

Roles of epithelial cell-derived periostin in TGF- β activation, collagen production, and collagen gel elasticity in asthma

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Periostin is considered to be a matricellular protein with expression typically confined to cells of mesenchymal origin. Here, by using *in situ* hybridization, we show that periostin is specifically up-regulated in bronchial epithelial cells of asthmatic subjects, and *in vitro*, we show that periostin protein is basally secreted by airway epithelial cells in response to IL-13 to influence epithelial cell function, epithelial–mesenchymal interactions, and extracellular matrix organization. In primary human bronchial epithelial cells stimulated with periostin and epithelial cells overexpressing periostin, we reveal a function for periostin in stimulating the TGF- β signaling pathway in a mechanism involving matrix metalloproteinases 2 and 9. Furthermore, conditioned medium from the epithelial cells overexpressing periostin caused TGF- β -dependent secretion of type 1 collagen by airway fibroblasts. In addition, mixing recombinant periostin with type 1 collagen in solution caused a dramatic increase in the elastic modulus of the collagen gel, indicating that periostin alters collagen fibrillogenesis or cross-linking and leads to stiffening of the matrix. Epithelial cell-derived periostin in asthma has roles in TGF- β activation and collagen gel elasticity in asthma.

airway fibrosis | fibroblasts | epithelial to mesenchymal transition | MMP-2 | MMP-9

In high-density microarray studies of gene expression in the airway epithelium in asthmatic subjects and healthy controls, we previously found that periostin is among the most highly differentially expressed genes in asthma (1). Full-length periostin is a secreted 90-kDa disulfide-linked protein composed of a typical signal sequence followed by a cysteine-rich EMI domain (believed to be important in multimerization), a tandem repeat of four fascilin-like (FAS1) domains—an evolutionarily conserved domain considered important in adhesion—and a variable C-terminal region (2–5). Periostin is considered a matricellular protein with expression confined to cells in connective tissues, including the periodontal ligament, tendons, heart valves, myocardium, skin, and bone (6, 7). Despite lacking an RGD domain, periostin is thought to interact with multiple integrins, including α V β 3, α V β 5, and α 6 β 4, to initiate a variety of biologic effects including cell proliferation, cell migration, epithelial to mesenchymal transformation, modulation of the biomechanical properties of connective tissues, and regeneration of cardiac myocytes after injury (4, 8, 9). Periostin also binds type 1 collagen to promote fibrillogenesis (10), and periostin-null mice show aberrant type 1 collagen fibrillogenesis in skin and poor integrity of the periodontal ligament in response to mechanical stress (11, 12).

Subepithelial fibrosis is an important pathologic feature of asthma, involving increased deposition of collagens and other ECM proteins in the lamina reticularis of the basement membrane zone (13–15). It is most prominent in patients with eosinophilia and severe disease (16, 17). Subepithelial fibrosis is thought to change the mechanical properties of the airway wall, alter the folding behavior of the airway mucosa, heighten bronchial reactivity, or narrow the airway and reduce airflow (18, 19).

Multiple cytokines, growth factors, and adhesion molecules, including IL-13 and TGF- β , have been implicated in the pathophysiology of subepithelial fibrosis in asthma (20, 21). Although periostin has been considered a mediator of inflammation and fibrosis in allergic diseases, these studies have focused on periostin derived from fibroblasts (22, 23); to our knowledge, the expression of periostin by epithelial cells has not been considered in mechanisms of airway fibrosis in asthma.

The “epithelial mesenchymal trophic unit” (EMTU) is comprised of opposing layers of epithelial and mesenchymal cells separated by the basement membrane zone (24). Reciprocal signaling from epithelial to mesenchymal cells in the EMTU is considered important for normal airway homeostasis, and alterations in the EMTU may result in aberrant autocrine and paracrine responses that could promote subepithelial fibrosis in asthma (25, 26). Relatively few mediators of epithelial mesenchymal communication have been studied in airway disease models, but IL-1 β , secreted by epithelial cells undergoing squamous differentiation, can induce a fibrotic response in adjacent airway fibroblasts (27). In considering our data for fourfold up-regulation of periostin in epithelial cells from asthmatics and the distinctive pattern of fibrosis and of periostin immunolocalization in asthma (1, 22), we considered the possibility that periostin is secreted basally by epithelial cells to influence fibrotic events in the underlying matrix. Furthermore, because periostin can bind to matrix proteins, we also investigated the effects of periostin on the biomechanical properties of these proteins.

Results

Periostin Expression in Airway Epithelial Cells in Asthmatic Subjects Is Correlated with Extent of Subepithelial Fibrosis. We hypothesized that epithelial cell-derived periostin has a role in the mechanism of subepithelial fibrosis in asthma. To begin to examine this hypothesis, we determined the relationship between periostin gene expression in epithelial brushings (measured by real-time RT-PCR) and subepithelial fibrosis in biopsy specimens (measured by stereology) in asthmatic subjects. For this analysis we were able to use previously published periostin gene expression data and subepithelial fibrosis data from paired samples of epithelial brushings and bronchial biopsies from 38 steroid-naïve asthmatic subjects with mild to moderate disease (1, 28). In directly analyzing the relationship between these outcomes, we found a strong and positive correlation between periostin gene

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Conflict of interest statement: P.G.W. and J.V.F. have submitted a provisional patent application for a three-gene signature profile for a molecular phenotype of asthma; one of the three genes in the signature is periostin.

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expression in epithelial brushings and measures of subepithelial fibrosis in biopsy specimens from the same subjects ($r = 0.52$; $P = 0.0008$; Fig. 1A). This result supports our hypothesis that periostin secreted by activated epithelial cells plays a role in the pathophysiology of subepithelial fibrosis in asthma.

Airway Epithelial Cells Express Periostin and Secrete Periostin in a Basal Direction. In previous immunostaining studies of airway biopsies from asthmatic subjects, we and others have reported that periostin staining is subepithelial with a decreasing gradient of expression into the adjacent stromal tissue (1, 22). Surprisingly, despite strong gene expression for periostin in airway epithelial brushings, we have not seen periostin immunostaining in epithelial cells themselves. This discordance between epithelial expression for periostin at the transcript and protein levels led us to hypothesize that periostin protein is rapidly secreted by airway epithelial cells. To test this hypothesis, we decided to confirm periostin gene expression in airway epithelial cells using in situ hybridization and to determine protein secretion by epithelial cells using human bronchial epithelial (HBE) cells grown in an air/liquid interface (ALI) on transwell permeable inserts (model depicted in Fig. 1F). In situ hybridization studies for periostin mRNA in tissue sections of airway biopsy specimens from asthmatic subjects and healthy controls clearly show that periostin is expressed in bronchial epithelial cells and that there is a significant increase in periostin transcripts in the epithelium of asthmatic subjects (Fig. 1B–E and Fig. S1). Fewer periostin transcripts were detected in the subepithelial region of the airways and no obvious changes in signal were observed between

asthmatic subjects and healthy controls in this region. These data show that, although airway epithelial cells are not the exclusive source of periostin in the airway mucosa, they are a major source. We have found previously that periostin gene expression is markedly up-regulated in HBE cells stimulated with recombinant IL-13 (1). Here we confirm and extend these earlier findings and show that IL-13 stimulation of primary HBE cells for 1, 2, or 4 d of treatment causes increases in periostin gene and protein expression at all three time points (Fig. S1). We were unable to detect periostin protein in apical washes from these cells, but we could easily detect abundant periostin protein levels in the basal medium (Fig. 1H). For later experiments we continued to use 4-d treatments in primary HBE cultures to model chronic asthma and to allow us to study mechanisms of remodeling.

