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Citation

Published Version
doi:10.1371/journal.pone.0028504

Accessed
April 27, 2018 5:56:30 AM EDT

Citable Link
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An RGS4-Mediated Phenotypic Switch of Bronchial Smooth Muscle Cells Promotes Fixed Airway Obstruction in Asthma

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Abstract
In severe asthma, bronchodilator- and steroid-insensitive airflow obstruction develops through unknown mechanisms characterized by increased lung airway smooth muscle (ASM) mass and stiffness. We explored the role of a Regulator of G-protein Signaling protein (RGS4) in the ASM hyperplasia and reduced contractile capacity characteristic of advanced asthma. Using immunocytochemical staining, ASM expression of RGS4 was determined in endobronchial biopsies from healthy subjects and those from subjects with mild, moderate and severe asthma. Cell proliferation assays, agonist-induced calcium mobilization and bronchoconstriction were determined in cultured human ASM cells and in human precision cut lung slices. Using gain- and loss-of-function approaches, the precise role of RGS proteins was determined in stimulating human ASM proliferation and inhibiting bronchoconstriction. RGS4 expression was restricted to a subpopulation of ASM and was specifically upregulated by mitogens, which induced a hyperproliferative and hypcontractile ASM phenotype similar to that observed in recalcitrant asthma. RGS4 expression was markedly increased in bronchial smooth muscle of patients with severe asthma, and expression correlated significantly with reduced pulmonary function. Whereas RGS4 inhibited G-protein-coupled receptor (GPCR)-mediated bronchoconstriction, unexpectedly RGS4 was required for PDGF-induced proliferation and sustained activation of PI3K, a mitogenic signaling molecule that regulates ASM proliferation. These studies indicate that increased RGS4 expression promotes a phenotypic switch of ASM, evoking irreversible airway obstruction in subjects with severe asthma.


Editor: Rory Edward Morty, University of Giessen Lung Center, Germany

Received: September 8, 2011; Accepted: November 9, 2011; Published: January 12, 2012

Competing Interests: The authors have declared that no competing interests exist.

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Introduction
Asthma, a common respiratory disease, manifests by airway inflammation, hyperresponsiveness and reversible luminal obstruction. Despite research efforts, 15%–25% of patients with asthma develop irreversible airway obstruction, which is refractory to steroids and bronchodilators, and disproportionately account for asthma morbidity and mortality [1,2]. In biopsies of the bronchial wall, increases in the mass of airway smooth muscle (ASM), the pivotal cell regulating bronchomotor tone, in part defines airway remodeling in severe asthma [2]. However, the contribution of increases in smooth muscle mass to irreversible airway obstruction remains controversial.

Phenotypic plasticity defines the ability of smooth muscle to switch between a contractile and synthetic state [3,4]. Although characteristic of smooth muscle in cell culture, phenotypic plasticity of smooth muscle in disease states remains controversial [3,4]. Proliferative smooth muscle manifests abundant organelles for protein and lipid synthesis, increased mitochondria and diminished expression of contractile apparatus and associated proteins [5,6,7]. Whether all or select populations of smooth muscle in vivo retain a proliferative capacity and whether distinct signaling pathways serve as master switches to promote smooth muscle growth and inhibit agonist-induced contraction remain unknown.

Regulators of G protein signaling (RGS) proteins inhibit GPCR function by binding activated (GTP-bound) Gα subunits and accelerating GTP hydrolysis by Gα [8]. This GTPase accelerating (GAP) activity hastens the return of Gα to an inactive (GDP-bound) form, promoting rapid termination of G protein signaling. Unrelated to their GAP function, RGS proteins of the R4 subfamily, which includes RGS1–5, 8, 10, 13, 18 and 21, regulate activity of PI3K by interacting with its regulatory p85α subunit [9]. The associated PI3K subunit p110 catalyzes the formation of phosphatidylinositol phosphate [PI(3)P] from membrane PI(2)P, activating signaling molecules involved in cell growth and synthetic function, such as Akt [10]. p85α, an adaptor, co-localizes with receptors and signaling complexes at the plasma
expression, near confluent ASM cells on sterile glass coverslips considered accessible as described previously [13]. For immuno-

