

Resistin-Like Molecule- β in Scleroderma-Associated Pulmonary Hypertension

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Scleroderma is a systemic, mixed connective tissue disease that can impact the lungs through pulmonary fibrosis, vascular remodeling, and the development of pulmonary hypertension and right heart failure. Currently, little is known about the molecular mechanisms that drive this condition, but we have recently identified a novel gene product that is up-regulated in a murine model of hypoxia-induced pulmonary hypertension. This molecule, known as hypoxia-induced mitogenic factor (HIMF), is a member of the newly described resistin gene family. We have demonstrated that HIMF has mitogenic, angiogenic, vasoconstrictive, inflammatory, and chemokine-like properties, all of which are associated with vascular remodeling in the lung. Here, we demonstrate that the human homolog of HIMF, resistin-like molecule (RELM)- β , is expressed in the lung tissue of patients with scleroderma-associated pulmonary hypertension and is up-regulated compared with normal control subjects. Immunofluorescence colocalization revealed that RELM- β is expressed in the endothelium and vascular smooth muscle of remodeled vessels, as well as in plexiform lesions, macrophages, T cells, and myofibroblast-like cells. We also show that addition of recombinant RELM- β induces proliferation and activation of ERK1/2 in primary cultured human pulmonary endothelial and smooth muscle cells. These results suggest that RELM- β may be involved in the development of scleroderma-associated pulmonary hypertension.

Keywords: pulmonary hypertension; scleroderma; resistin-like molecule β ; hypoxia-induced mitogenic factor; T helper type 2

Scleroderma, also known as systemic sclerosis, is a rare connective tissue disease in which uncontrolled deposits of collagen lead to fibrosis of the skin, internal organs, and vasculature (1). In the lung, a major target organ of scleroderma (2), fibrosis may result from chronic pulmonary inflammation that initiates collagen production from proliferating fibroblasts (3). This fibrosis results in reduced respiratory volume and impaired gas diffusion; approximately 5–15% of patients will develop the serious condition of pulmonary hypertension (for review see Ref. 4). Patients diagnosed with scleroderma-associated pulmonary hypertension have a reduced response to current therapies and a lower long-term survival rate than patients suffering from idiopathic pulmonary hypertension (5). The exact pathologic

CLINICAL RELEVANCE

We have demonstrated that resistin-like molecule (RELM)- β is up-regulated in the lungs of patients diagnosed with scleroderma-associated pulmonary hypertension. We also have shown that the addition of recombinant RELM- β induces proliferation of cultured pulmonary vascular smooth muscle cells and endothelial cells. Understanding the role of RELM- β in the development of pulmonary hypertension will provide insights for the future development of much-needed therapies for this disease.

mechanism for the development of pulmonary scleroderma is unknown, and current therapies only have limited effectiveness.

Inflammation plays an important role in the development of lung scleroderma. During the disease process, activated macrophages and T cells accumulate in the lungs, and several cytokines and growth factors become elevated in the bloodstream (6–11) and bronchoalveolar lavage fluid (BALF) (12). Increases in IL-1 and TNF- α may be the result of generalized inflammation, but increases in several other cytokines, including IL-4, IL-13, transforming growth factor- β , and monocyte chemotactic protein (MCP)-1, may be the primary mediators of the scleroderma disease process (3, 4). Production of excess collagen is essential to the etiology of scleroderma, and both IL-4 and MCP-1 have been shown to stimulate collagen production in lung fibroblasts *in vitro* (13, 14). It is interesting to note that increased levels of IL-4 mRNA detected in CD8⁺ T cells recovered from the BALF of patients with scleroderma correlates with decreased pulmonary function (12).

Recently, our laboratory described a gene that is up-regulated in the remodeling lung vasculature in the murine model of hypoxia-induced pulmonary hypertension (15). This molecule, known as hypoxia-induced mitogenic factor (HIMF), is a member of the newly described resistin gene family (16–18). In rodents, HIMF is also known as resistin-like molecule (RELM)- α , found in inflammatory zone (FIZZ) 1 (19), or murine ten-cysteine protein (mXCP)2 (20). There are three other rodent members of this gene family that are known as RELM- β /FIZZ2/mXCP3, resistin/FIZZ3/mXCP4, and RELM- γ /FIZZ4/mXCP1. We reported previously that HIMF expression is increased in the lungs of chronically hypoxic mice (15) and in the normal developing murine lung (21). We have also shown that HIMF introduced into murine lung induces angiogenesis and vascular thickening (22). Others have demonstrated that HIMF expression is increased in ovalbumin-induced asthma (19), bleomycin-induced pulmonary fibrosis (23, 24), compensatory growth after pneumectomy (25), and endotoxin-induced acute lung injury (26). Specifically, HIMF has been shown to be expressed by bronchial epithelium, type-II pneumocytes, pulmonary vascular smooth muscle cells, and endothelial cells under pathological conditions (15, 25). In particular, HIMF is coexpressed with

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proliferating cell nuclear antigen in the remodeling microvessels of our chronic hypoxia model of pulmonary hypertension (15). Previous studies from our laboratory have shown that recombinant HIMF has mitogenic, angiogenic, and vasoconstrictive effects in lung (15), and that HIMF possesses an antiapoptotic function in cultured murine embryonic lungs (21). It also has been reported that HIMF plays a role in type 2 T helper cell (Th)₂-mediated inflammation. Liu and colleagues (23) demonstrated that treatment of rat airway epithelial cells with recombinant IL-4 and IL-13 increases HIMF mRNA in a dose-dependent manner, whereas the addition of Th1-related inflammatory cytokines (e.g., TNF- α) induces no such effect. Several reports have indicated that HIMF and its related molecules play a key role in the alternative (Th₂) activation of macrophages (27, 28), and are involved in Th₂-dependent, asthma-induced airway remodeling (29). HIMF is also up-regulated in the newly described Th₂ model of pulmonary arterial remodeling (30). HIMF itself is activated by the Th₂ pathway via IL-4 and IL-13 activation of a Stat6 site in its promoter region (31).

