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Upregulated P-Rex1 exacerbates human airway smooth muscle hyperplasia in asthma

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Capsule Summary

P-Rex1, a Rac-selective activator is aberrantly upregulated in airway smooth muscle of subjects with asthma compared to non-asthmatics, and augments cell proliferation, thus contributing to airway smooth muscle hyperplasia and remodeling in severe asthma.

Keywords: asthma; cell proliferation; epidermal growth factor; human airway smooth muscle; hyperplasia; platelet-derived growth factor; P-Rex1; Rac1

To the Editor:

Patients with severe asthma often develop poorly reversible airway obstruction. While various factors such as abnormal airway smooth muscle relaxation may contribute to fixed airway obstruction, development of structural changes due to airway remodeling is commonly associated with this complication and poor clinical outcomes in patients¹. Human airway smooth muscle (HASM) hyperplasia is a major component of airway remodeling and cell proliferation plays an important role in HASM hyperplasia and remodeling². However, the molecular mechanisms regulating HASM cell proliferation in asthma are still poorly understood.

Platelet-derived growth factor (PDGF) is greatly increased in the lungs of asthmatic patients and is thought to promote proliferation of HASM cells via activation of tyrosine kinase receptor PDGFR. Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small GTPase that is required for PDGF-stimulated HASM cell proliferation³. However, little is known about how Rac1 activity is regulated in asthmatic HASM cells. P-Rex1 is a Rac-selective guanine nucleotide exchange factor (Rac-GEF) that catalyzes inactive GDP-bound Rac into active GTP-bound form⁴. We previously reported that aberrantly upregulated P-Rex1 promotes cancer metastasis by activating Rac1 signals⁵. Our report also identified the importance of P-Rex1 in cancer cell proliferation⁶. P-Rex1 cooperates with PDGFR to drive migratory and invasive phenotypes of cancer cells⁷, but its role in PDGF-stimulated HSAM cell proliferation is unknown.

Immunohistochemistry staining of formalin-fixed, paraffin-embedded lung samples from asthmatic and normal subjects (**Table E1**) revealed that P-Rex1 protein was only weakly expressed in epithelial layers and ASM cells of bronchioles in normal subjects (**Fig 1A, a and b**), whereas asthmatic patients had a much higher staining density of P-Rex1 protein (**Fig 1A, c and d**). Quantitative analysis of lung tissue samples using a “histo-score” (H-score)⁵ showed that P-

Rex1 protein expression in ASM bundles of asthmatic patients was 4-fold higher than that in normal subjects. In contrast, P-Rex-2, an isoform of P-Rex1, was barely detectable in ASM bundles and quantitative analysis showed no significant difference between normal and asthmatic patients (**Fig E1**). P-Rex1 upregulation was further confirmed in three primary ASM cell lines derived from asthmatic subjects, HASM-A, as compared to three primary cell lines isolated from non-asthmatic subjects, HASM-N (**Table E2** and **Fig 1B**, $^{**}P<0.01$). 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assays indicated that the stimulatory effects of PDGF in asthmatic HASM-A cells are 2-fold higher than that in HASM-N cells (23.2 ± 2.3 -fold vs. 10.9 ± 1.8 -fold, $^{**}P<0.01$). Treatment with Rac1 inhibitor NSC23766 ($50\mu\text{M}$) had no significant effects on HASM-N cell proliferation, but attenuated PDGF-stimulated proliferation of all three HASM-A cell lines by 40% (**Fig 2A**) ($^{*}P<0.05$), suggesting that active Rac1 promotes asthmatic HASM proliferation.

