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# Endothelial cells expressing endothelial and mesenchymal cell gene products in Systemic Sclerosis-associated interstitial lung disease lung tissues.

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Running Title: Endothelial cells in SSc-associated ILD express mesenchymal cell genes.

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**ABSTRACT.**

**Objective**. Examine whether lung endothelial cells (EC) from patients with Systemic Sclerosis (SSc)-associated interstitial lung disease (ILD) express mesenchymal cell markers and gene transcripts indicative of the occurrence of endothelial to mesenchymal (EndoMT) phenotypic transition.

**Methods**. Lung tissues from 6 patients with SSc-associated pulmonary fibrosis were examined by histopathology and immunohistochemistry. Confocal laser microscopy was employed to assess the simultaneous expression of EC and myofibroblast molecular markers. CD31+/CD102+ EC were isolated from lung tissues from two patients with SSc-associated ILD and from two normal lungs and the expression of EC and mesenchymal cell markers and other relevant genes were analyzed by quantitative PCR, immunofluorescence microscopy and Western blots.

**Results**. Immunohistochemistry showed cells expressing the CD31-specific EC marker in sub-endothelial, perivascular and parenchymal regions of lungs from patients with SSc-associated ILD. Confocal laser microscopy identified cells co-expressing endothelial and mesenchymal cell markers in SSc-associated ILD tissues but not in control lungs. CD31+/CD102+ EC isolated from SSc lung tissues displayed simultaneous expression of von Willebrand factor and $\alpha$-smooth muscle actin and expressed high levels of mesenchymal cell-specific genes (collagen I, collagen III and fibronectin), EC-specific genes (collagen IV and VE-cadherin), profibrotic genes (TGF-β and CTGF) and genes encoding EndoMT-related transcription factors (TWIST and SNAI2).

**Conclusion.** Cells co-expressing endothelial and mesenchymal cell-specific molecular markers are present in the lungs from patients with SSc-associated ILD. CD31+/CD102+ EC isolated from SSc-affected lungs expressed simultaneously mesenchymal and EC-specific transcripts.

Collectively, these observations strongly suggest involvement of EndoMT in the pathogenesis of

SSc-associated ILD.

**INTRODUCTION.**

Systemic Sclerosis (SSc) is a systemic autoimmune disease of unknown etiology characterized by progressive fibrosis of skin and multiple internal organs, and severe fibroproliferative vasculopathy affecting the microvasculature resulting in severe vessel narrowing or even complete obliteration (1-3). The pathogenetic mechanisms responsible for the fibrotic process and the severe vascular alterations in SSc are complex and have not been fully elucidated (4-6). Numerous recent studies have demonstrated that activated myofibroblasts are the cells ultimately responsible for the exaggerated deposition of extracellular matrix (ECM) macromolecules in skin, the parenchyma of affected organs such as the lungs and the heart, and the subendothelial space of small and medium sized arteries in SSc (7-9). The activated myofibroblasts are a unique class of mesenchymal cells characterized by specific biological functions including a motile phenotype, expression of α-smooth muscle actin (α-SMA), increased production of fibrillar collagens type I (COL1) and type III (COL3), and reduction in the expression of genes encoding ECM-degradative enzymes (9-11).

The activated myofibroblasts in fibrotic tissues emerge from several sources (12,13), including expansion of resident tissue fibroblasts (14), migration and tissue accumulation of bone marrow-derived circulating fibrocytes (15,16), and from epithelial cells, pericytes, and Gli1-positive perivascular progenitor cells that have undergone transition to a mesenchymal phenotype (17-19). More recently, it has been demonstrated that endothelial cells (EC) are also capable of undergoing a phenotypic change into mesenchymal cells in a process known as endothelial to mesenchymal transition or EndoMT (20-23). Although EndoMT was initially considered to occur only during cardiovascular embryonic development (24,25), accumulating