In humans, there are only two members of the resistin gene family, resistin and RELM- β . Currently, little is known about the closest human homolog to HIMF, RELM- β . It was initially described in the colon and small intestines (17, 19, 20), but recently was found to be expressed in other tissues, including the lung, heart, kidney, and adrenal glands (32). Here, we examine lung tissue from patients with scleroderma-associated pulmonary hypertension, patients with idiopathic pulmonary hypertension, and normal patients with no signs of pulmonary hypertension for RELM- β expression. We found that RELM- β was consistently expressed in the lungs of patients with scleroderma-associated pulmonary hypertension, particularly in the endothelium, smooth muscle cells (including myofibroblasts), T cells, and macrophages. In addition, the expression of RELM- β in lungs from normal patients and those with idiopathic pulmonary hypertension was much lower and more variable than that from patients with scleroderma-associated pulmonary hypertension. Furthermore, we found that RELM- β had mitogenic effects on both primary cultured pulmonary vascular endothelial and smooth muscle cells, and that this effect was, at least in part, mediated through ERK1/2 signaling.

MATERIALS AND METHODS

Human Tissue Samples

Lung tissue from patients with scleroderma was obtained through the Johns Hopkins University Scleroderma Center. To be included in this study, subjects had to meet the American College of Rheumatology classification criteria for scleroderma (33) or have three of five features of the CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) syndrome. For these studies, we obtained both paraffin-embedded and frozen human lung tissue from patients diagnosed with scleroderma-associated pulmonary hypertension ($n = 9$). For comparison, we also obtained lung tissue from patients diagnosed with idiopathic pulmonary hypertension ($n = 11$), and normal patients with no signs of pulmonary hypertension ($n = 6$). The lung tissue used for this study from patients with scleroderma-associated pulmonary hypertension and patients with idiopathic pulmonary hypertension was gathered after lung transplantation. Normal lung tissue was obtained from either organ donors ($n = 3$) or resections of lung tumors ($n = 3$). The clinical information of these patients is presented in Table 1. Colon tissues ($n = 3$) were obtained from resections of colon tumors. Approval from the Johns Hopkins Medicine Institutional Review Board was obtained before the start of these studies.

Immunohistochemistry

Paraffin blocks of lungs from normal patients, patients with idiopathic pulmonary hypertension, and those of patients with scleroderma-

TABLE 1. PATIENT DEMOGRAPHICS AND CLINICAL INFORMATION

Characteristics	Normal ($n = 6$)	SSc w/PH ($n = 9$)	IPH ($n = 11$)
Age, yr	59.33 \pm 7.03	51.67 \pm 2.83	38.91 \pm 2.78
Female, n (%)	5 (83.33)	7 (77.78)	9 (81.82)
Caucasian, n (%)	5 (83.33)	7 (77.78)	9 (81.82)
ACR criteria, n (%)	N/A	9 (100)	N/A
Limited SSc, n (%)	N/A	7 (77.78)	N/A
Disease duration, yr	N/A	10.23 \pm 1.89	6.14 \pm 0.63
mPAP, mm Hg	16.83 \pm 0.60	42.44 \pm 2.82	41.09 \pm 1.84
PVR, Woods' units	1.72 \pm 0.35	9.15 \pm 0.81	9.71 \pm 1.05
RA, mm Hg	4.33 \pm 0.33	11.78 \pm 0.76	10.55 \pm 0.76
CO, liter/min	4.80 \pm 0.26	3.50 \pm 0.16	3.34 \pm 0.16
PCWP, mm Hg	8.67 \pm 1.20	10.67 \pm 0.73	10.18 \pm 0.72
Epoprostenol treatment, n (%)	N/A	5 (55.56)	9 (81.82)
ETRA treatment, n (%)	N/A	3 (33.33)	3 (27.27)
Sildenafil treatment, n (%)	N/A	0 (0.00)	1 (9.09)
Diuretic treatment, n (%)	N/A	9 (100)	9 (81.82)
Never smoked, n (%)	3 (50.00)	6 (66.67)	7 (63.64)
Obese, n (%)	0 (0.00)	0 (0.00)	1 (9.09)
Hypertension, n (%)	1 (16.67)	3 (33.33)	4 (36.36)
High cholesterol, n (%)	0 (0.00)	3 (33.33)	6 (54.54)
Diabetes mellitus, n (%)	1 (16.67)	1 (11.11)	1 (9.09)

Definition of abbreviations: ACR, American College of Rheumatism classification; CO, cardiac output; mPAP, mean pulmonary artery pressure; ETRA, endothelin receptor antagonist; IPH, idiopathic pulmonary hypertension; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; RA, right atrial pressure; SSc, scleroderma.

Hemodynamic data (mPAP, PVR, RA, CO, PCWP) from normal patients was only obtained from organ donors ($n = 3$).

associated pulmonary hypertension were cut into 6- μ m sections and placed on clean glass slides. The slides were heated to 60°C for 30 minutes and then subjected to deparaffinization (100% xylene, 3 \times 15 min at room temperature [RT]) and rehydration by washes in decreasing concentrations of ethanol (2 \times 100%, 1 \times 95%, 1 \times 70%, and 1 \times 50% for 10 min each), followed by one 10-minute wash in ddH₂O and one 10-minute wash in PBS. Rehydrated slides were placed in antigen-unmasking solution (Vector Laboratories; Burlingame, CA) at 95°C for 20 minutes, then endogenous peroxidase was blocked with 3% H₂O₂ for 10 minutes. Endogenous avidin and biotin were blocked for 15 minutes each using the Avidin/Biotin Blocking Kit (Vector Laboratories). Nonspecific protein binding was blocked by treatment of the slides with normal goat serum for 30 minutes. After the final blocking step, the sections were treated with polyclonal rabbit anti-human RELM- β (1:200; Chemicon, Temecula, CA), or antibody diluent alone for 60 minutes at RT. The slides were washed with PBS and then treated with a goat anti-rabbit biotinylated secondary antibody (Vector Laboratories) and then an ABC horseradish peroxidase reagent for 30 minutes each at RT (Vectastain Elite ABC Kit; Vector Laboratories). The lung sections were then treated with the liquid diaminobenzidine plus substrate chromogen system for 1–2 minutes (DakoCytomation, Carpinteria, CA). Finally, the sections were dehydrated with ethanol (1 \times 50%, 1 \times 70%, 1 \times 95%, and 2 \times 100% for 10 min each), treated with xylene (3 \times 10 min), and mounted with Cytoseal60 (Richard-Allan Scientific, Kalamazoo, MI). Sections were visualized through a Nikon Eclipse TE2000-E microscope (Nikon \times 40, 0.75 numerical aperture Plan Fluor objective; Nikon Instruments, Inc., Melville, NY), and photographs were captured with a Nikon digital camera.