Western blot analysis indicated that PDGFR expression levels were similar comparing non-asthmatic and asthmatic HASM cell lines (**Fig 2A, inset**) and no association was found between PDGFR expression levels and PDGF-stimulated HASM cell proliferation. We next examined whether upregulated P-Rex1 is responsible for asthmatic HASM cell hyperplasia. As shown in **Fig 2B** (left panel), overexpression of recombinant P-Rex1 had no significant effects on the basal proliferation rate but significantly increased PDGF-stimulated proliferation of all three non-asthmatic HASM-N cell lines. The basal proliferation rate of HASM-N1 cells transfected with vector or recombinant P-Rex1 was $2.0 \pm 0.6\%$ and $2.5 \pm 0.3\%$, respectively. PDGF stimulation increased this value to $7.3 \pm 0.1\%$ and $13.1 \pm 0.4\%$, respectively. Similar results were obtained in two other HASM-N cell lines (N2 and N3). On average, overexpression of P-Rex1 increased PDGF-stimulated cell proliferation of three HASM-N cell lines by 1.9-fold ($^{*}P<0.05$) (**Fig 2B**,

right panel). P-Rex1-specific siRNA⁵ was used to reduce P-Rex1 protein levels in three asthmatic HASM-A cell lines (**Fig 2C**, inset). Silencing endogenous P-Rex1 had little effects on basal proliferation rate but significantly attenuated PDGF-stimulated cell proliferation of all three asthmatic HASM-A cell lines (**Fig 2C**, left panel). Treatment with PDGF increased the proliferation rate of HASM-A1 cells with scramble siRNA from $3.2 \pm 0.5\%$ to $29.7 \pm 3.1\%$, whereas the proliferation rate of HASM-A1 cells with P-Rex1 siRNA was only increased from $3.3 \pm 0.9\%$ to $14.2 \pm 2.1\%$ (**Fig 2C**, left panel). These results were confirmed in two other asthmatic HASM-A cell lines (A2 and A3). On average, knockdown of P-Rex1 expression by 70-80% attenuated PDGF-stimulated cell proliferation of three HASM-A cell lines by $45.0 \pm 3.8\%$ (* $P < 0.05$) (**Fig 2C**, right panel), indicating that upregulated P-Rex1 is required for PDGF-stimulated proliferation of asthmatic HASM-A cells. We also examined the importance of P-Rex1 in epidermal growth factor (EGF)-stimulated HASM cell proliferation (**Fig E2**). The stimulatory effect of EGF in asthmatic HASM-A1 cells was 2.5-fold higher than that in non-asthmatic HASM-N1 cells. P-Rex1 overexpression in HASM-N1 cells increased EGF-stimulated cell proliferation by 2-fold ($7.1 \pm 0.6\%$ vs. $15.6 \pm 1.9\%$), whereas silencing P-Rex1 in HASM-A1 cells reduced EGF-stimulated cell proliferation from $17.5 \pm 2.2\%$ to $4.2 \pm 1.1\%$. It should be noted that manipulations of P-Rex1 had little effect on PDGFR or EGF receptor expression in HASM cells (**Fig 2** and **E2**, insets).

In conclusion, our results in human tissues and primary cell lines demonstrate an aberrant upregulation of P-Rex1 in HASM of asthmatic patients. These data support a key role of P-Rex1 in the increased proliferation of asthmatic HSAM cells which is likely an essential component regulating ASM cell hyperplasia in asthma. Interestingly, activation of Rac1 by bronchoconstrictor G-protein coupled receptor (GPCR) ligands in ASM cells plays a major role

in airway hyper-responsiveness (AHR), the hallmark of asthma⁸. Since P-Rex1 is the only Rac-GEF synergistically activated by GPCRs and tyrosine kinase receptors⁴, upregulated P-Rex1 could also contribute to development of AHR. However, we are aware of some limitations of our study. Rac1 inhibitor NSC23766 at 100 μ M exhibits critical off-target effects *in vivo*⁹. Thus, the concentration used in our studies (50 μ M) cannot exclude Rac1-independent effects in asthmatic HASM cells. Further research is warranted to determine the functions and molecular mechanisms of upregulated P-Rex1 in asthma. Nonetheless, our data support the concept that P-Rex1 may represent a key target for treating both ASM hyperplasia and AHR, providing a novel approach to reverse airway remodeling and restore airway function in severe asthma.

