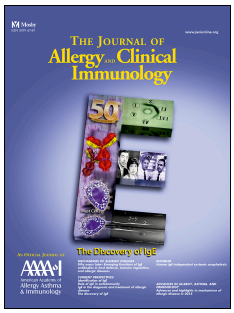
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RhoA/ROCK Signaling Modulates Lineage Commitment of Mesenchymal Stem Cells in Asthma through Lef1

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RhoA/ROCK Signaling Modulates Lineage Commitment of Mesenchymal Stem Cells in Asthma through Lef1

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Running title: RhoA/ROCK signaling and mesenchymal stem cells in asthma

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ABSTRACT

Rationale: Mesenchymal stem cells (MSCs) are increased in the airways after allergen challenge. RhoA/ROCK signaling is critical in determining the lineage fate of MSCs in tissue repair/remodeling.

Objectives: To investigate the role of RhoA/ROCK signaling in lineage commitment of MSCs during allergen-induced airway remodeling and delineate the underlying mechanisms.

Methods: Active RhoA expression in asthmatic lung tissues and its role in cockroach allergen-induced airway inflammation and remodeling were investigated. The RhoA/ROCK signaling-mediated MSC lineage commitment was assessed in an asthma mouse model using MSC lineage tracing mice (*nestin-Cre; ROSA26-EYFP*). The role of RhoA/ROCK in MSC lineage commitment was also examined by MSCs expressing constitutively active RhoA (RhoA-L63) or dominant negative RhoA (RhoA-N19). Downstream RhoA-regulated genes were identified using the stem cell signaling array.

Results: Lung tissues from asthmatic mice showed increased expression of active RhoA when compared with those from controls. Inhibition of RhoA/ROCK signaling with fasudil, a RhoA/ROCK inhibitor, reversed established cockroach allergen-induced airway inflammation and remodeling as assessed by more collagen deposition/fibrosis. Furthermore, fasudil inhibited MSC differentiation into fibroblasts/myofibroblasts, but promoted MSC differentiation into epithelial cells in asthmatic *nestin-Cre; ROSA26-EYFP* mice. Consistently, expression of RhoA-L63 facilitated the differentiation of MSCs to fibroblasts/myofibroblasts, whereas expression of RhoA-19 switched the differentiation toward epithelial cells. Gene Array identified the Wnt signaling effector Lef1 as the most up-regulated gene in RhoA-L63-transfected MSCs. Knockdown of Lef1 induced MSC differentiation away from fibroblasts/myofibroblasts but

towards epithelial cells.

Conclusions: These findings uncover a previously unrecognized role of RhoA/ROCK signaling in MSC-involved airway repair/remodeling in asthma.

Keywords: Asthma, Cockroach allergen, RhoA/ROCK, Mesenchymal stem cell, Lef1

Capsule summary

MSCs are critical in airway repair/remodeling in asthma. RhoA/ROCK signaling functions as a molecular switch for the lineage fate of MSCs during airway repair/remodeling. Targeting RhoA/ROCK signaling might be an effective therapeutic strategy for asthma.

Key Messages

- RhoA signaling is critical in airway inflammation and remodeling.
- RhoA signaling controls lineage commitment of MSCs in asthma.
- Wnt signaling effector Lef1 plays a role in RhoA signaling-mediated MSC differentiation.

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ABBREVIATIONS

CRE	Cockroach extract
BALF	Bronchoalveolar lavage fluid
CRE-ECM	Cockroach allergen-epithelial conditioned medium
MSC	Mesenchymal stem cell
RhoA	Ras homolog family member A
ROCK	Rho-associated protein kinase 1
Lef1	Lymphoid enhancer-binding factor 1
EMT	Epithelial-mesenchymal cell transition
α-SMA	Alpha-smooth muscle actin
WT	Wild-type
EV	Empty vector

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INTRODUCTION

Alterations within the structural cells of the airway, together with abnormal immune responses, lead to the full manifestation of asthma[1-3]. Airway epithelial damage and repair occur almost immediately after epithelial injury[4, 5], whereas airway remodeling, characterized by goblet cell hyperplasia, angiogenesis, smooth muscle hypertrophy, and sub-epithelial fibrosis[6], occurs after repeated allergen exposure, inflammation and damage[7-9]. Therefore, therapies that can prevent/inhibit fibrotic remodeling but accelerate airway epithelium repair are needed.

Stem/progenitor cells are known to participate in tissue repair and remodeling[10, 11]. Mesenchymal stem cells (MSCs) are adult connective tissue progenitor cells with the ability of self-renewal and differentiation into multiple cell types[12]. MSCs from bone-marrow/circulating blood can migrate to the sites of tissue damage[13-15]. Especially, we and others have shown that MSCs were significantly increased in the lungs after allergen challenge[16-20]. Nestin, originally detected in neuronal stem cells, has recently been used as a marker for bone marrow-derived MSCs[12, 21]. Nestin positive MSCs were recruited to the injured/diseased arteries, contributing to pathological arterial remodeling[22, 23] . This population of cells was also recruited to other injured/diseased sites such as the reactive stroma resulting in overgrowth of prostate tissues[24], subchondral bone of joints promoting osteoarthritis[25, 26], and lung tissues leading to the suppression of airway inflammation and remodeling[20, 27-29]. It has been revealed that MSCs have the ability to differentiate into a number of mature cell types in the lungs such as epithelial cells[4, 30-36] and fibroblasts/myofibroblasts[16-20, 37-39]. However, these studies were performed either in cultured MSCs or injected exogenous MSCs in mouse models of lung injury. Furthermore, the primary endogenous factors that control MSC differentiation at sites of airway damage as yet remain unclear. We have recently demonstrated that TGFβ1-activated RhoA/ROCK signaling in

MSCs is a determinant for the lineage commitment of MSCs[40]. RhoA is an intracellular signal transducer of the Rho family of small GTPases[41]. The RhoA-ROCK pathway is a central coordinator of tissue injury response. RhoA and its downstream effectors, ROCK1 and ROCK2, regulate a number of cellular processes, including actin cytoskeleton organization, cell adhesion, migration, proliferation, survival, and permeability[42-44]. RhoA/ROCK signaling is also essential for multiple aspects of VEGF-mediated angiogenesis[45]. Importantly, RhoA/ROCK signaling has been linked with pathophysiological processes of asthma, including repeated allergen challenge-induced airway smooth muscle contraction, formation of asthmatic airway hyper-responsiveness, and chemotactic aggregation of eosinophils to the airway, and allergic inflammation and airway remodeling[46-52]. These findings raise the possibility that RhoA/ROCK signaling may play a role in modulating MSC differentiation and subsequently airway repair/remodeling in allergic asthma.

Here, we tested the hypothesis that RhoA/ROCK signaling plays a role in allergen-induced airway repair/remodeling by controlling lineage commitment of MSCs. We provide evidence that RhoA is activated in asthmatic lung tissues, and inhibition of RhoA/ROCK signaling protects against cockroach allergen-induced airway inflammation and remodeling. By using MSC lineage tracing mouse model, we demonstrate that inhibition of RhoA/ROCK signaling suppresses MSC fibroblast/myofibroblast differentiation, but promotes MSC differentiation into airway epithelial cells. Importantly, we identified Wnt signaling effector Lef1 as the most up-regulated gene in MSCs by RhoA/ROCK signaling and a key factor for MSC lineage commitment.

METHODS

Animals

All animal experimentations were carried out using six- to eight-week-old mice. C57BL/6 wild-type (WT), B6.Cg-Tg (Nes-cre) 1Kln/J mice (Stock No: 003771) and B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$/J mice (Stock No: 006148) were purchased from Jackson Lab. All animals were maintained in the animal facility of the Johns Hopkins University School of Medicine. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University, Baltimore, MD.

Allergen-induced asthma mouse model

A chronic cockroach allergen-induced mouse model of asthma was generated. Mice were sensitized once a day with 20 µg cockroach extract (CRE, Greer Laboratory) in 50µl of PBS under light anesthesia on day 0, 7, and 14. Following sensitization, mice were treated three times a week with CRE for 6 continuous weeks (Fig 2, A). Control mice received PBS during the sensitization and challenge phases. In some cases, mice were treated with fasudil (LC Laboratories; Woburn, Massachusetts, USA) at a dose of 30 mg/kg dissolved in buffered saline by intra-peritoneal administration 30 min before CRE challenge for prevention or once a day for two consecutive weeks after the last challenge for treatment of the established mouse model. Vehicle-treated mice received water. Bronchoalveolar lavage (BAL) fluids were obtained with 2 flushes of 0.8 ml of PBS and BAL cells were collected for total and differential counts by flow cytometric analysis[53, 54]. Cell free BAL fluids were used for cytokine measurements. Lung tissues were dissected for histological analyses. Bloods were also taken to determine serum antibodies against CRE.

Analysis of lung inflammation

Lung inflammation was assessed as previously described[53-55]. Masson's Trichrome Staining was carried out using the kit from Sigma Aldrich according to the manufacture and the procedure is provided in the Online Repository.

ELISA

Concentrations of *IL-4, IL-5, IL-13, IL-17, TSLP, IL-33*, and *TGFβ1* in cell-free BAL fluids were measured by ELISA using the Ready-Set-Go! ELISA sets (ThermoFisher)[53-55]. Cockroach allergen-specific IgE and IgG1 serum levels were analyzed by ELISA as previously described [54].

Immunostaining

Immunostaining was performed as previously described[53-56]. The following primary antibodies were used: anti-active RhoA-GTPase (New East Biosciences), anti-E-cadherin (Cell Signaling), anti-αSMA (Sigma, clone SP171), anti-EYFP (Novus), anti-Vimentin (ThermoFisher, clone V9), and anti-Lef1 (Cell Signaling). The detailed information is provided in the Online Repository (Table E2). To determine the fluorescence signal in tissue sections, fluorescent positive cells in four different high-power fields from each lung section were quantified using ImageJ v1.50e (NIH) and presented as mean fluorescence intensity per square micrometer. Four to six lung sections from each sample were used for analysis.

Western blotting

Western blotting was assessed as previously described[53-56]. Primary antibodies include anti-active RhoA-GTPase, anti-αSMA, anti-COI, anti-E-cadherin, and anti-phospho-serine-42-Lef1 (Sigma, cat# SAB4504256), anti-Lef1, and β-actin (BioLegend, clone 2F1-1). Blots were washed

and probed with IRDye 800CW or IRDye 680RD-conjugated secondary antibodies (LI-COR). Detection was performed using a LI-COR Odyssey CLx imaging system and fluorescent intensities were quantified with Image Studio Lite version 5.2.5 (LI-COR).

MSC lineage tracing mouse model

To trace the lineage of MSCs, *nestin-Cre: ROSA26-EYFP* mice, where nestin^{+} cells and their progenitors express EYFP, were used. Fasudil was given 30 min prior to every single CRE challenge in some mice at a dose of 30 mg/kg dissolved in water by intra-peritoneal administration. Mice were sacrificed 4 days after the last CRE-challenge.

MSC differentiation *in vitro* assay

Mouse MSCs were cultured from bone marrow of C57BL/6J mice as previously described[40, 57] and characterized as described in the Online Repository. MSCs were transfected with a plasmid expressing a constitutively active RhoA (L63RhoA) or a dominant negative RhoA (N19RhoA) or empty vector (EV). Lef1 knockdown was accomplished by using MISSION® shRNA Plasmid (Sigma, TRCN0000225788). The methods for transfection and determination of transfection efficiency are provided in the Online Repository. For MSC differentiation into fibroblasts/myofibroblasts, these transfected MSCs were cultured in CRE-ECM or control medium for 7 days. Cells were fixed with 4% paraformaldehyde and immuno-stained for α-SMA. For MSC differentiation into epithelial cells, MSCs were cultured in Small Airway Epithelial Cell Growth Medium (Lonza, CC-3119 and CC-4124), or control medium for 14 days. Cells were then fixed with 4% paraformaldehyde and immune stained for E-cadherin.

Gene profiling of mesenchymal stem cells

For gene profiling, $5.0x10^5$ MSCs were seeded on tissue culture plate 24 h prior to transfection with a plasmid expressing a constitutively active RhoA (RhoA-L63) or empty vector (EV)

control using Lipofectamine 2000 (Thermo Fisher). Total RNA was isolated from the MSCs using Ribospin II RNA purification kit (GeneAll) and cDNA was synthesized using High Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher). The cDNA was used on the real-time RT^2 Profiler PCR Array (QIAGEN) specifically designed for Mouse Stem Cell Signaling (PAMM-047Z) in combination with RT^2 SYBR® Green qPCR Mastermix (QIAGEN). PCR Array data was analyzed using QIAGEN GeneGlobe Data Analysis Center and calculated fold change was done using the 2(-Delta Delta C(T)) method as described by Livak and Schmittgen[58].