Immunofluorescence Microscopy

Paraffin-embedded human lung tissue was sectioned, dewaxed, and rehydrated as described in IMMUNOHISTOCHEMISTRY. Antigen retrieval was also performed as stated above. Nonspecific protein binding was blocked by treatment of the slides with 5% normal horse serum for 30 minutes. After this blocking step, the sections were treated with rabbit anti-RELM- β (1:200) and mouse anti-von Willebrand factor (1:200;

DakoCytomation), α -smooth muscle actin (1:500; DakoCytomation), anti-CD68 (1:100; DakoCytomation), anti-CD3 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or preimmune rabbit IgG and preimmune mouse IgG for 60 minutes at RT. Next, the sections were incubated with AlexaFluor 488-labeled goat anti-rabbit IgG (H+L) (1:100; Invitrogen, Carlsbad, CA) and Cy3-conjugated donkey anti-mouse IgG (H+L) (1:100; Jackson ImmunoResearch Labs, Inc., West Grove, PA). Finally, the sections were washed in PBS, covered with a glass coverslip, and visualized through a Nikon Eclipse microscope (Nikon $\times 40$, 0.75 numerical aperture Plan Fluor objective). Photographs were captured with a Spot-RT monochrome digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and images were colorized using MetaMorph Offline version 6.1 (Molecular Devices, Downingtown, PA).

Human Cell Culture

Human lung microvascular endothelial cells (HMVEC-L; Lonza, Walkersville, MD) were cultured in endothelial cell basal medium-2 (Lonza) supplemented with 5% FBS, human epidermal growth factor, human vascular endothelial growth factor (VEGF), human fibroblast growth factor (with heparin), long R3 insulin-like growth factor I, hydrocortisone, ascorbic acid, gentamicin, and amphotericin B (Bulletedkit CC-3202; Lonza). Only endothelial cells from passages 5–10 were used. Human pulmonary artery smooth muscle cells (HPASMCs) (Lonza) were cultured in smooth muscle basal medium (Lonza) supplemented with 5% FBS, human epidermal growth factor, human fibroblast growth factor-B, insulin, gentamicin, and amphotericin B (Bulletedkit CC-4149; Lonza). Only HPASMCs from passages 6–9 were used.

Western Blot Analysis

Frozen human lung and colon tissue were homogenized on ice with a Brinkman Homogenizer (Polytron, Westbury, NY) in homogenization/lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM vanadate, 1 mM NaF, 10 mM pyrophosphate, and 1 mg/ml pepstatin A (all purchased from Sigma, St. Louis, MO), and one tablet of Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN) per 20 ml. HMVEC-Ls and HPASMCs were cultured to approximately 50% confluence before being serum and growth factor starved overnight and then treated with 100 ng/ml recombinant human (rh) RELM- β (Peprotech, Inc., Rocky Hill, NJ) or vehicle (0.1% BSA/PBS) for 5, 10, 15, 20, or 60 minutes. The cells were then lysed with ice-cold homogenization/lysis buffer (*described above*). The homogenates/lysates were centrifuged, and the supernatants were assayed for protein concentration with a standard Bio-Rad Protein assay kit (Bio-Rad Chemical Division, Hercules, CA). The samples were resolved by gradient 4–20% SDS-PAGE (Bio-Rad) and transferred onto nitrocellulose (Bio-Rad) membranes. The blots were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 hour, incubated with murine anti-human RELM- β (1:500; R&D Systems, Minneapolis, MN), rabbit anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; thr202/tyr204; 1:1,000; Cell Signaling Technologies, Beverly, MA), or rabbit anti-phospho-MEK1/2 (ser217/221; 1:1,000; Cell Signaling Technologies), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5,000; Bio-Rad). The blots were developed with enhanced chemiluminescence (Amersham, Piscataway, NJ) and exposed to X-ray film (Denville Scientific, Metuchen, NJ). To ensure equal protein loading and transfer, blots were stripped with Blot Restore Membrane Rejuvenation Kit (Chemicon) and reprobed with either murine anti- β -actin (Santa Cruz Biotechnology), rabbit anti-p44/42 MAPK (Cell Signaling Technologies), or anti-MEK1/2 (Cell Signaling Technologies). Bands of interest were quantified by laser densitometry (Molecular Dynamics; Sunnydale, CA), and normalized to either β -actin or p44/42 MAPK to ensure equal loading and transfer.

Direct Cell Counting Assay

HPASMCs or HMVEC-Ls were plated in six-well plates at a concentration of 10,000 cells/cm² and allowed to attach for 24 hours. The cells were then serum and growth factor starved for an additional 24 hours, according to a published method (34). Cells were then treated for 48 hours with rhRELM- β (25, 50, or 100 ng/ml) or media alone. To exclude the possibility of apoptosis due to serum starvation, these experiments were repeated in the presence of 2.5% FBS. To determine

if RELM- β -induced mitogenesis was ERK1/2-dependent, HMVEC-Ls or HPASMCs that were prepared as stated *above* were preincubated for 1 hour with U0126 (10 μ M), a pharmacologic ERK1/2 inhibitor (Cell Signaling Technologies). The preincubation was followed by a 48-hour exposure to 100 ng/ml rhRELM- β or vehicle. Positive control cells were exposed to medium supplemented with 5% FBS. After treatment, the cells were washed, trypsinized, and counted with a hemocytometer.

Statistical Analysis

Student's *t* test was used to compare laser densitometry results. ANOVA was used to compare the mean responses among experimental and control groups for the direct cell counting assays. The Dunnett and Scheffe *F* test was used to determine between which groups significant differences existed. A *P* value of less than 0.05 was considered significant for all experiments.

