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Opposing Actions of Stat1 and Stat6 on IL-13-Induced Up-Regulation of Early Growth Response-1 and Platelet-Derived Growth Factor Ligands in Pulmonary Fibroblasts[1]

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IL-13 is a key cytokine involved in airway remodeling in asthma. We previously reported that IL-13 stimulated the mitogenesis of lung fibroblasts via platelet-derived growth factor (PDGF)-AA. In this report, we show that IL-13 increases PDGF-A and PDGF-C mRNA levels through a dual intracellular cascade that requires coactivation of Stat6 and Stat1 to impact transcriptional regulation of the early growth response (Egr)-1 gene, which then drives PDGF expression. Increased levels of PDGF-AA and PDGF-CC protein were observed in vivo in the airways of IL-13 transgenic mice. IL-13 up-regulated PDGF-A and PDGF-C mRNA levels in lung fibroblasts isolated from three different background strains of mice. However, IL-13-induced PDGF-A and PDGF-C mRNA levels were significantly reduced in Stat6-deficient ($Stat6^{-/-}$) fibroblasts as compared with wild-type $Stat6^{+/+}$ fibroblasts. In contrast, IL-13-induced PDGF-A and PDGF-C mRNAs were enhanced in $Stat1^{-/-}$ fibroblasts as compared with $Stat1^{+/+}$ fibroblasts. IL-13 did not up-regulate PDGF-A or PDGF-C mRNA levels in $Egr\text{-}1^{-/-}$ fibroblasts. Moreover, IL-13 did not increase Egr-1 mRNA and protein levels in $Stat6^{-/-}$ fibroblasts and yet enhanced Egr-1 mRNA and protein levels in $Stat1^{-/-}$ fibroblasts. Our findings support the hypothesis that Stat6 and Stat1 exert stimulatory and inhibitory effects on Egr-1 and PDGF ligand mRNA transcription, respectively. This novel mechanism could aid in identifying molecular targets for the treatment of chronic airway remodeling and fibrosis in asthma. *The Journal of Immunology,* **2006, 177: 4141–4148.**

Interleukin-13 is a Th2 cytokine that plays a prominent role in mediating airway remodeling in asthma and other inflammatory airway diseases (1). Subepithelial fibrosis is an important feature of chronic airway remodeling. The proliferation of airway mesenchymal cells, as well as subsequent deposition of extracellular matrix, characterizes airway fibrogenesis (2). IL-13 has been shown to stimulate the mitogenesis of lung fibroblasts isolated from human subjects with mild asthma (3). We previously reported that one mechanism by which IL-13 stimulates proliferation of lung fibroblasts is through the autocrine release of platelet-derived growth factor (PDGF)-AA[3] (4). PDGF-AA is a potent mitogen and chemoattractant of lung fibroblasts (5), and IL-13-induced proliferation of lung fibroblasts is dependent on Stat6 signaling (4). PDGF-CC is closely similar to PDGF-AA but requires proteolytic activation (5). Both PDGF-AA and PDGF-CC have been implicated in fibroproliferative lung diseases and both ligands bind to and activate the PDGF α receptor (PDGFRα) tyrosine kinase to stimulate mesenchymal cell proliferation and migration (4–6). The signaling mechanisms through which IL-13 up-regulates PDGF expression remain unclear.

The Stat family of transcription factors are activated by IL-13 and represent potentially important signaling conduits for mediating the diverse biological effects of IL-13. Upon ligand binding, receptor kinases activate latent cytoplasmic Stats through tyrosine phosphorylation (7). The Stat proteins then homo- or heterodimerize and translocate to the nucleus, where they bind to DNA and modulate gene expression. Stat family members bind with varying affinities to a canonical palindromic sequence ($TTCN_{2-4}GAA$) in the promoters of their target genes (8). Stats play prominent roles in both pro- and anti-inflammatory processes, including cell proliferation, apoptosis, and differentiation (7). IL-13 induces phosphorylation of Stat6 in lung fibroblasts (4), and many of the characteristics of airway remodeling (eosinophilia, mucous cell metaplasia, and airway fibrogenesis) are absent in a model of allergic asthma in Stat6-deficient mice (9). Thus, Stat6 is required for many aspects of IL-13-induced airway remodeling. Stat1 is also activated by IL-13 in a variety of lung cell types (10) and yet negatively regulates IL-13-induced signaling in pulmonary cell types (11–13). We recently demonstrated that Stat1-deficient ($Stat1^{-/-}$) mice exhibited more severe pulmonary fibrosis after bleomycin injury compared with wild-type $Stat1^{+/+}$ mice (14). Therefore, Stat6 and Stat1 are both important modulators of airway remodeling but appear to exert opposing biologic effects on cytokine signaling.

A link between Stat signaling and PDGF gene expression has not been established, but growing evidence suggests that the early

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[3] Abbreviations used in this paper: PDGF, platelet-derived growth factor; Egr-1, early growth response-1 gene; SOCS, suppressor of cytokine signaling.

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growth response-1 (Egr-1) gene is important in regulating transcription of PDGF-A. Egr-1 is an 80- to 82-kDa zinc-finger transcription factor that regulates cell proliferation, differentiation, and apoptosis in response to growth factors, cytokines, oxidative stress, and injury (15–17). Egr-1 binding to DNA induces expression of isoforms of the PDGF family, including PDGF-A and PDGF-C (18, 19). While Egr-1 mediates PDGF expression, PDGF isoforms also stimulate expression of Egr-1, thereby indicating the potential for a mitogenic autocrine loop involving PDGF, PDGFRs, and Egr-1 (17, 18). Furthermore, much evidence suggests that Egr-1 may be involved in the pathogenesis of airway remodeling. Increased Egr-1 expression has been observed in patients with emphysema (20) or chronic obstructive pulmonary disease (16). Egr-1-deficient (Egr-$1^{-/-}$) mice exhibit elevated levels of IgE and airways hyporesponsiveness in a model of allergic asthma (21). In addition, Egr-1-mediated apoptosis was reported as a central feature of TGF-β_1-induced pulmonary fibrosis and alveolar remodeling in mice in vivo (22). Moreover, Egr-1 expression is induced by inflammatory cytokines such as IL-1, TNF-α, and IFN-γ (19, 20, 23). Finally, Egr-1 modulates expression of growth factors, extracellular matrix proteins, and mediators of the leukotriene biosynthesis pathway (24). Therefore, Egr-1 has been well characterized as an important transcription factor for regulation of inflammatory events in the lung. However, it remains unknown whether Egr-1 plays a role in IL-13-induced PDGF gene expression.