RT-PCR

Quantitative RT-PCR was performed as previously reported [53, 54, 56]. Primer sequences are listed in Table E1 in the Online Repository.

Statistical analysis

All data were analyzed with Graph Pad Prism statistical software program (GraphPad Inc., La Jolla, CA) and are expressed as mean ± SEM. The significance of differences among groups was determined by one-way ANOVA (nonparametric test), and between two groups was analyzed by an unpaired, 2-tailed Student's t-test. A $P<0.05$ was considered statistical significance.

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RESULTS

Expression of active RhoA is increased in asthmatic lung tissues

To test if RhoA is involved in the pathogenesis of asthma, we detected the expression of active RhoA in the lung tissues of asthmatic mouse model by immunofluorescence analysis. Increased expression of active RhoA (RhoA-GTP) was detected in the lung tissues of cockroach allergen-challenged mouse model relative to the PBS-treated mice as determined by immunostaining (Fig 1, A and B). The active RhoA was predominantly expressed in the airway epithelium as assessed by co-staining for both RhoA-GTP and Epi-CaM. The increased expression of RhoA was further supported by RT-PCR (Fig 1, C) and western blot analyses of lung tissues from these mice (Fig 1, D and E).

Inhibition of RhoA signaling reverses the established lung inflammation

We determined whether inhibiting RhoA signaling can reverse the established inflammatory phenotypes in a chronic mouse model of asthma (Fig 2, A)[20, 53, 57]. We found that cockroach allergen-challenged mice showed significantly increased lung inflammatory cell infiltration with goblet cell hyperplasia as assessed by H&E and PAS staining (Fig 2, B). In contrast, lungs from PBS control mice showed normal or undetectable airway inflammation. To determine whether inhibition of RhoA signaling reverses the established lung inflammation, fasudil, a selective RhoA/ROCK inhibitor[59], was used after the asthmatic mouse model was established. We found that treatment with fasudil significantly reversed the established lung inflammation with goblet cell hyperplasia. Moreover, these fasudil treated allergen-challenged mice showed reduced numbers of total inflammatory cells and specifically eosinophils in the BAL fluids (Fig 2, C). Furthermore, fasudil-treated mice showed lower levels of serum cockroach specific IgE (sIgE) (Fig 2, D) and reduced levels of *IL-4*, *IL-5*, *IL-13*, *TSLP*, *IL-33*, and *TGFβ1* (Fig 2, E) in the

BAL fluids. Next, we investigated whether inhibiting RhoA signaling can prevent cockroach allergen-induced inflammatory phenotypes by using fasudil before cockroach allergen challenge (Fig E1, A). Similarly, we found that treatment with fasudil before the allergen challenge also suppressed lung inflammation, including the recruitment of inflammatory cells to the lungs with goblet cell hyperplasia on histological examination (Fig E1, B), numbers of total inflammatory cells and specifically eosinophils in the BAL fluids (Fig E1, C), levels of serum cockroach specific IgE and IgG1 (Fig E1, D), and levels of *IL-4*, *IL-5*, *IL-13*, and *TGFβ1* in the BAL fluids (Fig E1, E). The results suggest that RhoA signaling participates in allergen-induced lung inflammation.

Inhibition of RhoA signaling reverses the established airway remodeling

Next, we asked whether RhoA signaling can reverse the established airway remodeling caused by chronic exposure to cockroach allergen. Compared to PBS-challenged mice, cockroach allergen-challenged mice showed increased airway thickening and more collagen deposition/fibrosis as assessed by Masson's trichrome staining (Fig 2, F). Similarly, treatment with fasudil after the establishment of asthmatic mouse model reversed the established airway thickening (Fig 2, G) and collagen deposition/fibrosis (Fig 2, H). The reversed airway remodeling was further confirmed by western blot (Fig 2, I) with the reduced expression of α-SMA (Fig 2, J) and Collagen 1(CoI1) (Fig 2, K) in fasudil-treated vs. vehicle-treated asthmatic mice. The same pattern was also observed for the asthma mouse model treated with fasudil before allergen challenge (Fig E2). The results indicate that RhoA signaling may be critical in allergen-induced airway remodeling.

Cockroach allergen-challenged epithelium conditioned medium (CRE-ECM) activates RhoA/ROCK signaling through TGFβ1

To investigate the underlying mechanisms by which active RhoA signaling exacerbates airway remodeling, we first determined whether cockroach allergen-challenged epithelium conditioned medium (CRE-ECM) induces the activation of RhoA/ROCK signaling in MSCs. Mouse bone-marrow-derived MSCs were cultured and characterized by positive expression of CD29, Sca1, nestin, and LepR, and negative expression of CD11b, CD34, CD31, and TER-119 (Fig 3, A)[40, 57]. MSCs were treated with CRE-ECM, and expression of active RhoA in MSCs was analyzed after 24 h. Expression of active RhoA in MSCs was markedly augmented upon exposure to CRE-ECM as detected by immunostaining with antibody against active RhoA (Fig 3, B). We further determined whether the CRE-ECM-induced activation of RhoA is through TGFβ1, because we previously demonstrated that CRE-ECM contains active TGFβ1 and activates TGFβ1 signaling in MSCs [20]. Interestingly, the augmented RhoA expression was significantly inhibited when TGFβ1 was removed from CRE-ECM by TGFβ1 neutralizing antibody (Fig 3, B). Similar results were also obtained by western blot (Fig 3, C).

Inactivation of RhoA/ROCK signaling promotes MSC differentiation into epithelial cells

To determine the role of RhoA/ROCK signaling in the lineage commitment of MSCs in asthma, we took advantage of the *nestin-Cre; ROSA26-EYFP* mice, where nestin$^+$ cells and their progeny permanently express EYFP. Compared to PBS-challenged mice, CRE-challenged mice showed increased double YFP$^+$/α-SMA$^+$ (Fig 4, A, *upper*) and YFP$^+$/vimentin$^+$ cells (Fig 4, A, *middle*) in the sub-epithelial layer, but not double YFP$^+$/E-cadherin$^+$ in the airway epithelial layer, indicating that these migrated YFP-labelled cells differentiate into fibroblasts/myofibroblasts.

Intriguingly, $YFP^+/\alpha\text{-}SMA^+$ or $YFP^+/vimentin^+$ cells were nearly undetectable in the sub-epithelial layer, whereas double $E\text{-}cad^+YFP^+$ cells were remarkably increased in the airway epithelial layer of mice with fasudil treatment (Fig 4, A, *lower*), indicating that these YFP-labelled cells are differentiated away from fibroblasts/myofibroblasts but toward airway epithelial cells after treatment with fasudil. Expression of α-SMA, vimentin, and E-cadherin in lung tissues from these mice were also analyzed by RT-PCR. Consistently, compared to these PBS treated mice, CRE-treated mice showed increased expression of α-SMA and vimentin, but was significantly inhibited by fasudil (Fig 4, B). In contrast, E-cadherin showed no change between PBS and CRE-treated group, but increased in fasudil-treated CRE-challenged mice.

The role of RhoA/ROCK signaling in controlling MSC lineage commitment was further confirmed with *in vitro* experiments. MSCs were cultured with CRE-ECM in the presence or absence of fasudil. We found that CRE-ECM promoted α-SMA expression in MSCs, but was significantly inhibited by fasudil (Fig 4, C and D). In contrast, E-cadherin was not expressed in the CRE-ECM-treated MSCs, but expressed in CRE-ECM-treated MSCs with fasudil (Fig 4, C and E). Similar pattern was observed for SAGM (small airway growth media). We found that SAGM induced E-cadherin expression, which was inhibited by CRE-ECM, but partially recovered by fasudil. As expected, α-SMA expression was not observed in SAGM-treated MSCs (Fig 4, C and D). To further validate these findings, we used MSCs expressing either a constitutively active RhoA (RhoA-L63) or dominant-negative (RhoA-N19). Compared to the cells transfected with control empty vector, MSCs expressing RhoA-L63 showed increased expression of α-SMA (Fig 4, F), but MSC expressing RhoA-N19 demonstrated decreased expression of α-SMA. Next, we tested whether inhibition of RhoA activation promotes MSCs

differentiation toward epithelial cells in MSCs cultured with SAGM. MSCs expressing RhoA-N19 showed increased expression of E-cadherin when compared with those transfected with empty vector (Fig 4, G), whereas MSCs expressing RhoA-L63 had no such effect. Collectively, inactivation of RhoA/ROCK signaling not only inhibits MSC fibroblasts/myofibroblast differentiation, but also triggers the switch of MSCs to differentiation into airway epithelial cells.

Identification of genes involved in RhoA-mediated MSC differentiation

To identify the molecules that mediate RhoA-induced MSC differentiation, we investigated the genes differentially expressed in mouse MSCs transfected with or without RhoA-L63 using the Stem Cell Signaling Array (PAMM-047Z, QIAGEN). This array profiles the expression of 84 key genes involved in signal transduction pathways important for embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) maintenance and differentiation (Fig 5, A). A total of 16 genes were differentially expressed in RhoA-L63 transfected MSCs compared to control cells with ranges from 2 to 56.61-fold change (Fig 5, B), including 8 genes up-regulated and 8 down-regulated (Fig 5, C). Of these, Lef-1 is the most up-regulated gene (56.61-fold), and the pluripotency maintenance pathway factor Lifr (leukemia inhibitory factor receptor) was the most down-regulated (13.55-fold). Especially, genes within TGF-β/BMP pathway and Wnt signaling factor showed the most up or down-regulation (Fig 5, D). Several selected up or down-regulated genes were further validated by qRT-PCR, including Lef1, Smad7, TGFβr3, and Lifr (Fig 5, E).

RhoA signaling regulates Lef1 activation

We then narrowed our focus down to Lef1 and investigated its regulation by RhoA signaling. Compared to PBS-treated mice, cockroach allergen-challenged mice showed significantly up-

315 regulated expression of Lef1 in lung tissues (Fig 6, A). The expression of Lef1 was attenuated if
316 mice were treated with fasudil before challenge. These findings were further supported by RT-
317 PCR analysis in lung tissues from these mice (Fig 6, B). Interestingly, although no difference
318 was found for total Lef1, increased expression was noted for phospho-Lef1 (p-Lef1) in MSCs
319 after exposure to CRE-ECM (Fig 6, C and D). The increased expression of p-Lef1 was blocked
320 when fasudil was added to CRE-ECM.
321
322 **Lef1 regulates MSC differentiation**
323 We tested whether the RhoA activated Lef1 plays a role in MSC differentiation by knocking
324 down Lef1 with targeted small interfering RNA. We first confirmed the transfection efficacy of
325 Lef1 shRNA using flow cytometry analysis (Fig 7, A). Moreover, a dose-dependent deletion of
326 Lef1 was detected by RT-PCR (Fig 7, B) and western blot (Fig 7, C). A time-dependent deletion
327 of Lef1 was also performed (Fig E3). While no difference was observed in these cells with or
328 without Lef1 knockdown after treatment with control medium (Fig 7, D to G), significant
329 changes on MSC differentiation were observed in cells cultured with either CRE-ECM or
330 SAGM. Particularly, MSCs with Lef1 knockdown display an inhibition of MSC differentiation
331 into fibroblasts compared to siRNA control-treated MSCs for α-SMA (Fig 7, D). The inhibition
332 was further supported by western blotting (Fig 7, E). By contrast, these MSCs with Lef1
333 knockdown showed an increased differentiation into epithelial cells with higher expression of E-
334 cadherin compared to shRNA control-treated MSCs (Fig 7, F) and western blot (Fig 7, G).
335 Collectively, our findings suggest that knockdown of Lef1 can suppress MSC differentiation into
336 fibroblasts, but promote differentiation of MSCs into epithelial cells.