RESULTS

RELM- β Expression Is Increased in the Lungs of Patients with Scleroderma

To establish the relationship between RELM- β and scleroderma-associated pulmonary hypertension, we evaluated patient lung samples by immunohistochemistry and Western blot, and compared the results with those from patients with no history of pulmonary hypertension. Demographic and clinical information for all patients is displayed in Table 1. All groups had similar demographic characteristics. Markers of pulmonary hypertension, such as mean pulmonary artery pressure and pulmonary vascular resistance, were similarly elevated in patients with scleroderma-associated pulmonary hypertension and patients with idiopathic pulmonary hypertension (Table 1). Immunohistochemical analysis revealed that RELM- β expression was consistently up-regulated in patients with scleroderma, and was extremely low in control subjects (Figure 1). Closer analysis revealed that this increase in RELM- β expression was localized to the vasculature, particularly plexiform lesions, and in pulmonary epithelia and inflammatory cells. For comparison, we also evaluated lung samples obtained from patients diagnosed with idiopathic pulmonary hypertension. In contrast to the lung samples from patients with scleroderma-associated pulmonary hypertension, the immunohistochemical stain for RELM- β in these samples was not consistent from patient to patient (Figure 2). In a few of the samples, we did observe strong staining for RELM- β in the borders of plexiform lesions (Figure 2B). In the rest of the lung, there was only mild to moderate expression in the vasculature (Figure 2C) or pulmonary septum (Figure 2D). To quantify RELM- β expression in lung tissue, and to determine its specificity to the disease process of scleroderma-associated pulmonary hypertension, we used Western blot to evaluate frozen lung samples from normal patients and those with scleroderma-associated pulmonary hypertension or idiopathic pulmonary hypertension (Figure 3A). Colon homogenates were used as positive control samples (17, 19, 20). Quantification of these immunoblots revealed an approximately twofold increase in RELM- β expression in scleroderma lungs when compared with control samples (Figure 3C). No such increases in RELM- β were observed in the lung tissue of patients with idiopathic pulmonary hypertension (Figures 3B and 3D). These results suggest a link between RELM- β expression and the development of scleroderma-associated pulmonary hypertension.

RELM- β Is Expressed in the Pulmonary Vasculature and Inflammatory Cells

To elucidate which specific cells express RELM- β in lungs from patients with scleroderma-associated pulmonary hypertension,

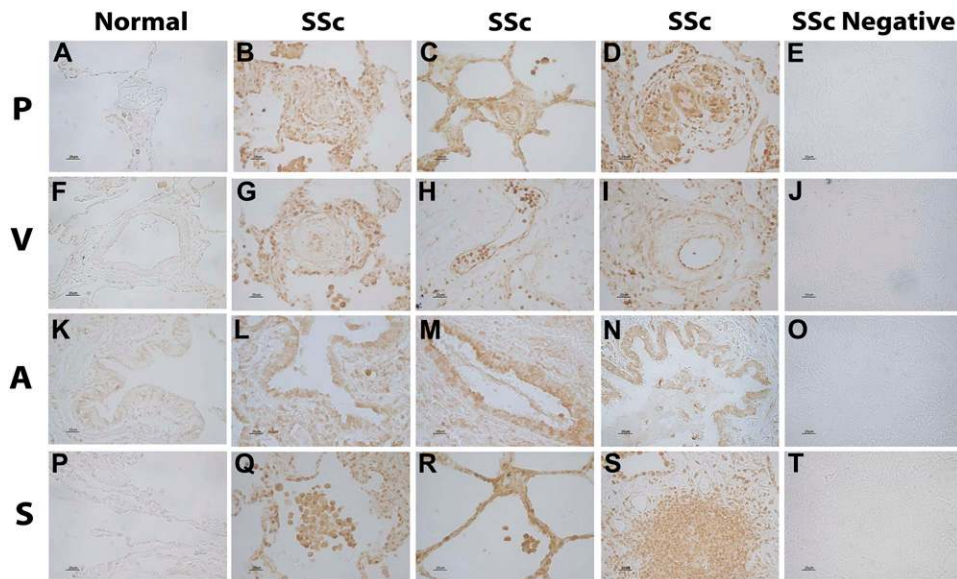


Figure 1. Immunohistochemical localization of resistin-like molecule (RELM)- β in human lungs. Paraffin-embedded sections from normal human lung (A, F, K, and P) and from lungs of patients with scleroderma-associated pulmonary hypertension (B–D, G–I, L–N, and Q–S) were stained with a rabbit anti-human RELM- β polyclonal antibody. In negative control samples, no primary antibody was added to serial sections (E, J, O, and T). A, airway; P, plexiform lesion; S, septum; SSc, scleroderma; V, vasculature. Scale bars, 25 μ m.

we used immunofluorescence colocalization to compare RELM- β expression with expression of cell-specific markers. The lung sections exhibited strong immunofluorescence to RELM- β in both the endothelium and the smooth muscle cells (Figure 4) of the vasculature (for additional images, see Figures E1 and E2 in the online supplement). Cells unassociated with the vasculature, that were positive for α -smooth muscle actin, also stained positive for RELM- β (Figure 4T). RELM- β also was expressed in macrophages and T cells (Figure 5). Neither B cells (marker, CD20) (Figure E3) nor mast cells (marker, mast cell tryptase) (Figure E4) appeared to express RELM- β .

Recombinant RELM- β Is Mitogenic for HMVEC-Ls and HPASMCs

When cells were grown in serum- and growth factor-free media, treatment with rhRELM- β (100 ng/ml) increased growth of HMVEC-Ls by approximately 1.6-fold ($P < 0.001$) and HPASMCs by approximately 1.4-fold ($P < 0.01$) compared with vehicle-treated control cells (Figures 6A and 6B). Pretreatment of both HMVEC-Ls and HPASMCs with the ERK1/2 inhibitor, U0126 (10 μ M), completely abolished the rhRELM- β effect (Figures 6A and 6B). As an additional control, both HMVEC-Ls and HPASMCs were cultured separately in media containing 2.5% FBS with or without rhRELM- β (100 ng/ml) for 48 hours. The presence of rhRELM- β increased the number of HMVEC-Ls by approximately 1.4-fold ($P < 0.01$) and HPASMCs by approximately 1.5-fold ($P < 0.01$) when compared with simultaneous vehicle control (Figure E5). These results indicate that rhRELM- β has mitogenic effects on human pulmonary microvascular endothelial cells and smooth muscle cells.