immunoelectron microscopy of ASM in the ASM bundle or adjacent to the

Ham’s F-12 medium supplemented with 10% FBS, 100 units/ml

inhibitor cocktails, protein quantification reagents and allied

Primers and reagents for RT-PCR were obtained commercially

Ham’s F-12 medium supplemented with 0.1%

were fixed in 3% paraformaldehyde (Sigma), blocked with 1%

BSA and stained with polyclonal RGS4 (N-16, 1:500), Ab or

isotype-matched Ab. Cells were then stained with Alexa Fluor

488-conjugated anti-goat Ab (1:1,000, Invitrogen) and counter-

stained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) to

identify nuclei.

Biochemical studies

Co-immunoprecipitation experiments were performed following

methodology described earlier [9,14]. Antibodies against

phosphorylated-p85, p85 were obtained from Santa Cruz

Biotechnology. For immunoprecipitation experiments, cleared cell

lysates were incubated overnight at 4°C with anti-RGS4 tagged

TrueBlot anti-goat Ig IP beads (biosciences). Protein transfer was

followed by overnight incubation with Ab against phosphorylated-

p85 (1:500) and p85 (1:500). After incubation with HRP-

conjugated secondary antibody, the signal was detected with the ECL reagent (Promega).

p-Akt dynamics and Akt kinase assay

Differential kinetics of PDGF-mediated Akt phosphorylation

(Ser-473) was assayed in total protein lysates (1 µg/ml) from

ShRGS4 and ShCnt using Akt phospho STAR ELISA Kit

(Millipore) following enclosed protocols. Akt-dependent phosphor-

ylation of GSK-3 in ShRGS4 and ShCnt cells was determined using an

Akt Activity Assay Kit (Abcam).

Cell counting experiments and cell cycle analysis

ShCnt and ShRGS4 ASM cells were seeded at equal densities

(96×103 cells/well) in 6-well plates and cultured for 7 days. Cells were

then serum deprived (Ham’s F-12 supplemented with 0.1%

BSA) for an additional 2 days before treatment with 10 ng/ml

PDGF for an additional 72 hours. Cultures were dissociated with

0.5% trypsin-EDTA (Invitrogen) solution and counted in triplicate

with the Coulter Z1 cell counter (Beckman Coulter). To analyze

cell cycle profiles, ShCnt and ShRGS4 ASM cells treated with/

without PDGF were prepared for propidium iodide staining using

CycleTest Plus Kit (BD Biosciences). Thereafter, DNA contents of

the stained nuclei were analyzed on a FACS Canto flow cytometer

and interpreted using Verity ModFit LT 3.0 Software.

Precision cut lung slices (PCLS) and small airway

responses

The smallest lobe of human lungs from lung tissue donors

(NDRI) was inflated with 2% (wt/vol) low-melting-point agarose

(Sigma), cored (8 mm in diameter) and sliced (250 µm thickness)

using a Krumdieck tissue slicer (Alabama Research and Develop-

ment) as described earlier [15]. Changes in small airway lumen to

increasing log concentration of CCh (10−6 to 10−4 mol/L) in vehicle

or PDGF-pre-treated (50 ng/ml for 8 hours) slices were recorded using a CCD camera (Nikon ECLIPSE Model

No. TE2000-U, magnification ×40) connected to a live video

feed (Evolution QEp, Model No. 32-0074A-130, video recorder). A

log half-maximum effective concentration (EC50) and maximum
drug effect (Emax) value for each airway were derived from a

concentration-response curve.