In this study, we sought to characterize the transcriptional regulation of PDGF-A and PDGF-C in response to IL-13 in lung fibroblasts. We demonstrate that Stat6 and Egr-1 are required for IL-13-mediated PDGF-A and PDGF-C gene expression. In contrast, Stat1 suppresses IL-13-induced PDGF ligand and Egr-1 gene expression. In addition, we show that PDGF-A and PDGF-C are abundantly expressed in airway epithelium and mesenchymal cells of IL-13-overexpressing transgenic mice in vivo. This study identifies Stat6 and Stat1 as key regulatory molecules in mediating IL-13-induced PDGF ligand transcription and provides a novel mechanism for understanding airway fibrosis in asthma.

Materials and Methods

Reagents

Recombinant human IL-13, phospho-Stat6 Ab and Stat6 Ab were obtained from R&D Systems. The MEK inhibitor, PD98059, was obtained from Calbiochem. Egr-1, PDGF-AA, and PDGF-CC Abs were purchased from Santa Cruz Biotechnology. Phospho-ERK and ERK Abs were purchased from Cell Signaling Technology. Phospho-STAT1 and STAT1 Abs were purchased from Cell Signaling Solutions. Gene expression assays for murine Egr-1, PDGF-A, PDGF-C, and β-actin were purchased from Applied Biosystems.

Mice

Stat6$^{-/-}$ mice and BALB/cJ (Stat6$^{+/+}$) mice were purchased from The Jackson Laboratory. Egr-$1^{-/-}$ mice and C57BL/6 (Egr-$1^{+/+}$) mice, as well as Stat$1^{-/-}$ mice and 129S6 (Stat$1^{+/+}$) mice, were purchased from Taconic Farms. Pulmonary tissue sections were obtained from transgene-negative and transgene-positive mice in which IL-13 is expressed in the lung. These mice have been described previously (25). All studies with mice were reviewed and approved by the Chemical Industry Institute of Technology Centers for Health Research Institutional Animal Care and Use Committee in accordance with the guidelines of the National Institutes of Health for the humane use of animals.

Isolation of mouse lung fibroblasts

Mouse lung fibroblasts were isolated from the lungs of three adult male mice from each of the following strains (Stat6$^{-/-}$, Stat6$^{+/+}$, Stat$1^{-/-}$, Stat$1^{+/+}$, Egr-$1^{-/-}$, or Egr-$1^{+/+}$). Fibroblasts were obtained by enzymatic digestion of finely divided lung tissue obtained after vascular perfusion with PBS and bronchoalveolar lavage. Tissue fragments were digested in DMEM containing 1 mg/ml collagenase, 2.5 mg/ml trypsin, and 2 mg/ml DNase I (Sigma-Aldrich) at 37°C in 5% CO_2. Digestion was conducted for three cycles at 30 min each, with removal of medium and free cells after each cycle and the addition of fresh medium for each succeeding cycle. The medium removed at the completion of each cycle was mixed with an equal volume of DMEM containing 10% FBS. Pooled, liberated cells were filtered through sterile gauze, centrifuged 400 $\times$ *g* for 10 min at 4°C to pellet cells, and resuspended in 10% FBS-DMEM. The cells were plated at $\sim 7 \times 10^4/cm^2$, grown to confluence, then trypsin-liberated and cryopreserved at 1×10^6/ml in 10% DMSO-DMEM. Fibroblasts were used at passage 2 out of cryopreservation. This procedure has been reliably shown to result in >90% fibroblasts that immunostained positive for vimentin and collagen I but negative for leukocyte common Ag and cytokeratin (4).

Preparation of cell lysates

Cultures of fibroblasts were grown to confluence in 100-mm dishes and growth arrested in serum-free defined medium for 24 h. Cell lysates were collected by washing the cells once with PBS on ice, and 200 μl of lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.25% sodium deoxycholate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium vanadate, and 1 mM sodium fluoride) was added to the cells. The cells were scraped from the dish in the lysis buffer. The lysed cells were sonicated for 1 min and spun in a microfuge at maximum speed for 5 min to fractionate insoluble DNA and chromatin proteins from the soluble cellular proteins in the lysis.

Western blot analysis

Samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blocked for 1 h in 5% nonfat milk in TBS (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20). The blot was then incubated at 4°C overnight in a 1/1000 dilution of primary Ab, followed by incubation for 1.5 h in a 1/2000 dilution of HRP- or alkaline phosphatase-conjugated secondary Ab. The immunoblot signal was detected and visualized through ECL or chemifluorescence. The blot was then stripped of Ab and signal by incubating the membrane in stripping buffer (62.5 mM Tris (pH 6.7), 2% SDS, and 100 mM 2-ME) for 30 min at 50°C. The blot could then be reprobed using a different primary Ab in the same procedure described above.

Real-time quantitative RT-PCR

Total RNA from mouse lung fibroblasts was isolated using the Qiagen RNAeasy Miniprep kit. One or two micrograms of total RNA was reverse transcribed at 48°C for 30 min using Multiscribe Reverse Transcriptase (Applied Biosystems) in 1× reverse transcriptase buffer, 5.5 mM $MgCl_2$, 0.5 μM of each dNTP, 2.5 μM of random hexamers, and 0.4 U/μl RNase inhibitor in a volume of 100 μl. One hundred nanograms of the reverse transcriptase product was amplified using TaqMan Gene Expression Assays specific for Egr-1, PDGF-A, PDGF-C, and β-actin on the Applied Biosystems 7700 Prism Sequence Detection System (Applied Biosytems). The PCR conditions and data analysis were performed according to the manufacturer's protocol described in user bulletin no. 2, Applied Biosystems Prism 7700 Sequence Detection System. All samples were run in triplicate. Gene expression was measured by the quantitation of cDNA converted from mRNA corresponding to Egr-1, PDGF-A, or PDGF-C relative to the untreated 0-min control groups and normalized to β-actin. Relative quantitation values ($2^{-\Delta\Delta CT}$) were expressed as fold change.