rhRELM- β Activates ERK1/2 in HMVEC-Ls and HPASMCs

In serum- and growth factor-starved HMVEC-Ls and HPASMCs, rhRELM- β (100 ng/ml) increased ERK1/2 phosphorylation. In HMVEC-Ls, this phosphorylation occurred as early as 10 minutes after exposure, with continued increases through 20 minutes (Figures 6C). In HPASMCs, these phosphorylation events initially occurred at 5 minutes after rhRELM- β treatment, and the ERK1/2 phosphorylation peaked at 15 minutes after exposure (Figure 6D). In both HMVEC-Ls and HPASMCs, phosphorylation appeared to return to baseline levels by 60 minutes after rhRELM- β exposure. To confirm that the ERK1/2 pathway was being activated by rhRELM- β , we

examined these same lysates for phosphorylation of the upstream molecule, MEK1/2 (Figure E6). Lysates from both HMVEC-Ls and HPASMCs treated with rhRELM- β (100 ng/ml) displayed increased MEK1/2 phosphorylation; the time course of MEK1/2 phosphorylation seemed to mirror the time course seen above.

DISCUSSION

Scleroderma is a disease characterized by widespread fibrosis, with the lungs being one of the main target organs (2). Pulmonary fibrosis in scleroderma can lead to vascular remodeling and the development of pulmonary hypertension. In the current study, we demonstrate, by both immunohistochemistry and Western blot analysis, that RELM- β is increased in the lungs of patients diagnosed with scleroderma-associated pul-

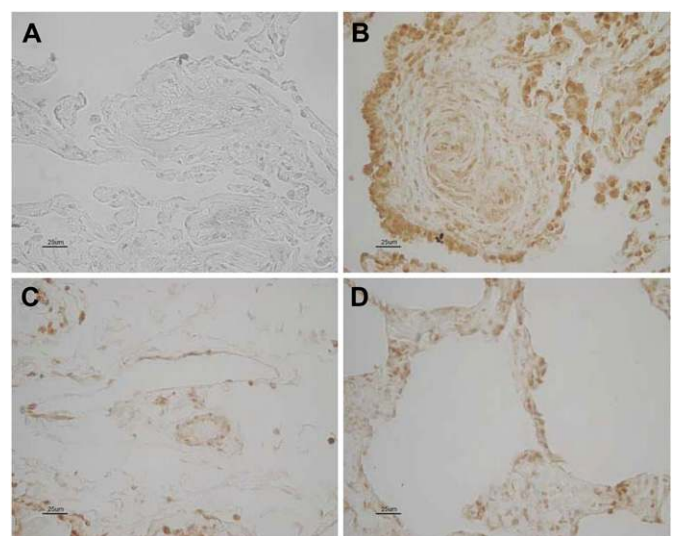


Figure 2. Immunohistochemical localization of RELM- β in lungs from patients with idiopathic pulmonary hypertension (IPH). Paraffin-embedded sections from normal human lung (A) and from lungs of patients with IPH (B–D) were stained with a rabbit anti-human RELM- β polyclonal antibody. Scale bars, 25 μ m.

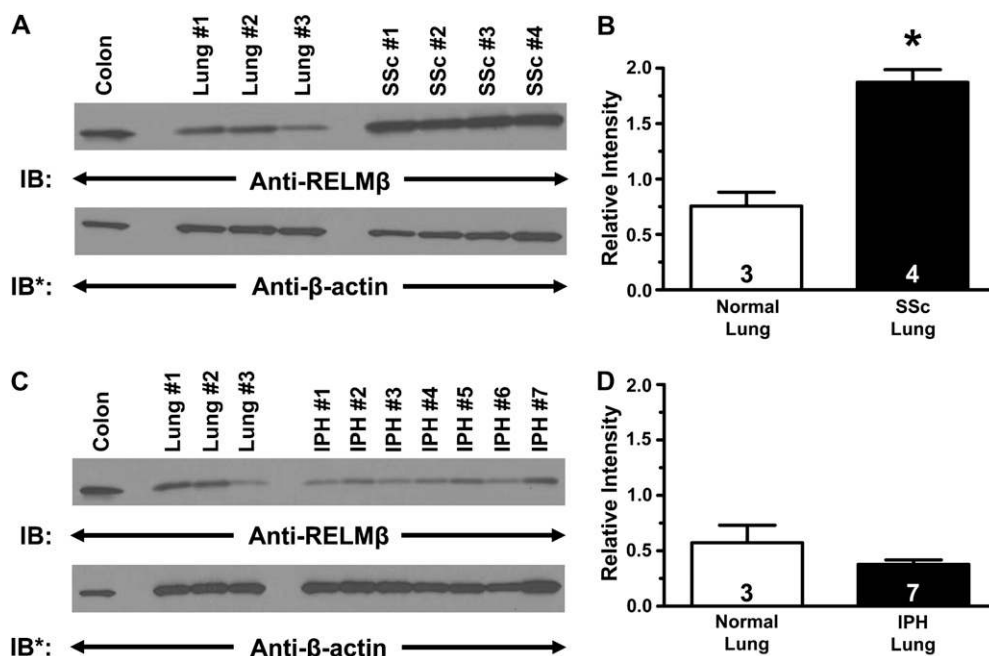


Figure 3. Quantification of RELM- β in lungs from patients with scleroderma-associated pulmonary hypertension (SSc) and IPH. (A and C) Frozen human colon (control) and lung tissue samples (SSc and IPH) were homogenized, resolved by 4–20% SDS-PAGE, and transferred to nitrocellulose membranes. The blots were then probed with mouse monoclonal RELM- β antibodies, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibodies, and developed with enhanced chemiluminescence (ECL). To ensure equal loading and transfer, blots were stripped and reprobed with anti- β -actin antibodies. IB, immunoblot; IB*, immunoblot after stripping. (B and D) Laser densitometry was used to quantify RELM- β levels in the lung samples. Data are normalized to β -actin expression and expressed as relative intensity (mean \pm SEM). * $P < 0.01$ compared with the normal lung.

monary hypertension. Immunohistochemical and Western blot analysis of lung tissue from patients diagnosed with idiopathic pulmonary hypertension did not reveal consistent increases in RELM- β expression. To the best of our knowledge, this is the first report describing human RELM- β in the lungs of patients with scleroderma. Our results specifically show that RELM- β is expressed in the endothelium, smooth muscle, macrophages, and T cells of these patients. Expression in these cell types may be key in determining the role of RELM- β in the development of scleroderma-associated pulmonary hypertension. We also show that rhRELM- β has mitogenic effects on both pulmonary vascular endothelial and smooth muscle cells; furthermore, rhRELM- β can activate the mitogenic signaling molecules, ERK1/2, in these cultured cells in a time-dependent manner. Together, these data suggest a role for RELM- β in the development of scleroderma-induced lung pathology and pulmonary hypertension.