Stable Transfection of BEAS2B Cells with a Human Recombinant Periostin Expression Vector. To explore the function of periostin in airway epithelial cells, we established an epithelial cell model of periostin overexpression. Specifically, we stably transfected BEAS2B cells (a SV40 large-T antigen transformed, but non-tumorigenic, bronchial epithelial cell line) with a human recombinant periostin expression vector (B2BPn) or control vector alone (B2BCTL). Periostin gene expression was typically eight-fold higher in B2BPn cells than in B2BCTL and parental cells (Fig. S2A); Western blot analysis of basal medium from cells grown in ALI confirmed increased periostin protein (Fig. S2B).

Periostin Up-Regulates Type I Collagen Secretion in B2BPn Cells. Previous studies have shown that periostin can regulate collagen

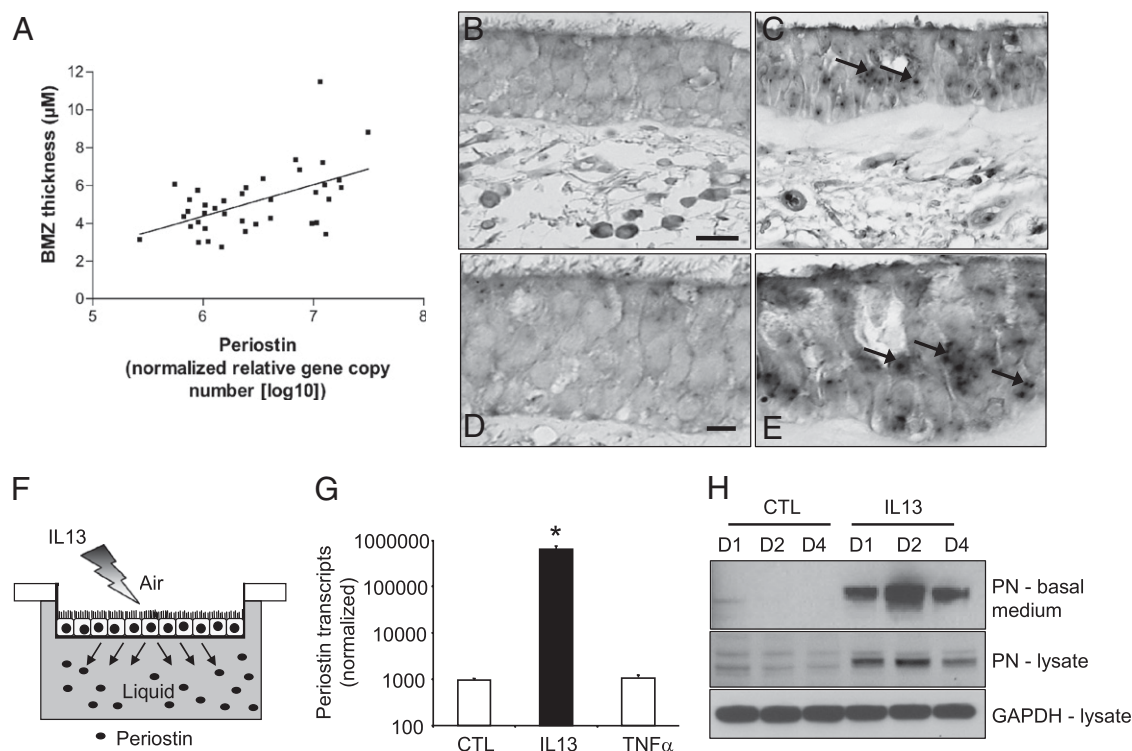


Fig. 1. IL-13 induces gene expression and basal secretion of periostin in primary HBE cells. (A) Periostin gene expression in epithelial brushings from asthmatic subjects correlates with the thickness of the basement membrane zone (BMZ) in the same subjects ($n = 38$; $r = 0.56$; $P = 0.0002$). (B–E) In situ hybridization analysis confirms periostin mRNA expression in epithelial cells in tissue sections taken from asthmatic and healthy subjects. Periostin staining is more intense in asthma epithelium (C; arrows) versus healthy controls (B) and the submucosa does not differ in staining. Higher-power images for healthy controls (D) and asthmatic subjects (E). (Scale bars: 20 μm in B and C; 7 μm in D and E.) (F) Diagram of ALI model used for the culture of primary bronchial epithelial cells. (G) Bar graphs show increased periostin gene expression in HBE cells after 4 d of treatment with IL-13 (10 ng/mL) compared with CTL (no treatment) or TNF- α (10 ng/mL); data are averaged from experiments in HBE cells from five different donors (* $P < 0.01$ vs. control). Error bars indicate SD. (H) Western blot analysis showing changes in periostin levels in basal medium [25 μL conditioned medium (CM)] and cell lysates (10 μg total protein) from HBE cells after 1, 2, and 4 d of treatment with IL-13 (10 ng/mL) compared with CTL (untreated cells). GAPDH was used as a loading control.

expression in osteoblasts and in fibroblasts from various tissues (5, 10, 29). We therefore examined if periostin could induce changes in collagen expression in airway epithelial cells. We initially found increased acid-soluble collagen levels in B2BPn cell lysates as measured by the Sircol assay (Fig. S2C) and then more specifically detected increased gene expression for type I collagen in B2BPn cells (Fig. S2D), and increases in type I collagen protein by Western blotting (Fig. S2E). The induction of type I collagen in airway epithelial cells by periostin led us to examine whether this effect of periostin occurs in the context of epithelial to mesenchymal transition (EMT). In the B2B cells overexpressing periostin, we examined several markers of EMT, including cell shape, E-cadherin, β -catenin, vimentin, and α -smooth muscle actin (α -SMA) expression. As can be seen in Fig. S3, the B2BPn cells show multiple markers of EMT. Light microscopy studies revealed that B2BPn cells displayed a striking change in cell shape, becoming more elongated and having reduced cell-cell contacts. Immunostaining, Western blotting, and TaqMan studies showed that B2BPn cells exhibited a loss in the epithelial markers E-cadherin and β -catenin and a gain in the mesenchymal markers vimentin and α -SMA (Fig. S3).

Type 1 Collagen Expression in B2BPn Cells is TGF- β -Dependent. TGF- β is implicated in mechanisms of collagen deposition in sub-epithelial fibrosis in asthma (30), and we therefore investigated the effects of periostin on the expression of TGF- β isoforms. We found that periostin overexpression in BEAS2B cells caused increased expression of all three TGF- β isoforms (Fig. 2 A–C). To establish that periostin promotes activation of TGF- β , we used the transformed mink lung cell (TMLC) reporter system (31) in co-culture with B2B, B2BCTL, or B2BPn cells separated by a permeable membrane. This system uses cells stably transfected with a TGF- β -responsive plasminogen activator inhibitor 1 promoter/luciferase construct and the B2BPn cells displayed increased luciferase reporter activity (Fig. 2D). To determine whether periostin-induced expression of type I collagen is a functional consequence of TGF- β signaling, we used SB431542 to inhibit TGF- β tyrosine kinase activity. We found a significant inhibition of type I collagen gene expression in B2BPn cells (Fig. 2E); these gene expression results were confirmed at the protein level using Western blots (Fig. 2F).