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FIGURE LEGENDS

FIG 1 P-Rex1 protein expression is elevated in asthmatic human lung tissues and isolated primary asthmatic HASM cells. **A**, Left panel: Representative immunohistochemistry staining of P-Rex1 protein in human lung tissue of normal subjects (a and b) and asthmatic patients (c and d). Small boxes indicate ASM cells. Inserted boxes are a 2 times enlarge of small boxes. Right panel: Analysis of P-Rex1 protein expression in HASM cells using H-score index on immunohistochemically stained tissue sections from normal and asthmatic subjects (n=3) (right). **P<0.01. **B**, Left: A representative western blot of P-Rex1 protein in three non-asthmatic HASM-N cell lines and three asthmatic HASM-A cell lines. Right: Mean P-Rex1 band density was normalized to β -actin $\pm$ S.E., **P<0.01.

FIG 2 Upregulated P-Rex1 exacerbates asthmatic HASM hyperplasia. **A**, Serum-starved HASM cells were stimulated with 10 ng/ml of PDGF in the absence and presence of 50 μ M Rac1 inhibitor NSC23766 for 36 h, and the cell proliferation was assessed using a BrdU incorporation assay. Inset: Western blot analysis of PDGFR expression levels in three non-asthmatic HASM-N1-3 and three asthmatic HASM-A1-3 cell lines. **B**, Non-asthmatic HASM-N cell lines were transfected with empty vector or vector encoding recombinant P-Rex1. **C**, Asthmatic HASM-A cell lines were transfected with scramble or P-Rex1-specific siRNA using lipofectamine RNAiMAX. Cells were treated with 10 ng/ml of PDGF for 24 h and then subjected to western blot analysis of P-Rex1 and PDGFR protein expression (Insets) or BrdU incorporation assay. Quantitative analysis of percent BrdU-positive cells was performed by counting BrdU-positive cells compared to total DAPI stained cells. Data are means $\pm$ S.E. from at least 100 cells. *P<0.05 and **P<0.01. All experiments were independently repeated 3 times.

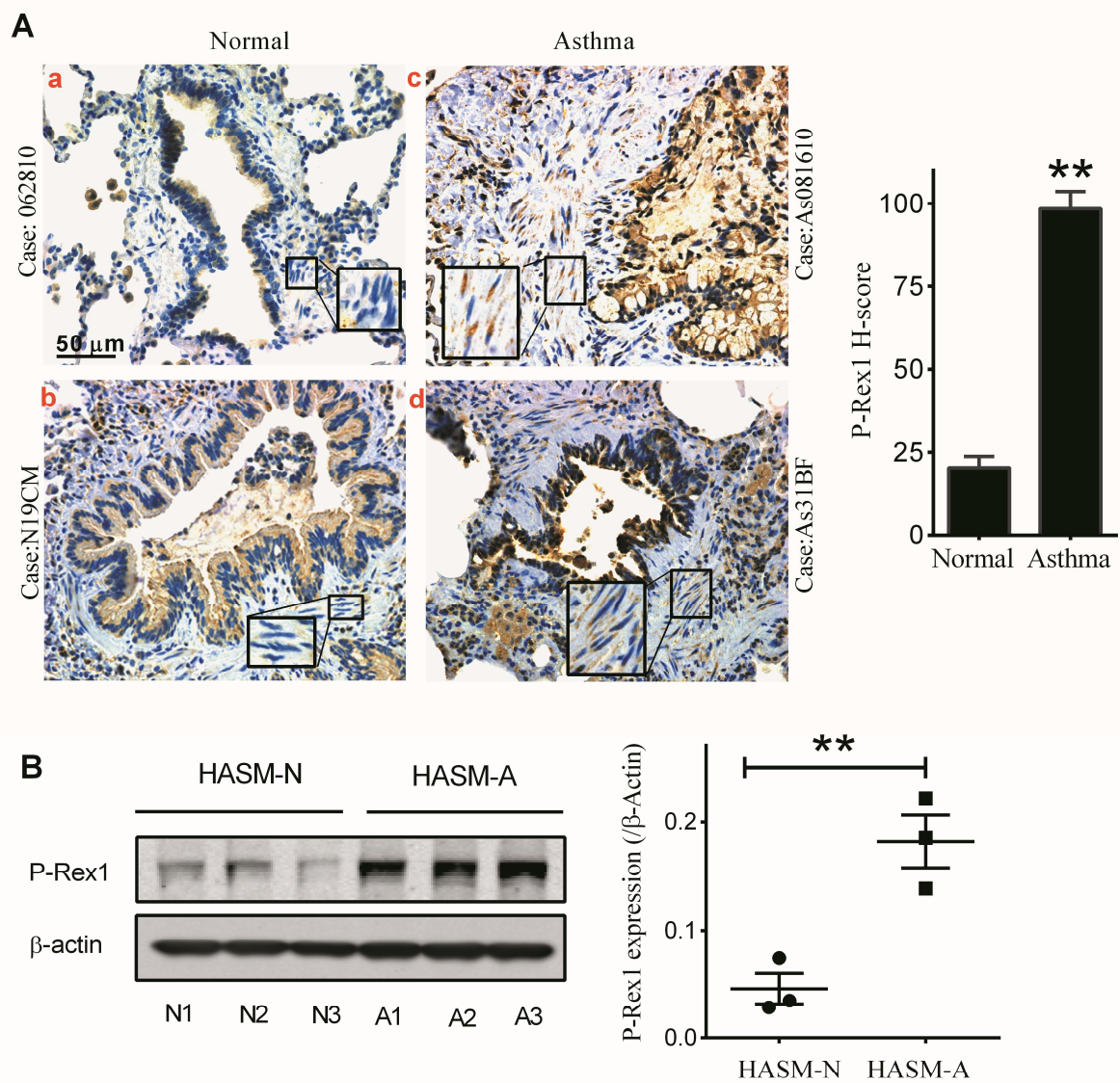
Figure 1 (Huang et al. JACI-D-18-00219R1)