DISCUSSION

In this study, we made a novel finding that TGFβ1 activated RhoA/ROCK signaling functions as a molecular switch for the lineage fate of the recruited MSCs in asthma: activation of the RhoA/ROCK signaling drives MSC differentiation toward fibroblasts/myofibroblasts and enhances airway remodeling, whereas inactivation of this signaling pathway diverts MSC differentiation away from fibroblasts/myofibroblasts to epithelial cells for airway repair. Our study also revealed that the RhoA/ROCK signaling-mediated MSC lineage commitment /differentiation is, at least partially, through Lef1. The results suggest that Lef1 is a novel key downstream target of RhoA signaling. Our findings provide evidence for the potential development of RhoA/ROCK inhibitor as a therapeutic target for asthma.

Studies have suggested that active TGFβ1 released from the injured tissues is essential for the recruitment of MSCs to the damaged tissues and tissue repair/remodeling[20, 22, 40, 60]. Our recent finding that TGFβ1-activated RhoA/ROCK signaling functions as a molecular switch regarding the fate of MSCs in arterial repair/remodeling after injury[40], which led us to hypothesize that TGFβ1-activated RhoA/ROCK signaling may control the lineage commitment of MSCs in airway repair/remodeling in asthma. Indeed, numerous studies have suggested that RhoA is required for B cell[61] and Th2 cell differentiation[51], and is associated with pathophysiological processes of asthma[46-52]. Data presented in this study adds new understanding on the involvement of MSCs in the pathogenesis of asthma and reveals a novel role of RhoA in airway remodeling.

Our studies offered several lines of evidence to support the biological role of RhoA/ROCK signaling in asthma. First of all, we found an activated RhoA signaling in the lung tissues of cockroach allergen-induced mouse model of asthma. Next, we showed that

RhoA/ROCK signaling is an important player in allergic inflammation, and specifically, inhibition of RhoA/ROCK signaling can not only reverse the established cockroach allergen-induced inflammation, but also prevent the development of allergen-induced inflammation. However, we noted that inhibition of RhoA/ROCK signaling did not "completely" block the cockroach allergen-induced lung inflammation. Although the reason is unclear, we believe that RhoA signaling is not sole signaling activated by cockroach allergen that contributes to the cockroach allergen-induced lung inflammation. In particular, we have recently demonstrated that mannose receptor modulates macrophage polarization and allergic inflammation through miR-511-3p[54], and miR-155 modulates cockroach allergen and oxidative stress-induced cyclooxygenase-2 in asthma[55]. PAR2 signaling has also been shown to play a key role in cockroach allergen-induced airway hyper-responsiveness and airway inflammation/remodeling in a chronic mouse model[62]. Furthermore, we showed that inhibition of RhoA/ROCK signaling reversed the established cockroach allergen-induced collagen deposition and airway wall thickening, an index of airway remodeling. These findings are consistent with several recent reports that inhibition of RhoA/ROCK signaling attenuates hyperoxia-induced pulmonary fibrosis in neonatal rats[63], prevents and reverses intestinal fibrosis[64], and contributes to the development of experimental pulmonary fibrosis[65]. Collectively, our studies provide further evidence that inhibition of RhoA/ROCK signaling could prevent or recover the cockroach allergen-induce chronic inflammation and remodeling in asthma.

One of the most important mechanisms elucidated from this study is that RhoA/ROCK signaling controls lineage commitment of the recruited MSCs during asthma. MSCs can differentiate into a number of mature cell types such as epithelial cells[30-35] and fibroblasts/myofibroblasts[38, 39]. Particularly, we hypothesized that the migrated MSCs at early

383 stages may suppress inflammation and repair the damaged airway through cell differentiation
384 and paracrine effects (growth factors and cytokines at sites of tissue injury)[15, 20, 35, 66-68], but when
385 there are repetitive allergen exposures and sustained release of local growth factors, MSCs may
386 contribute to progressive fibrosis and pathological remodeling due to their differentiation into
387 myofibroblasts[38]. Indeed, this hypothesis was well-supported by our findings with the use of the
388 *nestin-Cre; ROSA26-EYFP* mice, which directly trace MSC migration and differentiation. We
389 found increased double $YFP^+/\alpha\text{-}SMA^+$ or $YFP^+/vimentin^+$ cells in the sub-epithelial layer after
390 being repeatedly exposed to cockroach allergen, whereas these cell populations were reduced
391 when these mice were pre-treated with fasudil. In contrast, YFP^+ cells were increased in the
392 epithelial layer which were also positive for airway epithelial marker E-cadherin. These findings
393 were further supported by *in vitro* experiments with MSCs expressing a constitutively active
394 RhoA or dominant negative RhoA. Thus, this study provides a proof of concept that MSCs can
395 differentiate into airway epithelial cells, and inactivation of RhoA/ROCK signaling not only
396 inhibits MSC fibroblast/myofibroblast differentiation and airway remodeling but also triggers the
397 switch of MSCs to participate in epithelium repair after airway damage.

398 The airway epithelium is the lung's first line of defense against inhaled allergens and
399 pollutants. Once injury occurs, airway epithelium must retain a robust capacity for self-repair.
400 Airway basal progenitor cells are one of the most important factors involved with repair[69, 70]. A
401 recent study has suggested that multipotent stem cell population in airway submucosal glands
402 can proliferate, migrate, and transdifferentiate to repair surface epithelium following injury[71].
403 Furthermore, alveolar type 2 (AT2) cells also function as alveolar stem cells, and single-cell Wnt
404 signaling niches maintain stemness of AT2[72]. These studies mainly focused on the local resident
405 progenitor cells, but migrated stem cells like bone marrow-derived MSCs are not considered.

Thus, our studies filled the gap and illustrated that MSCs can migrate from bone marrow into airway and differentiate into airway epithelial cells after airway injury. However, future studies are needed to track the lineage commitment/differentiation of MSCs in asthma mouse model over time using an inducible MSC lineage tracing mouse model. Particularly, it would be of interest to determine the differentiation of MSCs into different subtypes of airway epithelial cells in these mouse models. While we focused on MSCs that may serve as progenitor cells for the epithelium, local epithelial cells (e.g., basal cells, club cells and AT-II)[70, 73] are also critical as stem/progenitor cells for the epithelial repair. We assume that airway repair/remodeling in early stages may be due to local airway basal progenitor cells or local MSCs with ultimate loss of their proliferative and differentiation capacity ("progenitor exhaustion")[70], but in late stages is due to migrated bone-marrow-derived MSCs following repeated challenges. Our studies here further recognized the downstream event of RhoA/ROCK signaling in MSC lineage commitment and demonstrated that genes within TGF-β/BMP pathway and Wnt signaling are the most up or down-regulation in RhoA over-expressed MSCs. Particularly, we identified Lef1 as a Wnt signaling factor to be the most up-regulated. Lef1 as a transcription factor belongs to a family of proteins that share homology with the high mobility group protein-1(HMG1)[74]. Interestingly, Lef1 can directly induce epithelial-mesenchymal cell transition (EMT)[75] and is also required for the transition from mesenchymal to epithelial cells in the mouse embryonic mammary gland[76] and lineage commitment of myoepithelial cells (stem cells for regenerating tracheal epithelium)[77]. Our studies provide novel evidence for the requirement of Lef1 in lineage commitment/MSCs to either myofibroblasts/fibroblasts or epithelial cells. While no difference was observed between untreated MSCs with or without Lef1 knockdown, it is likely that the trigger (s) or the activation of RhoA signaling is essential in Lef1-mediated MSC differentiation.

These findings suggest that Lef1 is critical in regulating MSC differentiation, and blocking Lef1 can be an alternative approach to interfere with the TGFβ1-RhoA signaling pathway for airway repair or suppression of airway remodeling in asthma. However, the underlying mechanism regarding Lef1-mediated MSC differentiation remains unknown. β-catenin, a coactivator for transcription factors of the TCF/LEF family[78], plays a role in proliferation, self-renewal, and differentiation of hematopoietic stem cells[35, 75, 79], raising the possibility that β-catenin may mediate the RhoA-Lef1 interaction during MSC differentiation. In addition, TGFβ transcriptional coactivator Smad3, co-localized with Lef1 at canonical Wnt-responsive elements, directly interacts with Lef1[80]. Furthermore, Lef1 has been demonstrated to bind the promoter regions of E-Cadherin[81] or VEGF[82] and regulate cell differentiation[75, 83]. Lastly, downstream genes are required for the Lef1-regulated MSC differentiation (e.g., vimentin[84], Oct4 and nanog[85]) (Fig 8).

In summary, our observation in a chronic asthma mouse model yield important findings: RhoA/ROCK signaling plays a critical role in allergen-induced airway inflammation and remodeling, and activation of the RhoA/ROCK signaling drives MSC differentiation toward fibroblast/myofibroblasts and enhances airway remodeling. Inactivation of the RhoA/ROCK signaling promotes MSC differentiation into epithelial cells for airway repair through the Wnt signaling factor Lef1. These findings provide new understanding of the signaling pathways that control MSC lineage commitment in airway repair/remodeling in asthma and recognize a novel therapeutic target for patients with asthma.

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452

453 **Author contributions**

454 X. K., D.D., C. L., Y. Z. performed experiments and analyzed data. M. K. identified and
455 provided lung tissue sections from human subjects. X.K., D.D., and P.G. wrote the manuscript.
456 P.G., M. W., and Q. F. designed and supervised the study. All authors read and approved the
457 final manuscript.

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686 **FIGURE LEGENDS**

687 **FIG 1**. Expression of active RhoA (RhoA-GTP) in asthmatic lung tissues. **A**, Expression of
688 active RhoA (green) in the airway epithelium (red) and lung tissue samples from CRE and PBS
689 treated mice. **B**. Quantification of RhoA expression in lung sections using ImageJ v1.50e (NIH).
690 **C**, Expression of RhoA was detected by RT-PCR. **D**, Representative Immunoblots for RhoA-
691 GTP in lung tissues of CRE and PBS-treated mice. **E**, Densitometric analysis for RhoA
692 expression. Data represent mean ± SEM, n=6/group. ***P*<0.01.

693 **FIG 2.** Inhibition of RhoA signaling reverses the established lung inflammation. **A**, Protocol for
694 cockroach allergen-induced mouse model of asthma. CRE: cockroach extract, i.t., intra-tracheal,
695 i.p., intra-peritoneal. **B**, Paraffin tissue sections of lung were stained with H&E (upper panel) and
696 periodic acid-Schiff (PAS, lower panel). **C**, Bronchoalveolar lavage (BAL) total and eosinophil
697 cell counts as assessed by flow cytometry. **D-E**, ELISA analyses for serum levels of cockroach
698 allergen specific IgE and IgG1 (**D**) and BAL levels of cytokines (**E**). **F**, Representative images of
699 the Masson's trichrome staining of collagen on lung sections. **G,** Analyses of the epithelial layer
700 thickness. **H**, airway collagen/fibrosis (blue) presented as fold change (blue/total area in each
701 group normalized to PBS group). **I**, Representative immunoblots for α-SMA and collagen 1 (Col
702 1) in lung sections. *β-actin* was used as a control. n=3/group. **J-K**, Densitometric analysis for α-
703 SMA (**J**) and Col 1 (**K**). **C-H**, n=6-8/group. Data represent mean ± SEM. **P*<0.05, ***P*<0.01.
704 **FIG 3**. Characteristics of mesenchymal stem cells and CRE-ECM-induced RhoA activation. **A**,
705 MSCs were characterized by positive expression of CD29, Sca1, nestin, and LepR, and negative
706 expression of CD11b, CD34, CD31, and TER-119. **B**, Representative images of
707 immunofluorescence staining for GTP-RhoA (red) in MSCs. **C**, Representative immunoblots for

RhoA-GTP in MSCs. *β-actin* was used as a control. Data represent mean ± SEM of three independent experiments. ***P*<0.01.

FIG 4. RhoA signaling regulates the lineage commitment of MSCs in asthma mouse model and in vitro. **A**, Representative image of double immunofluorescence staining with the primary antibodies against YFP, α-SMA (top), vimentin (meddle), and E-cadherin (bottom). n=6/group Nuclei were counterstained with DAPI. AL: airway lumen, BV: bronchial vein. ASM: airway smooth muscle. **B**, Expression of α-SMA, vimentin, and E-cadherin in lung tissues were analyzed by RT-PCR, n=6/group. **C**, Representative immunoblots for α-SMA and E-cadherin in MSCs. *β-actin* was used as a control. **D**-**E**, Densitometric analysis for α-SMA (**D**) and E-cadherin (**E**). **F**, Immunofluorescence staining of CRE-ECM-treated MSCs expressing empty vector (EV), RhoA-L63, and RhoA-N19 with the primary antibody against α-SMA. **G**, Immunofluorescence staining of SAGM-treated MSCs expressing EV, RhoA-L63, and RhoA-N19 with the primary antibody against E-cadherin. Data represent mean ± SEMs of three independent experiments. **P*<0.05, ***P*<0.01.