Inflammation is an important pathologic event in the progression of pulmonary scleroderma. Cytokines, particularly the Th2 type, are expressed in the BALF of patients with scleroderma (12). It has been previously demonstrated that the murine molecule, HIMF, is involved in Th2 signaling associated with bleomycin-induced pulmonary injury and fibrosis. A recent report by Liu and colleagues (23) showed that pulmonary bleomycin-induced HIMF expression is completely abolished in IL-4/IL-13 double-knockout mice. These mice also displayed less lung fibrosis than the wild-type counterparts. Furthermore, recombinant IL-4 and IL-13 can induce HIMF expression in various pulmonary cells *in vitro*. In human scleroderma, Th2 responses, including IL-4 and IL-13 production, are primary mediators of the disease process. It is interesting to note that Artis and colleagues (35) found that RELM- β mRNA increased in a human colon cancer cell line (LS174T) after treatment with recombinant IL-4 or IL-13. A specific relationship appears to exist between HIMF and IL-4 and IL-13, but the exact role that it plays in human scleroderma is currently unclear.

Abnormal endothelial cell growth and organization are important factors in the pathology of human scleroderma-associated pulmonary hypertension. One such factor is the development of

tumor-like structures, also known as plexiform lesions. These structures can be described as uncontrolled, intraluminal, endothelial cell growth, and are seen in both primary and secondary cases of pulmonary hypertension. The proangiogenic molecule, VEGF, appears to be critical in the development of plexiform lesions. Patients with pulmonary hypertension have elevated serum VEGF and high levels of VEGF mRNA and protein in the plexiform lesion itself. We previously demonstrated that HIMF causes endothelial proliferation and tubule formation, both in culture and in *in vivo* mouse lung (15, 22). We also have shown that, in microvascular endothelial cells and in murine lung organ culture, the addition of recombinant HIMF induces VEGF expression (22). Plexiform lesions can be duplicated in animal models only by VEGF receptor 2 inhibition. Interestingly, we have also shown that intravenous injection of recombinant HIMF into a mouse reduces VEGF receptor 2 expression in the lung (22). In our current study, we observed strong RELM- β immunostaining in the plexiform lesions of the scleroderma lung tissue samples, as well as of some of the idiopathic pulmonary hypertension tissue. In addition, we showed that RELM- β is mitogenic for HMVEC-L *in vitro*. It is conceivable that mitogenic activity of RELM- β *in vivo* could contribute to the endothelial cell proliferation seen in plexiform lesions.

Pulmonary vascular smooth muscle cell proliferation and migration are major events that lead to the development of pulmonary hypertension in both clinical situations and animal models (e.g., chronic hypoxia). In this study, we show that RELM- β is expressed in the pulmonary vascular smooth muscle cells of patients with scleroderma-associated pulmonary hypertension, and that the addition of rhRELM- β to primary cultured HPASMCs induces cellular proliferation as well as activation of the ERK1/2 pathway. The concentration of rhRELM- β used for the current study was similar to what was used to demonstrate HIMF-induced smooth muscle cell proliferation (15), as well as endothelial cell proliferation (22), in our animal studies. In human aortic smooth muscle cells, Calabro and colleagues (34) found that a dose of 10–100 ng/ml rhResistin was able to elicit a promitogenic response. Mu and colleagues (36) found that a dose of 10–80 ng/ml rhResistin was able to induce a physio-

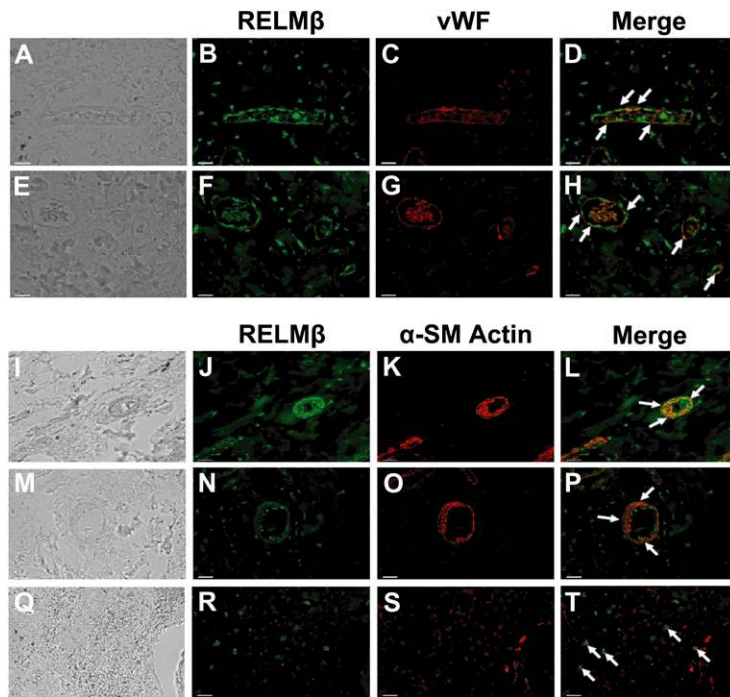


Figure 4. (A–H) Colocalization of RELM-β with pulmonary vascular endothelium in patients with scleroderma-associated pulmonary hypertension. (A and E) Light micrograph of fluorescence images to show structure. Paraffin-embedded lung sections were dual stained with a rabbit anti-human RELM-β polyclonal antibody that was visualized by an Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody (B and F) and a mouse anti-von Willebrand (vWF) factor monoclonal antibody visualized by a Cy3-conjugated donkey anti-mouse IgG antibody (C and G). The *arrows* in the merged images demonstrate colocalization of RELM-β with the pulmonary vascular endothelium (D and H). (I–T) Colocalization of RELM-β with pulmonary vascular smooth muscle in patients with scleroderma-associated pulmonary hypertension. (I, M, and Q) Light micrograph of fluorescence images to show structure. Paraffin-embedded lung sections were dual stained with a rabbit anti-human RELM-β polyclonal antibody that was visualized by an Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody (J, N, and R), and with a mouse anti-α-smooth muscle actin monoclonal antibody that was visualized by a Cy3-conjugated donkey anti-mouse IgG antibody (K, O, and S). The *arrows* in the *merged images* demonstrate colocalization of RELM-β with pulmonary vascular smooth muscle (L and P) and with myofibroblasts (T). Scale bar, 25 μm.

