembly in ROCK1 $^{low}$, ROCK2 $^{low}$ and ROCK1+2 $^{low}$ lung fibroblasts for the first time. The main conclusion from these experiments is that either of ROCK isoforms are sufficient for αSMA expression on soft matrix but both isoforms are required for αSMA stress fibre assembly and maintaining the αSMA protein level on stiff matrix suggesting the differential role of ROCK isoforms in myofibroblast differentiation on soft and stiff matrix. More importantly, one unexpected finding is that the absence of one ROCK isoform on stiff matrix, as opposed to knocking down both isoforms simultaneously, leads to a fibroblast phenotype with increased level of αSMA with thick αSMA stress fibre assembly. This is opposite to what was observed on a soft matrix where αSMA expression was dramatically reduced after knocking down only one ROCK isoform.

Additionally, the aggressive myofibroblast features are more prominent in the absence of ROCK 2 isoform suggesting a more dominant role for ROCK1 isoform in controlling αSMA gene expression and fibre assembly. Together these findings highlight the importance of ROCK isoform balance in maintaining fibroblasts normal phenotype and the fact that inhibiting individual ROCK isoforms (as opposed to simultaneous inhibition of both) could accelerate myofibroblast transformation on an

already stiff matrix (e.g. fibrotic lung).   Therefore, since on a stiff matrix, the total loss of αSMA assembly could be achieved only in the absence of both isoforms, it is reasonable to suggest that targeting both ROCK isoforms would be a safer pharmacological intervention for controlling myofibroblast in fibrosis.

Interestingly our data show that knocking down both ROCK isoforms in cells cultured on stiff matrix do not reduce the αSMA protein expression back to the levels observed in cells cultured on soft matrix. This finding is inconsistent with our observations when using ROCK inhibitor Y27632 where αSMA protein level was reduced significantly within 24hours treatment with Y27632. The possible explanation is that Y27632 has additional effect in controlling αSMA synthesis and breakdown through other kinases rather than ROCK signalling.  Furthermore we can argue that even though ROCK isoform gene silencing achieved high efficiency about 75% in our experiments, the stimulus from the stiff matrix was strong enough to activate the low level of ROCK kinase in myofibroblast to maintain the αSMA expression at high level. These data highlight the importance of a continuous drive from surrounding stiff matrix in influencing myofibroblasts phenotype. Additionally, the loss of αSMA fibre assembly correlated with diminished F actin polymerisation not only in the absence of both ROCK isoforms but also in Y27632 treated samples highlighting the critical role of ROCK activation in the contractile function of myofibroblast by controlling both αSMA fibre assembly and F actin fibre polymerisation on a stiff matrix.

MRTF-A activation has shown to be involved in stiffness induced myofibroblast transformation as actin dynamics sensor (14). Our data showing an increase in nuclear translocation of MRTF-A in the absence of one ROCK isoform in myofibroblasts on stiff matrix suggest the possible link between increased αSMA expression with mature αSMA fibre assembly and changes in actin dynamics in these conditions. One explanation is that ROCK isoform imbalance on stiff matrix increased F - G actin ratio that favours the MRTF-A nuclear localisation and trans activates the αSMA gene expression. As Rho-ROCK-MRTF-A signalling pathway has been implicated in controlling myofibroblast differentiation in lung fibrosis, our data further highlights the association between MRTF-A and ROCK isoform in stiffness induced myofibroblast differentiation.  Further investigations are required to explain how MRTFA nuclear translocation affects increase in αSMA profile when one ROCK isoform is depleted in myofibroblast on stiff matrix.

In summary, our study provides a detailed understanding of the role of ROCK isoforms in the regulation of stiffness induced myofibroblast transformation and highlight the importance of ROCK isoform balance in lung fibrosis. This new insight could inform future attempts for developing therapeutic strategies that target ROCK activation for the treatment of lung fibrosis.

**Acknowledgement**

**Author Disclosure**

No conflicts of interest are declared by the authors.

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