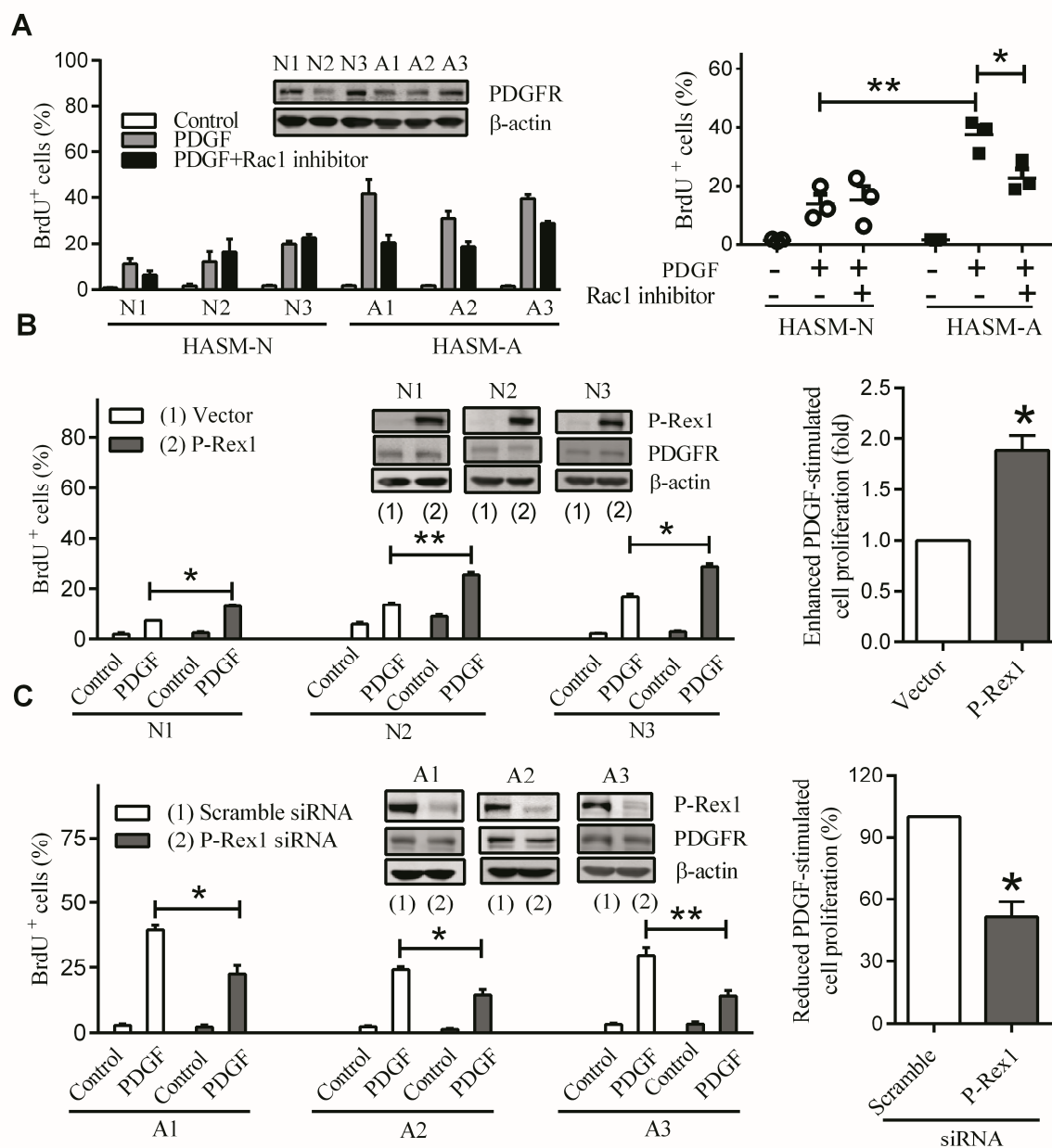
Figure 2 (Huang et al. JACI-D-18-00219R1)