Primary HBE Cells Treated with Recombinant Periostin Display Increased TGF- β Bioactivity. To confirm the data from the B2BPn cells indicating that periostin can up-regulate TGF- β expression and increase collagen in epithelial cells, we repeated key experiments using primary HBE cells grown at an ALI and stimulated with recombinant human periostin. Five different donors of primary HBE cells grown in an ALI were treated with recombinant periostin (2 μ g/mL) for 4 d with or without SB431542. Treatment of

HBE cells with recombinant human periostin led to statistically significant increases in gene expression of TGF- β isoform 1 (Fig. S4A), but no significant changes in gene expression were observed in TGF- β isoforms 2 and 3 (Fig. S4B and C). Confirming results in the BEAS2B cells, we found that periostin treatment of HBE cells led to an increase in gene expression for type 1 collagen that was inhibited by SB431542 (Fig. 4D); these gene expression results were confirmed at the protein level using Western blots (Fig. S4E). We next examined if periostin induces EMT in primary HBE cells. For these experiments, we treated HBE cells from three donors with recombinant periostin (2 μ g/mL) for 4 d. Of the three donors examined, one exhibited a clear loss in E-cadherin expression with periostin stimulation whereas gains in vimentin were observed from two of three donors (Fig. S5). These results are consistent with other studies in primary airway epithelial cells that show that EMT responses to TGF- β activation in primary airway epithelial cells are more variable than the responses seen in BEAS2B cells (32). It is not surprising that highly differentiated HBE cells are more resistant than BEAS2B cells to EMT induction.

Role of Integrins and Metalloproteinases in Periostin-Induced TGF- β Activation. TGF- β can be activated by integrin up-regulation (α v β 6 and α v β 8) and also by metalloproteinases (MMPs), most often MMP-2 and MMP-9 (33, 34), and we investigated whether these mechanisms were relevant to periostin-induced TGF- β activation in airway epithelial cells. We examined α v β 6 and α v β 8 integrin expression levels in B2BPn cells. FACs analysis showed down-regulation of both α v β 6 and α v β 8 in these cells compared with B2BCTL cells (Fig. S6A). We also assessed the effect of blocking Abs to both α v β 6 and α v β 8 (20 μ g/mL for 48 h) on markers of EMT (vimentin and α -SMA) and collagen I as surrogate read-outs of TGF- β activation in B2BPn cells. The integrin blocking Abs had no significant effect on the markers examined (Fig. S6B). Taken together, these data suggest that α v β 6 and α v β 8 are not involved in periostin-driven TGF- β activation in these cells.

In examining MMP-2 and MMP-9 expression in B2B cells, we found a marked up-regulation in gene expression for MMP-2 and MMP-9 (Fig. 3A). To determine if MMP-2 and MMP-9 are involved in mechanisms of TGF- β up-regulation by periostin, we measured TGF- β 1 protein in conditioned media from B2BPn cells and controls. We found no TGF- β 1 in the conditioned media of the control cells but average concentrations of 200 pg/ml in the B2BPn cells (Fig. 3). These data confirm gene expression data and activity data for TGF- β in Fig. 2. Notably, we found that a potent cyclic peptide inhibitor of MMP-2 and MMP-9 (MMP-2/MMP-9 inhibitor III) reduced TGF- β 1 release from the B2BPn cells by approximately 50% (Fig. 3). Taken together, these data point to important roles for MMP-2 and MMP-9 in the mechanism of periostin-induced activation of TGF- β in airway epithelial cells.

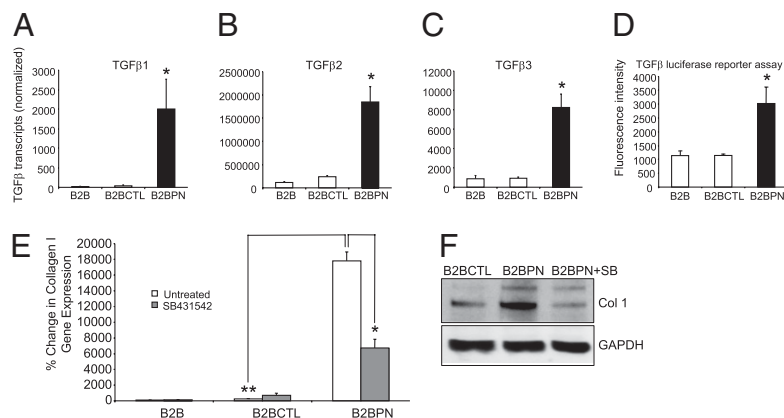


Fig. 2. Periostin up-regulates TGF- β signaling in BEAS2B cells. (A–C) B2BPn cells up-regulate gene expression of TGF- β isoforms 1, 2, and 3 compared with B2BCTL and untreated parental cells. Asterisk indicates a significant difference from controls for TGF- β isoforms 1 ($P < 0.048$), 2 ($P < 0.02$), and 3 ($P < 0.02$). (D) Increased luciferase reporter activity indicating higher levels of bioactive TGF- β in TMLC cocultured (24 h) with B2BPn cells compared with B2BCTL and parental cells ($*P < 0.001$ vs. control). (E) Collagen I gene expression in B2BPn cells is mediated by TGF- β . SB431542 (10 μ M) down-regulates periostin-mediated collagen I gene expression ($*P < 0.001$ vs. control; $**P < 0.001$ vs. periostin without treatment). y axis reflects percent change relative to untreated and nontransfected BEAS2B cells. Error bars indicate SD. (F) Western blots confirm that collagen I levels are reduced in lysates (10 μ g) of B2BPn cells treated with SB431542 (10 μ M) for 2 d. Data in A–E are averaged from three separate experiments performed under the same conditions. y axis reflects percent change relative to untreated and nontransfected BEAS2B cells. Error bars indicate SD.

B2BPn Cells Can Induce Collagen Gene Expression in Airway Fibroblasts via TGF- β Our discovery that periostin could induce a substantial increase in type 1 collagen expression in bronchial epithelial cells led us to examine whether it could stimulate type 1 collagen expression in airway fibroblasts. Fibroblasts are thought to be the principal sources of collagen in peribronchial fibrosis in asthma (20). Surprisingly, recombinant periostin (2 $\mu\text{g/mL}$ for 48 h, the same dose of recombinant periostin used to successfully to activate primary HBE cells) did not cause any significant change in gene expression for type 1 collagen in primary lung fibroblasts (Fig. 4A). However, conditioned medium from B2BPn cells was able to elicit a significant up-regulation of type 1 collagen gene expression in fibroblasts from these donors ($n = 5$) at a 48-h time point (Fig. 4B), an effect that was attenuated by inhibitors of TGF- β (Fig. 4B). Western blot and densitometric analysis confirmed these changes in type 1 collagen protein levels (Fig. 4 C and D).

Periostin Increases the Elastic and Viscous Moduli of Collagen Gels.

The high concentrations of periostin protein in the basal conditioned medium of IL-13 activated epithelial cells also led us to consider the possible biological effect of periostin in the sub-epithelial matrix. Periostin has been shown to bind to matrix proteins, but the effects of this binding on the biomechanical properties of matrix proteins have not been directly measured. Newly synthesized collagens form fibrils that are greatly strengthened by the formation of covalent cross-links between lysine residues of the constituent collagen molecules. We considered the possibility that periostin acts as a cross-linker of collagen, and used rheologic measures to test this possibility *in vitro*. Rheological measurements of viscosity and elasticity elucidate the microstructure of fluids (35, 36), and rheometers work by measuring the response of a fluid to an imposed force or an imposed deformation (Fig. 5A). A fluid's capacity to respond to deformation by flowing reflects its viscosity, whereas its capacity to resist deformation by storing energy and recoiling reflects its elasticity. Increased cross-linking of polymers will be reflected by an increase in the elastic response. A representative set of rheological data from analyses of collagen mixed

with periostin is shown in Fig. 5B. For collagen solutions (0.35 mL of 2.5 mg/mL solution), we found that the elastic modulus (G') predominated over the viscous modulus (G''). This rheological signature is characteristic of cross-linked collagen polymers in a gel; however, the very low values for both G' and G'' indicate a very lightly cross-linked gel. In type 1 collagen solutions mixed with periostin, the G' again predominated over G'' , but the values for G' and G'' were markedly higher, and the G' and G'' curves become parallel over a broader range of frequency, indicating transformation of the lightly cross-linked collagen gel into one that is more densely cross-linked (Fig. 5B). The data for G' and G'' at one frequency in the sweep is summarized for multiple experiments in Fig. 5C, which shows that type 1 collagen mixed with periostin markedly increases both the viscous and elastic moduli of the collagen gel, whereas control proteins (albumin and fibronectin) have no effect. We hypothesize that this effect of periostin is a result of periostin dimers forming physical cross-links between adjacent collagen fibrils. Indeed, when we performed immunoblotting of the recombinant periostin used in these experiments, we found that it forms dimers in solution (Fig. 5D). However, it is also possible that the effect of periostin is a result of enhanced collagen fibrillogenesis. Although we favor the cross-linking mechanism, our methods do not distinguish between cross-linking and fibrillogenesis as the mechanism of the marked effect of periostin on the elasticity of the collagen gel.

