

# Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration

Hesam Movassagh, MSc,<sup>a</sup> Lianyu Shan, MSc,<sup>a</sup> Andrew J. Halayko, PhD,<sup>b</sup> Michael Roth, PhD,<sup>c</sup> Michael Tamm, MD,<sup>c</sup> Jamila Chakir, PhD,<sup>d</sup> and Abdelilah S. Gounni, PhD<sup>a</sup> Winnipeg, Manitoba, and Quebec City, Quebec, Canada, and Basel, Switzerland

**Background:** Chronic airway diseases, including asthma, are characterized by increased airway smooth muscle (ASM) mass that is due in part to growth factor-mediated ASM cell proliferation and migration. However, the molecular mechanisms underlying these effects are not completely understood.

Semaphorin 3E (Sema3E) has emerged as an essential mediator involved in cell migration, proliferation, and angiogenesis, although its role in ASM cell function is not investigated.

**Objectives:** We sought to determine the expression of Sema3E receptor, plexinD1, in human ASM cells (HSMCs); effect of Sema3E on basal and platelet-derived growth factor (PDGF)-induced proliferation and migration; and underlying signaling pathways.

**Methods:** Expression of plexinD1 in HSMCs was studied with RT-PCR, immunostaining, and flow cytometry. The effect of Sema3E on HASMC proliferation and migration was evaluated by 5-ethynyl-2'-deoxyuridine (EdU) incorporation, cell count, and Boyden chamber assay. Sema3E-mediated intracellular signaling was investigated with fluorescent microscopy, flow cytometry, Rac1 activation, and Western blot analysis.

**Results:** HSMCs from healthy persons expressed plexinD1 more than HSMCs from asthmatic patients. Sema3E increased plexinD1 expression in HSMCs from asthmatic patients. Recombinant Sema3E inhibited PDGF-mediated HASMC proliferation and migration, which was associated with F-actin depolymerization, suppression of PDGF-induced Rac1 guanosine triphosphatase activity, and Akt and extracellular signal-regulated kinase 1 and 2 phosphorylation. Bronchial biopsies from patients with mild asthma displayed immunoreactivity of plexinD1, suggesting the potential *in vivo* role of Sema3E–PlexinD1 axis in HASMC function.

**Conclusion:** This study provides the first evidence that Sema3E receptor is expressed and plays functional roles in HSMCs. Our data suggest a regulatory role of Sema3E in PDGF-mediated proliferation and migration, leading to downregulation of ASM remodeling. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

**Key words:** Airway smooth muscle cell, migration, platelet-derived growth factor, plexinD1, proliferation, semaphorin 3E

Asthma is a complex and heterogeneous syndrome, which is characterized by reversible airway obstruction, inflammation, hyperresponsiveness, and remodeling.<sup>1</sup> Human airway smooth muscle cells (HSMCs) are a key cell type in asthma because of their ability to contract in response to inflammatory cell products. Because of their intrinsic plasticity, HSMCs also exhibit the capacity for multifunctional behavior and are actively involved in local inflammation and airway remodeling.<sup>2</sup>

Increased airway smooth muscle (ASM) mass, one of the hallmarks of airway remodeling, is commonly observed in patients with chronic airways diseases, including asthma and chronic obstructive pulmonary disease.<sup>3–5</sup> This pathology is believed to occur via multiple mechanisms such as cell proliferation and migration.<sup>6,7</sup> The increase in proliferation rate of HSMCs obtained from asthmatic patients compared with nonasthmatic subjects is remarkable.<sup>8</sup> HASMC proliferation is increased in response to allergen challenge, growth factors, and inflammatory mediators such as platelet-derived growth factor (PDGF)-BB,<sup>9</sup> epidermal growth factor (EGF),<sup>10</sup> and leukotriene B<sub>4</sub>,<sup>11</sup> suggesting an important role of these factors in HASMC hyperplasia and airway remodeling. In addition, increased accumulation of ASM cells in asthma is not solely because of HASMC proliferation, and it might be partly attributable to migration of their progenitors from outside the muscle toward the lumen or immigration of proliferating cells within the muscle bundles.<sup>12</sup> Previous studies have reported promigratory effects of various growth factors and inflammatory mediators, including PDGF, TGF-β, and IL-8, on HSMCs.<sup>13,14</sup>

PDGF-BB modulates the contractile phenotype of HSMCs to a proliferative phenotype and stimulates their proliferation and migration *in vitro*.<sup>14</sup> Furthermore, Hitora et al<sup>15</sup> have shown mitogenic effect of this growth factor *in vivo*, whereby PDGF-BB overexpression in mice airways induced ASM hyperplasia and changed lung mechanics. However, the precise regulatory mechanisms underlying PDGF-induced HASMC proliferation and migration have remained elusive.

Semaphorins are a family of conserved-secreted and membrane-associated proteins originally discovered as axon guidance cues in neuronal development. More recently, their role in processes other than neuronal guidance was documented,

From the Departments of <sup>a</sup>Immunology and <sup>b</sup>Physiology, University of Manitoba, Winnipeg, Manitoba; <sup>c</sup>University Hospital Basel, University of Basel, Basel; and <sup>d</sup>Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie du Quebec, Université Laval, Quebec City, Quebec.

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Corresponding author: Abdelilah S. Gounni, PhD, Department of Immunology, Faculty of Medicine, University of Manitoba, 419 Apotex Center, 750 McDermot Ave, Winnipeg, Manitoba, R3E 0T5, Canada. E-mail: [gounni@cc.umanitoba.ca](mailto:gounni@cc.umanitoba.ca). 0091-6749/\$36.00

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**Abbreviations used**

|         |                                               |
|---------|-----------------------------------------------|
| ASM:    | Airway smooth muscle                          |
| EdU:    | 5-Ethynyl-2'-deoxyuridine                     |
| EGF:    | Epidermal growth factor                       |
| ERK:    | Extracellular signal-regulated kinase         |
| FACS:   | Fluorescence-activated cell sorting           |
| GTPase: | Guanosine triphosphatase                      |
| HASMC:  | Human airway smooth muscle cell               |
| MAPK:   | Mitogen-activated protein kinase              |
| MFI:    | Mean fluorescence intensity                   |
| PDGF:   | Platelet-derived growth factor                |
| PI3K:   | Phosphatidylinositol 3-kinase                 |
| Rac1:   | Ras-related C3 botulinum toxin substrate 1    |
| Sema3E: | Semaphorin 3E                                 |
| VEGFR2: | Vascular endothelial growth factor receptor 2 |

which encompasses angiogenesis, differentiation, cell proliferation, and migration.<sup>16</sup> Semaphorin 3E (Sema3E) was previously described as an intrinsic mediator involved in axon path finding<sup>17</sup> and vascular patterning.<sup>18</sup> Sema3E interacts with its receptor, plexinD1, with high affinity and regulates migratory functions. Sema3E–plexinD1 axis has also emerged as a pivotal pathway in cell migration and angiogenesis in immune and endothelial contexts, respectively.<sup>19,20</sup> The antiangiogenic effect of Sema3E is mediated through inhibition of endothelial cell proliferation.<sup>20</sup> Therefore, it is a likely candidate to play a significant role in pathogenesis of airways diseases, where the mechanisms regulating these processes are impaired.

In this study we aimed to investigate the expression of Sema3E receptor on HASMCs and whether it affects basal and PDGF-induced HASMC proliferation and migration. We demonstrated that both normal and asthmatic HASMCs constitutively express Sema3E high-affinity receptor, plexinD1, importantly to a lesser extent in HASMCs from patients with asthma than from healthy subjects. Furthermore, Sema3E enhances plexinD1 surface expression in HASMCs from asthmatic patients. Sema3E significantly inhibited PDGF-BB–induced HASMC proliferation and migration via mechanisms that involve downactivation of Ras-related C3 botulinum toxin substrate 1 (Rac1) guanosine triphosphatase (GTPase), Akt, and extracellular signal-regulated kinases 1/2 (ERK1/2). PlexinD1 immunoreactivity was detected within the ASM bundle in bronchial sections of patients with mild allergic asthma. Our data suggest that Sema3E and its receptor are involved in the regulation of ASM remodeling in chronic airway diseases such as asthma.

**METHODS**

For details on the methods used in this study, please see this article's *Methods* section in the Online Repository at [www.jacionline.org](http://www.jacionline.org).