Intracellular Ca2+ responses

Fura-2-loaded ShCnt and ShRGS4 ASM cells cultured on

coverslips were mounted onto an open slide chamber, placed

onto an inverted microscope and excited at 340 and 380 nm

wavelength, and emissions were collected at ±450 nm wavelength

using a CCD camera (Nikon/Photon Technology International).
Figure 1. Human airway smooth muscle (HASM) mitogens induce RGS4 expression. (A) HASM cells express RGS mRNAs. RGS expression was determined by real-time PCR in HASM cells left untreated or treated with PDGF for 2 h. Data presented are mean ± SEM of 3 separate experiments performed in triplicate using Gadph as an internal control, relative to RGS2 in untreated cells, set as ‘1’. (B) Analysis of RGS4 expression in HASM cells treated with mitogens such as EGF (1 ng/ml), PDGF (10 ng/ml), thrombin and cytokines by real-time PCR. Data (mean ± SEM) of 5 independent experiments. (C) Kinetics of PDGF-mediated RGS4 mRNA expression as a function of time. Values (mean ± SEM of 3 separate
After subtracting the background signal obtained with Mn$^{2+}$ and ionomycin, the ratio of fluorescence intensities at 340 and 380 nm wavelength was determined using IgorPro (WaveMetrics). The net Ca$^{2+}$ responses to contractile agonists were calculated by subtracting the basal from that of the peak ratio values.

**Statistical analysis**

Graph Pad Prizm software was used to determine statistical significance evaluated by a paired Students $t$-test for two groups or analysis of variance (ANOVA) for multiple groups. $P$ values of <0.05 were considered significant.

**Results**

Mitogens selectively induce expression of RGS proteins

Human ASM cells predominantly expressed RGS2–5 (Figure 1A). Because RGS transcription often dynamically responds to environmental cues, we analyzed RGS expression in HASM treated with growth factors or cytokines associated with airways dysfunction in asthma by real-time PCR. Exposure of HASM to PDGF more effectively induced RGS4 mRNA and protein expression (Figure 1A). Increased RGS4 transcripts were also detected in cells exposed to EGF, thrombin, interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) compared to untreated cells (Figure 1B). PDGF-induced RGS4 expression was dose- and time-dependent, with maximum expression occurring at 6 hours (Figure 1C), and required active transcription as it was inhibited by actinomycin D (Figure S1A). PDGF-elicited RGS4 transcription also required activity of PI3K and ERK1/2 but not p38 mitogen-activated protein (MAP) or Janus (JAK) kinases (Figure S1B). Immunofluorescent staining with an RGS4-specific antibody demonstrated minimal RGS4 expression in quiescent cells whereas PDGF induced RGS4 expression in nearly all HASM cells (Figure 1D). Consistent with prior studies of other cell types [16], RGS4 localized in the cytoplasm and at the plasma membrane of HASM cells.

RGS4 expression in ASM increases with asthma disease severity

The selective induction of RGS4 by HASM mitogens suggested a potential function in the ASM hyperplasia and fixed airway obstruction associated with severe asthma. To characterize RGS4 expression in bronchial ASM bundles, we evaluated endobronchial biopsies from patients with asthma and age-matched healthy

| Table 1. Clinical and sputum characteristics of patients categorized according to GINA. |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Normal          | Mild-Moderate Asthma (GINA 1 = 11, 2 = 3, 3 = 2) | Severe Asthma (GINA 4 = 11, 5 = 4) |
| Number                         | 13              | 16              | 15              |
| Age*                           | 47 (4)          | 49 (4)          | 52 (3)          |
| Male/Female                    | 8/5             | 6/9             | 5/10            |
| Never/current/ex-smokers       | 11/0/2          | 12/0/4          | 12/0/3          |
| Pack years*                    | 0.5 (0.4)       | 2.9 (1.5)       | 1.5 (0.9)       |
| Atopy n (%)                    | 7 (54)          | 11 (69)         | 10 (67)         |
| PC$_{20}$FEV$_1$ (mg/ml)$^\dagger$ | >16            | 0.37 (0.14–0.97)$^{**}$ | 1.1 (0.3–3.6)$^{**}$ |
| FEV$_1$% predicted*            | 97 (4)          | 83 (6)          | 79 (7)$^{**}$   |
| Pre-BD FEV$_1$/FVC %*          | 83 (2)          | 72 (2)          | 68 (4)          |
| BD response (%)                | X               | 12 (4)          | 9 (3)           |
| Inhaled corticosteroids (BDP/day) | 0              | 300 (139)       | 1645 (197)      |
| Oral corticosteroids n (%)     | 0               | 0               | 4 (27)          |
| LABA n (%)                     | 0 (0)           | 2 (13)          | 15 (100)        |