Discussion

Previous genome-wide profiling studies by our group identified periostin as the second most highly expressed gene in airway epithelial brushings from asthmatic subjects (1). In the work presented here, we describe experiments designed to reveal the consequences of periostin overexpression in airway epithelial cells. We demonstrate that activated airway epithelial cells secrete large quantities of periostin basally into the underlying matrix, where it has autocrine effects on epithelial cell function, including MMP-2- and MMP-9-mediated activation of TGF- β and up-regulation of collagen in the context of an overall EMT. We also show that periostin secreted by epithelial cells causes TGF- β -mediated activation of airway fibroblasts. Finally, we show that recombinant periostin mixed with type I collagen causes marked increases in the elasticity of the collagen gel. These data identify periostin as a previously unsuspected regulator of TGF- β and as a protein product of airway epithelial cells that can influence events in the underlying matrix, including fibroblast activation and the biomechanical properties of matrix proteins (Fig. S7).

Periostin is considered to be expressed primarily in mesenchymal tissues or in malignant epithelial cells. Although intense immunostaining for periostin has been observed in the subepithelial region in asthma (1, 22), airway epithelial cells themselves have not shown positive staining. In this study, we demonstrate that airway epithelial cells are a major source of periostin. We validate prior microarray and TaqMan quantitative PCR results with *in situ* hybridization studies that clearly show that periostin mRNA signal is up-regulated in the bronchial epithelial cells of asthmatic subjects. Furthermore, by using an *in vitro* model of the airway epithelium, we show that IL-13-activated epithelial cells secrete large quantities of periostin in a basal direction. Thus, the difficulty that we and others have had in detecting periostin in epithelial cells is most likely a result of rapid basal secretion of periostin from epithelial cells. Importantly, however, we unequivocally identify periostin as a protein that is synthesized and secreted by nonmalignant airway epithelial cells, and we show that its secretion is markedly up-regulated by IL-13, a key cytokine in allergic asthma.

Subepithelial fibrosis is a characteristic of airway remodeling in asthma and TGF- β is considered an important mediator of this pathology (30, 37–39). Despite its importance, the mechanisms of TGF- β up-regulation in the airway in asthma are incompletely understood. Here we provide evidence that periostin is an up-

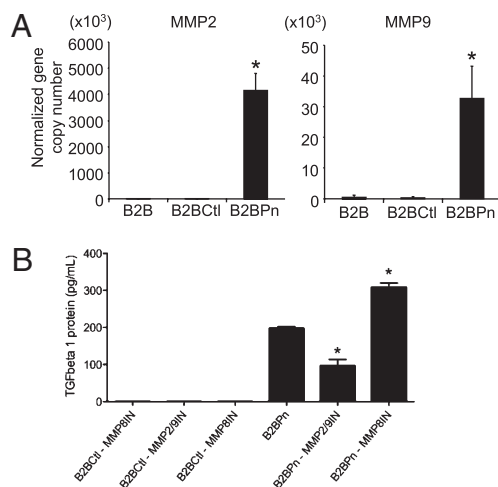


Fig. 3. MMP-2 and MMP-9 mediate periostin-induced TGF- β up-regulation in airway epithelial cells. (A) Bar graphs display increased MMP-2 and MMP-9 gene expression in B2BPn cells compared with B2BCTL and parental cells. Data are averaged from three separate experiments performed under the same conditions ($*P < 0.0001$ and $P < 0.006$ vs. control, respectively). (B) TGF- β 1 ELISA shows a marked increase in TGF- β 1 in conditioned media from B2BPn cells after 8 h of culture and significant inhibition by the MMP-2/9 inhibitor III (20 μ M); in contrast, the MMP-8 inhibitor I (8 nM) increased TGF- β 1 release under these conditions. Data are averaged from six separate experiments performed under the same conditions ($*P < 0.0001$ vs. B2BPn). Error bars indicate SD.

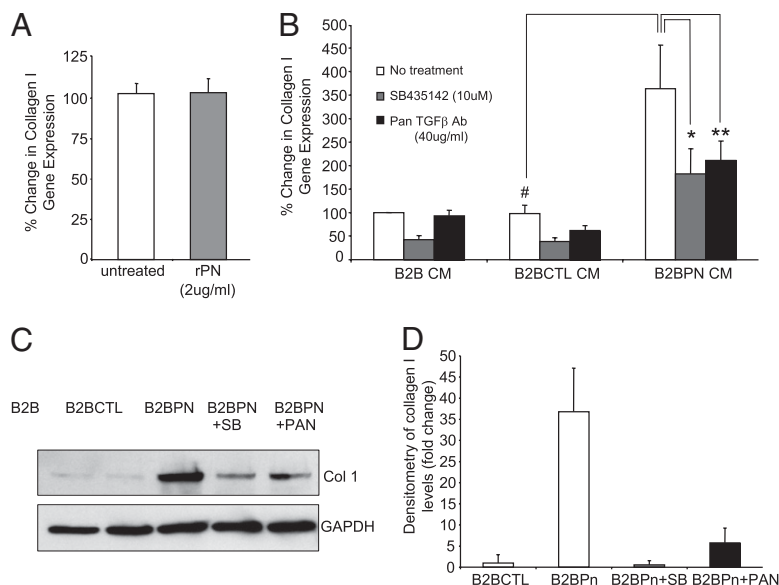


Fig. 4. TGF- β signaling mediates periostin-induced type I collagen production in airway fibroblasts cultured with conditioned medium (CM) from B2BPn cells. (A) Recombinant periostin treatment alone (2 μ g/mL for 48 h) does not stimulate collagen I gene expression in airway fibroblasts (data pooled from five different primary fibroblast donors). (B) Collagen I gene expression is up-regulated in airway fibroblasts (data pooled from five donors) after culture with CM from B2BPn cells compared with B2BCTL and parental cells (48 h). However, in the presence of anti-pan-TGF- β blocking Ab (1D11; 40 μ g/mL) or SB435142 (10 μ M) collagen gene expression is decreased. (* P < 0.003, ** P < 0.004, # P < 0.007). y axis reflects percent change relative to fibroblast cells treated with CM from B2B cells. (C) Typical Western blot confirming changes in collagen I levels in lysates (5 μ g) from airway fibroblasts after treatment with CM. (D) Bar graph depicting densitometric analysis of changes in collagen levels in fibroblast lysates after treatment with B2B, B2BCTL, or B2BPn with or without anti-pan-TGF- β Ab (40 μ g/mL) or SB435142 (10 μ M). Data are presented as fold change from B2BCTL alone. Data in A, B, and D are averaged from experiments using human airway fibroblasts from five different donors.