TABLE E1. Subject profiles of archived formalin-fixed, paraffin-embedded lung tissue blocks

	Cell	Cause of death	Pulmonary disease	Cancer	Medication
Non-Asthma	N1	Gunshot wound neck	None	None	None
	N2	Head/chest trauma caused by vehicle accident	None	None	None
	N3	Anoxic brain injury	None	None	NA
Asthma	A1	Anoxia and asthma attack	Asthma	None	Albuterol, Advair
	A2	Anoxia and asthma attack	Asthma	None	Albuterol
	A3	Anoxia and asthma attack	Asthma	None	Albuterol

	Tissue Case number	Cause of death	Pulmonary disease	Cancer	Medication
Non-Asthma	062810	Cerebral vascular accident	None	None	Hypertension medication
	N19CM	Head gunshot wound	None	None	None
	090710	Overdose on Tylenol-intentional	None	None	Xanax, Lisinopril
Asthma	As081610	Anoxia and asthma attack	Asthma	None	Advair, Combivent
	As31BF	Anoxia and asthma attack	Asthma	None	Albuterol
	As0726	Anoxia and asthma attack	Asthma	None	Singulair

TABLE E2. Subject profiles of isolated non-asthmatic and asthmatic human airway smooth muscle cells

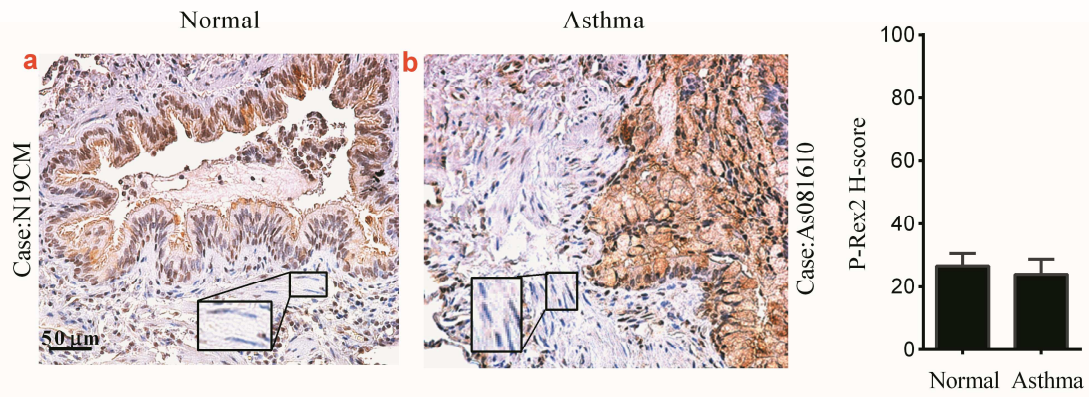
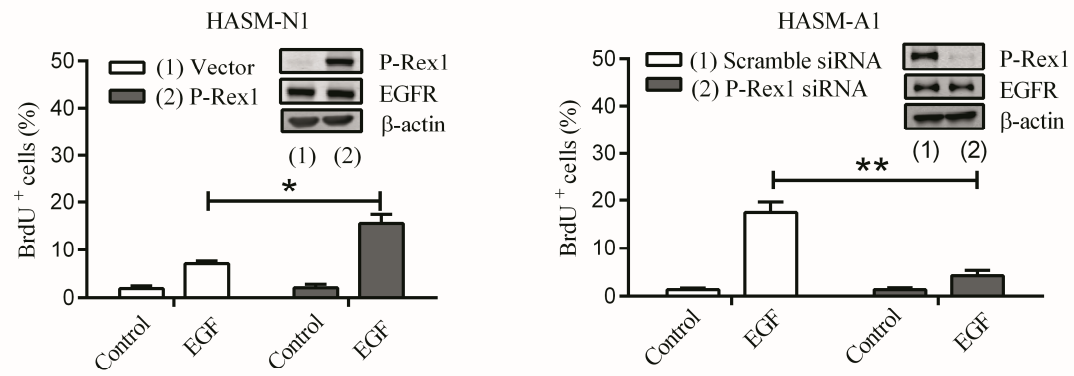
Figure E1 (Huang et al. JACI-D-18-00219R1)

Figure E2 (Huang et al. JACI-D-18-00219R1)

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METHODS

Immunohistochemistry analysis of human lung tissues

Archived formalin-fixed, paraffin-embedded lung tissue blocks of subjects with asthma (n=3) and healthy controls (n=3) were from Dr. Reynold A. Panettieri. These tissue samples were obtained during open lung resection from patients at the time of death (**Table E1**)^{E1}. Tissue sections were stained