Immunohistochemistry

Slides were deparaffinized through three changes of xylene each for 3 min, then rehydrated through descending grades of ethanol (100, 95, and 70%) to water. Ag recovery was performed using a solution of 0.001 M EDTA (pH 8.0). Slides were placed in Biotek staining containers in recovery solution in a Decloaking Chamber (Biocare Medical) containing 500 ml of distilled water and heated to 105°C for 10 min. After heating was completed, slides were removed from the chamber and allowed to cool for 15 min at room temperature. Endogenous peroxidases were blocked using 3% H_2O_2 for 5 min. Slides were rinsed in PBS and incubated with normal goat serum for 5 min. Primary Ab (Santa Cruz Biotechnology) PDGF-A (I-20) and PDGF-C (C-17) were diluted 1/100 and 1/125, respectively, for 40 min at room temperature. Slides were rinsed in PBS and incubated with biotinylated anti-rabbit IgG (Vector Laboratories) diluted 1/200 for 30 min. Slides were rinsed in PBS and incubated with streptavidin HRP (Zymed Laboratories) diluted 1/200 for 30 min. Slides were then rinsed in distilled water and reacted with 3-amino-9-ethylcarbazole chromogen (Zymed Laboratories) for 5 min, rinsed in tap water, and counterstained with Dako hematoxylin for 30 s, followed by a rinse in PBS. Slides were then rinsed in tap water and allowed to air-dry before coverslipping with an aqueous mounting medium.

Statistics

Data are expressed as mean ± SD as indicated. A two-way ANOVA was used to analyze differences between strains and treatment groups with posthoc comparisons by paired Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Overexpression of IL-13 in transgenic mice increases PDGF-AA and PDGF-CC in vivo

Targeted transgenic overexpression of IL-13 in the distal airways of mice results in airway remodeling and fibrosis (25). PDGF is a key mediator of fibrosis (5). To define the effects of IL-13 on PDGF ligand expression in vivo, sections of lung tissue isolated from CC10-IL-13 transgene-positive and transgene-negative littermate controls were analyzed by immunohistochemistry. PDGF-AA immunostaining was observed in type 2 pneumocytes of IL-13 transgene-negative mice, along with relatively weak immunostaining in the distal airway epithelium (Fig. 1*A*). PDGF-AA immunostaining was strongly expressed in the lungs of IL-13 transgene-positive mice and localized to the airway epithelium, airway smooth muscle, and interstitial cells (Fig. 1, *B–E*). PDGF-CC immunostaining was weakly expressed in the distal airway epithelium of transgene-negative mice (Fig. 1*F*). PDGF-CC immunostaining was strongly expressed in the lungs of IL-13 transgene-positive mice and localized to airway epithelium, type II pneumocytes, alveolar macrophages, and fibroblasts (Fig. 1*G*). These results clearly showed that PDGF-AA and PDGF-CC were up-regulated by IL-13 overexpression. Also, while some differences were observed in the spatial expression of PDGF-AA and PDGF-CC, both ligands were expressed in epithelial and mesenchymal cells.

Induction of PDGF-A and PDGF-C mRNA in lung fibroblasts by IL-13 requires Stat6

We hypothesized that Stat6 would play a role in IL-13-induced transcriptional regulation of PDGF-A and PDGF-C mRNA expression since we previously reported that IL-13-induced proliferation of mouse lung fibroblasts was Stat6 dependent (4). IL-13-induced PDGF-A and PDGF-C mRNA levels were significantly reduced in fibroblasts isolated from the lungs of $Stat6^{-/-}$ mice compared with fibroblasts isolated from the lungs of the wild-type $Stat6^{+/+}$ mice (Fig. 2, *A* and *B*). These data showed that stimulation of PDGF-A and PDGF-C expression in response to IL-13 required Stat6.

Stat1-signaling suppresses IL-13-stimulated PDGF expression

Stat1 has been implicated in the negative regulation of Th2 cytokine signaling (11–13). Therefore, we hypothesized that Stat1 may play a role in IL-13-induced PDGF expression. First, we observed that IL-13 caused tyrosine phosphorylation of Stat1 in $Stat6^{-/-}$ and $Stat6^{+/+}$ fibroblasts (Fig. 3, *A* and *C*). Next, we observed that Stat6 was tyrosine phosphorylated in $Stat1^{-/-}$ and $Stat1^{+/+}$ fibroblasts (Fig. 3, *B* and *D*). These data indicated that Stat1 and Stat6 phosphorylation in response to IL-13 were independent of one another. In contrast to Stat6 deletion, Stat1 deletion resulted in increased PDGF-A and PDGF-C mRNA levels (Fig. 4, *A* and *B*). These data indicate that Stat1 suppresses the IL-13-induced increase in PDGF-A and PDGF-C mRNA levels.

Egr-1 is required for IL-13-stimulated PDGF-A and PDGF-C expression

Egr-1 has been characterized as a transcriptional regulator of both PDGF-A and PDGF-C gene expression (18, 19). We sought to