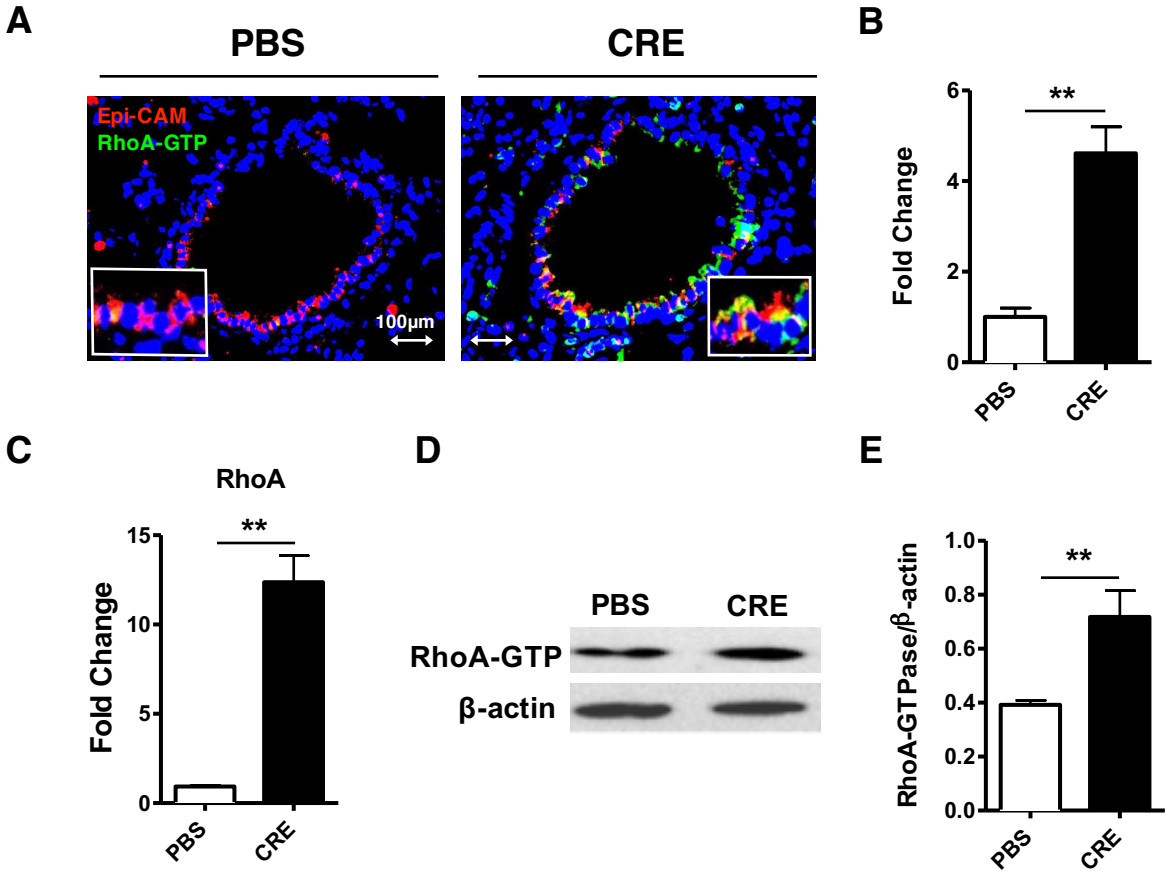
FIG 5. Identification of RhoA downstream genes by using Stem Cell Signaling Array. **A**, Differentially expressed genes in MSCs transfected with empty vector (EV) or RhoA-L63, n=4/group. **B**, Heatmap of gene expression analysis (Fold changes ≥2.0, *P*<0.01). **C**, Relative fold change of top up (red) or down (blue)-regulated genes. **D**, Most differentially expressed genes in TGFβ/BMP and WNT pathways. **E**, qRT-PCR analysis for Lef1, Smad7, Tgfbr3, and Lifr mRNA expression in MSCs expressing empty vector (EV) and RhoA-L63. Data represent mean ± SEMs of three independent experiments. **P*<0.05, ***P*<0.01.

FIG 6. RhoA signaling induces Lef1 activation in MSCs. **A**, Representative image of immunofluorescence staining for lef1 in lung section (n=5/group). **B**, RT-PCR analysis of Lef1

731 expression in lung tissues (n=5/group). **C**, Representative immunoblots for total Lef1 and
732 phosphote-Lef1 (p-Lef1) in MSCs. *β-actin* was used as a control. n=3. **D**, Densitometric analysis
733 for Lef1. Data represent mean ± SEMs. **P*<0.05, ***P*<0.01.
734 **Figure 7**. Lef1 regulates MSC differentiation. **A**, Transfection efficacy of Lef1 shRNA was
735 confirmed using flow cytometry analysis. **B-C**, A dose-dependent deletion of Lef1 was detected
736 by RT-PCR (**B**) and western blot (**C**). **D**, Immunofluorescence staining of MSCs transfected with
737 control shRNA and Lef1 shRNA with the primary antibody against α-SMA. **E**, Representative
738 immunoblots for α-SMA in MSCs transfected with control shRNA and Lef1 shRNA. **F**,
739 Immunofluorescence staining of MSCs transfected with control shRNA and Lef1 shRNA with
740 the primary antibody against E-cadherin. **G**, Representative immunoblots for E-Cadherin in
741 MSCs transfected with control shRNA and Lef1 shRNA. Data represent mean ± SEMs of three
742 independent experiments. **P*<0.05, ***P*<0.01.
743 **Figure 8**. Schematic showing the role of TGFβ1-RhoA signaling in MSC recruitment, lineage
744 commitment/differentiation, and its underlying molecular mechanism.

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Fig.1


A
PBS
CRE
Epi-CAM
RhoA-GTP
100μm
B
Fold Change
**
PBS
CRE
C
RhoA
Fold Change
**
PBS
CRE
D
PBS
CRE
RhoA-GTP
β-actin
E
RhoA-GTPase/β-actin
**
PBS
CRE