stream regulator of TGF- β activation. In both periostin-over-expressing epithelial cells and primary airway epithelial cells stimulated with recombinant periostin, we found indicators of TGF- β up-regulation, including changes in TGF- β gene expression, TGF- β 1 protein, and TGF- β bioactivity. The mechanism of periostin-induced activation of TGF- β did not depend on integrins α v β 6 and α v β 8, but we did find evidence for roles for MMP-2 and MMP-9, at least in the BEAS2B cells. Furthermore, although 48-h treatments with periostin in the same dose that activated epithelial cells did not up-regulate expression of type I collagen in primary human airway fibroblasts, we found that fibroblasts incubated for 48-h with conditioned medium from B2BPn cells did, and that this effect was decreased, at least in part, by inhibitors of TGF- β . Taken together, these data show that epithelial-cell-derived periostin up-regulates MMP-2 and MMP-9 in an auto-crine manner to activate TGF- β leading to up-regulation of collagen in airway epithelial cells and fibroblasts. However, the inability of inhibitors to TGF- β to fully block periostin-mediated

collagen I expression in these cells suggest that other molecules are also involved in this pathway.

By using rheological methods, we found that recombinant periostin markedly increases the elastic modulus of gels formed by type I collagen. The elastic modulus is a measure of polymer cross-linking and entanglement, and we show that the addition of periostin to type I collagen transforms it from a lightly cross-linked gel to a more densely cross-linked gel. The importance of collagen crosslinking in modulating tissue fibrosis in cancer has recently been highlighted (40), and although others have shown that periostin can bind to collagen and other matrix proteins (10, 22), we show here that periostin can cross-link collagen, as evidenced by an increase in elastic modulus. Indeed, these data support a role for periostin as an epithelial cell protein that can alter the biomechanical properties of the subepithelial matrix. Thus, in the airway, the secretion of periostin by epithelial cells in response to activation signals could allow epithelial cells to regulate the stiffness of the subepithelial matrix and the distensibility of the airway. This mechanism provides a way in which airway

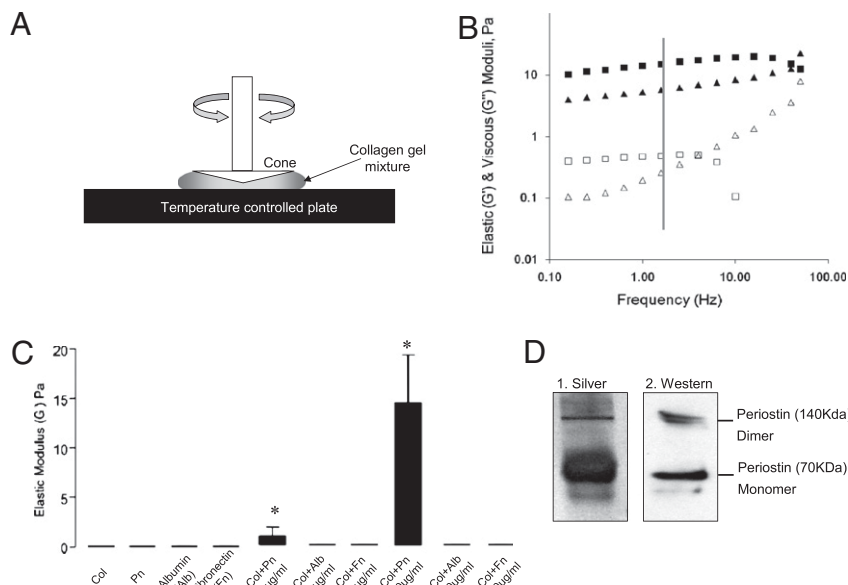


Fig. 5. Periostin alters the biomechanical properties of gels formed by type 1 collagen. (A) Measurement of viscoelastic properties of a gel using an AR2000 shear stress rheometer. (B) Frequency sweep for type I collagen alone (open triangles and squares) and for type I collagen mixed with periostin (closed triangles and squares). The vertical line indicates data at 1.0 Hz, which is used as a summary data point for C. (C) The addition of periostin increases the elastic modulus (G') of the collagen gel by as much as 100-fold. In contrast, the addition of albumin or fibronectin to type I collagen has no effects on the G' of the collagen gel (* P < 0.001 vs. all collagen albumin and fibronectin controls). (D) Recombinant periostin (Biovendor) is present in monomeric and dimeric form in solution. Lane 1 shows detection by silver staining, lane 2 shows detection by an antiperiostin rabbit polyclonal antibody.

epithelial cells—the first-line sensors of inhaled toxins—can regulate airway physiologic function to defend the airway. In diseases such as asthma, the persistence of epithelial cell activation because of persistent IL-13 signaling could lead to inappropriate and prolonged periostin secretion, which could account for chronic subepithelial fibrosis and chronic reductions in airway distensibility.

In summary, we identify periostin as an epithelial cell-derived protein that plays a pivotal role in the regulation of the EMTU. Periostin secreted by airway epithelial cells has autocrine effects, which include activation of TGF- β and up-regulation of type I collagen, and paracrine effects, which include TGF- β -mediated increases in type I collagen production in fibroblasts. In addition, periostin can increase the elastic modulus of gels formed by type I collagen, which could change the biomechanical properties of the airway. Persistent up-regulation of periostin in the airway epithelium in asthma is likely to contribute to mechanisms of increased airway fibrosis and decreased airway distensibility.

Methods

Reagents and Assays. Antibodies and immunoreagents are described in *SI Methods*, as are methods for immunoassays, collagen assays, and gene expression profiling. TGF- β protein was assayed using the TGF- β 1 DuoSet ELISA (R&D Systems)

Cell Culture. Primary HBE cells were cultured as described in *SI Methods*, as are the culture methods for BEAS2B cells and B2BPn cells, which was previously described (41). Normal primary human airway fibroblasts were obtained

from S. Nishimura (University of California, San Francisco, San Francisco, CA) (42). The TGF- β reporter cell line TMLC containing TGF- β -responsive plasminogen activator inhibitor 1 promoter/luciferase construct (gift from Daniel B. Rifkin, New York University, New York) was cultured as previously described (43).

In Situ Hybridization. A standard nonradioactive in situ hybridization protocol was used as described in *SI Methods*.

FACS. Details of FACS procedures are provided in *SI Methods*. Anti- α V β 6 (3G9) and α V β 8 (14E5) were a gift from Dean Sheppard (University of California, San Francisco, CA).

Rheology. Type 1 collagen in a 2.5 mg/mL solution was mixed with periostin or control proteins (albumin or fibronectin) in ratios of approximately 1:10,000 (20 μ g/mL) and approximately 1:1,000 (200 μ g/mL). Mixing was done for 4 h at 37 °C and rheological measurements were made with a sensitive cone-and-plate rheometer (AR2000; TA Instruments) as described in *SI Methods*.

Statistics. Details of statistics in the present study are provided in *SI Methods*.

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Supporting Information

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SI Methods

Antibodies and Immunoreagents. Antibodies used were rabbit anti-periostin (Biovendor), mouse anti-GAPDH (Millipore), mouse anti-E-cadherin, mouse anti- β -catenin (BD Biosciences), mouse antivimentin (Sigma), mouse anti- α smooth muscle actin (Thermo Scientific), rabbit anticollagen I, anti-pan-TGF- β (1D11), integrin antibodies to α v β 6 (3G9) and α v β 8 (37E1 and 14E5) obtained from S. Nishimura and D. Sheppard (University of California, San Francisco, CA). Secondary Abs were obtained from Jackson ImmunoResearch. Antimouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies were used for Western blots. Recombinant or purified human proteins used were periostin (Biovendor), IL-13, TNF- β (R&D Systems), collagen type I (BD Biosciences), albumin (Sigma), and fibronectin (Millipore). SB431542 hydrate, a chemical inhibitor of TGF- β type I receptor kinases was obtained from Sigma. MMP-2/9 inhibitor III and MMP-8 inhibitor I were obtained from EMD Chemicals.