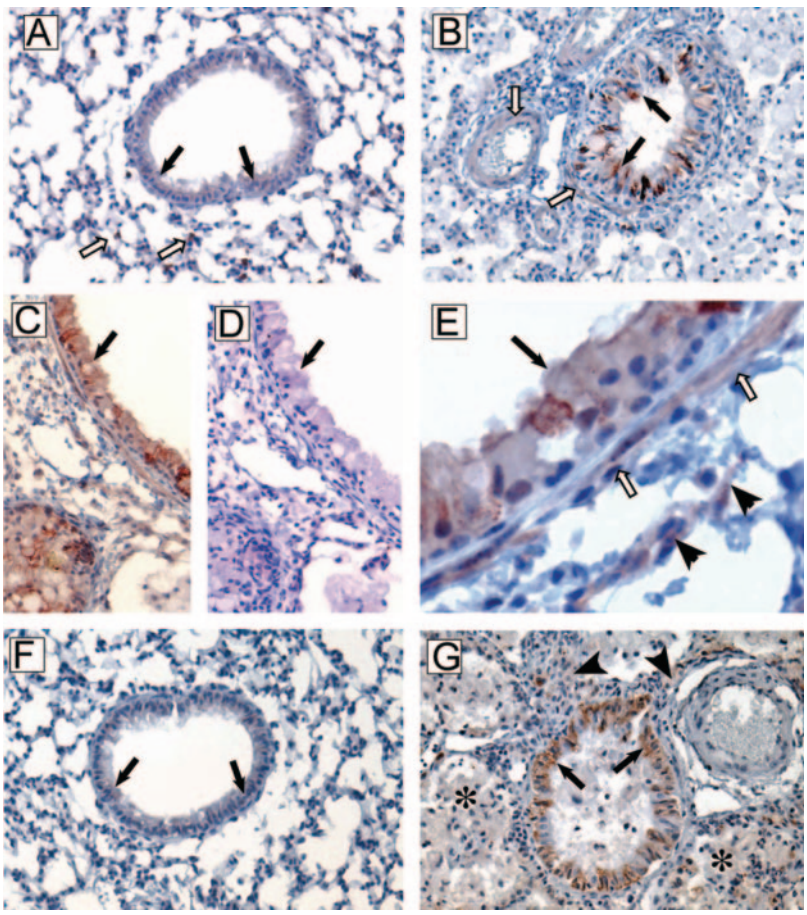

FIGURE 1. PDGF-AA and PDGF-CC protein expression in the lungs of IL-13 transgenic mice. *A*, PDGF-AA immunostaining in the lungs of transgene-negative mice (solid arrows denote airway epithelium; open arrows denote type 2 cells). Original magnification, ×40. *B*, PDGF-AA immunostaining in the lungs of IL-13 transgene-positive mice (solid arrows identify airway epithelium; open arrows identify airway and vascular smooth muscle). Original magnification, ×40. *C* and *D*, Comparison of PDGF-AA Ab (*C*) with nonspecific IgG control (*D*). *E*, PDGF-AA in airway epithelium (solid arrow), airway smooth muscle (open arrows), and interstitial cells (arrowheads) of IL-13 transgene-positive mice. Original magnification, ×160. *F*, Weak PDGF-CC immunostaining in transgene-negative mice (solid arrows identify airway epithelium). Original magnification, ×40. *G*, Strong expression of PDGF-CC in airway epithelium (solid arrows), interstitial cells (arrowheads), and alveolar macrophages (asterisks) of IL-13 transgene-positive mice. Original magnification, ×40.

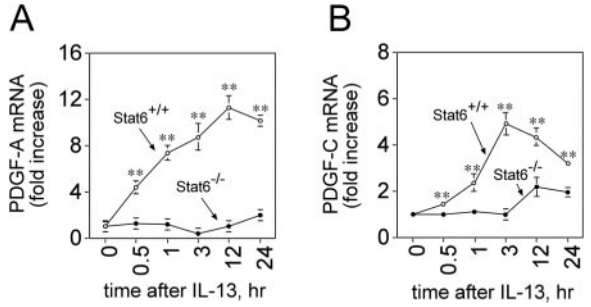

FIGURE 2. Requirement of Stat6 for IL-13-induced up-regulation of PDGF-A and PDGF-C mRNA. RT-PCR of (*A*) PDGF-A or (*B*) PDGF-C mRNA expression in lung fibroblasts isolated from Stat6$^{-/-}$ mice (●) or wild-type Stat6$^{+/+}$ mice (○). PDGF-A and PDGF-C mRNA levels were normalized to β-actin and expressed as fold change relative to untreated control at time 0. Data are presented as mean values ± SD from triplicate samples and are representative of three independent experiments with similar results. **, $p < 0.001$ compared with Stat6$^{-/-}$.

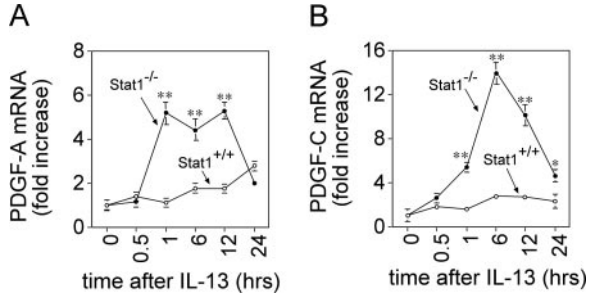

FIGURE 4. Suppression of IL-13-stimulated PDGF-A and PDGF-C mRNA expression by Stat1. RT-PCR of (*A*) PDGF-A or (*B*) PDGF-C mRNA expression in lung fibroblasts isolated from Stat1$^{-/-}$ mice (●) or wild-type Stat1$^{+/+}$ mice (○). PDGF-A and PDGF-C mRNA data were normalized to those of β-actin and expressed as fold change relative to untreated control at time 0. Data are presented as mean values ± SD from triplicate samples and are representative of three independent experiments with similar results. *, $p < 0.01$; **, $p < 0.001$ compared with Stat1$^{+/+}$.

determine whether Egr-1 played a role in IL-13-induced up-regulation of PDGF-A or PDGF-C. IL-13-stimulated PDGF-A and PDGF-C mRNA levels were markedly reduced in Egr-1$^{-/-}$ fibroblasts compared with Egr-1$^{+/+}$ fibroblasts (Fig. 5, *A* and *B*). These observations indicated that Egr-1 is required for induction of PDGF-A and PDGF-C by IL-13 in lung fibroblasts.

IL-13-induced Egr-1 expression requires Stat6

We next determined whether Stat6 was required for IL-13-induced Egr-1 mRNA and protein. IL-13 increased Egr-1 mRNA levels in a biphasic manner in Stat6$^{+/+}$ fibroblasts; early induction of Egr-1 mRNA was observed 30 min following exposure to IL-13 and declined after 3–6 h (Fig. 6*A*). Egr-1 protein was increased in Stat6$^{+/+}$ fibroblasts as compared with Stat6$^{-/-}$ fibroblasts from 30 min to 3 h after IL-13 treatment (Fig. 6, *B* and *C*). A late peak of Egr-1 mRNA occurred 12–24 h after IL-13 treatment but was not accompanied by an increase in Egr-1 protein. Egr-1 mRNA levels were nearly abolished in Stat6$^{-/-}$ fibroblasts compared with Stat6$^{+/+}$ fibroblasts (Fig. 6*A*), and Egr-1 protein levels were also reduced in Stat6$^{-/-}$ fibroblasts compared with Stat6$^{+/+}$ fibroblasts (Fig. 6, *B* and *C*).