**RESULTS****PlexinD1 is constitutively expressed by HASMCs *in vitro***

It has been previously shown that Sema3E binds plexinD1 with high affinity directly in neuronal and cardiovascular systems.<sup>17,21</sup> *In vitro* expression of Sema3E receptor in HASMCs was evaluated. As shown in Fig 1, A, mRNA for plexinD1 was expressed in primary HASMCs from 4 different donors. Human

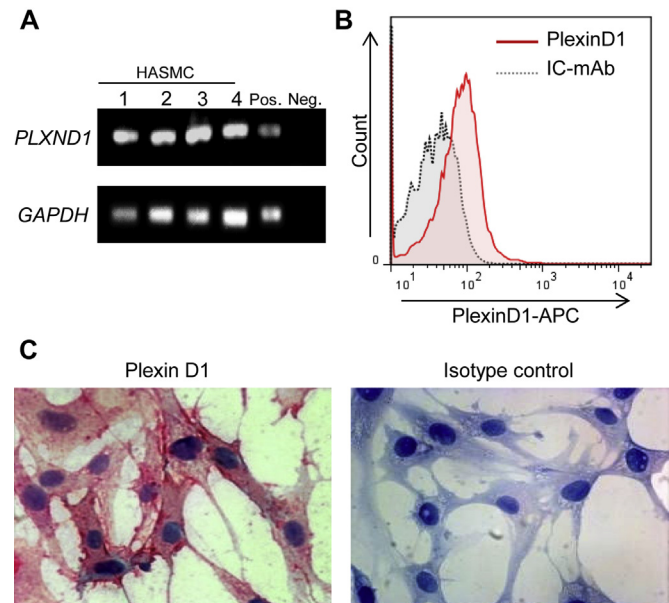


**FIG 1.** Expression of plexinD1 on HASMCs. Expression of plexinD1 on primary HASMCs was examined by RT-PCR with the use of specific primers (A), flow cytometry (B), and immunocytochemistry (C) by specific antibodies. Staining with isotype control antibody showed no immunoreactivity (B and C). RNA and protein expression studies were performed on at least 3 different HASMCs under the same conditions. APC, Allophycocyanin; GAPDH, glyceraldehyde phosphate dehydrogenase gene; IC, isotype control; PLXND1, plexin D1 gene.

Universal Reference Total cDNA (Clontech, Calif) was used as a positive control in all RT-PCR experiments, and no cDNA was served as negative control tubes (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). In parallel, flow cytometry with the use of specific mAb directed against human plexinD1 indicated its surface expression on HASMCs (Fig 1, B). We further confirmed expression of plexinD1 by performing immunocytochemistry on HASMCs (Fig 1, C).

**PlexinD1 is expressed in allergic asthma with a differential surface expression between normal and asthmatic HASMCs**

To examine whether plexinD1 is expressed *in vivo*, we performed immunostaining in bronchial tissue sections obtained from allergic asthmatic persons (n = 5). The clinical characteristics of the patients are provided in Table I. Fig 2, A–C, indicates that plexinD1 is expressed in ASM bundles and adjacent endothelium (red staining). Tissue sections stained with isotype antibody showed no immunoreactivity (Fig 2, D). Immunostaining of bronchial sections from healthy nonatopic subjects indicated plexinD1 expression in ASM bundles and airway endothelium (n = 5; data not shown) in a level comparable with asthmatic patients. However, fluorescence-activated cell sorting (FACS) analysis (Fig 2, E and F) found a significant decrease in plexinD1 surface expression on asthmatic versus normal bronchial HASMCs. Interestingly, treatment of HASMCs obtained from asthmatic patients with Sema3E, as a ligand, significantly increased surface expression of its receptor, plexinD1, to a level comparable with HASMCs from healthy donors (n = 3; *P* < .05; see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

**TABLE I.** Clinical characteristics of mild asthmatic persons subjected to bronchoscopy

| Mild asthma | Sex    | Age (y) | Smoking status | Allergy | Medication | Dose (mg/d) | PC <sub>20</sub> (mg/mL) | FEV <sub>1</sub> (%) |
|-------------|--------|---------|----------------|---------|------------|-------------|--------------------------|----------------------|
| Subject 1   | Female | 19      | No             | Yes     | Salbutamol | PR          | 0.26                     | 71                   |
| Subject 2   | Male   | 26      | No             | Yes     | Salbutamol | PR          | 0.43                     | 94                   |
| Subject 3   | Female | 28      | Ex             | Yes     | Salbutamol | 200         | 3.7                      | 111                  |
| Subject 4   | Male   | 21      | Ex             | Yes     | Salbutamol | PR          | 0.04                     | 100                  |
| Subject 5   | Female | 19      | No             | Yes     | Salbutamol | PR          | 0.41                     | 93                   |

Ex, Ex-smoker; PC<sub>20</sub>, provocative concentration of methacholine causing a 20% fall in FEV<sub>1</sub>; PR, per need.

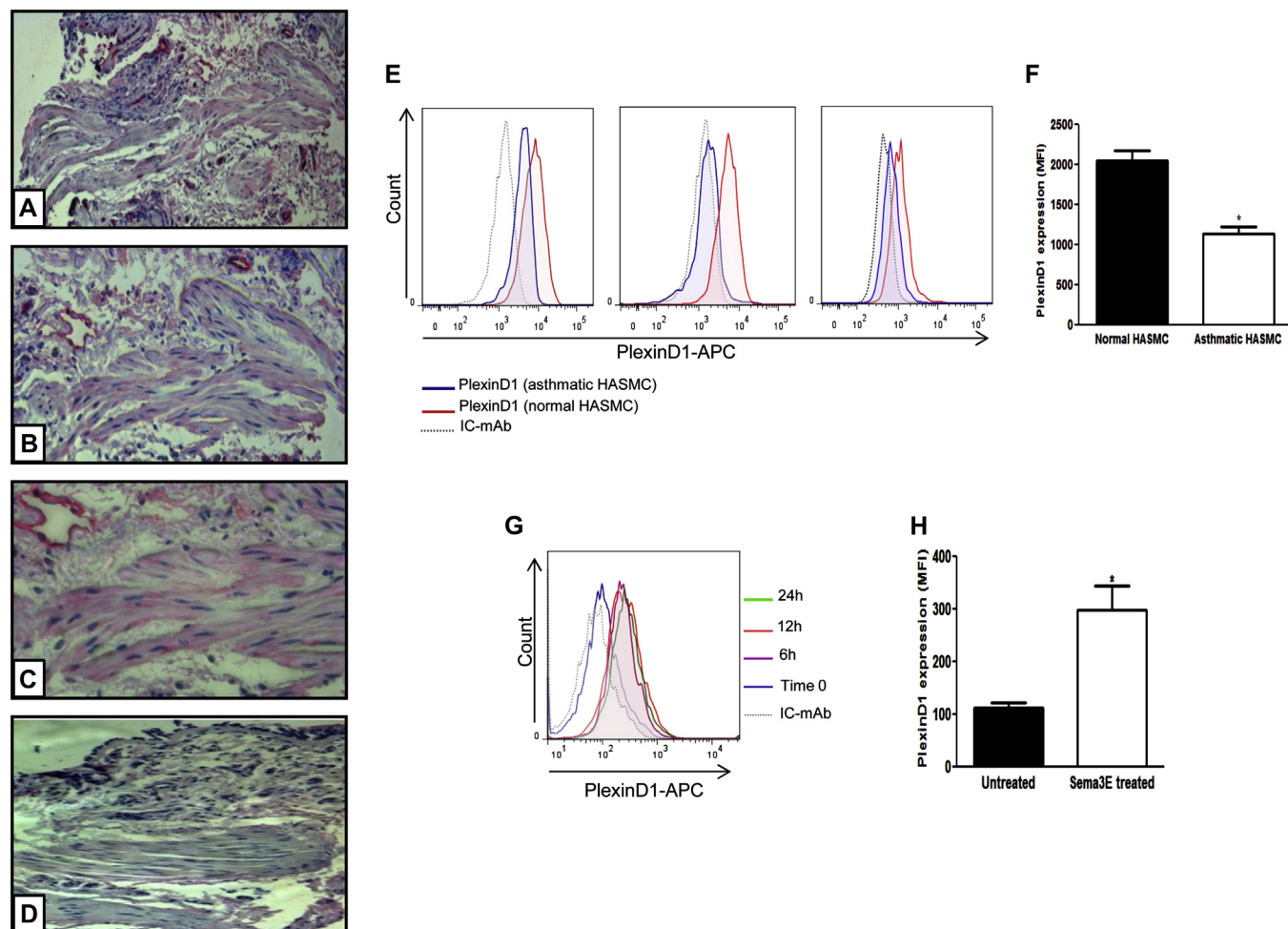


**FIG 2.** Immunohistochemical and FACS analysis of plexinD1 expression. Bronchial sections of mild allergic asthmatic subjects were stained with anti-plexinD1 antibody and visualized by magnification at  $\times 100$  (A),  $\times 200$  (B), and  $\times 400$  (C) ( $n = 5$ ). D, No immunoreactivity was observed after isotype antibody staining. E and F, Surface expression of plexinD1 was compared between asthmatic and normal bronchial HASMCs by FACS. G and H, Asthmatic HASMCs were treated with Sema3E and plexinD1, and surface expression was studied at indicated time points ( $n = 3$ ;  $P < .05$ ). APC, Allophycocyanin; IC, isotype control. \* $P < .05$ .