**Sputum Cell Counts**

| TCC*                           | 1.2 (0.2)       | 2.4 (0.5)       | 4.7 (1.2)$^{**}$ |
| Eosinophils %$^\ddagger$       | 0.3 (0.8)       | 1.0 (5.7)       | 2.8 (48)         |
| Neutrophils %$^*\$             | 47 (13)         | 51 (8)          | 58 (8)           |
| Macrophages %$^*$              | 51 (9)          | 38 (6)          | 25 (6)$^{**}$    |
| Lymphocytes %$^*$              | 1.8 (0.8)       | 1.0 (0.2)       | 1.5 (0.7)        |
| Epithelial cells %$^*$          | 3 (2)           | 4 (1)           | 7 (3)            |

GINA: Global Initiative for Asthma; BD: bronchodilator; LABA: long-acting bronchodilator; FEV$_1$: forced expiratory volume in one second; TCC: total cell counts; BDP: beclomethasone dipropionate; PC$_{20}$FEV$_1$: provocative concentration of methacholine to induce a 20% decrease in FEV$_1$;

$^*$mean (SE);

$^\dagger$geometric mean (95% CI);

$^\ddagger$median (IQR);

$^{**}$P < 0.05 compared to control.

doi:10.1371/journal.pone.0028504.t001
controls by immunohistochemistry. We categorized patients into 3 groups according to criteria established by the Global Initiative for Asthma (GINA, http://www.ginasthma.com): healthy, mild-moderate asthma and severe asthma (Table 1). Smooth muscle bundles in bronchi from those with asthma had markedly increased numbers of RGS4⁺ ASM cells compared to those with mild-moderate asthma or healthy subjects (Figures 2A–C). RGS4⁺ cells were found at the periphery of the ASM bundle although some cells within the bundles also stained positively for α-smooth muscle actin and RGS4. Notably, the number of RGS4⁺ cells correlated inversely with pulmonary function as assessed by the forced expiratory volume in 1 second (FEV₁) (Figure 2D).

RGS4 expression is required for mitogen-induced myocyte proliferation

Since RGS4 expression increased in proportion to ASM mass in asthma and correlated with the severity of disease, we next addressed whether ASM proliferation requires RGS4 expression. To test this directly, we extinguished RGS4 expression in cultured HASM cells using siRNA and measured proliferation in the presence and absence of PDGF. RGS4 amounts were reduced 64±6% in cells expressing an RGS4-specific shRNA relative to cells expressing a scrambled control shRNA (Figure 3A). Unexpectedly, PDGF-evoked proliferation was profoundly reduced in RGS4-depleted cells compared to control in the presence...
or absence of PDGF (Figure 3B). RGS4 deficiency led to a 20-fold increase in the percentage of cells in G2 phase of the cell cycle compared to control, indicating growth arrest (P<0.0005) (Figure 3C). To exclude the possibility that reduced cell numbers were due to apoptosis or necrosis, we measured caspase 3 activity or LDH levels, respectively. While caspase activity in HASM apoptosis as evidenced by a 10-fold increase in caspase 3 activity, minimal caspase 3 activity was detected in control or RGS4-deficient cells in the presence or absence of PDGF (Figure S2A). Accordingly, assessment of LDH levels showed no significant differences in cell viability across all study groups (Figure S2B).