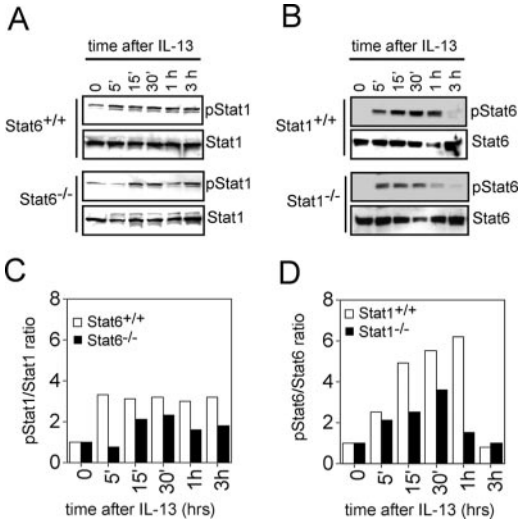

FIGURE 3. Phosphorylation of Stat6 and Stat1 by IL-13 in mouse lung fibroblasts with Stat6 or Stat1 deletions. *A*, Western blot analysis of IL-13-induced tyrosine phosphorylation of Stat1 (pStat1) in Stat6$^{-/-}$ or wild-type Stat6$^{+/+}$ fibroblasts. *B*, Western blot analysis of IL-13-induced tyrosine phosphorylation of Stat6 (pStat6) in Stat1$^{-/-}$ or wild-type Stat1$^{+/+}$ fibroblasts. *C* and *D*, Densitometric evaluation of the data in *A* and *B* showing the ratio of phosphorylated Stat1 or Stat6 normalized against total Stat1 or Stat6, respectively. The blots are representative of three independent experiments.

Stat1-signaling suppresses IL-13-stimulated Egr-1 expression

We next evaluated whether Stat1 signaling would suppress IL-13-stimulated Egr-1 expression in the same manner as observed for PDGF-A and PDGF-C. Stat1$^{-/-}$ fibroblasts produced an enhanced peak of IL-13-induced Egr-1 mRNA at 30 min as compared with Stat1$^{+/+}$ fibroblasts (Fig. 7*A*). Egr-1 protein levels were increased in Stat1$^{-/-}$ fibroblasts compared with Stat1$^{+/+}$ fibroblasts from 1 to 24 h after IL-13 treatment (Fig. 7, *B* and *C*).

ERK1/2 suppresses IL-13-stimulated Stat6 phosphorylation, Egr-1 protein levels, and PDGF ligand mRNA levels in lung fibroblasts

MAPKs, particularly ERK1 and ERK2, are central intracellular signaling intermediates in growth factor-induced cell proliferation and have been reported to potently stimulate Egr-1 expression (17, 19). We hypothesized that ERK1/2 could mediate the induction of Egr-1 and PDGF ligand expression in lung fibroblasts in response

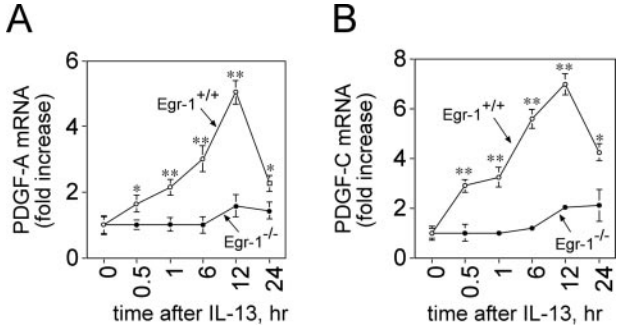

FIGURE 5. Requirement of Egr-1 for IL-13-induced up-regulation of PDGF-A and PDGF-C. RT-PCR of PDGF-A (*A*) or PDGF-C mRNA (*B*) expression in lung fibroblasts isolated from Egr-1$^{-/-}$ mice (●) or wild-type Egr-1$^{+/+}$ mice (○). The PDGF-A and -C mRNA data were normalized to those of β-actin and expressed as fold change relative to untreated control at time 0. Data are presented as mean values ± SD from triplicate samples and are representative of three independent experiments with similar results. *, $p < 0.01$; **, $p < 0.001$ compared with Egr-1$^{-/-}$.

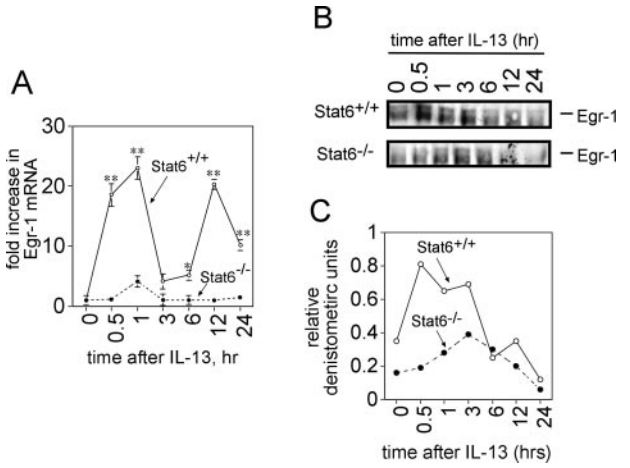

FIGURE 6. Requirement of Stat6 for IL-13-induced Egr-1 mRNA and protein expression. *A*, RT-PCR of Egr-1 mRNA in lung fibroblasts from Stat6$^{-/-}$ mice (●) or wild-type Stat6$^{+/+}$ mice (○) in response to IL-13. The Egr-1 mRNA data were normalized to those of β-actin and expressed as fold change relative to untreated control at time 0. Data are presented as mean values ± SD from triplicate samples and are representative of three independent experiments with similar results. **, $p < 0.01$; **, $p < 0.001$ compared with Stat6$^{-/-}$. *B*, Western blot analysis of IL-13-induced Egr-1 protein in Stat6$^{-/-}$ or wild-type Stat6$^{+/+}$ fibroblast cell lysates. *C*, Densitometry of IL-13 induced Egr-1 protein levels shown in *B*.

to IL-13. To test our hypothesis, mouse lung fibroblasts were pretreated with a MEK inhibitor (PD98059) for 90 min before a time course exposure to IL-13. Western blot analysis demonstrated that PD98059 blocked IL-13-induced phosphorylation of ERK1/2 (Fig. 8*A*) yet enhanced the phosphorylation of Stat6 and increased protein expression of Egr-1 in a time-dependent manner after IL-13 treatment (Fig. 8, *B* and *C*). PD98059 treatment had no effect on IL-13-stimulated Stat1 phosphorylation (data not shown). PD98059 pretreatment enhanced IL-13-induced up-regulation of