Epithelial Cell Culture and Conditioned Medium Preparation. Primary HBE cells were obtained from Lonza or from S. Nishimura and W. Finkbeiner (University of California, San Francisco, San Francisco, CA). Passage 1 (P1) cells were grown to near-confluence in T25 flasks in modified Grey medium (no BSA added) (1) and then plated onto ALI wells (Costar). A minimum of five donors were used for each experiment. Cells were cultured until confluent (as indicated by their ability to resist fluid movement although the epithelial layer and as measured by changes in resistance (voltmeter; World Precision Instruments) and then used in experiments. Apical secretions from HBE cells were prepared by washing the surface of the wells with $2 \times 250 \mu\text{L}$ of PBS solution and concentrated approximately 10-fold using Amicon 30K MWCO columns (Millipore). Modified Gray medium (without bovine pituitary gland extract and BSA) was used for the collection of conditioned medium from HBE cells from the last 24 h of culture.

BEAS2B cells were originally obtained from American Type Culture Collection. Cells were grown in RPMI medium 1640 with 10% FBS. To determine the function of periostin in epithelial cells, we stably transfected BEAS2B cells with a human periostin expression vector (B2BPn) previously described (2) or control vector alone (pCDNA3.1; B2BCTL). Stably transfected cell populations were selected for, on the basis of neomycin resistance using Geneticin (Life Technologies). For Western blots on basal conditioned medium from BEAS2B cells, medium was prepared from cells grown in ALI in serum free conditions from 12 h of culture. All experiments were carried out at least in triplicate on three separate stably transfected populations.

Fibroblast Cultures. Normal primary human airway fibroblasts were obtained from S. Nishimura (University of California, San Francisco, CA) (3). Cells were cultured in DMEM H21 medium with 10% FBS and used from P1 to P4. Five primary fibroblast donors were used in experiments to model the epithelial-mesenchymal trophic unit. Conditioned medium from epithelial cells was prepared in serum free conditions for 24 h (as outlined earlier) and mixed with DMEM H21 plus 1% FCS at a 75%/25% ratio, respectively, and placed into wells containing fibroblasts with or without TGF- β pathway inhibitors SB431542 (10 μM) or 1D11 (40 $\mu\text{g/mL}$). Conditioned medium was replaced at time 0 and at 24 h and RNA and lysates were prepared from fibroblasts at 48 h.

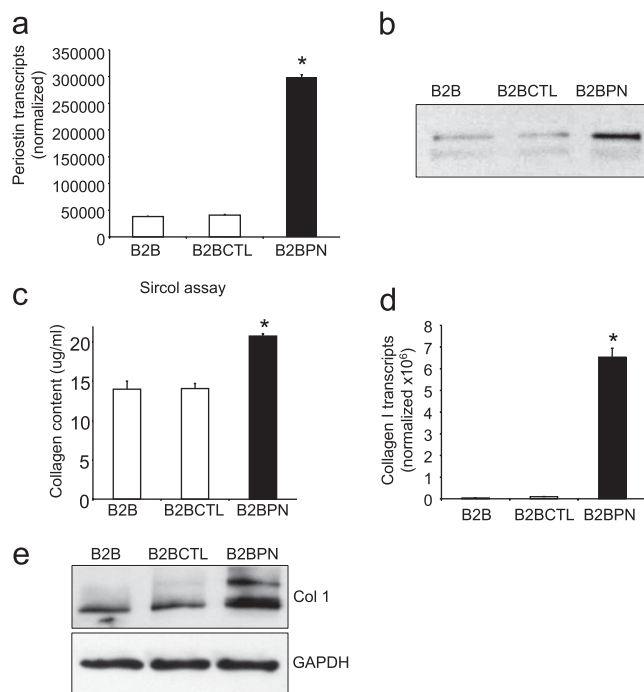
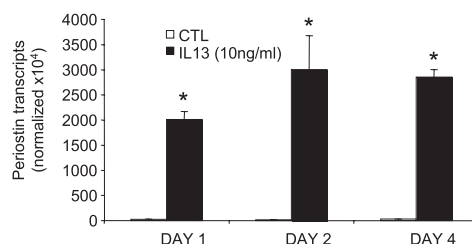
RNA Extraction and Quantitative RT-PCR. RNA was extracted using RNeasy minikit (Qiagen) as previously described (4). Briefly,

TaqMan primers and probes were designed using Primer Express (Applied Biosystems; Table S1). Messenger RNA detection for specific genes of interest was achieved using an adaptation of a two-step real time RT-PCR method described previously (1). Briefly, single-stranded cDNA generated from 2.5 ng of total RNA was subjected to 20 cycles of PCR using 5 pmol of each RTF/RTR primer listed in Table S1. A volume of this reaction equivalent to 2.5 fg of total RNA was used in a 5' nuclease assay using TaqMan primers and probes. A panel of four house-keeping genes (EEF1A1, RPL13A, PPIA, GAPDH) was quantified simultaneously and normalization factors were calculated using the two most stably expressed genes (5).

Collagen Assays. Total soluble collagen content in cell lysates was measured using Sircol soluble collagen assays as described in the manufacturer's protocol (Biocolor). Briefly, cells were prepared by washing with PBS solution, treated with 0.5 M acetic acid, freeze/thawed, and digested with pepsin overnight at 4 °C. Samples were centrifuged to remove insoluble debris then incubated with Sircol dye reagent for 30 min at RT on a shaker. Samples then spun to pack the insoluble collagen-dye complex and excess liquid decanted off. One milliliter of alkali reagent was then added and mixed to dissolve bound dye and samples were assayed at 540 nm on a SpectraMax Plus spectrophotometer and data analysis was carried out using Softmax Pro software (Molecular Devices). Collagen standards were used as controls.

TGF- β Bioassays. The TGF- β reporter cell line TMLC containing TGF- β -responsive plasminogen activator inhibitor 1 promoter/luciferase construct (gift from Daniel B. Rifkin) was cultured as previously described (6). TGF- β bioactivity was measured after coculturing TMLC with bronchial epithelial cells. Epithelial cells were plated in upper inserts of a transwell plate and grown to confluency, then transferred to transwell plates in which TMLC had been cultured on the lower surface. Cells were cocultured for 24 h in SFM. Cells were washed in ice-cold PBS solution, then lysates prepared using Promega lysis buffer. Lysates were frozen at -80 °C, thawed, then analyzed using Promega luciferase assay reagents. Luciferase reporter activity was measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

Western Blot Analyses and Silver Staining. Cell lysates were prepared by washing the cells in PBS solution before lysing in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) containing a mixture of Complete (proteinase inhibitors) and Phospho-STOP (Roche). For Western blot analyses, equal protein concentrations were loaded based on quantification with the BCA Protein Assay (Pierce) following the manufacturer's protocol. For basal-conditioned medium 25 μL of neat samples (taken from 1 mL total volume in a 12-well plate) were used for Western blotting. Samples were electrophoresed under reducing conditions (or nonreducing conditions for collagen I) in 10% TrisHCl Ready Gels (Bio-Rad) and transferred to Hybond nitrocellulose paper (Amersham). Nonspecific binding sites were blocked by washing blots in 5% nonfat milk (Nestle) in PBS containing 0.1% Tween-20 (Sigma) for 1 h with shaking. Blots were incubated with primary antibody overnight at 4 °C, washed in PBS solution with Tween-20, and incubated with HRP conjugated secondary antibody for 1 h at room temperature. Blots were washed again before visualization of immunocomplexes using ECL Western blotting kit (GE) and exposing to Hyperfilm (Amersham). GAPDH was used