These results suggest that, in contrast to the inhibition of PI3K-mediated proliferation of neoplastic cells by RGS16, mitogen-induced cell cycle progression of primary HASM cells unexpectedly required RGS4. Among several critical signaling events, robust and durable activation of PI3K and its downstream effector S6K1 is required for HASM mitogenesis [1,17]. We hypothesized that RGS4 mediates PDGF-elicited HASM growth by interacting with the phosphorylated p85α subunit of PI3K and modulating activity of the PI3K signaling pathway. PDGF induced tyrosine phosphorylation in HASM cells (Figure 3D). Consistent with our previous studies with mast cells [9], we observed equivalent p85α phosphorylation in control and RGS4-depleted HASM cells. To characterize RGS4-p85α interactions in HASM, we immunoprecipitated proteins from untreated or PDGF-treated cells. We detected RGS4 specifically in immunoprecipitates of phospho-p85α from PDGF-treated but not untreated cells (Figure 3E). Similarly, immunoprecipitation of RGS4 from lysates of PDGF-treated HASM cells also extracted p-p85α PI3K.

To determine the molecular consequence(s) of RGS4-phospho-p85α binding for PI3K signaling in HASM cells, we analyzed Akt phosphorylation quantitatively by enzyme-linked immunosorbent assay (ELISA). Growth factors including PDGF elicit Akt phosphorylation at Thr-308 and Ser-473 residues, resulting in Akt kinase activity in cells expressing RGS4 shRNA compared to control. Finally, to determine the requirement of RGS4 for PDGF-induced PI3K- and Akt-dependent cell cycle progression, we evaluated expression of the Akt target gene cyclin D1 by quantitative real-time PCR [19,20,21]. As expected, RGS4 depletion decreased PDGF-evoked cyclin D1 expression (Figure 3H). The requirement of RGS4 for mitogen-induced cell growth was specific to the PI3K pathway, as siRNA-mediated extinction of RGS4 had no effect on PDGF-evoked ERK phosphorylation in HASM (Figure S3). Collectively, these studies demonstrate that PDGF induction of RGS4 expression has a critical function in mitogen-induced ASM hyperplasia through regulation of the PI3K-Akt signaling axis.

Mitogen-induced expression of RGS4 attenuates ASM excitation-contraction coupling

Since RGS4 expression is upregulated in failing human hearts [22], and overexpression of RGS4 impairs cardiac myocyte contractility and increases heart failure in a mouse model [23], we next examined whether mitogens attenuate agonist-induced contractile responses in an RGS4-dependent manner. GPCR agonists including carbachol, thrombin and histamine induce ASM contraction through a Gαq-dependent pathway that increases intracellular Ca2+ concentrations. We treated human precision cut lung slices (PCLS) with PDGF and measured airway contraction by supravital microscopy. PDGF dramatically upregulated RGS4 expression in lung slices (Figure 4A). Compared to untreated slices, PDGF reduced the potency [log EC50] of carbachol, an agonist of the M3 muscarinic receptor, as well as maximal carbachol-evoked bronchostriiction (βmax) (Figure 4A). PDGF also diminished histamine-induced contraction, suggesting that PDGF modulates contractile responses downstream of receptor activation through RGS4 upregulation.

To test this directly, we treated cultured HASM cells with PDGF and measured intracellular Ca2+ flux in quiescent and agonist-treated cells. Acetylcholine (M3 receptor agonist), histamine or thrombin induced a rapid increase in intracellular Ca2+ levels, and PDGF pre-treatment markedly blunted these responses (Figure 4B). In approximately 15% of HASM cells, PDGF rendered cells completely unresponsive to agonists. To determine whether PDGF inhibition of bronchoconstriction required RGS4, we compared agonist-induced Ca2+ responses in control and RGS4-depleted cells. Knockdown of RGS4 completely reversed PDGF-induced inhibition of histamine-evoked Ca2+ flux (Figure 4C). In parallel, we also determined that PDGF had little effect on isoproterenol-induced bronchodilation of carbachol-stimulated bronchoconstriction as shown in Figure 4D.