logic response in several types of human endothelial cells. In addition, Sunden-Cullberg and colleagues (37) described mean serum resistin levels of 44 ng/ml in patients with severe sepsis, and 78 ng/ml in patients with septic shock. Based on these studies, we were able to use rhRELM-β concentrations that may be similar to *in vivo* levels. It is interesting to note that resistin also can induce proliferation of HPASMCs in an ERK1/2-dependent manner (34). Hypoxia can induce RELM-β expression in cultured human HPASMCs, and transfection of these cells with RELM-β cDNA induces significant proliferation (32); however, whether RELM-β contributes to the control of proliferation of these cells in scleroderma, at least in part, remains unclear.

In the scleroderma lung tissue that we studied, large numbers of individual cells unassociated with the vascular or airway structures stained positive for both RELM-β and α-smooth muscle actin, strongly suggesting that these cells are myofibroblasts. Recent data have suggested a role for myofibroblasts in the fibrosis associated with scleroderma (38). In addition, Liu and colleagues (24) reported recently that HIMF converts fibroblasts into myofibroblasts *in vitro*. In that study, rat fibroblasts that were cocultured with HIMF-expressing airway epithelial cells developed marked increases in α-smooth muscle actin and collagen type I, both markers of myofibroblast differentiation (24). Our findings, combined with those of Liu and colleagues (24), provide strong evidence that RELM-β

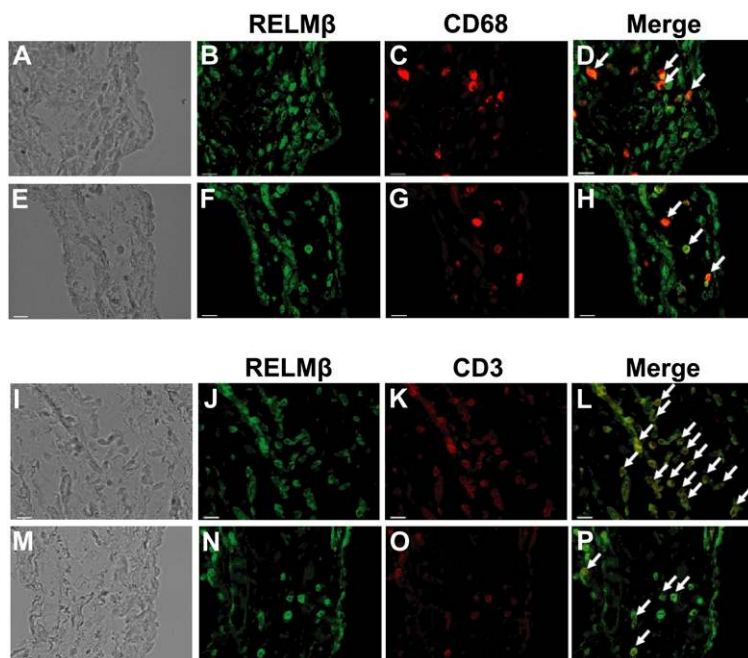


Figure 5. (A–H) Colocalization of RELM-β with macrophages in patients with scleroderma-associated pulmonary hypertension. (A and E) Light micrograph of fluorescence images to show structure. Paraffin-embedded lung sections were dual stained with a rabbit anti-human RELM-β polyclonal antibody that was visualized by an Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody (B and F) and with a mouse anti-CD68 monoclonal antibody that was visualized by a Cy3-conjugated donkey anti-mouse IgG antibody (C and G). The *arrows* in the merged images demonstrate colocalization of RELM-β with the pulmonary macrophages (D and H). (I–P) Colocalization of RELM-β with T cells in patients with scleroderma-associated pulmonary hypertension. (I and M) Light micrograph of fluorescence images to show structure. Paraffin-embedded lung sections were dual stained with a rabbit anti-human RELM-β polyclonal antibody that was visualized by an Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody (J and N) and with a mouse anti-CD3 monoclonal antibody that was visualized by a Cy3-conjugated donkey anti-mouse IgG antibody (K and O). The *arrows* in the *merged images* demonstrate colocalization of RELM-β with T cells (L and P). Scale bar, 25 μm.