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evidence indicates that this process occurs in various experimentally-induced models of tissue fibrosis (20-23,26-29), and may play a role in the pathogenesis of certain human fibrotic and vascular disorders (30-34). However, the possibility that EndoMT might be an important source of the mesenchymal cells responsible for the fibroproliferative vasculopathy and the fibrotic process in SSc has not been considered until very recently (35). Demonstration that EndoMT may participate in the generation of activated myofibroblasts in SSc would be of substantial value as it may provide an important and currently unexplored target for the development of novel disease-modifying therapeutic interventions for this currently incurable disease (35). Here, we provide immunohistological and confocal microscopy evidence demonstrating that cells displaying specific EC molecular markers are present in the subendothelial, perivascular and parenchymal regions of lungs from patients with SSc-associated interstitial lung disease (ILD) and that EC expressing myofibroblast-specific molecular markers can be identified in the endothelium and subendothelial space of small arteries of lungs from patients with SSc-associated ILD. We further show that CD31+/CD102+ EC isolated from SSc-affected lungs produce numerous mesenchymal cell specific proteins and display the simultaneous expression of mesenchymal and EC-specific-transcripts. These observations provide strong support to the hypothesis that a population of activated myofibroblasts involved in the progressive intimal fibrosis, vascular occlusion and pulmonary fibrosis in SSc-associated ILD originate from lung EC through the EndoMT process. This novel mechanism may represent an important and novel therapeutic target for the severe and currently fatal complications of SSc –associated fibroproliferative vasculopathy and pulmonary fibrosis.

**MATERIALS AND METHODS.**

**Tissue samples.** Lung tissues from six patients with SSc-associated ILD were studied. Two of the lung samples were from surgical open lung biopsies obtained at Thomas Jefferson University Hospital (Patients 1 and 2 in Table I) and the other four lung samples were from patients who had undergone lung transplantation at the University of Pittsburgh Medical Center. The surgical biopsies and the lung transplants were performed following informed consent and according to IRB approved protocols from Thomas Jefferson University and the University of Pittsburgh Medical Center. Two normal lung samples obtained at necropsy served as controls for all procedures. Both of the donors of normal lung tissues died from cerebrovascular accidents. One of the normal controls was from the University of Pittsburg Medical Center and the other was obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). These two patients did not have a clinical history of pulmonary pathology and chest radiographs of both subjects obtained before their demise were normal.

**Histopathology, immunohistochemistry and confocal laser microscopy.** Paraffin embedded tissue slides were examined histopathologically employing hematoxylin-eosin and Masson's trichrome staining, and by immunohistochemical staining with specific endothelial and mesenchymal (myofibroblast) cell markers. The SSc and normal lung tissue samples were also examined by confocal laser microcopy as described previously (36,37). In all immunohistochemical and confocal laser microscopy studies samples incubated without primary antibody were used as negative controls. For immunohistochemistry the following primary antibodies were used: anti-CD31 (Neomarkers, Fremont, CA), anti-von Willebrand Factor (vWF, Dako, Denmark), anti-COL1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-COL3 (Fitzgerald, Acton, MA), and anti-α-SMA (Abcam, Cambridge, MA). For single antibody labeling,

paraffin sections were immunoassayed by the peroxidase method using the indicated

antibodies. For confocal laser microscopy paraffin samples were de-paraffinated and

dehydrated following antigen retrieval with a citric acid buffer as described (36,37). Slides were

first incubated with blocking IgG solution for 1 h and then overnight with a 1:50 dilution of anti

CD31, or a 1:200 dilution of anti α-SMA, or a 1:50 dilution of anti-vWF, or a 1:200 dilution of

anti COL1 or a 1:200 dilution of anti-COL3 antibodies. IgG binding was revealed following

incubation with (Fab')-sheep anti-rabbit Cy3 antibody and (Fab')-sheep anti-mouse FITC

antibody (Sigma, St. Louis, MO) for 1 h. Nuclei were counterstained with 4,6-diamidino-2-

phenylindole ( DAPI, Jackson ImmunoResearch Laboratories, West Grove, PA). Samples were

examined with a Zeiss 51 confocal laser microscope (Zeiss, Thronwood, NY) to evaluate the co-

localization of immunoreactivity with polyclonal and monoclonal antibodies in paired

combinations of either CD31 or vWF with either α-SMA, COL1, or COL3.

**Isolation of human lung EC.** CD31+/CD102+ EC were isolated from the lungs of two patients

with SSc-associated pulmonary fibrosis (Patients 3 and 4 on Table I) and from the two normal

lungs employing a modification of previously published methods (38,39) as described previously

(40). Briefly, lung tissue samples were minced with a scalpel and enzymatically digested with

clostridial collagenase (30 mg/100ml in 0.1% BSA, Worthington, Lakewood, NJ) at 37°C for 1h to

obtain a single cell suspension. The isolated cell suspension was immunoprecipitated with CD45

microbeads (MilTenyi BioTec, Auburn, CA) to remove non-EC as described (39). Following

immunomagnetic removal of CD45+ cells, the remaining cells were then employed for EC

isolation and purification employing immunomagnetic immunoselection with rabbit anti-human