### Recombinant Sema3E inhibits PDGF-induced HASMC proliferation and migration

According to previous studies, antiangiogenic effect of Sema3E is mediated through inhibition of endothelial cell proliferation.<sup>20</sup> However, no study indicated the inhibitory effect of Sema3E on proliferation of other cell types. To study effects of recombinant Sema3E on HASMC proliferation, serum-deprived HASMCs were treated with different concentrations of recombinant Sema3E in the presence or absence of PDGF-BB (10 ng/mL). Then, incorporation of a fluorescent-

labeled thymidine analogue, 5-ethynyl-2'-deoxyuridine (EdU), into newly synthesized DNA was measured as an indicator of proliferation. PDGF-induced proliferation was inhibited by Sema3E in human telomerase reverse transcriptase-immortalized bronchial (Fig 3, A) and primary tracheal (Fig 3, C) HASMCs ( $n = 4$ ;  $P < .01$ ). Sema3E was also able to inhibit proliferation of primary bronchial HASMCs isolated from asthmatic persons (Fig 3, E) (see Table E2 for clinical characteristics of asthmatic subjects in whom their HASMC proliferation was studied). As indicated in Fig 3, B, D, and E, Sema3E inhibited cell

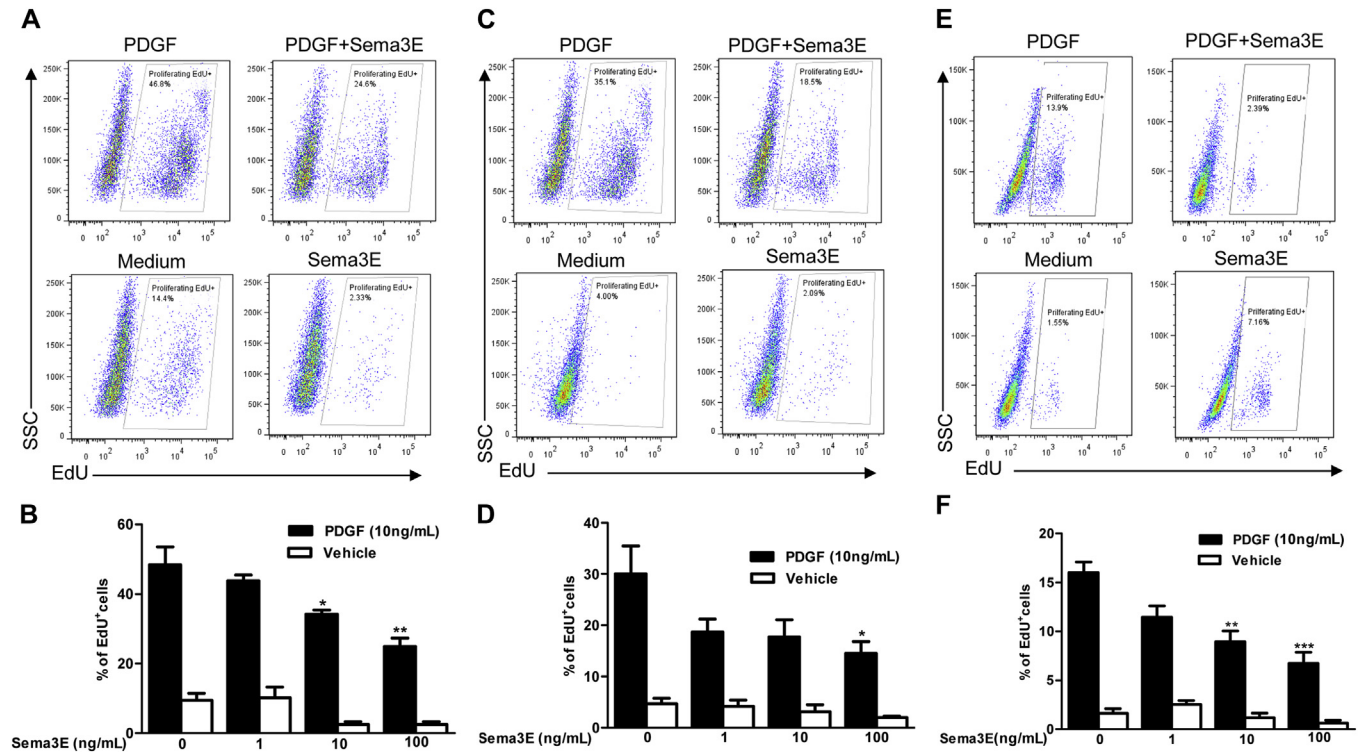


**FIG 3.** Inhibition of HASMC proliferation in response to Sema3E. Basal and PDGF-mediated proliferation of immortalized bronchial (A), primary tracheal (C) normal HASMCs, and also primary bronchial asthmatic HASMCs (E) was studied by EdU incorporation assay 48 hours after stimulation. The results were quantified and statistically compared (B, D, and F). The graphs are based on at least 3 independent proliferation experiments. SSC, Side scatter. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

proliferation in a dose-dependent manner in immortalized bronchial and primary tracheal HASMCs as well as asthmatic bronchial ones, respectively. In a different set of experiments, antiproliferative effect of Sema3E on HASMCs was further confirmed by performing cell count 4 days after Sema3E  $\pm$  PDGF stimulation of HASMCs (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org);  $n = 4$ ). In addition, Sema3E + PDGF costimulation of HASMCs negatively affected cell cycle distribution and led to a decreasing trend of DNA synthesis in the presence of PDGF as a mitogen. However, the results were not statistically significant (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Inhibitory effect of Sema3E was not restricted to PDGF. As such, Sema3E significantly inhibited EGF-induced HASMC proliferation, another important growth factor that promotes ASM hyperplasia and airway remodeling (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org);  $n = 3$ ;  $P < .05$ ).

To ascertain whether the Sema3E effect in HASMCs is mediated through plexinD1, cells were treated with exogenous human recombinant plexinD1 and stimulated with PDGF or PDGF combined with Sema3E.<sup>20</sup> Human plexinD1 recombinant protein significantly reversed the antiproliferative effect of Sema3E on HASMCs ( $n = 3$ ;  $P < .05$ ) as indicated by the EdU incorporation assay (Fig 4, A and B) and manual cell count (Fig 4, C).

Next, we evaluated the effect of recombinant Sema3E on HASMC migration with the use of a Boyden chamber assay. Cultures were treated with Sema3E  $\pm$  PDGF, and the number of migrated cells was counted after 4 hours. Fig 5, A and B, indicates a significant

inhibitory effect of Sema3E on PDGF-induced HASMC migration in a dose-dependent fashion ( $n = 5$ ;  $P < .001$ ). Sema3E concentrations used in migration assays are based on previous studies.<sup>19</sup>

Collectively, our functional studies suggest that Sema3E regulates PDGF-mediated HASMC proliferation and migration.