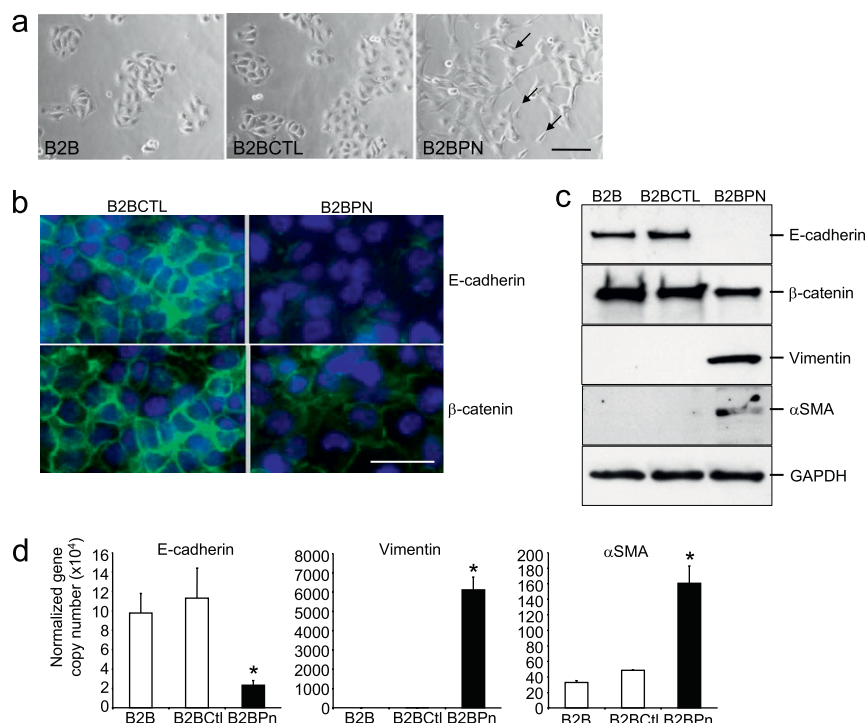


Fig. S3. B2BPn cells display an EMT phenotype. (A) Comparison of cell morphology of BEAS2B cells stably expressing periostin or vector control. Bright-field picture of cells 48 h after plating. Overexpression of periostin leads to changes in cell shape, with more elongated and spindle-shaped with increased lamellipodia formation (arrows). (Scale bar: 100 μm .) (B) Immunofluorescence staining depicting loss of E-cadherin and β -catenin in B2BPn cells compared with CTL-transfected cells. (Scale bar: 40 μm .) (C) Western blot analysis of various EMT markers in B2B, B2BCTL, and B2BPn cells lysates (10 μg). B2BPn cells show a loss of epithelial markers, E-cadherin, and β -catenin with a gain in mesenchymal markers vimentin and α -SMA. GAPDH was used as a loading control. (D) Gene expression analysis of E-cadherin, vimentin, and α -SMA levels in B2BPn compared with B2BCTL and parental cells ($P < 0.01$ vs. control). Error bars indicate SD.

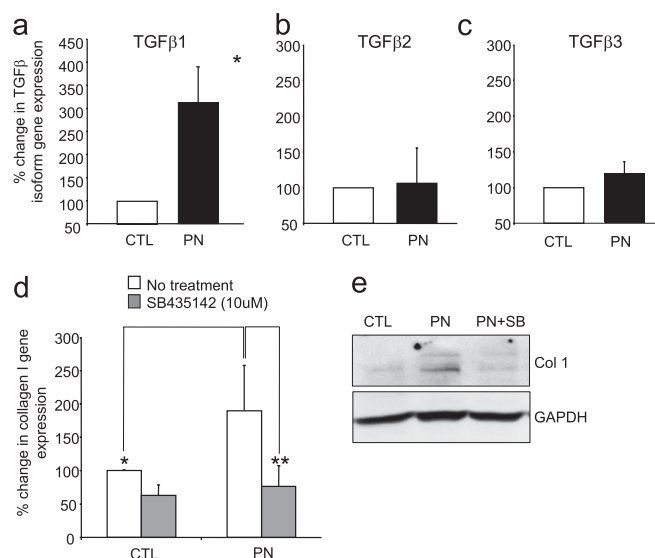


Fig. S4. Periostin up-regulates TGF- β signaling in primary HBE cells grown in an ALI. (A–C) Increased gene expression of TGF- β isoform 1, but not isoforms 2 and 3, in HBE cells treated with rPeriostin (2 μ g/mL for 4 d) * P < 0.027, data pooled from five different primary donors. (D) HBE cells treated with periostin (2 μ g/mL for 4 d) upregulate collagen I gene expression levels, but this effect is inhibited by the TGF- β pathway inhibitor SB431542 (10 μ M). y axis reflects percent change relative to untreated HBE cells. Data in A–D are averaged from experiments in HBE cells from five different donors (** P < 0.001 vs. periostin without treatment). (E) Typical Western blot of type I collagen levels in lysates from a HBE donor after periostin treatment (2 μ g/mL) with or without SB431542 (10 μ M).

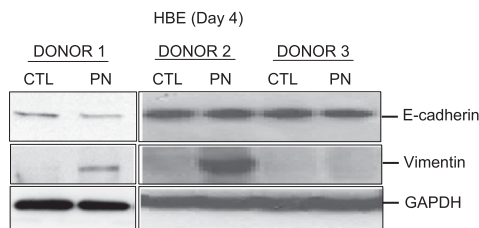


Fig. S5. HBE cells treated with recombinant periostin display some mesenchymal characteristics. Western blots of HBE lysates (10 μ g) from three different donors show changes in E-cadherin and vimentin expression levels after treatment with rPN (2 μ g/mL) for 4 d. GAPDH was used as a loading control.