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Fig.2

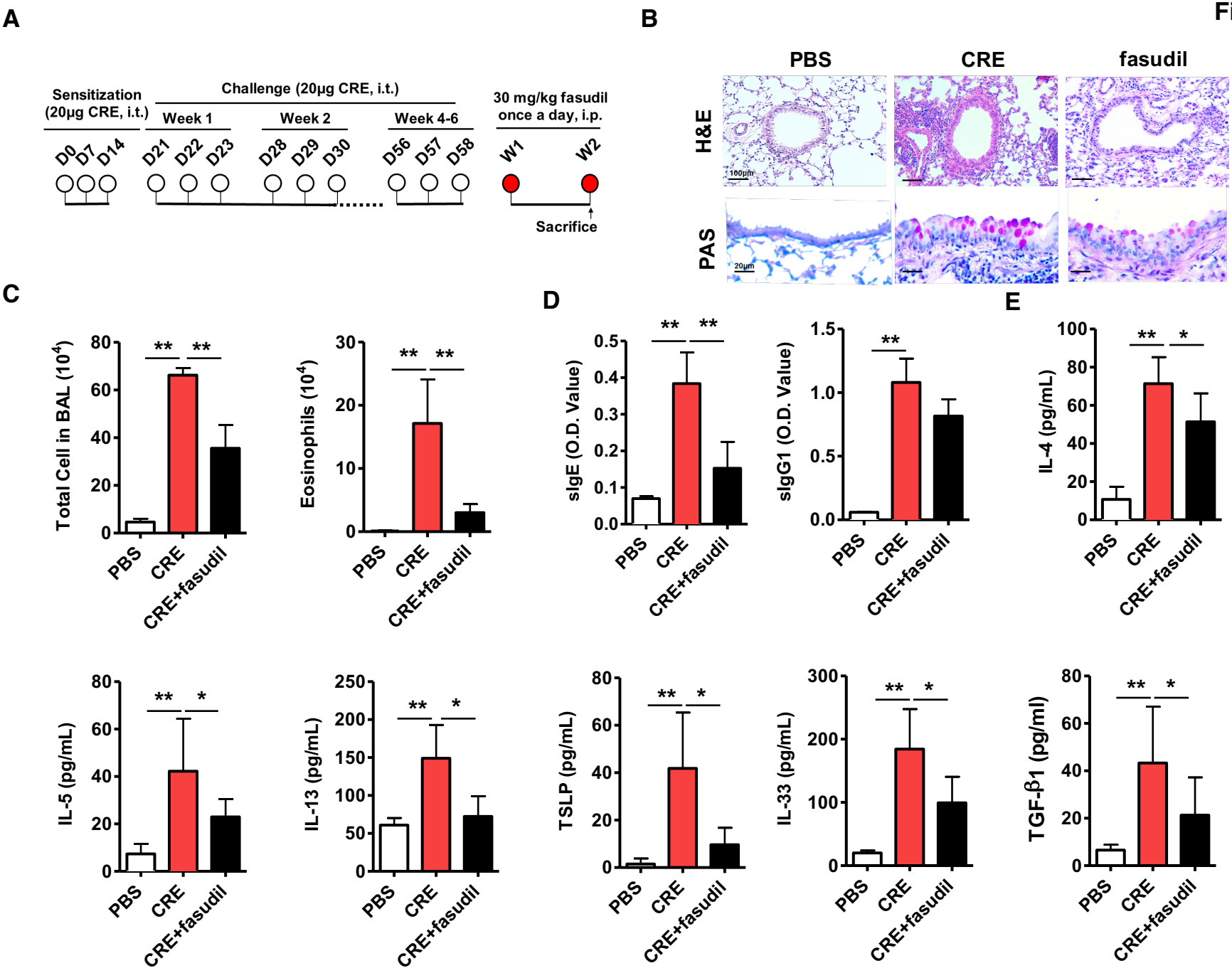


Fig. 2

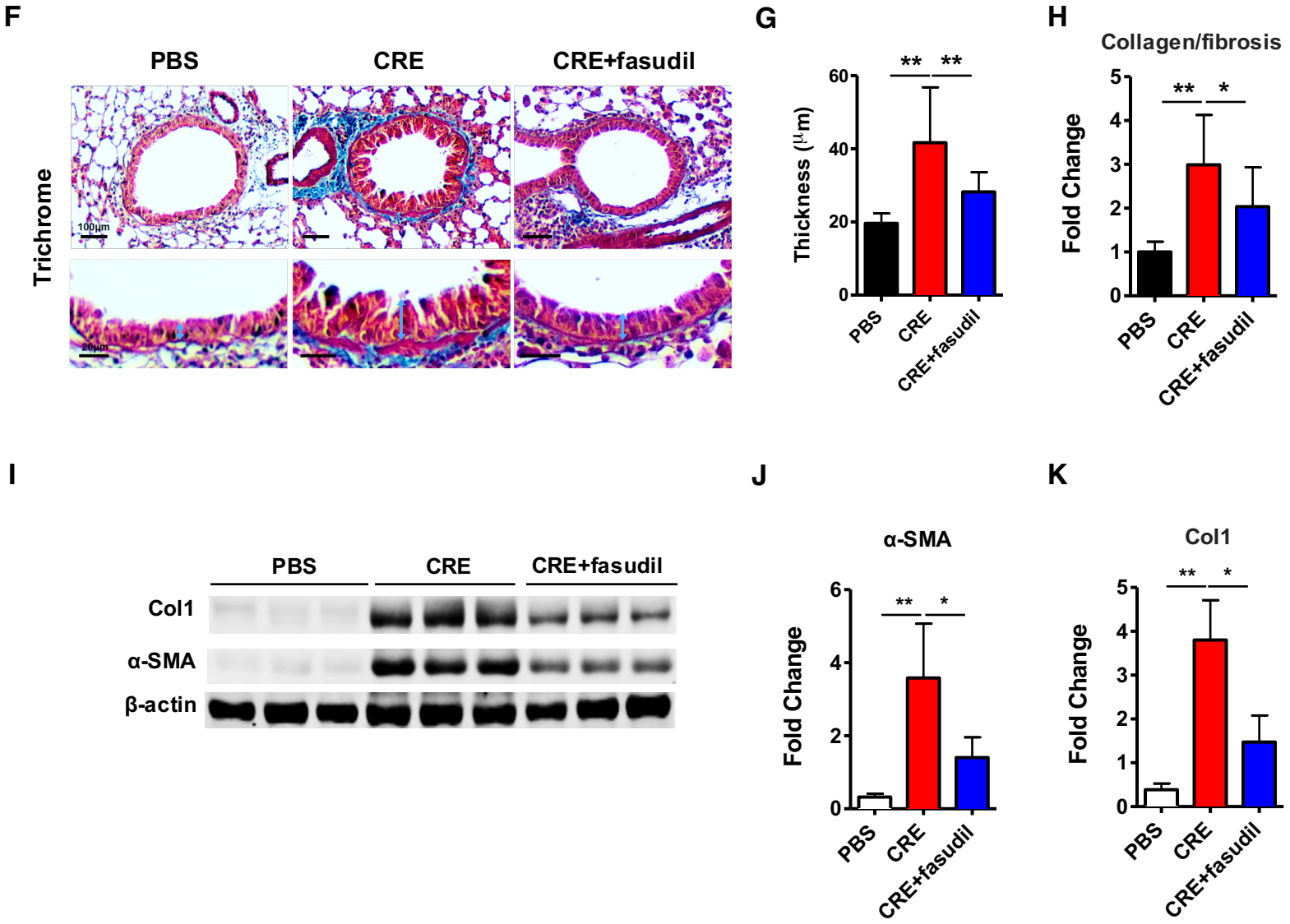

F
PBS
CRE
CRE+fasudil
Trichrome
100μm
20μm
G
Thickness (μm)
60
40
20
0
**
**
PBS
CRE
CRE+fasudil
H
Collagen/fibrosis
Fold Change
5
4
3
2
1
0
**
*
PBS
CRE
CRE+fasudil
I
PBS
CRE
CRE+fasudil
Col1
α-SMA
β-actin
J
α-SMA
Fold Change
6
4
2
0
**
*
PBS
CRE
CRE+fasudil
K
Col1
Fold Change
5
4
3
2
1
0
**
*
PBS
CRE
CRE+fasudil

Fig.3

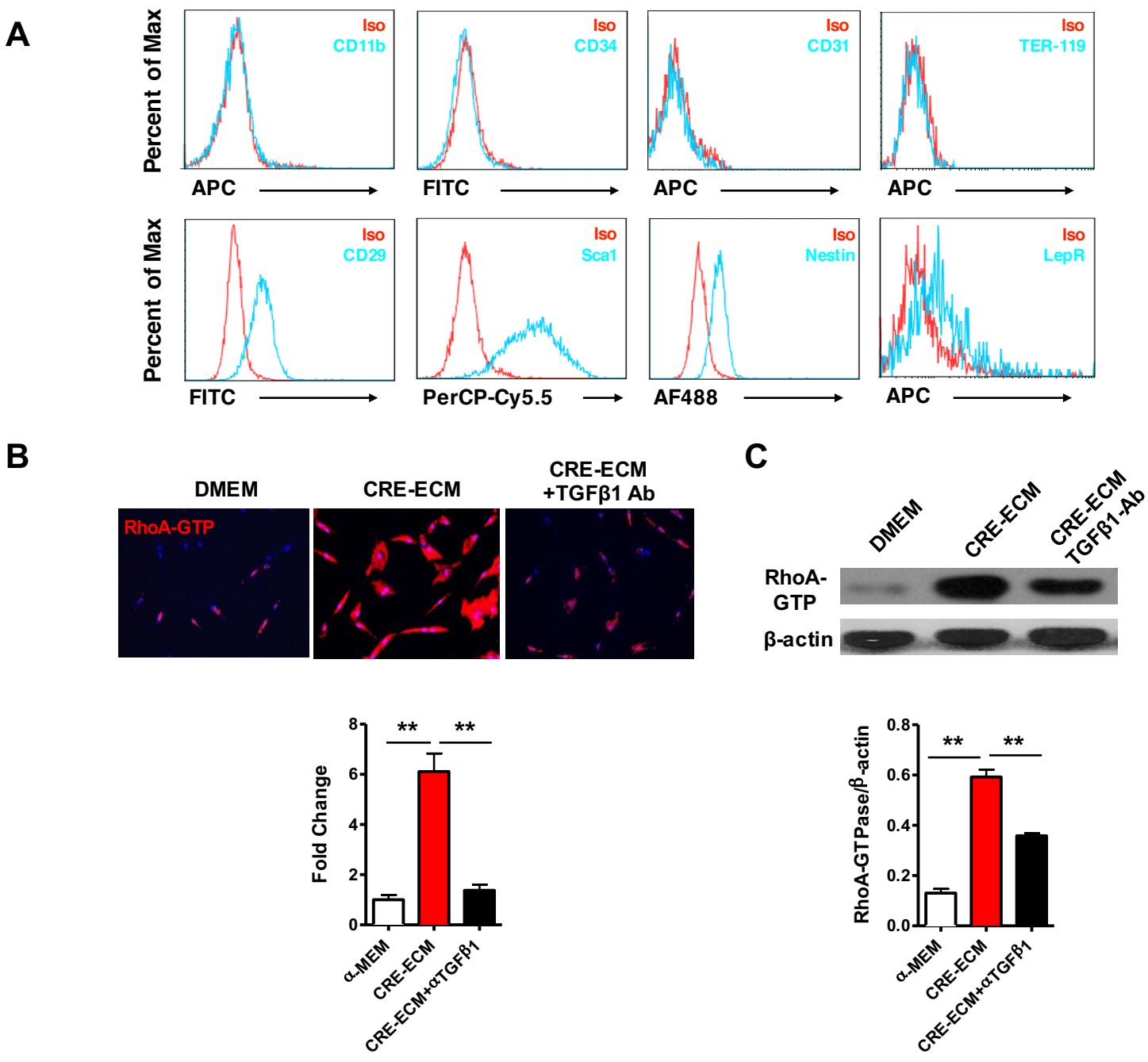

A
Percent of Max
Iso
CD11b
APC
Iso
CD34
FITC
Iso
CD31
APC
Iso
TER-119
APC
Percent of Max
Iso
CD29
FITC
Iso
Sca1
PerCP-Cy5.5
Iso
Nestin
AF488
Iso
LepR
APC
B
DMEM
CRE-ECM
CRE-ECM
+TGFβ1 Ab
RhoA-GTP
Fold Change
8
6
4
2
0
**
**
α-MEM
CRE-ECM
CRE-ECM+αTGFβ1
C
DMEM
CRE-ECM
CRE-ECM
TGFβ1-Ab
RhoA-
GTP
β-actin
RhoA-GTPase/β-actin
0.8
0.6
0.4
0.2
0.0
**
**
α-MEM
CRE-ECM
CRE-ECM+αTGFβ1

Fig.4

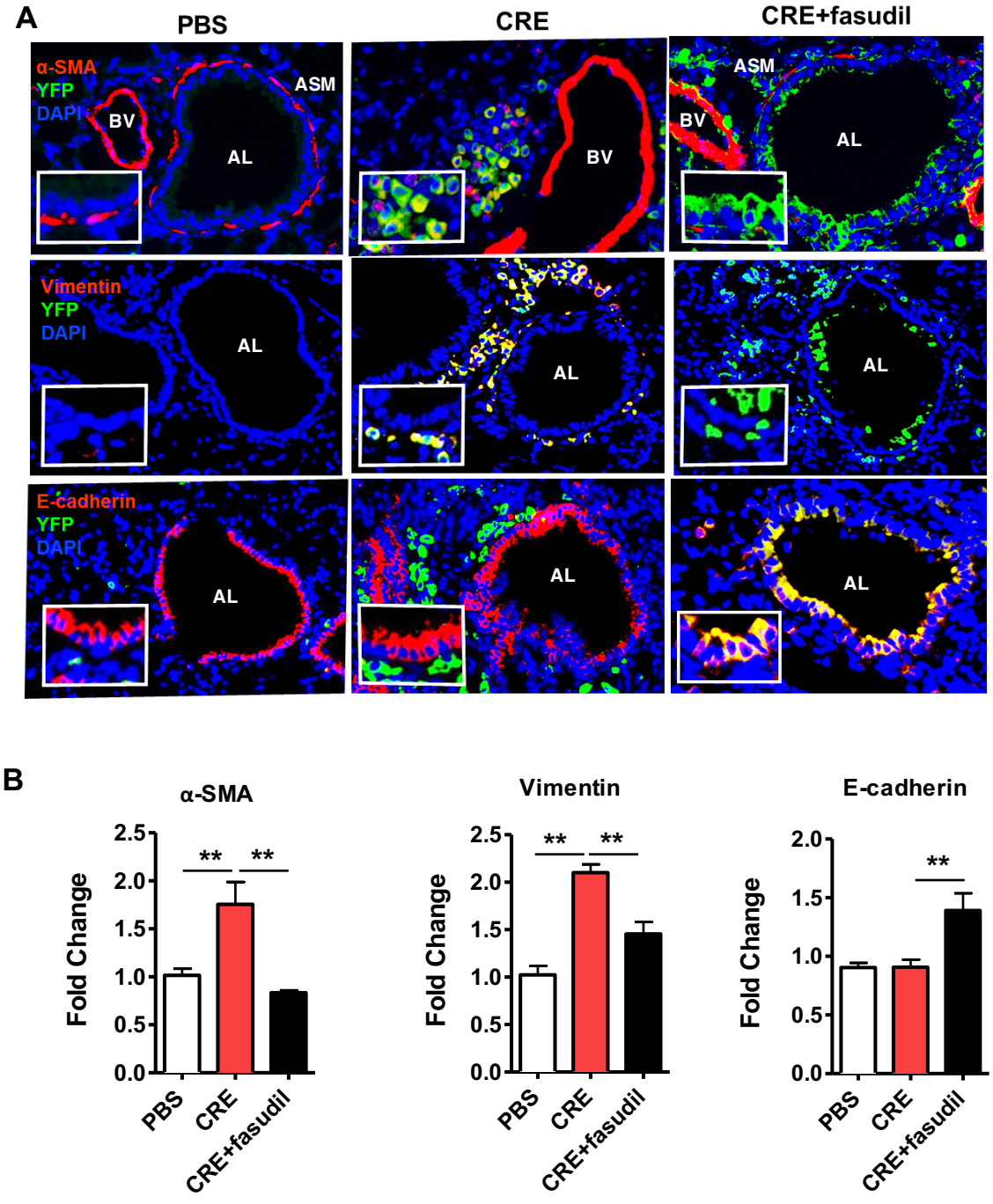

A
PBS
CRE
CRE+fasudil
α-SMA
YFP
DAPI
ASM
BV
AL
Vimentin
E-cadherin
B
α-SMA
Vimentin
E-cadherin
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
**
PBS
CRE
CRE+fasudil