### Actin depolymerization is induced after Sema3E treatment in HASMCs

On the basis of the canonical importance of actin dynamics in ASM cell migration<sup>22</sup> and also the effect of Sema3E on actin depolymerization,<sup>23,24</sup> F-actin alterations were studied by phalloidin staining. HASMCs were stimulated with Sema3E, PDGF, or both for 0 to 30 minutes, stained with Alexa Fluor 488-phalloidin, and visualized by immunofluorescence microscopy. Fig 5, C, depicts reduced F-actin content, as found by decreased immunofluorescence signal, on Sema3E stimulation with or without PDGF. Actin depolymerization was also confirmed with flow cytometric quantification and represented as mean fluorescence intensity (MFI) values. HASMCs indicated a substantial decrease in F-actin content in response to Sema3E  $\pm$  PDGF in a time-dependent manner (Fig 5, D and E;  $n = 3$ ;  $P < .01$ ; see also Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### Sema3E suppresses PDGF-induced Rac1 GTPase activity in HASMCs

To consider the role of Rac1 signaling in PDGF-induced smooth muscle proliferation and migration,<sup>9,25</sup> Rac1 GTPase

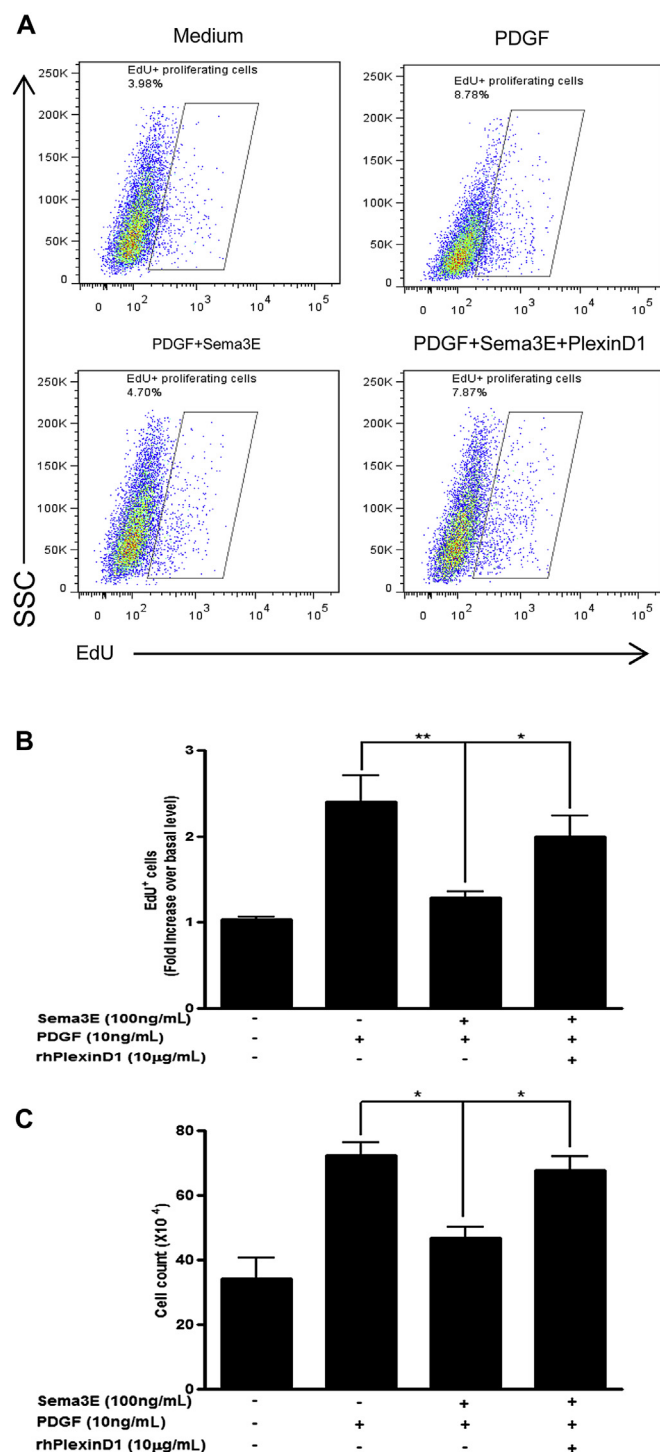


**FIG 4.** PlexinD1-mediated fashion of Sema3E effect on HASMC proliferation. HASMCs were stimulated with Sema3E and PDGF in the presence or absence of recombinant human plexinD1, followed by EdU incorporation assay (**A** and **B**) and cell count (**C**). Sema3E inhibitory effect on PDGF-induced proliferation was significantly abrogated in the presence of exogenous plexinD1 in 3 independent experiments. SSC, Side scatter. \* $P < .05$  and \*\* $P < .01$ .

activity was measured at indicated time points to determine the mechanism by which Sema3E inhibits PDGF-induced HASMC proliferation and migration. As shown in Fig 6, PDGF induced a rapid and robust increase of Rac1 GTPase activity 1 minute after

stimulation, which was reduced to the basal level of activity in the presence of Sema3E ( $n = 5$ ;  $P < .01$ ). Despite a decreasing trend in Rac1 GTPase activity 1 minute after stimulation with Sema3E alone, it was not significantly different compared with time 0 (Fig 6). It is in line with reduced, but not significant, basal proliferation (Fig 3; see also Fig E1) and migration (Fig 5, A and B) on Sema3E treatment alone.

### Sema3E inhibits PDGF-mediated phosphorylation of PI3K/Akt and MAPK/ERK1/2 in HASMCs

To further elucidate the signaling pathways leading to inhibitory effect of Sema3E on HASMC function, we investigated the role of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/ERK1/2 by Western blot analysis with the use of specific Abs. PDGF induced a dramatic and reproducible increase in Akt and ERK1/2 phosphorylation in HASMCs at indicated time points after stimulation. Combination of PDGF with Sema3E treatment reduced PDGF-induced phosphorylation of Akt and ERK1/2 significantly. Sema3E alone did not induce noticeable phosphorylation in HASMCs (Fig 7, A and B).

These data suggest that Akt and ERK1/2 are involved in Sema3E inhibition of PDGF-mediated HASMC proliferation and migration.

### DISCUSSION

Our study is the first to describe the expression of plexinD1, high-affinity receptor for Sema3E, and functional consequences of its ligation with exogenous recombinant Sema3E on HASMC proliferation and migration. We found that Sema3E significantly inhibits PDGF-induced HASMC proliferation and migration. This effect is associated with depolymerization of F-actin, downregulation of Rac1 GTPase activity, as well as a decrease in MAPK/ERK1/2 and PI3K/Akt phosphorylation. HASMCs from patients with asthma displayed less plexinD1 surface expression than HASMCs from healthy donors which was increased on Sema3E treatment.

Understanding the regulatory mechanisms of HASMC proliferation and migration is of potential clinical value and may provide further insight into the new treatment strategies of airway diseases associated with smooth muscle hyperplasia such as asthma and chronic obstructive pulmonary disease.

Semaphorins are multifaceted proteins involved in the pathogenesis of several diseases from different types of cancer to autoimmune and neurologic disorders.<sup>26-30</sup> It was previously shown that Sema3E contributed to tumor progression,<sup>31</sup> ischemic retinopathy,<sup>32</sup> and congenital defects<sup>33</sup> through its effect on angiogenesis, cell proliferation, and migration. However, expression and function of this neuronal guidance molecule in asthmatic and other allergic conditions has remained to be addressed.

We have shown that plexinD1 is expressed by and present on HASMCs. Unlike other class 3 semaphorins, Sema3E interacts directly with plexinD1. However, gating of Sema3E–plexinD1 complex by neuropilin 1 or vascular endothelial growth factor receptor 2 (VEGFR2) switches axonal repulsion to attraction.<sup>34,35</sup> Because neuropilin 1 and VEGFR2 are abundantly expressed in asthma and chronic rhinosinusitis compared with healthy subjects,<sup>36,37</sup> differential interaction of Sema3E with them on asthmatic HASMCs and the cell response should be further investigated. Nevertheless, Sema3E-repulsive effect on asthmatic