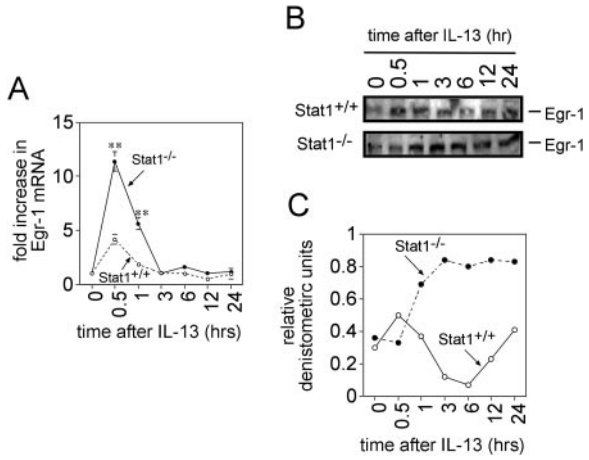

FIGURE 7. Enhanced IL-13-induced Egr-1 mRNA and protein expression in Stat1$^{-/-}$ lung fibroblasts. *A*, RT-PCR of IL-13-induced Egr-1 mRNA levels in lung fibroblasts isolated from Stat1$^{-/-}$ mice (●) or wild-type Stat1$^{+/+}$ mice (○). The Egr-1 mRNA data were normalized to those of β-actin and expressed as fold change relative to untreated control at time 0. Data are presented as mean values ± SD from triplicate samples and are representative of three independent experiments with similar results. **, $p < 0.01$; **, $p < 0.001$ compared with Stat1$^{+/+}$. *B*, Western blot analysis of IL-13-induced Egr-1 protein in Stat1$^{-/-}$ or wild-type Stat1$^{+/+}$ fibroblast cell lysates. *C*, Densitometry of IL-13 induced Egr-1 protein levels shown in *B*.

PDGF-A and PDGF-C mRNA levels (Fig. 9). Finally, we observed that ERK was phosphorylated in response to IL-13 treatment in Stat6$^{-/-}$, Stat1$^{-/-}$, or Egr-1$^{-/-}$ fibroblasts (data not shown). These data indicate that ERK1/2 is activated by IL-13 independently of Stats and Egr-1 and serves to suppress phosphorylation of Stat6 and downstream Egr-1 and PDGF ligand expression.

Discussion

In this study, we report that PDGF-AA and PDGF-CC are increased in the airways of IL-13 transgenic mice. Moreover, our in vitro experiments with fibroblasts isolated from the lungs of Stat6 and Stat1 null mice indicate that IL-13 induces coordinate phosphorylation of Stat6 and Stat1 to regulate opposing effects on PDGF transcription. Although Stat6 appears to be an important factor for mediating increased IL-13-induced Egr-1 and subsequent PDGF production, Stat1 appears to suppress Egr-1 and PDGF production. Furthermore, Egr-1 was identified as a key link between IL-13-induced Stat signaling and the transcription of PDGF-A and PDGF-C mRNAs. Our postulated mechanism of IL-13-induced PDGF production is illustrated in Fig. 10.

We observed that transgenic overexpression of IL-13 in the distal airways of the lung stimulated PDGF-AA and PDGF-CC production in a variety of cell types, including airway epithelial cells, fibroblasts, smooth muscle cells, and macrophages. For several reasons, we chose to use lung fibroblasts to dissect the intracellular events leading to PDGF-AA and PDGF-CC production. First, while expression of PDGF-AA and PDGF-CC was observed in the airway epithelium, the isolation of relatively pure cultures of mouse airway epithelial cells is problematic, especially when dealing with limited numbers of Stat-1$^{-/-}$, Stat6$^{-/-}$, or Egr-1$^{-/-}$ mice. Second, our previous work demonstrated that IL-13 stimulates the mitogenesis of lung fibroblasts through autocrine production of PDGF-AA (4). Finally, fibroblasts play a central role in fibrogenesis and serve not only as the principal source of secreted collagen but also as an important source of profibrogenic growth factors, including PDGFs.

A central finding of this study is that Stat6 and Stat1 mediate stimulatory and suppressive effects on IL-13-induced PDGF mRNA levels, respectively (Figs. 2 and 4). Several studies have reported that Stat factors mediate opposing effects on cell proliferation. For example, Stat3 and Stat5 activated by growth stimulatory cytokines promote cell proliferation and transformation through antiapoptotic pathways while Stat1 mediates growth arrest and apoptosis (26, 27). Often, Stat factors with opposing action can be activated by the same cytokine or growth factor. Simultaneous activation of Stat1 and Stat3 in the Me180 tumor cell line resulted in resistance to IFN-γ-induced apoptosis (28). Yu et al. (12) reported that IL-4 stimulates activation of both the Stat1 and Stat6 pathways in Th2 cells and that IL-4-induced Stat6-mediated cell proliferation and differentiation are negatively regulated by Stat1 activation. These studies support our observations of dual activation of Stat1 and Stat6 by IL-13 in pulmonary fibroblasts and the opposing actions of Stat factors on growth factor expression.

Our investigation revealed a key role for Egr-1 in mediating IL-13-stimulated transcription of PDGF-A and PDGF-C. Egr-1 has been reported as a transcriptional regulator of PDGF-A and PDGF-C (4, 19). A recent report by Cho et al. (29) identified a pivotal role for Egr-1 in IL-13-mediated airway fibrosis. They showed that IL-13 transgenic mice crossed with Egr-1 null mice had reduced airway fibrosis due to IL-13 overexpression in distal airways. We showed that IL-13-induced PDGF-A and PDGF-C mRNA expression was nearly abolished in Egr-1-deficient mouse lung fibroblasts in vitro compared with wild-type lung fibroblasts (Fig. 5). Moreover, our data indicate that IL-13-stimulated Egr-1