with the 6F12 anti-P-Rex1 antibody (Millipore sigma, MA) or anti-P-Rex2 antibody (Cat. NBP1-84275, Novus Biologicals), counterstained with hematoxylin. The negative control used nonimmune rabbit IgG as the primary antibody. The intensity of P-Rex1 protein staining was determined as an average optical density by Image-Pro Plus in 5 randomly chosen airways for each sample. A “histo-score” (H-score) was calculated by multiplying the percentage (P) of positive cells by the average intensity (I) as described previously^{E2}.

Cells and culture

Human airway smooth muscle (HASM) cells from asthmatic (A1-3) and non-asthmatic (N1-3) donors were established by Dr. Rey Panettieri's laboratory using human lung tissue samples obtained from non-asthmatic subjects and asthmatic subjects during open lung resection at the time of death (**Table E2**). The samples had no identifiers, and the protocol for cell isolation was previously approved by the University of Pennsylvania Institutional Review Board^{E3}. Cells were cultured at 37°C with 5% CO₂ in 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) (GE, UT) and Ham's F-12 Nutrient Mixture (F12) (Corning, VA) supplemented with 10% FBS and were used at passage <10 for experiments.

Expression of recombinant P-Rex1

Non-asthmatic HASM-N cells (2.5×10^5) were electroporated with 2 µg of either pcDNA3.1 control vector or vector encoding-P-Rex1 using the Nucleofector system as per manufacturer's protocol. Transfected cells were cultured for 24 h and then re-seeded for proliferation assays or western blot analysis as described below.