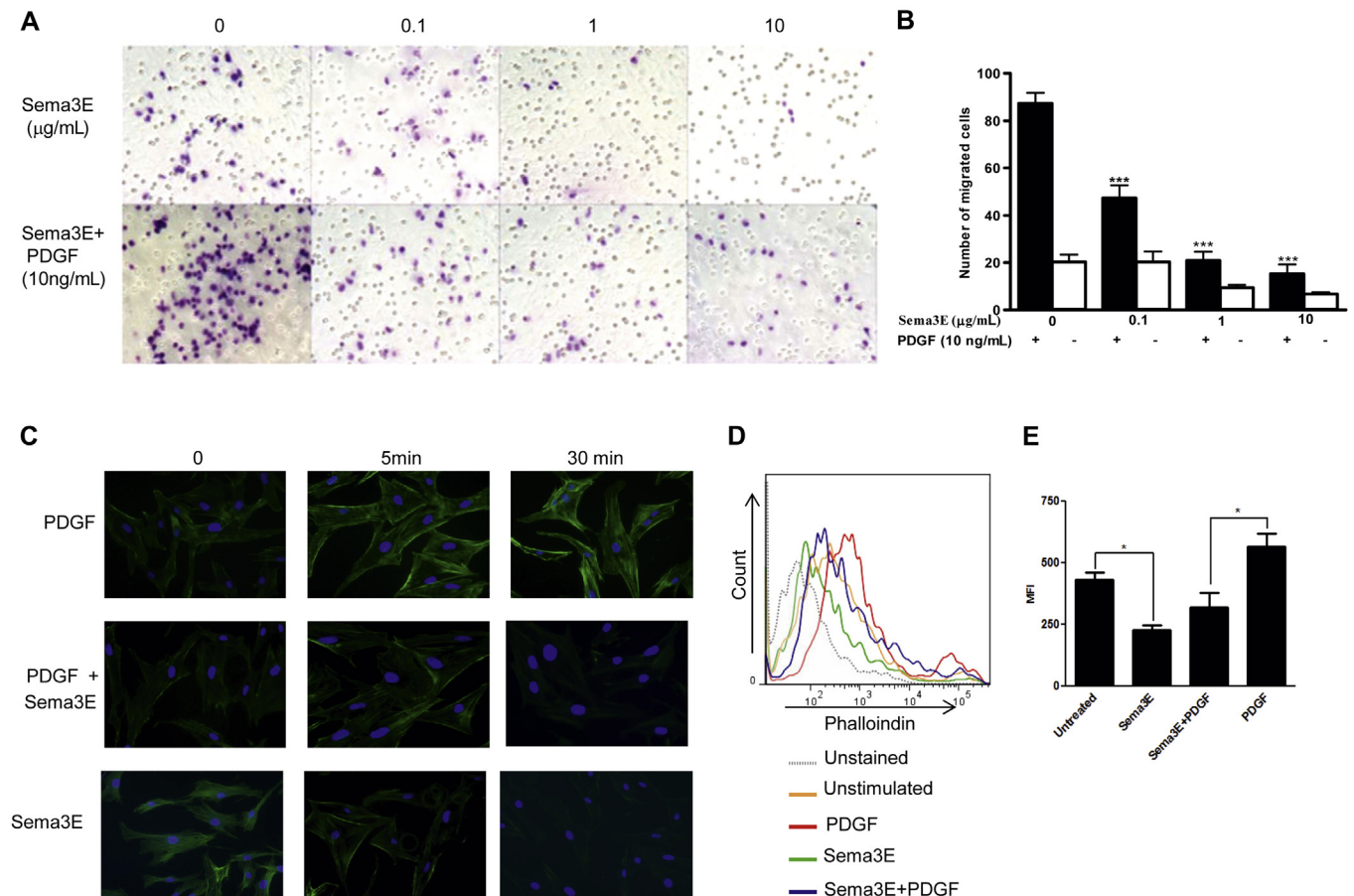


**FIG 5.** A and B, Inhibitory effect of Sema3E on HASMC migration. HASMC migration toward Sema3E  $\pm$  PDGF was studied in a double-blind manner. C-E, Phalloidin staining was performed to detect F-actin content of HASMCs after treatment with Sema3E, PDGF, or their combination. \* $P < .05$  and \*\*\* $P < .001$ .

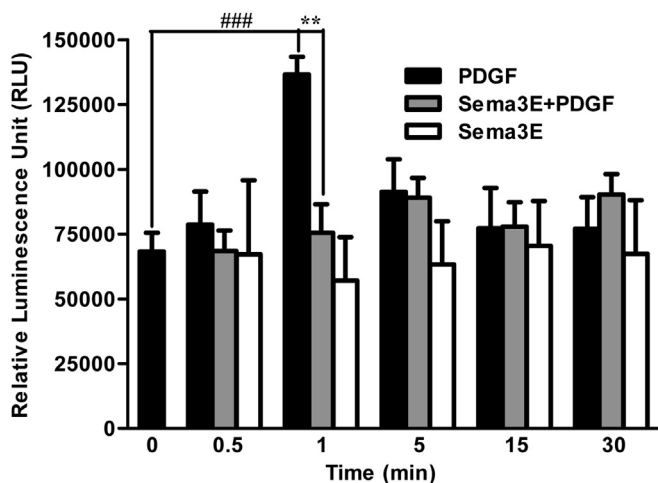


**FIG 6.** Suppression of PDGF-induced Rac1 GTPase activity by Sema3E in HASMC. Rac1 GTPase activity was measured after Sema3E  $\pm$  PDGF stimulation by G-LISA. Sema3E suppressed PDGF-induced Rac1 GTPase activity 1 minute after costimulation without significant effect on the basal activity. Data represent mean  $\pm$  SEM of 5 independent experiments performed in the same conditions. \*\* $P < .01$  and ### $P < .001$ .

HASMC proliferation and also reversion of this effect by recombinant plexinD1 treatment indicate that Sema3E signaling in HASMCs may be mediated exclusively through plexinD1.

We confirmed expression of plexinD1 on human ASM bundles in asthmatic and nonasthmatic tissue sections *in vivo*. Decreased surface expression of plexinD1 on primary bronchial HASMCs from asthmatic subjects compared with healthy ones suggests that Sema3E–plexinD1 inhibitory axis might be abrogated under pathologic circumstances such as airway remodeling in which HASMC functions are highly dysregulated. However, we have found here that HASMCs isolated from asthmatic patients responded to exogenous Sema3E through plexinD1 activation, by induction of receptor expression on ligand stimulation as reported for other surface receptors.<sup>38-40</sup>

Our functional studies have indicated that Sema3E inhibited PDGF-induced HASMC proliferation and migration. Although basal proliferation and migration of HASMCs in response to Sema3E showed a decreasing trend compared with unstimulated HASMCs, the differences were not statistically significant. Sema3E-mediated inhibition of HASMC migration was associated with F-actin depolymerization. Therefore, it may be speculated that the antimigratory effect of Sema3E on HASMCs is mediated by actin remodeling that is in line with previous findings in endothelial cells and macrophages.<sup>23,24</sup> It should be noted that semaphorins are more likely to provoke repulsion of target cells than attraction and that an individual semaphorin can induce either repulsive or attractive responses in a cell type-specific manner.<sup>41</sup> As a bifunctional semaphorin, Sema3E-repulsive effect can be reversed by proteolytic processing<sup>42</sup> and

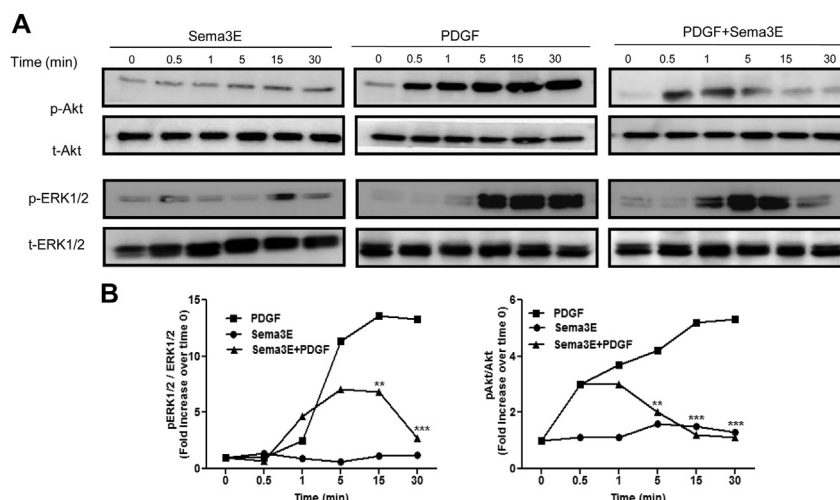


**FIG 7.** Kinetic analysis of signaling pathways downstream of Sema3E stimulation in HASMCs. **A**, Sema3E effect on HASMCs was associated with a dramatic decrease in Akt and ERK1/2 phosphorylation. **B**, Densitometric analysis was performed on Akt and ERK1/2 phosphorylation and presented as the ratio of phosphorylation over total compared with time zero. Data represent mean  $\pm$  SEM of 3 independent experiments.  $^{**}P < .01$  and  $^{***}P < .001$ .

its interaction with coreceptors.<sup>33,34</sup> However, in our *in vitro* system, Sema3E negatively regulated HASMC proliferation and migration.

In an effort to understand the mechanism of action of Sema3E in HASMCs, considering involvement of Rac1 in smooth muscle proliferation and migration<sup>9,25</sup> as well as unknown function of Rac1 in Sema3E–plexinD1 signaling, we investigated the role of Rac1 GTPase in Sema3E-mediated HASMC repulsion. As a novel finding, we found that Sema3E induced inactivation of Rac1 GTPase in the presence of PDGF, which has been shown to strongly stimulate Rac1 GTPase activity in HASMCs. Semaphorin signaling is mainly converged to small GTPases, including Ras homology (Rho) proteins such as Rac1. When bound to GTP, small GTPases are activated and attach to effector proteins to promote cell proliferation and migration.<sup>43</sup> Along with the study by Sakurai et al<sup>23</sup> on human umbilical vein endothelial cells, we found that treatment of HASMCs with Sema3E alone did not significantly affect the basal Rac1 GTPase activity in HASMCs. Considering the physical interaction and nuclear colocalization of signal transducer and activator of transcription 3 and Rac1 in response to PDGF in HASMCs,<sup>9</sup> signal transducer and activator of transcription 3 (STAT3) as an essential signaling component of ASM cell proliferation may also be negatively affected by Sema3E treatment and needs to be further investigated.