Fig.4

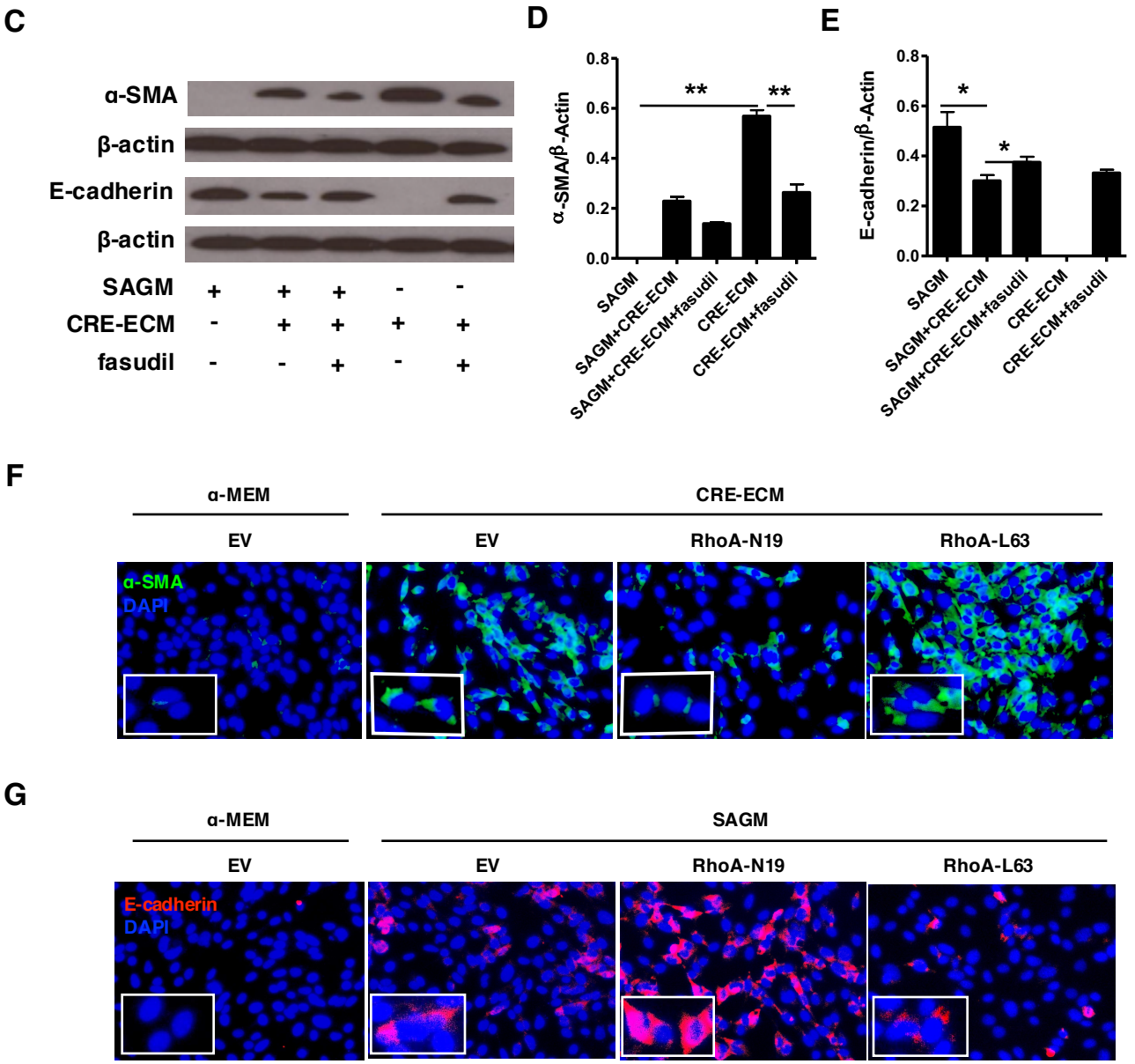
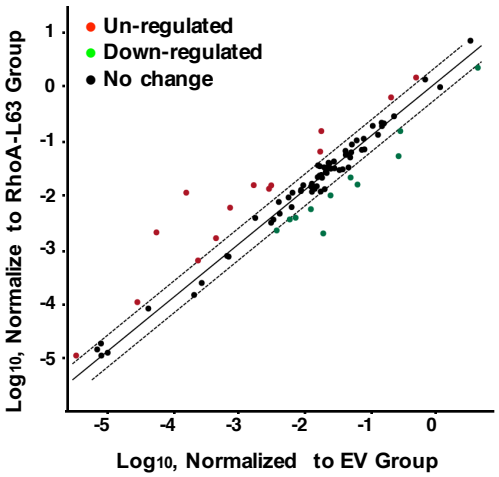
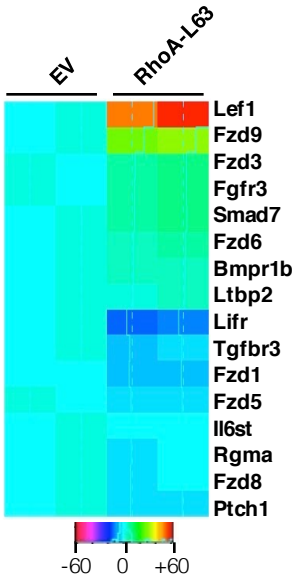

C
α-SMA
β-actin
E-cadherin
β-actin
SAGM + + + - -
CRE-ECM - + + + +
fasudil - - + - +
D
α-SMA/β-Actin
0.8
0.6
0.4
0.2
0.0
**
**
SAGM
SAGM+CRE-ECM
SAGM+CRE-ECM+fasudil
CRE-ECM
CRE-ECM+fasudil
E
E-cadherin/β-Actin
0.8
0.6
0.4
0.2
0.0
*
*
SAGM
SAGM+CRE-ECM
SAGM+CRE-ECM+fasudil
CRE-ECM
CRE-ECM+fasudil
F
α-MEM
CRE-ECM
EV
EV
RhoA-N19
RhoA-L63
α-SMA
DAPI
G
α-MEM
SAGM
EV
EV
RhoA-N19
RhoA-L63
E-cadherin
DAPI

Fig.5

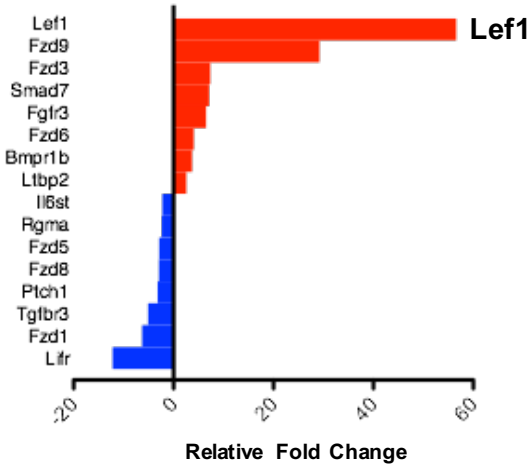
A



B



C



D

Pathway	Gene	Fold Change	Description
TGFβ/BMP Pathway	*Tgfbr3*	-5.07	Transforming growth factor, beta receptor III
	Rgma	-2.38	Repulsive guidance molecule family member A
	Ltbp2	2.57	Latent transforming growth factor beta binding protein 2
	Smad7	7.13	SMAD family member 7
WNT Signaling	*Fzd8*	-2.89	Frizzled class receptor 8
	Fzd9	29.2	Frizzled class receptor 9
	Lef1	56.61	Lymphoid enhancer binding factor 1

E

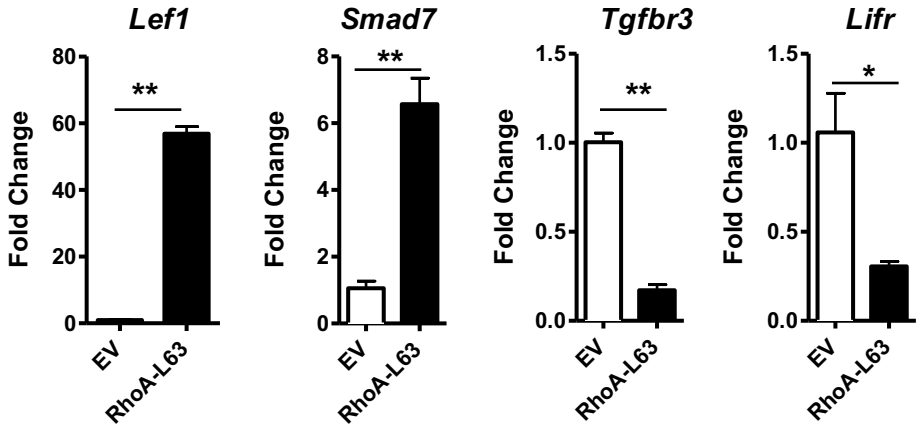
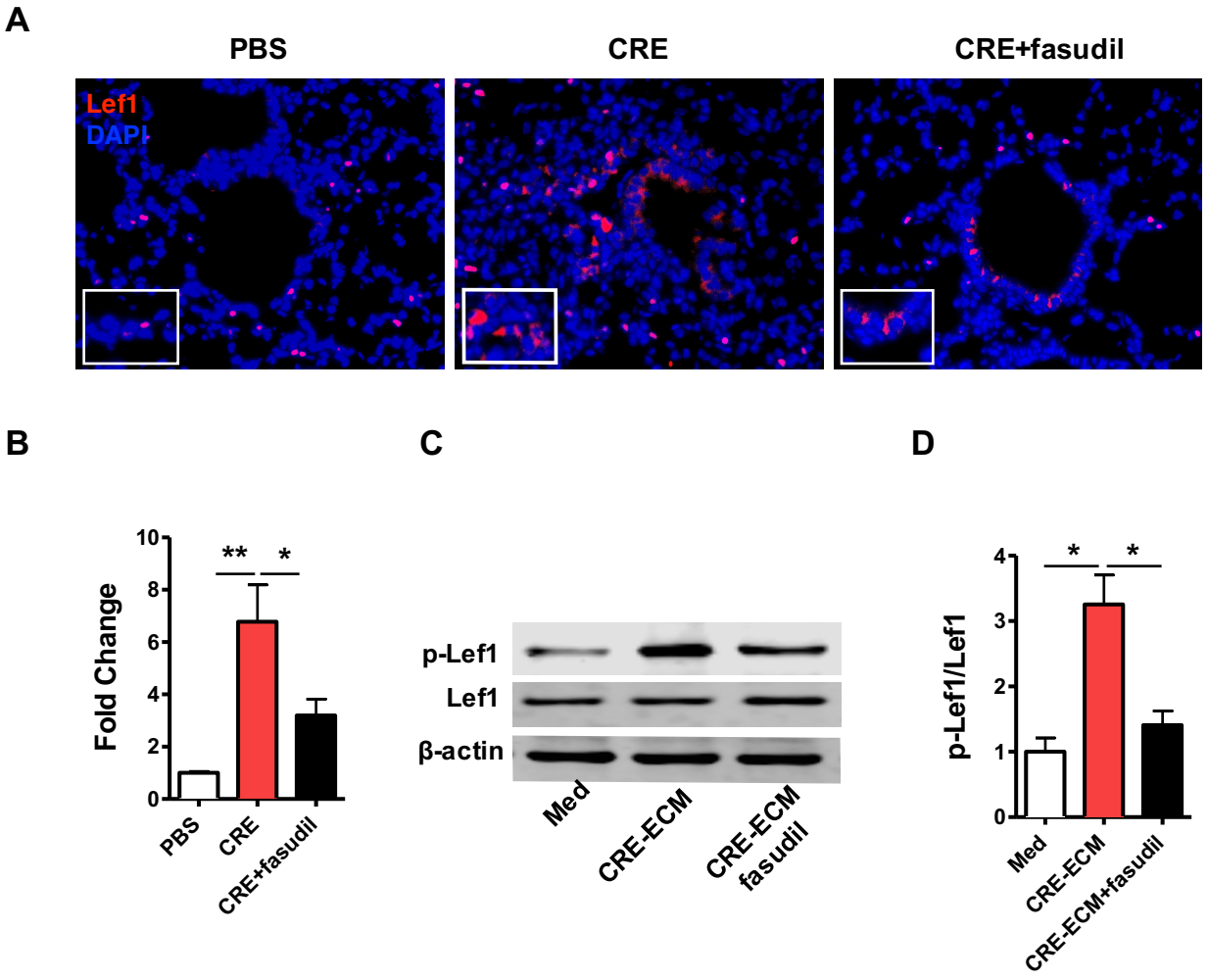


Fig.6


A
PBS
CRE
CRE+fasudil
Lef1
DAPI
B
Fold Change
10
8
6
4
2
0
**
*
PBS
CRE
CRE+fasudil
C
p-Lef1
Lef1
β-actin
Med
CRE-ECM
CRE-ECM fasudil
D
p-Lef1/Lef1
4
3
2
1
0
*
*
Med
CRE-ECM
CRE-ECM+fasudil