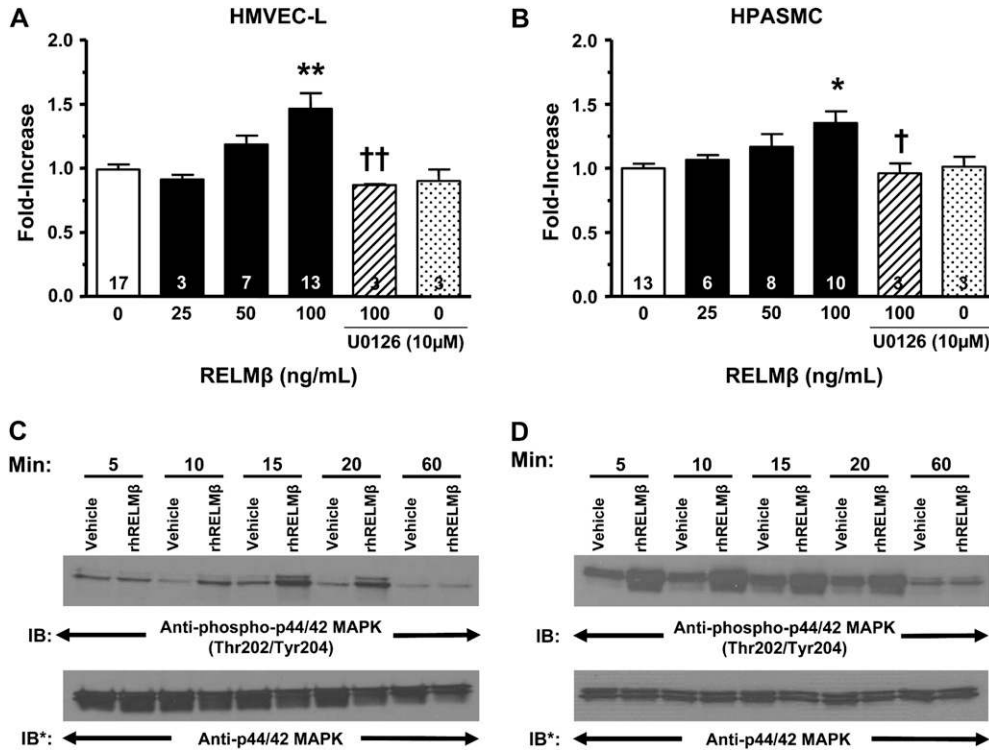


Figure 6. Recombinant human (rh) RELM-β has mitogenic effects on human lung microvascular endothelial cells (HMVEC-Ls) and human pulmonary artery smooth muscle cells (HPASMCs) and activates ERK1/2. HMVEC-Ls (A) and HPASMCs (B) were serum and growth factor starved for 24 hours before being treated with various concentrations of rhRELM-β (0 [control], 25, 50, 100 ng/ml) with or without U0126 (10 μM) for 48 hours. After treatment, the cells were washed, trypsinized, and counted with a hemacytometer. Results are reported as mean (±SEM) of fold increase compared with control. **P* < 0.01, ***P* < 0.001 compared with control; †*P* < 0.05, ††*P* < 0.01 compared with rhRELM-β (100 ng/ml). HMVEC-Ls (C) and HPASMCs (D) were cultured to approximately 50% confluence, serum and growth factor starved overnight, and then exposed to rhRELM-β (100 ng/ml) or vehicle (0.1% BSA/PBS) for up to 60 minutes. Cells were lysed and resolved with 4–20% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes

were probed with rabbit anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; thr202/tyr204), followed by HRP-conjugated anti-rabbit IgG antibodies, and developed with ECL. To ensure equal loading and transfer, blots were stripped and reprobed with anti-p44/42 MAPK antibodies. IB, immunoblot; IB*, immunoblot after stripping.

plays a role in the pathologic disease process associated with myofibroblasts.

Inflammation—specifically, the accumulation of macrophages and T cells in the lungs—is key to the scleroderma disease process. Here, we demonstrate that RELM-β is expressed in both macrophages and T cells in the scleroderma lung. These results are not surprising, as several animal models of alternatively activated macrophages have shown significant increases in HIMF production (27, 28). Using an HIMF antibody that we developed, Mora and colleagues (39) found that HIMF was expressed by these macrophages in an animal model of herpes virus-induced lung fibrosis. In addition, macrophages collected from the BALF of mice experimentally infected with *Pneumocystis pneumonia* showed consistent up-regulation of HIMF concomitant with the development of fibrosis (40). Several cytokines and chemokines seem to be responsible for the attraction of these inflammatory cells to the lungs. IL-4 and IL-13 are critical Th2 cytokines involved in the development of lung scleroderma. Both are profibrotic; IL-4 can induce collagen production *in vivo* and *in vitro*. In the skin-tight mouse model of scleroderma, the absence of IL-4 or Stat6 completely blocked the fibrosis seen in this model (41). In fact, a recent rodent study by Daley and colleagues (30) demonstrated Th2-mediated vascular remodeling, and the presence of HIMF in the inflammatory cells of these same remodeling vessels. Another important chemokine is MCP-1, which regulates the migration of monocytes as well as Th2 cells. MCP-1 can also stimulate collagen production by lung fibroblasts *in vitro* (13, 14). We have previously demonstrated in mice that HIMF can stimulate MCP-1 production in pulmonary vascular endothelial cells and in lung organ culture (22), and our laboratory now has data to suggest that HIMF can stimulate IL-4 production in rodent lung (unpublished data), suggesting

that it may be an amplifier of the Th2 response. It is possible that RELM-β induces multiple cytokines and chemokines that then act on the diseased lung.

Currently, no receptor(s) for human RELM-β or resistin has been identified, and receptors for the rodent versions of these proteins have been elusive as well. We have previously identified that murine HIMF increases rodent vascular smooth muscle cell proliferation *in vitro*, and this proliferation is inhibited by pharmacological inhibition of phosphoinositol-3-kinase (PI3K) (15). Further investigation revealed that recombinant HIMF induced phosphorylation of Akt, a downstream target of PI3K, in a time-dependent manner (15). It has also been shown that HIMF can activate the ERK1/2 signaling pathway. Chung and colleagues (42) have recently demonstrated that HIMF can activate ERK1/2 in cultured murine fibroblasts. Little is known about the signaling pathways associated with human RELM-β. In this article, we demonstrate that rhRELM-β activates ERK1/2 signaling in a time-dependent manner in both HMVEC-Ls and HPASMCs. In addition, pharmacological inhibition of this pathway blocks rhRELM-β-dependent cell growth. A few studies have provided some insight regarding the signaling pathways associated with human resistin. Calabro and colleagues (34) found that the smooth muscle cell proliferation induced by human resistin could be inhibited by PI3K- and ERK1/2-selective inhibition. In addition, Mu and colleagues (36) have demonstrated that human resistin promotes angiogenesis in human endothelial cells and activates the ERK1/2 signaling pathway. Although there is little known of the signaling pathways associated with RELM-β, we have shown evidence to suggest a role in the development of scleroderma-associated pulmonary hypertension.

Scleroderma-induced pulmonary hypertension is a complex disease with multiple etiologies. In this study, we have identified

a novel pathway that may be responsible, at least in part, for the fibrosis and vascular remodeling associated with the disease. We have demonstrated that RELM- β is expressed in the remodeling vasculature and inflammatory cells associated with pulmonary scleroderma, and that it has mitogenic effects in cultured HMVEC-L and HPASMCs. These findings could lead to a better understanding of the pathophysiology of this disease and the development of novel therapies for scleroderma-associated pulmonary hypertension.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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