As previously reported in human umbilical vein endothelial cells, Sema3E decreased growth factor-induced activation of Akt and ERK1/2,<sup>20</sup> which are essential mediators of HASMC proliferation and migration.<sup>14,44,45</sup> Therefore, based on our findings, Rac1, Akt, and ERK1/2 seem to play a role in the signaling events that are influenced on Sema3E + PDGF costimulation in HASMCs. However, it seems Sema3E alone does not affect these pathways, which may explain the nonsignificant effect of Sema3E on basal HASMC proliferation and migration in agreement with findings in endothelial cells.<sup>23</sup> The alternative signaling mechanisms underlying Sema3E effects on PDGF-induced HASMC proliferation and migration might be mediated through PDGF receptor (PDGFR). Antiproliferative effect of Sema3E

on endothelial cells, which eventually leads to repressing VEGF-induced angiogenesis, is associated with a decrease in ligand-mediated phosphorylation of VEGFR2.<sup>20</sup> Thus, we would speculate that phosphorylation of PDGFR may be decreased on costimulation of HASMCs with Sema3E and PDGF. In fact, Sema3E–plexinD1 axis may prevent PDGF–PDGFR signaling by inhibiting PDGFR activity or its accessibility for the ligand through an unknown mechanism. Because Sema3E inhibited EGF-induced HASMC proliferation (Fig E2), EGF receptor signaling needs to be further addressed in this context.

In summary, our study provides the evidence that semaphorins may contribute to asthma pathology by affecting ASM cell function and airway remodeling. As a novel approach, our results suggest a new mechanism through which Sema3E inhibits HASMC proliferation and migration, giving a new clue to control airway remodeling and clinical manifests of allergic asthma.

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**Clinical implications: Antiproliferative and antimigratory effect of Sema3E on human airway smooth muscle may provide a novel therapeutic approach to minimize airway remodeling in chronic airway diseases.**

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## METHODS

### Ethics statement

Bronchial and tracheal HASMCs used in this study were obtained from healthy persons without asthma and persons with asthma in accordance with procedures approved by the Human Research Ethics Board of the University of Manitoba, Winnipeg, Manitoba, Canada, and the Ethics Committee at University Hospital Basel, Basel, Switzerland. Written informed consent was obtained from each person. The clinical characteristics of asthmatic patients are described in Table E2.

Bronchoscopy was performed in accordance with procedures approved by the Human Research Ethics Board of Laval University, Quebec City, Quebec, Canada, after the receipt of the subjects' written informed consent. Clinical features of asthmatic patients from whom bronchial biopsies were obtained have been categorized in Table I.

### Reagents

Recombinant human Sema3E, plexinD1, PDGF-BB, EGF, allophycocyanin (APC)-conjugated mouse anti-human plexin D1 monoclonal antibody and goat anti-human plexin D1 affinity purified polyclonal antibody were purchased from R&D Systems (Minneapolis, Minn). Unlabeled and APC-conjugated isotype control antibodies were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada) and eBiosciences (San Diego, Calif), respectively. Cell culture media were obtained from Invitrogen (Burlington, Ontario, Canada). FBS and insulin-transferrin-selenium-X (ITS-X) were purchased from HyClone Laboratories (Logan, Utah) and Invitrogen, respectively. Alkaline phosphatase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa). All other reagents are from Sigma-Aldrich unless otherwise indicated.

### Isolation and culture of HASMCs

HASMCs were obtained as described previously.<sup>E1,E2</sup> In brief, primary HASMCs were isolated from lower tracheal section of healthy donors from whom recipient cannot be matched. Asthmatic primary bronchial HASMCs were isolated from dissected airway muscle bundles of the bronchial biopsies from patients with mild-to-moderate asthma. The patients did not manifest exacerbations and received no medication 1 week before bronchoscopy.<sup>E3</sup> Cells retain  $\alpha$  smooth muscle-specific actin (filament form), SM22, calponin, and/or SMHC protein expression, as well as negative staining for fibronectin and E-cadherin and mobilize intracellular  $\text{Ca}^{2+}$  in response to acetylcholine.<sup>E3,E4</sup> Primary HASMCs were used at passages 2 to 5 in all experiments. Bronchial HASMCs (D9) immortalized by stable ectopic expression of human telomerase reverse transcriptase was used at passages 15 to 20 and was described in detail.<sup>E5</sup>

HASMCs were grown on uncoated plastic dishes in complete Dulbecco modified Eagle medium (supplemented with 100  $\mu\text{g}/\text{mL}$  streptomycin, 100 U/mL penicillin, and 10% FBS). Unless otherwise mentioned, cells were grown to a subconfluent (~60%-70%) condition and serum starved to synchronize for 48 hours in Ham's F12 supplemented with  $1 \times \text{ITS}$  (5  $\mu\text{g}/\text{mL}$  insulin, 5  $\mu\text{g}/\text{mL}$  transferrin, and 5 ng/mL selenium), 100  $\mu\text{g}/\text{mL}$  streptomycin, and 100 U/mL penicillin and before each experiment.

### RNA extraction and RT-PCR

Total RNA was extracted from HASMCs (passages 2-5) by using TRIzol (Invitrogen). RNA (2  $\mu\text{g}$ ) was subjected to MultiScribe Reverse Transcriptase to synthesize cDNA according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif). Expression of the plexin D1 and glyceraldehyde phosphate dehydrogenase genes was analyzed by RT-PCR. PCR products were run on 2% wt/vol agarose gel electrophoresis and visualized by ethidium bromide staining. PCR conditions and specific primers have been mentioned in Table E1.

### Flow cytometric analysis of receptor expression

Primary cultured confluent HASMCs were detached with Versene (0.02% EDTA; Lonza, Walkersville, Md) without trypsinization. The single cell suspensions ( $1 \times 10^5$  cells) were separately incubated with APC-conjugated

mouse anti-human plexinD1 or isotype control Ab (IgG2b subclass) for 30 minutes at 4°C. The cells were washed twice with flow buffer, resuspended in PBS containing 0.1% BSA, and analyzed on FACScan (BD Biosciences, San Jose, Calif). FACS analysis was performed by FlowJo software (Tree Star, Ashland, Ore). In some experiments, serum-deprived asthmatic HASMCs were treated with Sema3E (100 ng/mL) for 0, 6, 12, and 24 hours, and then plexinD1 surface expression was studied as described.

### Immunocytochemistry

HASMCs were seeded onto sterile glass coverslips and grown to 70% confluence. Cells were then fixed in 4% paraformaldehyde for 20 minutes and permeabilized, and nonspecific Ab binding was blocked. Immunolabeling was performed with goat anti-human plexinD1 Ab, as well as appropriate control goat IgG at 4°C overnight. Cells were incubated with biotin-labeled rabbit anti-goat IgG as secondary antibody, washed, and followed by streptavidin-alkaline phosphatase-conjugated secondary Abs for 1 hour at room temperature. The signals were detected by adding Fast Red, and then cells were counterstained with modified Mayer hematoxylin (Fisher Scientific, Fair Lawn, NJ). Coverslips were mounted with crystal mount and visualized by AxioVision software (Carl Zeiss, Inc, Thornwood, NY).

### Immunohistochemistry

Bronchial biopsies were obtained from mild asthmatic patients fulfilling the American Thoracic Society selection criteria.<sup>E6</sup> Patients with mild asthma used no inhaled corticosteroids, had symptoms <2 days per week, and experienced rare exacerbations. These atopic nonsmokers had not received long-acting  $\beta_2$ -agonists or inhaled corticosteroids in the past 3 months (see Table I).