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Fig 7

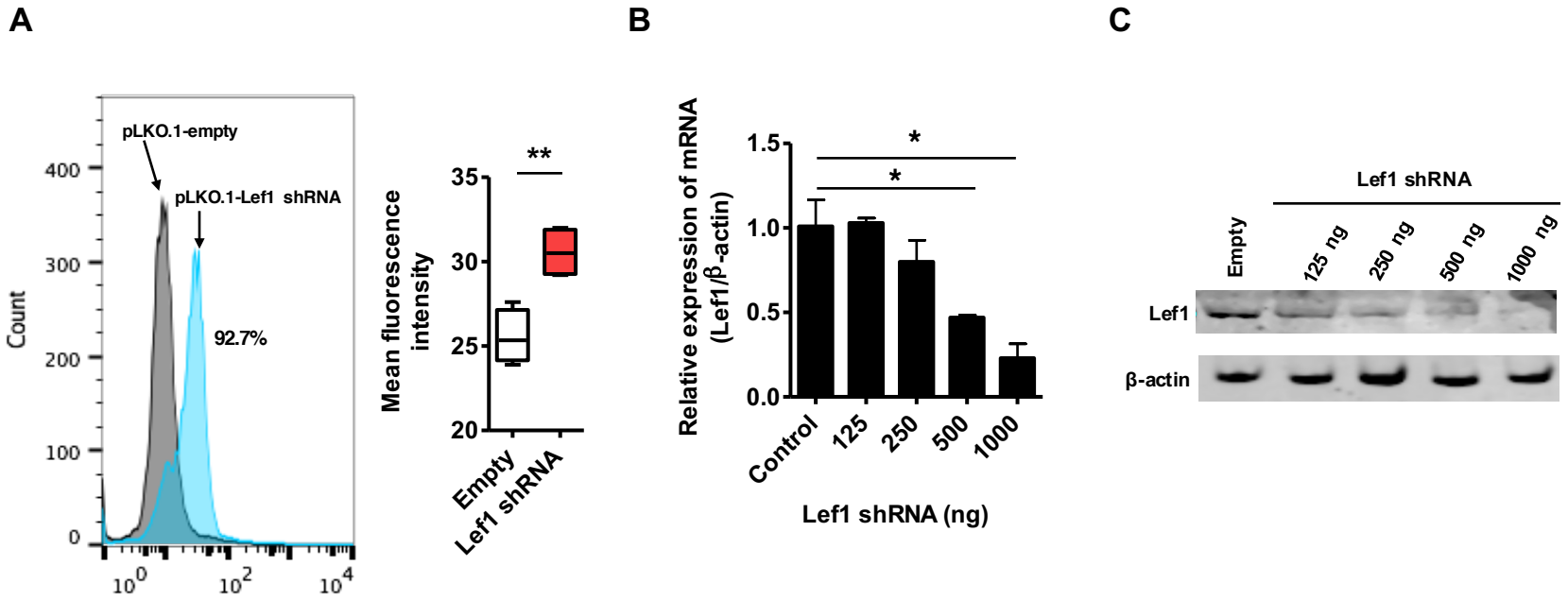


Fig 7

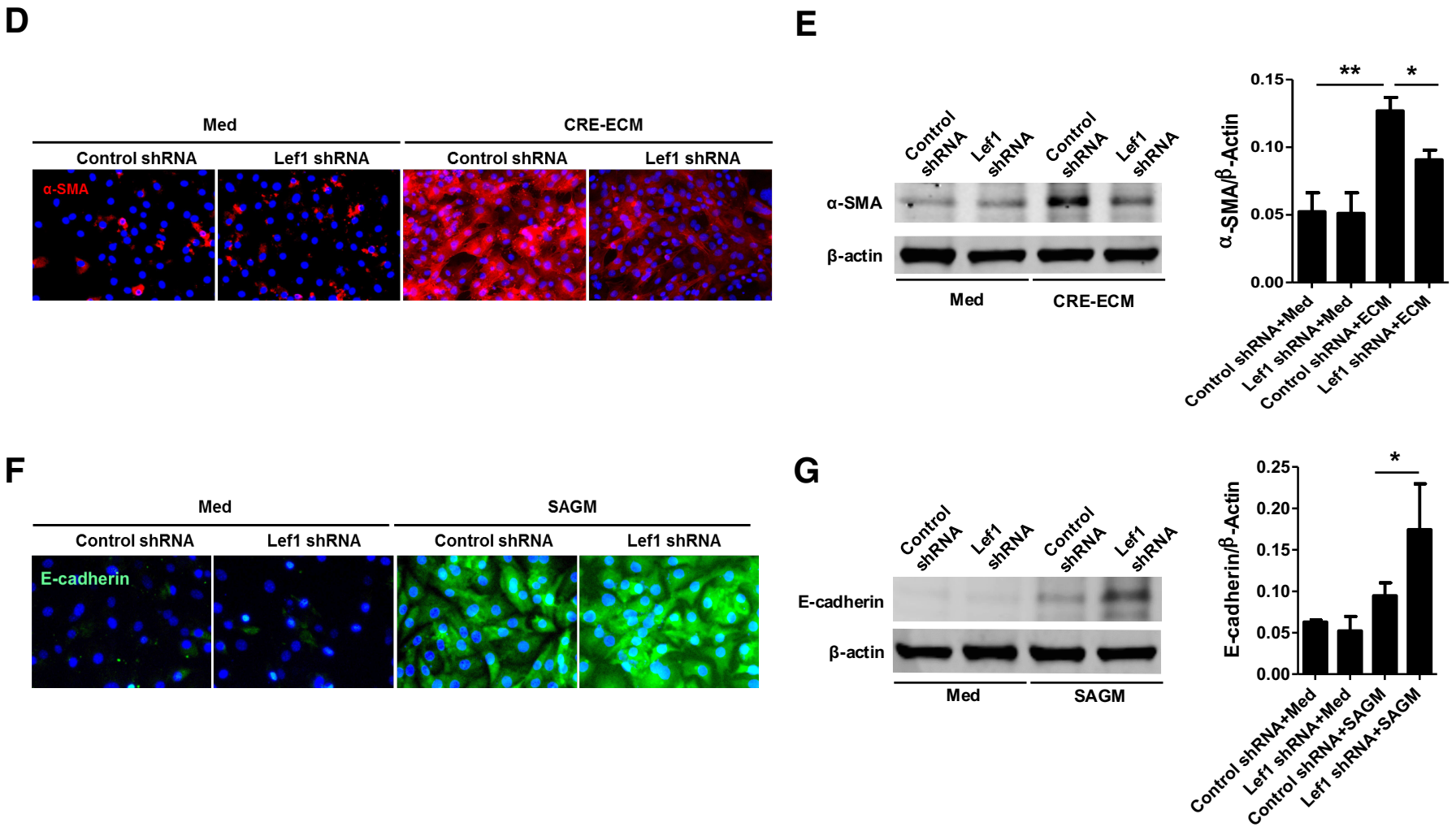
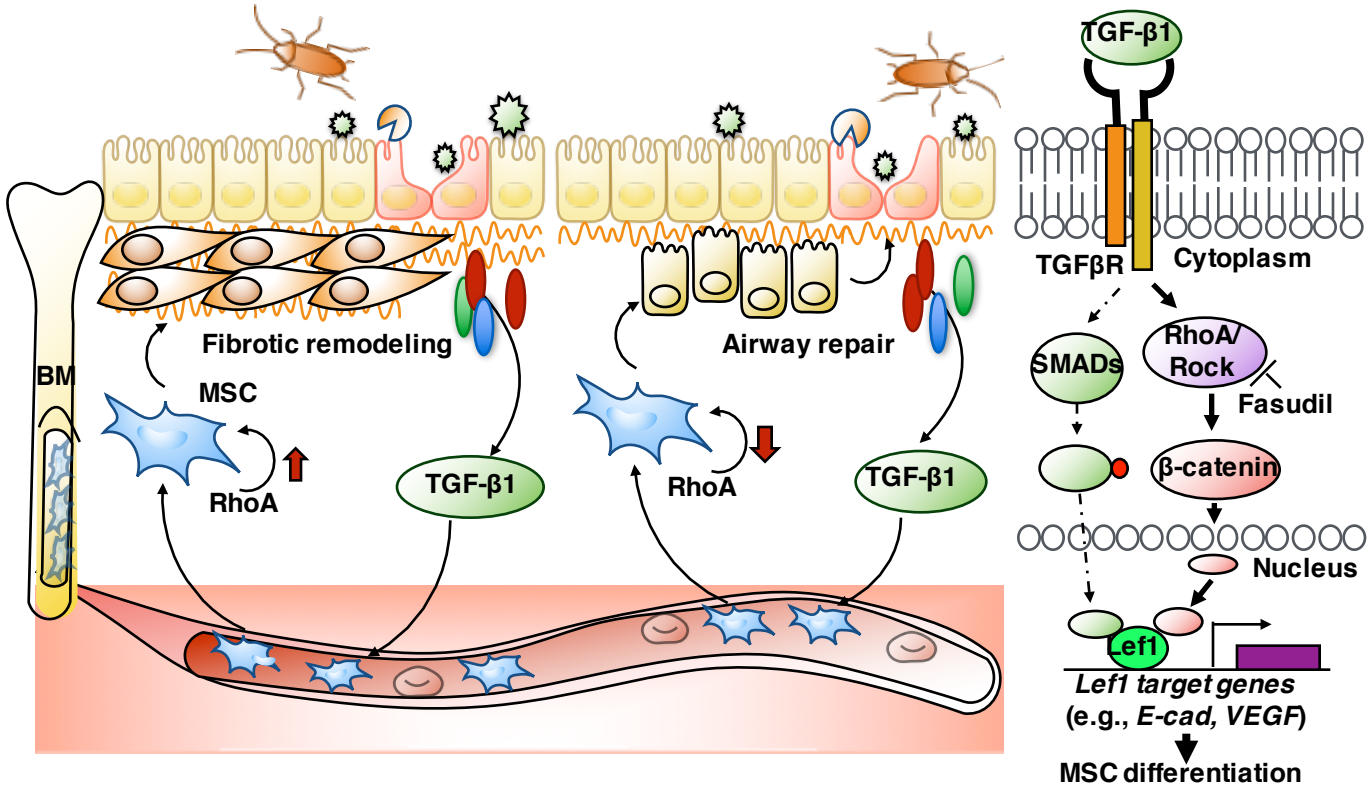
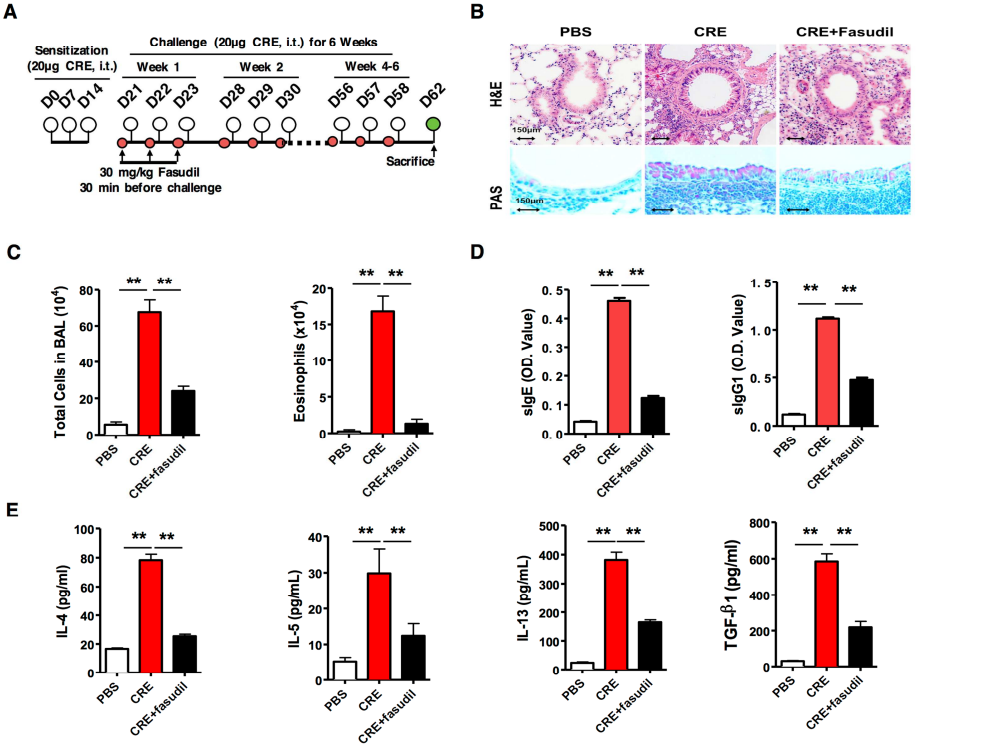

D
Med
CRE-ECM
Control shRNA
Lef1 shRNA
Control shRNA
Lef1 shRNA
α-SMA
E
Control shRNA
Lef1 shRNA
Control shRNA
Lef1 shRNA
α-SMA
β-actin
Med
CRE-ECM
α-SMA/β-Actin
0.15
0.10
0.05
0.00
**
*
Control shRNA+Med
Lef1 shRNA+Med
Control shRNA+ECM
Lef1 shRNA+ECM
F
Med
SAGM
Control shRNA
Lef1 shRNA
Control shRNA
Lef1 shRNA
E-cadherin
G
Control shRNA
Lef1 shRNA
Control shRNA
Lef1 shRNA
E-cadherin
β-actin
Med
SAGM
E-cadherin/β-Actin
0.25
0.20
0.15
0.10
0.05
0.00
*
Control shRNA+Med
Lef1 shRNA+Med
Control shRNA+SAGM
Lef1 shRNA+SAGM