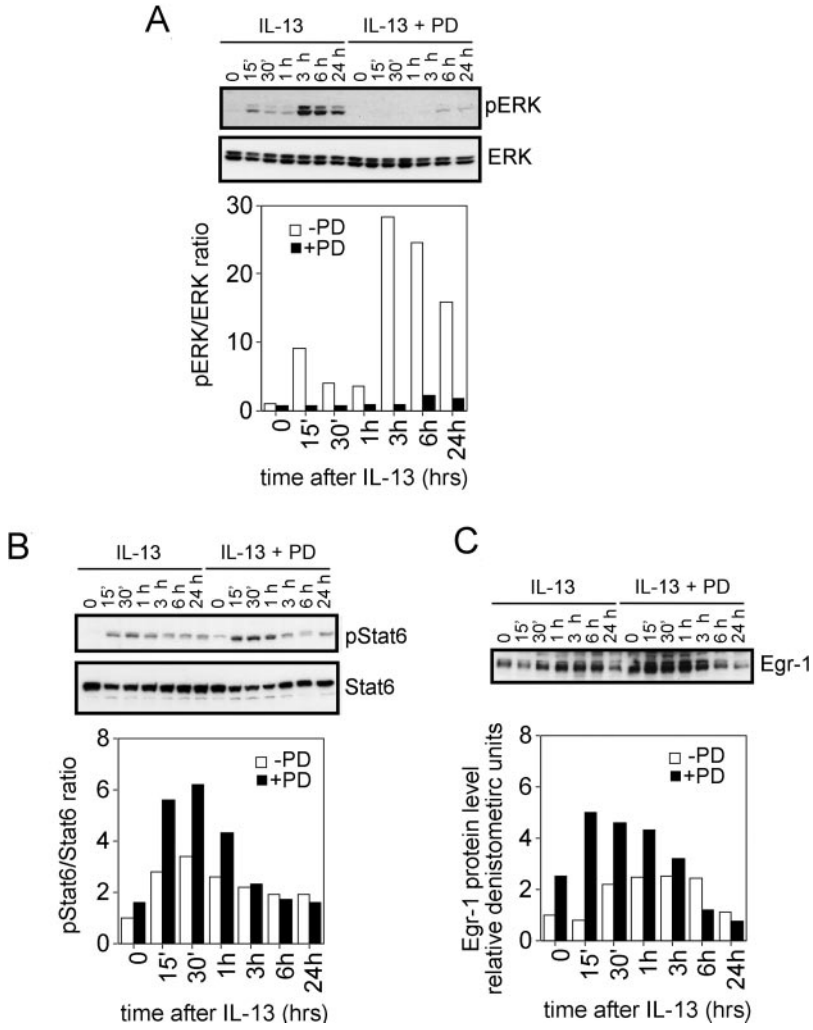

FIGURE 8. Inhibition of the ERK1/2 pathway enhances IL-13-induced Stat6 phosphorylation and increases levels of Egr-1 protein in lung fibroblasts from wild-type C57BL6 mice. *A*, Western blot analysis of IL-13-induced phosphorylation of ERK1/2 in the absence or presence of 10 μM PD98059 (PD) pretreatment for 90 min and densitometric evaluation of the data showing a signal for phosphorylated ERK (pERK) normalized for total ERK protein (ERK). *B*, IL-13 induced phosphorylation of Stat6 with or without PD98059 pretreatment, and densitometric evaluation showing phosphorylated Stat6 (pStat6) normalized for total Stat6 protein (Stat6). *C*, IL-13 up-regulation of Egr-1 protein levels in the presence or absence of PD98059 pretreatment and densitometry of protein levels.

expression is dependent upon Stat6 signaling because the IL-13-induced increase in Egr-1 mRNA and protein were nearly absent in Stat6-deficient lung fibroblasts (Fig. 6). These findings are in agreement with those of Cho et al. (29), who showed that Egr-1 was not expressed in IL-13 transgenic mice that contained a Stat6 mutant locus. Additionally, these investigators showed that IL-13 induction of Egr-1 was not significantly altered in IL-13 transgenic mice in which dominant-negative MEK-1 was also expressed, indicating that IL-13-stimulated Egr-1 expression was ERK independent. Our data also indicate that IL-13-induced Egr-1 is ERK independent, yet ERK activation serves to suppress IL-13-stimulated Egr-1, Stat6, and PDGF production (Figs. 8 and 9). In contrast to our findings with Stat6-deficient lung fibroblasts, we found that Stat1-deficient lung fibroblasts had enhanced IL-13-induced Egr-1 mRNA and protein (Fig. 7).

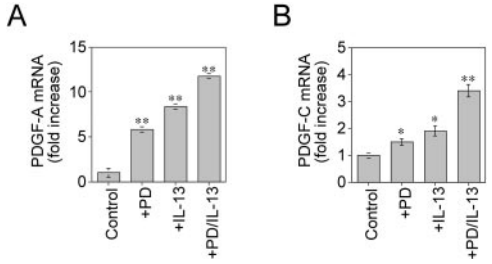

FIGURE 9. Inhibition of the ERK1/2 pathway enhances IL-13-induced up-regulation of PDGF mRNA in lung fibroblasts from wild-type C57BL/6 mice. RT-PCR of PDGF-A mRNA levels (*A*) and PDGF-C mRNA levels (*B*) 6 h after IL-13 treatment with our without 90-min pretreatment with PD98059 (PD). The PDGF-A and -C mRNA data were normalized to those of β-actin and expressed as fold change relative to untreated control. Data are presented as mean values ± SD from triplicate samples and are representative of three independent experiments with similar results. **, $p < 0.01$; **, $p < 0.001$ compared with untreated control.

Computational analysis of the Egr-1 promoter using Transcription Element Search Software from online (⟨www.cbil.upenn.edu/tess⟩) identified a putative Stat factor-binding element. This core palindromic sequence matches the consensus sequence for DNA binding used by all of the Stat transcription factors, including Stat6 and Stat1. Recently, Yamada et al. (30) found that both Stat1 and Stat6 bind to the same Stat factor binding element in the promoter of the IL-4- and IL-13-responsive *Ugrp2* gene and that activation of Stat1 decreased the binding of Stat6 to the site. One mechanism to explain our findings of the opposing effects of Stat1 and Stat6 on IL-13-induced PDGF-A and PDGF-C is that there exists a competition between Stat1 and Stat6 for binding to the Stat factor-binding element in the Egr-1 promoter. Such a mechanism was described by Ohmori and Hamilton (31) as a means of IL-4-stimulated suppression of IFN-γ-induced IRF-1 gene expression. Future studies of Stat factor binding to the Egr-1 promoter should address the mechanism of transcriptional regulation of Egr-1 in response to IL-13 in pulmonary fibroblasts.