Paraffin sections were stained as described previously.<sup>E2</sup> Briefly, formalin-fixed tissues were paraffin-embedded, and 5- $\mu\text{m}$ -thick sections were prepared, deparaffinized in xylene, and rehydrated through graded concentrations of alcohol to water and then boiled with microwave for 10 minutes in sodium citrate buffer (pH 6). Sections were washed and then incubated with blocking solution (1% BSA, 0.1% cold fish skin gelatin in TRIS-buffered saline [TBS]) for 60 minutes at room temperature. Goat anti-human plexin D1 Ab or control goat IgG (both at 10  $\mu\text{g}/\text{mL}$ ) were added, and sections were incubated overnight at 4°C. Slides were then washed twice with TBS followed by incubation for 1 hour at room temperature with biotin-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc). Slides were then washed extensively with TBS and incubated with streptavidin alkaline phosphatase for 30 minutes at room temperature. After washing with TBS, the slides were developed with Fast Red and counterstained with modified Mayer hematoxylin (Fisher Scientific).

### EdU cell proliferation assay and cell cycle analysis

Serum-deprived HASMCs were stimulated with the aforementioned concentrations of Sema3E with or without PDGF. Click-iT EdU flow cytometric assay kit (Invitrogen) was used to further investigate the Sema3E effect on HASMC proliferation. Briefly, EdU reagent was added at a 10- $\mu\text{mol}/\text{L}$  concentration 40 hours after stimulation, and cells were harvested 8 hours later. Cells were collected, fixed, and then incubated with saponin-based permeabilization buffer. Click-iT reaction cocktail containing copper sulfate and fluorescent dye azide (Alexa Fluor 488) was freshly prepared and added to the samples. Finally, cell cycle was analyzed with propidium iodide staining. EdU incorporation into DNA and cell cycle distribution were assessed with flow cytometry.

### Cell count

HASMCs were seeded at  $5 \times 10^4$  cells/well in triplicates in 6-well plates and maintained in Dulbecco modified Eagle medium to become 50% to 70% confluent. After serum starvation in F12 medium that contained ITS-X and sodium pyruvate for 48 hours, cells were treated with Sema3E (0, 1, 10, and 100 ng/mL) with or without PDGF (10 ng/mL). Two days later, cells were collected and counted with a hemocytometer, and cell viability was determined by trypan blue exclusion. Cell count experiments were performed

double-blind and twice by 2 independent persons. Proliferation assays were also performed with EGF (10 ng/mL) instead of PDGF at the same conditions.

### Cell migration assay

A Boyden chamber (Neuro Probe Inc, Gaithersburg, Md) assay developed specifically to study smooth muscle cell migration was used as described previously.<sup>E7</sup> Sema3E (0, 0.1, 1, and 10  $\mu$ g/mL) with or without PDGF (10 ng/mL) was added to the wells of the bottom chamber in triplicate. Then, collagen-coated filter membrane was placed on the bottom chamber, and serum-deprived HASMCs ( $5 \times 10^5$  cells/mL) were added to the upper chamber wells. After 4 hours of incubation in 37°C, nonmigrated cells that did not pass through the membrane pores were wiped out from the top side of the insert membrane, and then migrated cells in each well were stained with Protocol Hema 3 stain (Biochemical Sciences Inc, Swedesburg, NJ) and counted in random fields.

### Phalloidin staining and immunofluorescence

The cells cultured on sterile coverslips were serum-starved and treated with Sema3E (100 ng/mL) for 0 to 5 minutes, fixed with 4% paraformaldehyde in PBS for 20 minutes, and permeabilized with 0.05% Triton X-100. Filamentous (F) actin was visualized with Alexa Fluor 488 Phalloidin (Invitrogen). Samples were mounted in gold anti-fade mounting medium (Invitrogen) and visualized under fluorescent microscope.

### Quantification of F-actin content by flow cytometry

HASMCs were seeded in triplicate in 12-well plates, serum starved, and stimulated with Sema3E (100 ng/mL) for 0 to 5 minutes. Cells were fixed, permeabilized, and stained with Alexa Fluor 488 Phalloidin as described earlier. Then, intracellular fluorescence was determined with flow cytometry. After appropriate gating to remove cell debris from analysis, histograms of cell number versus log fluorescence intensity and forward angle light scatter were recorded. Relative F-actin content after Sema3E treatment was expressed as the MFI and was compared with that of nonstimulated HASMCs.

### Measurement of Rac1 GTPase activity

Rac1 GTPase activity was measured in snap-frozen cell lysates harvested from unstimulated or Sema3E  $\pm$  PDGF-stimulated HASMCs by using a luminometric-based G-LISA Rac1 Activation Assay Kit (Cytoskeleton, Inc, Denver, Colo). Briefly, cell lysates were subjected to Rho binding domain of Rac1 in a Rac-GTP affinity 96-well plate. Then, Rac1-GTP was detected with specific primary Ab, followed by horseradish peroxidase-conjugated secondary Ab incubation and development with a chemiluminescent reagent. A constitutively active Rac1 provided in the kit was used as positive control in all experiments.

### Assessment of MAPK/ERK1/2 and PI3K/Akt activation

Protein lysates obtained from G-LISA experiments were investigated for downstream signaling with the use of Western blot analysis. Protein lysates (10  $\mu$ g) were loaded on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, blocked, and then incubated overnight at 4°C with Abs specific for phosphorylated Akt (S473) and ERK1/2 (T202/Y204). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary Abs, and bands were revealed with electrochemiluminescence reagents. Total anti-Akt and ERK1/2 were used as loading control. Densitometric analysis was performed by using AlphaEase FC software (Alpha Innotech, San Leandro, Calif), and integrated density value was presented as the fold-increase in phosphorylated over total compared with time zero.

### Statistical analysis

GraphPad Prism 5.0 software (GraphPad Inc, San Diego, Calif) was used for statistical analysis, and values were presented as the mean  $\pm$  SEM of at least 3 independent experiments. Depending on the number of groups and treatments, data were analyzed by unpaired *t* test, 1-way or 2-way ANOVA, followed by the Bonferroni multiple comparison *post hoc* test. Differences were considered to be statistically significant at \**P* < .05, \*\**P*  $\leq$  .01, and \*\*\**P*  $\leq$  .001.

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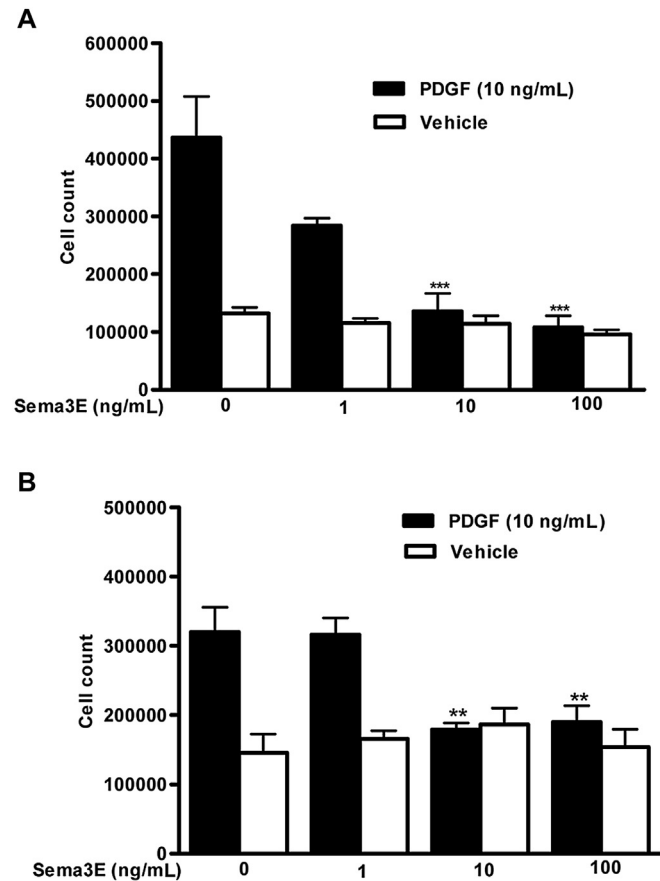


**FIG E1.** Inhibition of HASMC proliferation in response to Sema3E. Basal and PDGF-mediated proliferation of HASMCs was studied by manual cell count 4 days after stimulation. The graphs are based on 4 independent proliferation experiments on bronchial (**A**) and tracheal (**B**) HASMCs. \*\* $P < .01$  and \*\*\* $P < .001$ .