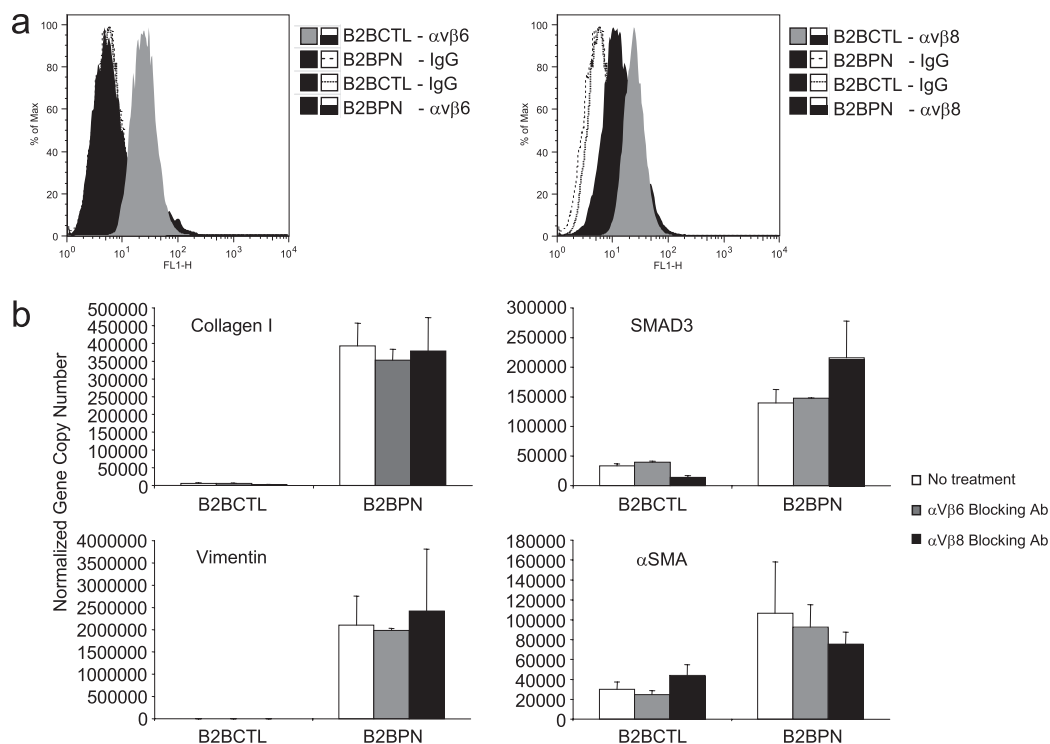


Fig. S6. Periostin-mediated EMT and TGF- β activity is not inhibited by blocking antibodies to integrins α v6 and α v8. (A) FACS analysis of α v6 and α v8 staining of B2BCTL and B2BPN cells. B2BPN cells displayed a reduction in both α v6 and α v8 staining. (B) Gene expression analysis of type 1 collagen, α -SMA, and vimentin from RNA extracted from B2BCTL and B2BPN cells treated with blocking Abs to integrin α v6 (20 μ g/mL) or α v8 (20 μ g/mL) for 24 h. No statistical differences were observed. Error bars indicate SD.

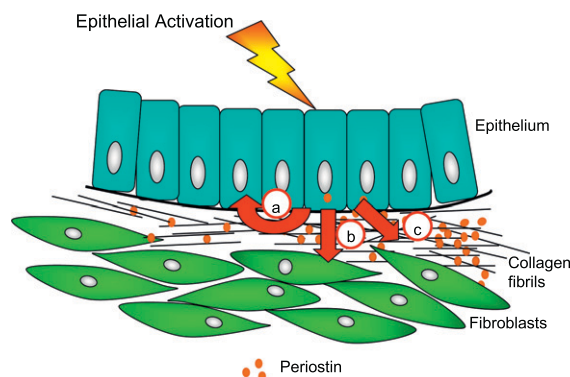


Fig. S7. Summary model of periostin functions in airways. Potential in vivo consequences of basally secreted epithelial-cell derived periostin in asthma include: (A) epithelial feedback and activation of TGF- β signaling, (B) stimulate collagen production in adjacent fibroblasts and (C) increased collagen fibrillogenesis or cross-linking, leading to stiffening of the extracellular matrix.

Table S1. Primers and probes used in quantitative PCR

Gene/type	Sequence
Eukaryotic translation elongation factor 1 α1 (EEF1A1)	
RT-forward	TGCTAACATGCCTTGGTTCAAG
RT-reverse	TTGGACGAGTTGGTGGTAGGAT
Taqman-forward	CCTTGGTTCAAGGGATGGAA
Taqman-reverse	GCCTCAAGCAGCGTGGTT
Probe	CACTGGCATTGCCATCCTTACGGG
Ribosomal protein L13A (RPL13A)	
RT-forward	GGACCGTGCGAGGTATGCT
RT-reverse	TTCAGACGCACGACCTTGAG
Taqman-forward	TATGCTGCCCCACAAAACC
Taqman-reverse	TGCCGTCAAACACCTTGAGA
Probe	CAGAGCGGCTGGCCTCGCT
Peptidylprolyl Isomerase A (PPIA)	
RT-forward	ATGAGAACTTCATCCTAAAGCATACG
RT-reverse	TTGGCAGTGCAGATGAAAACT
Taqman-forward	ACGGGTCTGGCATCTTGT
Taqman-reverse	GCAGATGAAAACTGGGAACCA
Probe	ATGGCAAATGCTGGACCCAACACA
GAPDH	
RT-forward	CAATGACCCCTTCATTGACCTC
RT-reverse	CTCGCTCTGGAAGATGGTGAT
Taqman-forward	GATTCCACCCATGGCAAATTC
Taqman-reverse	GGGATTTCATTGATGACAAGC
Probe	CGTTCTCAGCCTTGACGGTGCCA
Periostin (POSTN)	
RT-forward	GCAAACCACCTTCACGGATCT
RT-reverse	TTATTCACAGGTGCCAGCAAAG
Taqman-forward	CGGATCTTGTGGCCCAATT
Taqman-reverse	AGGTGCCAGCAAAGTGATTCTC
Probe	CTTGGCATCTGCTCTGAGGCC
Collagen, type I, α1 (COL1A1)	
RT-forward	ATGTGGCCATCCAGCTGAC
RT-reverse	GTTGGAGCCCTTGAGGAGC
Taqman-forward	ATGTGGCCATCCAGCTGAC
Taqman-reverse	CTTGCACTGGTAGGTGATGTTCTG
Probe	TCCTGCGCCTGATGTCCACCG
TGFβ1	
RT-forward	CCCTCTCTGCCTCCTCCTG
RT-reverse	GGCACTTCAACAGTGCCCA
Taqman-forward	CCTGCCTGTCTGCACTATTCC
Taqman-reverse	AGTGTTCCCCACTGGTCCC
Probe	TTGCCCGGCATCAAGGCACA
TGF-β2	
RT-forward	GATGGCACCTCCACATATACCA
RT-reverse	TTCCACCCTAGATCCCTCTTG
Taqman-forward	GTTATTGCCCTCTACAGACTTGAG
Taqman-reverse	AATAGGCCGCATCCAAAGC
Probe	CACAACAGACCAACCGGCGGAAGA
Vimentin (VIM)	
RT-forward	CTGCCAACCGGAACAATGAC
RT-reverse	CATTGGTTCTTTAAGGGCATC
Taqman-forward	CAGGCAAAGCAGGAGTCCA
Taqman-reverse	TAAGGGCATCCACTTCACAGG
Probe	TGAGTACCGGAGACAGGTGCAGTCCC
E-cadherin (CDH1)	
RT-forward	GGCCTGAAGTGACTCGTAACG
RT-reverse	CAGTATCAGCCGCTTCAGATT
Taqman-forward	TGAGTGTCCCCGGTATCTTC
Taqman-reverse	CAGCCGCTTTCAGATTTTCAT
Probe	CTGCCAATCCCGATGAAATTGGAAATTT
Actin, α-2, smooth muscle (ACTA2)	
RT-forward	GTGTTGCCCCCTGAAGAGCAT
RT-reverse	CCGCCTGGATAGCCACATAC

Table S1. Cont.

Gene/type	Sequence
Taqman-forward	GAAGAGCATCCCACCCTGC
Taqman-reverse	ATTTTCTCCCGTTGGCCT
Probe	ACGGAGGCACCCCTGAACCCC
Matrix metalloproteinase 2, gelatinase A (MMP-2)	
RT-forward	TTTGATGGCATCGCTCAGATC
RT-reverse	ATCAATCTTTCCGGGAGCTC
Taqman-forward	CGCTCAGATCCGTGGTGAG
Taqman-reverse	TGTCACGTGGCGTCACAGT
Probe	CTTCAAGGACCGGTTTCATTTGGCG
Matrix metalloproteinase 9, gelatinase B (MMP-9)	
RT-forward	ACCTCGAACTTTGACAGCGAC
RT-reverse	CATAGGGTACATGAGCGCCTC
Taqman-forward	CCCGGACCAAGGATACAGTTT
Taqman-reverse	GAATGATCTAAGCCCAGCGC
Probe	CCTCGTGGCGGCGCATGAG