We found that IL-13-stimulated Egr-1 mRNA expression was biphasic, an observation that was consistent over several repeated

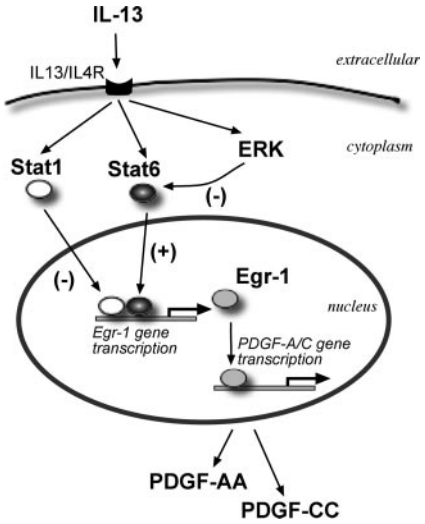

FIGURE 10. Postulated mechanism of transcriptional regulation of PDGF ligand production by IL-13 in lung fibroblasts. IL-13 binds to receptors on the lung fibroblast cell surface, which activate Stat6 and Stat1. Activation of the ERK1/2 pathway by IL-13 suppresses Stat6 signaling. The Stat transcription factors modulate Egr-1 expression that serves to regulate transcription of PDGF-A and PDGF-C and subsequent production of functional PDGF-AA and PDGF-CC dimeric proteins.

experiments. Egr-1 has been characterized as an immediate and early expressed gene, and we observed early expression of IL-13-induced Egr-1 mRNA (within 30 min) in wild-type Stat6$^{+/+}$ fibroblasts (Fig. 6*A*). However, a late transient peak of Egr-1 was also observed at 12 h following IL-13 treatment. We postulate that the early peak of Egr-1 expression is driven directly by IL-13, whereas the late peak is due to autocrine PDGF. Despite a late peak of Egr-1 mRNA at 12 h, we did not observe a concurrent late peak of Egr-1 protein. This could be due to coexpression of other mediators at 12 h that affect Egr-1 translation in a negative manner. Others have reported a biphasic time course of Egr-1 expression. For example, Yan et al. (15) reported early induction of Egr-1 in mouse lung within 0.5 h following hypoxia, but Egr-1 expression dissipated by 6 h followed by another peak of Egr-1 expression at 24–48 h.

Several studies have shown that ERK regulates Egr-1-mediated PDGF-A and PDGF-C expression (19, 32, 33). We found that inhibition of the ERK signaling pathway with the MEK inhibitor PD98059 did not reduce IL-13-stimulated Egr-1 production but instead increased Stat6 phosphorylation and resulted in increased Egr-1 protein expression (Fig. 8). Additionally, IL-13-stimulated PDGF-A and PDGF-C mRNA expression were enhanced with addition of the MEK inhibitor, PD98059 (Fig. 9). These data indicate that ERK1/2 activation negatively regulates IL-13-initiated Stat6 signaling, Egr-1 production, and PDGF gene expression in cultured lung fibroblasts. Since PDGF is a well-known activator ERK, our data suggest that ERK could function in a feedback loop to down-regulate PDGF production initiated by IL-13. Indeed, we observed biphasic phosphorylation pattern of ERK following IL-13 treatment (Fig. 8*A*), where the delayed peak of ERK activation corresponded temporally to PDGF production (Figs. 2, 4, and 5).

ERK1/2 activation by IL-13 or PDGF ligands leads to up-regulation of suppressor of cytokine signaling (SOCS) proteins. These proteins serve as negative regulators of JAK/STAT signaling responses to cytokines, and specifically, SOCS-1 was reported to inhibit Stat6 activation (12). Moreover, both IL-13 and PDGF-AA were recently shown to induce SOCS-1 expression (34–36). Therefore, it is possible that SOCS-1 could serve to mediate the suppressive effect of ERK in IL-13-induced PDGF production.

Lee et al. (37) recently reported that the systemic administration of the MEK inhibitor PD98059 in vivo or use of transgenic mice in which a dominant-negative MEK1 construct was expressed inhibited IL-13-induced inflammation and alveolar remodeling. They concluded that ERK is activated by IL-13 in the lung in a Stat6-independent manner where it contributes to IL-13-induced inflammation and optimal IL-13 stimulation of specific chemokines. Therefore, while our in vitro data in the present study indicates that ERK suppresses IL-13-induced PDGF production, ERK may also coordinately stimulate the expression of specific chemokines during IL-13-induced airway inflammation in mice.

While PDGF and its receptors are established mediators of pulmonary fibrosis (5), PDGF has been implicated only recently as having an important role in the pathogenesis of asthma. Aubert et al. (38) reported that PDGF-B and PDGF-Rβ expression did not correlate closely with the structural changes in diseased airways from patients with asthma. However, a later study by Lewis et al. (39) indicated that airway fibroblasts isolated by endobronchial biopsy from severe asthmatics had increased levels of PDGF-Rβ. More recent evidence suggested that PDGF-AA and its receptor, PDGF-Rα, could be more important than PDGF-B and PDGF-Rβ in asthma. First, PDGF-AA is up-regulated by IL-13 in human lung fibroblasts (4). Second, the cell surface PDGF-Rα, but not PDGF-Rβ, is up-regulated by a variety of mediators implicated in asthma, including IL-1β, fibroblast growth factor-2, and LPS (40–42). Finally, a recent report by Leung et al. (43) showed that PDGF-AA is increased in the exhaled breath condensate from asthmatic children with severe airflow limitation. Collectively, these studies suggest that PDGF-AA could be a mediator of asthma pathogenesis. Although PDGF-CC is up-regulated in pulmonary fibrosis (6), to our knowledge there is no clinical data to implicate PDGF-CC in asthma and future studies should elucidate the role of this PDGF ligand.

In summary, we have shown for the first time that IL-13 activates Stat6 and Stat1 to exert opposing inductive and suppressive effects on Egr-1 expression, respectively. Egr-1 then drives the expression of two potent PDGF ligands that are secreted and bind the PDGFRα to promote lung fibroblast replication. Since lung fibroblast proliferation is a key feature that contributes to the progression of airway fibrosis, the signaling events reported in this study provide potential targets for treatment of chronic airway remodeling in asthma.

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Disclosures

The authors have no financial conflict of interest.

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