Fig 8


BM
MSC
RhoA
TGF-β1
Fibrotic remodeling
RhoA
TGF-β1
Airway repair
TGF-β1
TGFβR
Cytoplasm
SMADs
RhoA/
Rock
Fasudil
β-catenin
Nucleus
lef1
Lef1 target genes
(e.g., E-cad, VEGF)
MSC differentiation

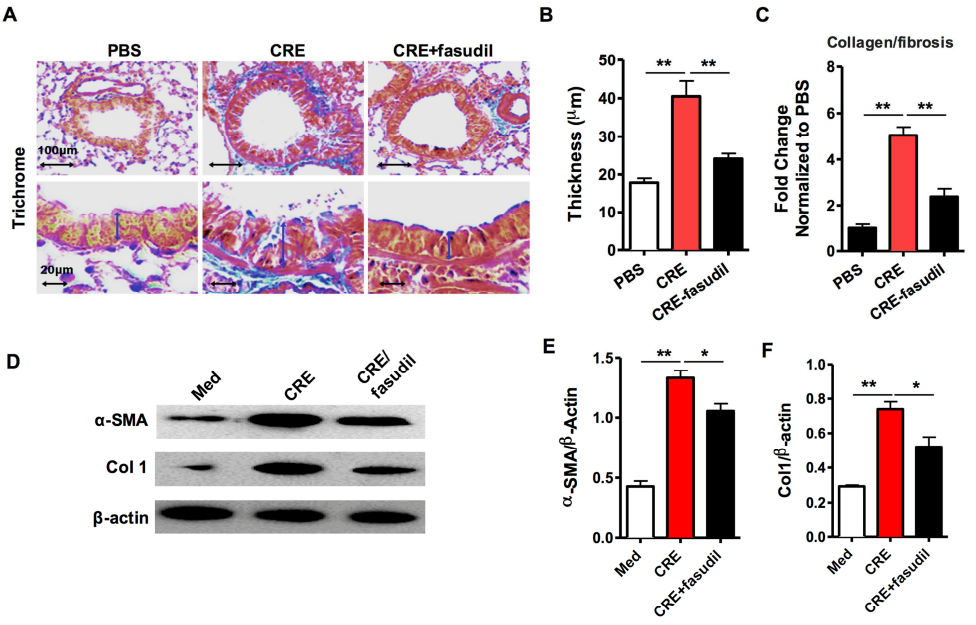
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FIG E1



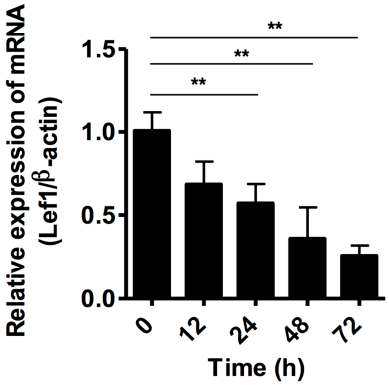
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FIG E2



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FIG E3



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Online Repository

RhoA/ROCK Signaling Modulates Lineage Commitment of Mesenchymal Stem Cells in Asthma through Lef1

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[*]Contributed equally to this work.

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METHODS

Masson's Trichrome staining

The *Masson Trichrome staining procedure stains* the collagen-rich *fibrotic* regions in blue, it is suited to assess and visualize the extent of *fibrosis* in lung tissues. Masson's Trichrome was conducted according to the guideline of the agent kit from Sigma (HT15, St. Louis, MO, USA). Briefly, frozen slides were fixed in Bouin's solution overnight at room temperature. After incubation in Weigert's Iron Hematoxylin Solution, the slides was stained with Biebrich Scarlet-Acid Fuchsin and Aniline Blue and dehydrated in ethanol and xylene. Extensive washes were done between each staining. Images where taken using a Nikon ETi microscope. The collagen fibers were stained blue, the nuclei were stained black and lung tissues were stained red. Quantification of collagen fiber from Masson Trichrome stained lung sections (n=4-6/group) were carried out using ImageJ v1.50e (NIH) with installed plugins, deconvolution of the color images, and image calculator. Airway thickness were also measured using ImageJ based on given length-to-pixel ratio.

Epithelial cell-derived conditioned medium

Cockroach allergen-induced epithelial conditioned medium (CRE-ECM) was prepared by treating TC-1, a mouse lung epithelial cell-line, with CRE (50 μg/ml) for 72 h in serum free DMEM. Cell free CRE-ECM was collected, filtered through a 0.22 μm filter (Millipore) and stored at -80^{o}C until used.

Bone marrow-derived mesenchymal stem cell and characterization.

MSCs were cultured from bone marrow of C57BL/6J mice as previously described (E1, E2). MSCs were cultured in α-MEM containing 20% FBS (Gibco). MSCs (passage 6-8) were characterized by flow cytometry using the following markers: CD11b (Thermo Fisher, clone

M1/70), CD34 (Thermo Fisher, clone RAM34), CD31 (Thermo Fisher, clone 390), TER-119 (Thermo Fisher), CD29 (Thermo Fisher, clone eBioHMb1-1), Sca-1 (Thermo Fisher, clone D7), Nestin (ThermoFisher, clone 1A4), and LepR (Thermo Fisher). These samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences) as previously described (E2).

MSC transfection

Approximately $1.0x10^5$ MSCs were seeded onto a 6-well tissue culture plate 24 h prior transfection with a plasmid expressing a constitutively active RhoA (L63RhoA) or a dominant negative RhoA (N19RhoA) or empty vector (EV) control using FuGENE 6 (Promega) as instructed by the manufacturer. Transfection efficiency was assessed by GFP expression using flow cytometry.

Lef1 knockdown

Lef1 knockdown was accomplished by transfecting $1x10^5$ MSCs with 0.5 μg of MISSION® shRNA Plasmid (Sigma, TRCN0000225788) expressing Lef1 specific shRNA using FuGENE 6 (Promega). pLKO.1-puro non-target shRNA (Sigma, SHC016) was used as a control. Lef1 knockdown was confirmed by RT-PCR and Western blot.

RT-PCR

Total RNA was extracted from CK14 ceramic beads (Cayman Chemical) homogenized tissues and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcriptase kit (Thermo Fisher). Quantification PCR assay was performed in triplicate using 7300 Real Time PCR System (Applied Biosciences) with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher) with the appropriate primers (Table E1). Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ methods as described by Livak and Schmittgen (E3). The mRNAs were normalized to the internal control gene *β-actin*. A 'no template' sample was used as a negative control.

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REFERENCES

E1: Li C, Zhen G, Chai Y, Xie L, Crane JL, Farber E, et al. RhoA determines lineage fate of mesenchymal stem cells by modulating CTGF-VEGF complex in extracellular matrix. Nat Commun 2016; 7:11455.

E2: Xu T, Zhou Y, Qiu L, Do DC, Zhao Y, Cui Z, et al. Aryl Hydrocarbon Receptor Protects Lungs from Cockroach Allergen-Induced Inflammation by Modulating Mesenchymal Stem Cells. J Immunol 2015; 195:5539-50.

E3: Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8.

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FIG E1. Inhibition of RhoA signaling reverses cockroach allergen-induced lung inflammation. **A**, Protocol for cockroach allergen-induced mouse model of asthma. CRE: cockroach extract, i.t., intra-tracheal. **B**, Paraffin tissue sections of lung were stained with H&E (upper panel) and periodic acid-Schiff (PAS, lower panel). Scale bars represent 150 μm. **C**, Bronchoalveolar lavage (BAL) total and eosinophil cell counts as assessed by flow cytometry. **D-E**, ELISA analyses for serum levels of cockroach allergen specific IgE and IgG1 (**D**) and BAL levels of cytokines (**E**). **C-E**, 10 to 12 mice were pooled from two independent experiments. Data represent mean ± SEM. **P*<0.05, ***P*<0.01.

FIG E2. Inhibition of RhoA signaling reverses cockroach allergen-induced airway remodeling. **A**, Representative images of the Masson trichrome staining of collagen on lung sections. **B,** Analyses of the epithelial layer thickness. **C**, airway collagen/fibrosis (blue) presented as fold change (blue/total area in each group normalized to PBS group). **D**, Representative immunoblots for α-SMA and collagen 1 (Col 1) in lung sections. *β-actin* was used as a control. n=3. **E-F**, Densitometric analysis for α-SMA (**E**) and Col 1 (**F**). **A-C,** n=4-6/group. Quantification data represent mean ± SEM. **P*<0.05, ***P*<0.01.

FIG E3. Lef1 knockdown in MSCs at different times was assessed by RT-PCR. ***P*<0.01.

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Gene	GenBank Accession	Primer Sequence	Amplicon Size	PrimerBank ID*
Acta2	NM_007392	Fwd: 5'GTCCCAGACATCAGGGAGTAA Rev: 5'TCGGATACTTCAGCGTCAGGA	102	6671507a1
Actb	NM_007393	Fwd: 5' GGCTGTATTCCCCTCCATCG Rev: 5' CCAGTTGGTAACAATGCCATGT	154	6671509a1
Cdh1	NM_009864	Fwd: 5'CAGGTCTCCTCATGGCTTTGC Rev: 5'CTTCAAGAAGGCTGTCC	175	6753374a1
Lef1	NM_010703	Fwd: 5'TGTTTATCCCATCACGGGTGG Rev: 5'CATGGAAGTGTCGCCTGACAG	67	27735019a1
Lifr	NM_013584	Fwd: 5'TACGTCGGCAGACTCGATATT Rev: 5'TGGGCGTATCTCTCTCTCCTT	113	4379217a1
Rhoa	NM_016802	Fwd: 5'AGCTTGTGGTAAGACATGCTTG Rev: 5'GTGTCCCATAAAGCCAACTCTAC	138	31542143a1
Smad7	NM_001042660	Fwd: 5'GGCCGGATCTCAGGCATTC Rev: 5'TTGGGTATCTGGAGTAAGGAGG	125	6678778a1
Tgfβr3	NM_011578	Fwd: 5'GGTGTGAACTGTCACCGATCA Rev: 5'GTTTAGGATGTGAACCTCCCTTG	125	33469109a1
Vimentin	NM_011701	Fwd: 5'CGGCTGCGAGAGAAATTGC Rev: 5'CCACTTTCCGTTCAAGGTCAAG	124	31982755a1

TABLE E1: Primers used for RT-PCR assays

*Primer identification number given by PrimerBank, a PCR primer database for quantitative gene expression analysis (https://pga.mgh.harvard.edu/primerbank/).

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TABLE E2. Antibodies used for flow cytometry and immunohistochemical studies

Antibody	Clone	Species	Application	Source
CD11b	M1/70	Rat	FC (0.25 µg/10^5 cells)	BioLegend
CD29	HMβ1-1	Hamster	FC (0.25 µg/10^5 cells)	BioLegend
CD31	390	Rat	FC (0.25 µg/10^5 cells)	BioLegend
CD34	HM34	Hamster	FC (0.25 µg/10^5 cells)	BioLegend
Collagen 1	NB600-408	Rabbit	WB (1:1000)	Novus Biologicals
E-cadherin	24E10	Rabbit	IHC (1:200) ICC (1:200) WB (1:1000)	Cell Signaling Technology
Epi-CAM	G8.8	Rat	IHC (1:100)	ThermoFisher
EYFP	GF28R NB600-303	Mouse Rabbit	IHC (1:100) IHC (1:100)	ThermoFisher Novus Biologicals
Lef1	C12A5	Rabbit	IHC (1:200)	Cell Signaling Technology
LepR	BAF497	Goat	FC (1:50)	R&D Systems
Nestin	1D11H2	Mouse	FC (1:100)	Proteintech
pLef1	SAB4504256	Rabbit	WB (1:1000)	Sigma-Aldrich
RhoA-GTPase	26904	Mouse	IHC (1:100) ICC (1:50) WB (1:500)	New East Biosciences
Sca1	D7	Rat	FC (0.25 µg/10^5 cells)	BioLegend
TER-119	BAM1125	Rat	FC (1:50)	R&D Systems
Vimetin	V9	Mouse	IHC (1:50)	ThermoFisher
α-SMA	SP171	Rabbit	IHC (1:100) ICC (1:100) WB (1:1000)	Sigma-Aldrich

IHC, Immunohistochemistry; ICC, Immunocytochemistry; WB, Western blotting; FC, Flow cytrometry