Figure 3. RGS4 is required for PI3K and Akt-dependent HASM proliferation. (A) Gel photographs of RT-PCR analysis of RGS4 and GAPDH expression in untreated and PDGF-treated HASM cells infected with lentiviruses encoding either control (ShCnt) or RGS4-specific (ShRGS4) shRNAs. (B) RGS4 depletion attenuates PDGF-mediated HASM proliferation. Untreated ShRGS4 and ShCnt ASM cells were serum-starved for 24 h followed by treatment with serum-free medium or medium containing PDGF for an additional 72 h. Total cell numbers were determined using a Beckman Cell Coulter counter (mean ± SEM of 6 separate experiments performed in 2 cell lines). (C) PDGF-induced cell cycle traversal after 24 h analyzed by FACS analysis of propidium iodide-stained nuclei isolated from ShRGS4 and ShCnt HASM cells. (D) Analysis of p85α phosphorylation assessed by immunoblotting of lysates of untreated or PDGF-treated ShCnt or ShRGS4 expressing HASM cells. Blots are representative of 3 separate experiments performed in 2 cell lines. (E) Left: Immunoblot analysis of p-p85 PI3K, RGS4 and total p85 PI3K expression in untreated or PDGF-treated cells. Right: Immunoblot analysis of lysates of untreated or PDGF-treated cells immunoprecipitated with indicated antibodies. Blots are representative of 3 separate experiments performed in 3 cell lines. (F) Kinetics of PDGF-mediated Akt phosphorylation were analyzed in lysates of ShRGS4 or ShCnt HASM cells by ELISA. Data (mean ± SEM) are expressed as fold change over basal, set as 1” in 5 separate experiments. (G) Akt kinase activity in ShRGS4 or ShCnt HASM cells in untreated or PDGF-treated cells. p-Akt (Ser-473) was immunoprecipitated from total cell lysates using a specific antibody followed by incubation with recombinant GSK3. Phospho-GSK-3β (Ser-21/9) (Ser-21/9) was quantified by colorimetric assay. Data (mean ± SEM) are fold-change over vehicle-treated ShCnt cells determined in 4 independent experiments measured in triplicate. (H) Total RNA was extracted from ShRGS4 and ShCnt HASM cells treated with PDGF or diluent for 8 h followed by analysis of relative cyclin D1 expression by real-time PCR. Data are mean ± SEM of 6 independent experiments using 2 cell lines. doi:10.1371/journal.pone.0028504.g003
Discussion

Severe asthma encompasses a variety of phenotypes that are characterized by ages of onset [24], duration of disease, degree of airflow impairment, presence of co-morbidity and types of inflammation [25,26]. The majority of subjects with severe asthma manifest a degree of irreversible airway obstruction despite maximal bronchodilation and, in some, a lack of methacholine responsiveness [25,27,28]. Severe asthma patients also experience more frequent and sustained exacerbations as compared with that of mild/moderate patients [24,25,26,27,28]. Given the irreversible component of the disease, investigators have suggested in part that airway remodeling and ASM hyperplasia may contribute to fixed airway obstruction [24,26,27,28]. We now show that human ASM proliferation requires expression of RGS4 protein, which interacts with the p85 subunit of PI3K. RGS4 also inhibits agonist-induced bronchoconstriction and calcium mobilization. Further, RGS4 expression in ASM cells is associated with increasing disease severity and may serve as a unique biomarker and/or therapeutic target to abrogate ASM hyperplasia and irreversible airway obstruction in asthma.