Silence of endogenous P-Rex1 by siRNA

Asthmatic HASM-A cells (2.5×10^5) were transfected with 50 µM of scramble siRNA (negative control #1 siRNA, Ambion TX) or synthesized P-Rex1 siRNA (target sequence of human P-Rex1 gene:

5'-GCAACGACTTCAAGCTGGTGGAGAA-3')^{E2} using Lipofectamine RNAiMAX reagent (Invitrogen) as per manufacturer's protocol. The final concentration of siRNA was 50 nM. Transfected cells were cultured for 24 h and then re-seeded for proliferation assays or western blot analysis.

Western blot

Cell protein extraction and western blot were conducted as described previously^{E4}. Protein was extracted from cells using 1x RIPA lysis buffer (Santa Cruz, Dallas, TX). Samples were electrophoresed and subjected to western blot using primary antibodies against P-Rex1 (mouse, clone 6F12), PDGF receptor (Cat.3162S, Cell Signaling Technology, MA), EGF receptor (Cat.06-847, Millipore Sigma, MA) and the loading control β -actin (Santa Cruz, TX). IRdye700 or IRdye800 labeled secondary antibodies (LI-COR Biosciences) (Lincoln, NE) were used for protein band detection. The images were captured with a LI-COR Odyssey infrared imaging system (LI-COR Biosciences). For the quantification of the western blot results, the intensity of each band was divided by the β -actin intensity of that same sample and subsequently divided by the corrected intensity of the untreated sample of the same blot.

5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay

HASM cells were seeded on coverglasses in 12-well plates (5×10^3 cells/well) and cultured in serum-free medium for 24 h. Serum-starved HASM cells were stimulated with PDGF-BB (10 ng/ml) or EGF (100 ng/ml) for 24 h. To test the effect of a Rac1 inhibitor on HASM cells, 50 μ M Rac1 inhibitor NSC 23766 (Tocris, MN) was added to serum-starved HASM cells 1 h prior to PDGF-BB or EGF treatment. Then 10 μ M BrdU (BD Pharmingen, CA) was added to cultured cells for 18 h. Cells were fixed with 70% ethanol, permeabilized, blocked and then stained for nuclei and BrdU using 4',6-diamidino-2-phenylindole (DAPI) and anti-BrdU antibody (Cell Signaling, MA). BrdU incorporation into newly synthesized DNA, as an indicator of cell proliferation, was measured by fluorescence microscopy. Results are expressed as the percentage of DAPI stained cells that were also BrdU-positive.

Statistical analysis

Data are expressed as means $\pm$ S.E. Groups were compared using a Student's t-test for unpaired observations. A probability level (p) < 0.05 was considered statistically significant.

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FIGURE LEGENDS

FIG E1. P-Rex2 expression in human normal and asthmatic lung tissues. Left panel: Representative immunohistochemistry staining of P-Rex2 protein in human lung tissue of normal subjects (a) and asthmatic patients (b). Small boxes indicate ASM cells. Inserted boxes are a 2 times enlarge of small boxes. Right panel: Analysis of P-Rex2 protein expression in HASM cells using H-score index on immunohistochemically stained tissue sections from normal and asthmatic subjects (n=3).

FIG E2. Up-regulated P-Rex1 exacerbates EGF-induced HASM cell proliferation. Non-asthmatic HASM-N1 cells were transfected with empty vector or vector encoding recombinant P-Rex1 (Left panel) whereas asthmatic cell HASM-A1 was transfected with scramble or P-Rex1-specific siRNA (Right panel) for 48 h. Cells were treated with 100 ng/ml EGF for 24 h and then subjected to western blot analysis of P-Rex1 and EGFR proteins (Insets) or BrdU incorporation assay. Quantitative analysis of BrdU-positive cells was performed by counting the percentage of BrdU-positive cells compared to total DAPI stained cells. Data are means $\pm$ S.E. from at least 100 cells. *P<0.05 and **P<0.01. All experiments were independently repeated 3 times.