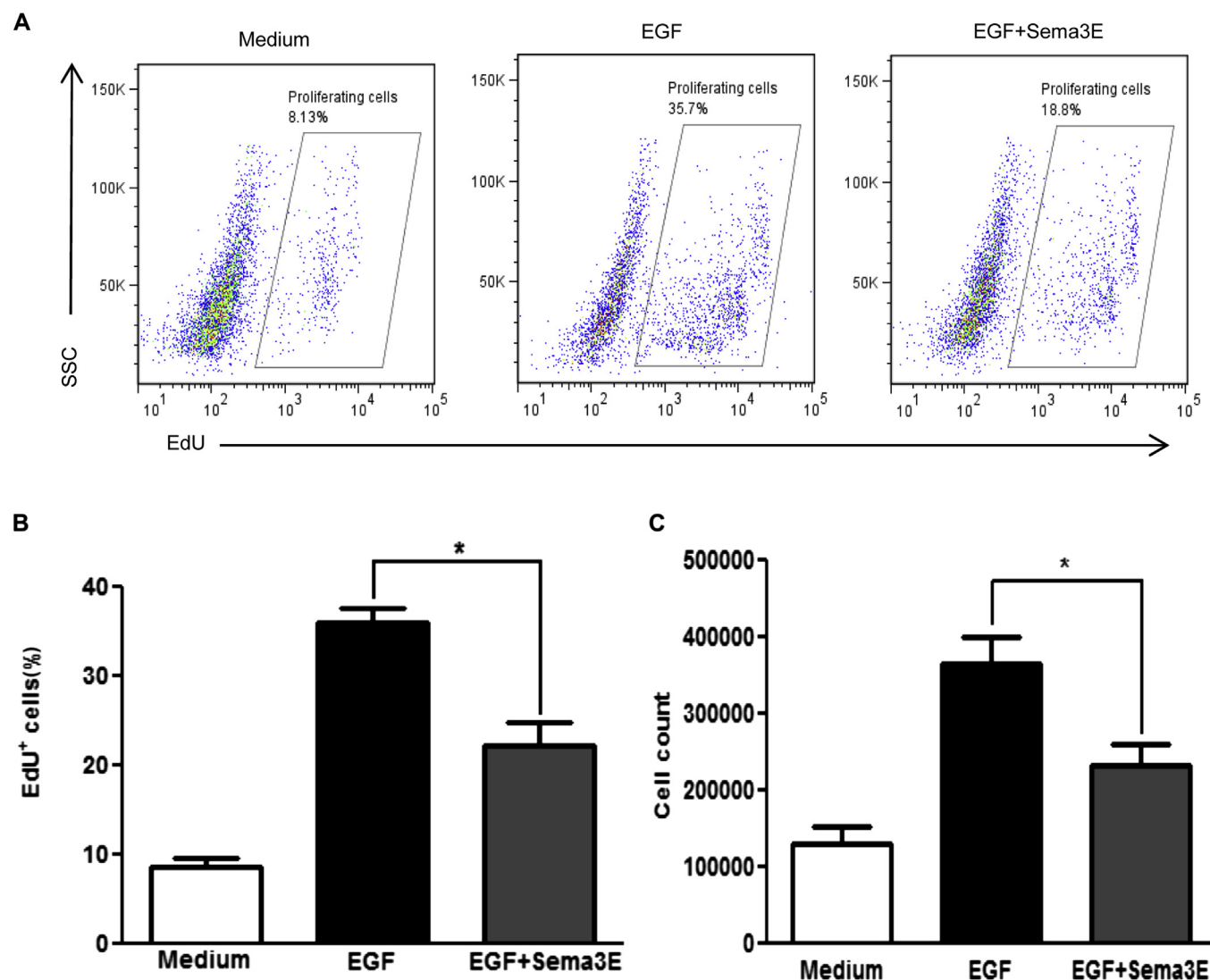


**FIG E2.** Effect of Sema3E on EGF-induced HASMC proliferation. HASMC proliferation in response to EGF (10 ng/mL) at the presence of Sema3E (100 ng/mL) was studied by EdU incorporation assay (**A** and **B**) and cell count (**C**) 2 and 4 days after stimulation, respectively. The graphs are based on 3 independent proliferation experiments. \* $P < .05$ .

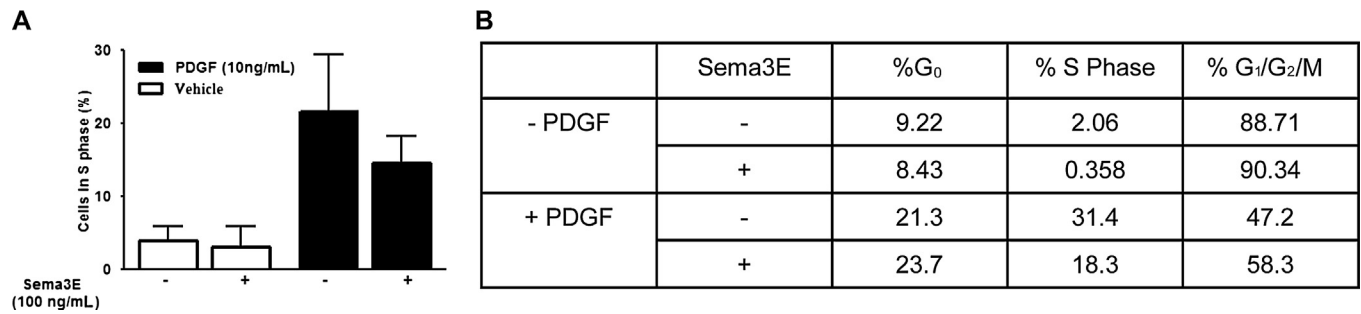


**FIG E3.** Analysis of HASMC cycle after Sema3E stimulation. Cell cycle distribution was assessed on Sema3E with or without PDGF treatment by propidium iodide staining on EdU incorporation assay. The graph (**A**) and table (**B**) are the results obtained from 3 independent experiments. SSC, Side scatter.

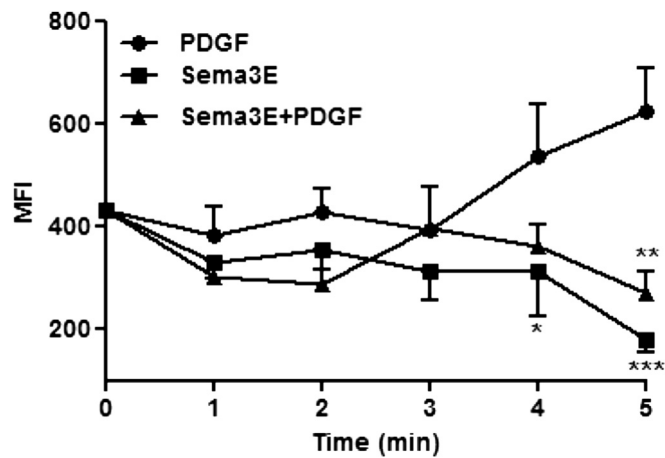


**FIG E4.** Quantification of F-actin depolymerization on Sema3E treatment. HASMCs were simulated with PDGF, Sema3E, and their combination for 0 to 5 minutes. Alexa Fluor 488-Phalloidin-labeled F-actin was quantified with flow cytometry at the indicated time points by measuring MFI. The graphs represent at least 3 different experiments. \*\* $P < .01$  and \*\*\* $P < .001$ .

**TABLE E1.** Primer sequence, amplicon length, and RT-PCR conditions

| Primer sequences                                                                   | Amplicon size (bp) | Annealing temperature (°C) | Cycles (no.) |
|------------------------------------------------------------------------------------|--------------------|----------------------------|--------------|
| PLXND1:<br>Fwd: 5'-TGGATGTCGCAGCTTACTTG-3'<br>Rev: 5'-CCCCAACCCACAGTTCTCTA-3'      | 156                | 58                         | 30           |
| GAPDH:<br>Fwd: 5'-AGCAATGCCTCCTGCACCACCAAC-3'<br>Rev: 5'-CCGGAGGGGCCATCCACAGTCT-3' | 137                | 60                         | 30           |

*Fwd*, Forward; *GAPDH*, glyceraldehyde phosphate dehydrogenase gene; *PLXND1*, plexin D1 gene; *Rev*, reverse.

**TABLE E2.** Clinical profile of asthmatic patients used for bronchial HASMC isolation

| Patient   | Sex  | Age (y) | FEV <sub>1</sub> (%) | Medication | MEF <sub>25</sub> | MEF <sub>50</sub> | MEF <sub>75</sub> |
|-----------|------|---------|----------------------|------------|-------------------|-------------------|-------------------|
| Subject 1 | Male | 30      | 81                   | Seretide   | 39                | 59                | 59                |
| Subject 2 | Male | 42      | 80                   | Symbicort  | 28                | 43                | 85                |
| Subject 3 | Male | 42      | 78                   | Symbicort  | 25                | 48                | 79                |

MEF, Maximal expiratory flow.