The most well-known function of RGS proteins is to reduce signaling output from GPCR activation. The importance of RGS proteins in the dynamic control of signaling is supported by...
changes in mRNA for these proteins under a variety of conditions [14,29]. Different RGS proteins interact with varying preference with members of the G_{i/o} and/or G_{q} families to reduce signaling. In addition to the RGS domain, RGS proteins have a variety of domains for non-GPCR protein-protein interactions, and thus selectivity for activation of particular pathways may be obtained by scaffolding mechanisms. Although few investigators have explored whether RGS proteins modulate RTK signaling pathways [30,31,32,33], our data suggest that RGS4 is essential to regulate RTK-mediated ASM growth. RGS expression is highly tissue- and cell-specific and, as such, imparts unique control of cellular function [29,34].

However, although the RGS family includes GPCR kinases (GRKs) such as β-adrenergic receptor kinase, about which much is known, the function of the smallest RGS molecules, namely, the B/R4 subfamily (RGS1–5, 8, 13, 16, 18, 21), remains unclear. In HASM, we show that the B/R4 RGS3, 4 and 5 proteins are the dominant RGS molecules expressed. Since RGS proteins can profoundly modulate GPCR signaling downstream from the receptor, conceptually B/R4 RGS proteins may modulate agonist-induced ASM contractility at multiple levels. In aortic smooth muscle, knockdown of RGS3 increased muscarinic 3 receptor (M3R)-dependent ERK activation but had no effect on angiotensin II-evoked signaling, suggesting receptor selectivity [35,36].

Given the necessity of RGS4 in regulating ASM proliferation, we posited that patients with severe asthma, who manifested marked increases in bronchial smooth muscle mass and irreversible airway obstruction, would manifest increased numbers of RGS4* ASM cells. The numbers of RGS4* ASM cells correlated with increasing disease severity. Interestingly, the RGS4* myocytes localized in discrete areas of the bronchial smooth muscle bundle suggesting heterogeneity of expression among myocytes. The recent development of bronchial thermoplasty, which delivers a thermal injury to the bronchial wall, appears to decrease ASM mass and improve clinical outcomes in severe asthma [37,38]. The location of the RGS4* ASM cells and the requirement of RGS expression in mediating ASM proliferation suggest a particular susceptibility of these cells to such an injury. Since the ASM cells fail to regenerate after the thermal injury, RGS4* cells may represent a unique population of ASM akin to skeletal myoblasts that serve to replenish differentiated muscle.

Although phenotypic plasticity of smooth muscle has been recognized for decades, the precise signaling pathways that inhibit contractile responses and that promote smooth muscle growth remain unknown. In cultured vascular smooth muscle, mostly RGS2 expression, but also 1, 3 and 4, are increased after stimulation with angiotensin II, PDGF, IL-1β or TGFβ [30,31]. RGS5 is expressed in rat and human aortic smooth muscle but not coronary or venous myocytes [35]. In cardiac muscle, RGS4 plays a critical role in regulating the chronotropic actions of acetylcholine. Lack of RGS4 enhances sensitivity to carbachol-induced bradycardia and evokes arrhythmias [39]. Others reported that increases in cardiac muscle expression of RGS4 decreases cardiac inotropy that promotes heart failure [22]. In myometrial smooth muscle, RGS12 expression is markedly increased at term while other RGS proteins are unchanged [40,41]. We now show that RGS4 promotes mitogen-induced ASM growth through regulation of PI3K activity yet inhibits agonist-induced contractile function by decreasing calcium mobilization stimulated by agonists. Importantly, PDGF had little effect on β-agonist-induced bronchodilation. Increases in RGS4 expression by growth factors, which enhance ASM mitogenesis, may impart a susceptibility to ASM hyperplasia in asthma. In our study, cultured ASM cells were derived from the proximal airway while the contractile responses were measured in the distal airway. To address whether the proximal and distal ASM responded differentially to agonists, cytosolic calcium mobilization to agonists was also studied in the cultured ASM and demonstrated that PDGF modulated agonist-induced calcium levels in the cultured ASM. Despite these studies, we recognize that proximal and distal ASM in vivo may undergo differential growth responses, and further experiments are needed to definitively demonstrate whether growth factors modulate RGS4 expression, contraction and proliferation in vivo.

Although asthma is considered a disease of reversible airway obstruction and inflammation, patients with severe disease experience irreversible airflow obstruction refractory to current therapies. Given the strikingly increased morbidity seen in this subset of patients compared to those with mild/moderate asthma, the need for new therapeutic approaches remains dire. We have identified growth factor-mediated upregulation of RGS4 in ASM as a deleterious event in severe asthma. RGS4 was required for ASM hyperplasia and rendered cells poorly contractile, consistent with a maladaptive phenotypic switch as shown in Figure 5. Although asthma is characterized by airway hyperresponsiveness, the data herein suggest that in severe disease, ASM becomes less responsive, which fixes the airway luminal diameter. Therapeutic approaches that decrease RGS4 expression or antagonize RGS4 function may prevent ASM hyperplasia and irreversible airway obstruction while promoting a more responsive smooth muscle phenotype.

Supporting Information

Figure S1 Mitogens induce RGS4 expression that requires PI3K and ERK activation. (A) Mitogens transcriptionally induce RGS4 in ASM cells. Pre-treatment of ASM cells with actinomycin (5 μM) for 1 h abrogated PDGF-, EGF- and thrombin-mediated RGS4 enhancement. (B) Assessment of signaling mechanisms mediating PDGF-induced RGS4 transcription. Real-time PCR analysis of HASM cells pre-treated with pharmacological inhibitors of PI3K (10 μM), ERK (10 μM), p38 MAPK (10 μM) or JAK (100 nM) for 1 h prior to treatment with PDGF for an additional 6 h. Data are mean ± SEM of 4 separate experiments performed in triplicate. (TIF)

Figure S2 Inhibition of ASM proliferation by silencing RGS4 has little effect on ASM apoptosis. (A) Apoptosis as assessed by caspase 3 activity in PDGF or diluent-treated untransfected (Untfr) shCat- or shRGS4-expressing HASM cells after 72 h. As a positive control, ceramide (40 μM) was used as an inducer of caspase-dependent apoptosis. (B) LDH levels as a measure of cell viability in Untfr shCat or shRGS4 HASM cells. As a positive control, Triton X-100 (3%) was used as an inducer of cell toxicity. Data are mean ± SEM of 4 separate experiments performed in triplicate. Values (mean ± SEM of 3 separate experiments performed in triplicate) are relative to those of vehicle-treated Untfr cells, set as ‘1’. (TIF)

Figure S3 RGS4 depletion has no effect on PDGF-induced ERK phosphorylation. Total cell lysates from PDGF- or diluent-treated shCat- or shRGS4-expressing HASM cells were immunoblotted by using p-ERK1/2. Total ERK1/2 expression was used as a control for protein loading. Blots are representative of 3 separate experiments performed in 2 cell lines. (TIF)
Figure S4 PDGF has little effect on isoproterenol-induced bronchodilation. PCLS were treated for 6 h with PDGF [50 ng/ml], then bronchoconstricted with carbachol, and cumulative additions of isoproterenol then added. As shown, PDGF had little effect on isoproterenol-induced bronchodilation. These experiments were performed in 3 slices obtained from 3 donors, and the data represent mean ± standard deviations.

Acknowledgments
We thank M. McNichol for her assistance in the preparation of the manuscript.

Author Contributions
Conceived and designed the experiments: KMD TH RAP RJS. Performed the experiments: GD PRC YA CEB TH. Analyzed the data: GD PRC TH RAP. Contributed reagents/materials/analysis tools: KMD VPK YA CEB. Wrote the paper: GD RAP RJS.

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