CD31 antibody followed by magnetic bead separation using goat anti-rabbit IgG-conjugated

microbeads (1:5, Miltenyi Biotec). The isolated CD31+ EC were cultured in EC culture medium (ScienCell Research Laboratories, Carlsbad, CA) containing 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in 2% gelatin pre-coated tissue culture dishes for 5-10 days. Following expansion, the cells were re-suspended and the EC were subsequently further purified employing a second immunologic separation using rabbit anti-human CD 102 antibody to obtain a highly purified preparation of CD31+/CD102+ EC. The purified CD31+/CD102+ EC were plated on 2% gelatin coated plastic dishes, their morphology assessed by phase contrast microscopy, and their EC phenotype confirmed by evaluating the cellular uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil)-acetylated LDL (Dil-AcLDL, Biomedical Technologies, Stoughton, MA) as previously described (40). All studies were performed with cells in early passage (less than passage 5) to assure the preservation of their original phenotype.

**Immunofluorescence staining.** CD31+/CD102+ lung EC were seeded onto glass culture slides and were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 3 min. Slides were washed with PBS and blocked with PBS containing 1% BSA at room temperature for 1 h, and then they were incubated with primary antibodies against α-SMA (1:200), CD31 and vWF epitopes. Slides were then incubated with Cy3-conjugated secondary antibodies (1:500) followed by DAPI for nuclear staining.

**Western blot analysis.** Aliquots of CD31+/CD102+ lung EC culture media were processed for Western blot under denaturing conditions. Secreted proteins in the culture media were resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). Blots were blocked for 1 h in Odyssey Blocking buffer (LI-

COR, Lincoln, NE). The membranes were then incubated overnight at 4°C with a polyclonal anti-COL1 antibody (Southern Biotech, Birmingham, AL), or a polyclonal anti-COL3 antibody (Sigma-Aldrich, St. Louis, MO) in the same blocking buffer. Membranes were then washed with PBS-Tween (PBS, 0.2% Tween 20), and incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, UK) diluted 10,000-fold in Odyssey Blocking buffer. Signals were detected and quantitated employing the Odyssey Imagen System (LI-COR).

**Quantitative reverse transcription (RT)-PCR.** CD31+/CD102+ lung EC preparations from two normal and two SSc-associated ILD samples were cultured in duplicate wells of 12 well gelatin-treated plastic tissue culture dishes for 72 h and harvested with a cell lifter, washed in cold PBS and processed for RNA extraction (RNeasy kit; Qiagen, Valencia, CA) including a genomic DNA digestion step. Total RNA (1µg) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) to generate first strand cDNA. EC transcript levels were quantified using SYBR Green real time PCR as described (41). The primers employed are listed in Supplementary Table I. The differences in the number of mRNA copies in each PCR were corrected for the 18S endogenous control transcript levels; levels in control experiments were set at 100 and all other values expressed as multiples of control values. Target gene expression was estimated by the ΔΔCt method (41). The specificity of the primers was established at the end of the PCR amplification employing melt curve analyses. These studies displayed a single melt peak of the amplified product in all cases.

**Statistical analysis.** Comparative CT (ΔΔCt) analysis was performed employing DataAssist 3.0 Software ( Applied Biosystems) as described previously (41). The data obtained from SSc lung

EC from each of the two patients was compared with the average of the data obtained from the two normal lung EC preparations and expressed as fold change.

**RESULTS.**

**Clinical and demographic characteristics of SSc-associated ILD patients studied.** The clinical features of the patients and normal controls whose lung tissues were studied are shown in Table I. None of the patients had clinical features of pulmonary arterial hypertension (PAH) at the time the tissue samples were obtained and all were tested for the presence of PAH employing transthoracic echocardiograms and right heart catheterizations which were normal. One patient developed clinical PAH 10 months after the lung biopsy sample was obtained.

**Histopathology and immunohistology**. All six lung tissue samples from the SSc-associated ILD patients displayed varying degrees of interstitial fibrosis along with a mononuclear cell inflammatory infiltrate pattern. Numerous small and medium size arteries in all samples showed marked intimal proliferation resulting in narrowing of the vessel lumen and sometimes complete obliteration of the affected vessel as illustrated in Figure 1A. The endothelial cell markers CD31 and vWF were employed to identify EC. As expected, CD31 positive EC were found lining the vessel lumen as illustrated in Figure 1B-D. However, CD31 positive cells were also observed beneath the endothelial layer embedded within the subendothelial space which contained numerous elongated mesenchymal cells (Figure 1B and 1C) as well as in the perivascular tissue and within the parenchyma, often near small arteries and arterioles as illustrated in Figure 1D.

**Confocal laser microscopy**. Confocal laser microscopy showed co-localization of vWF with α-SMA in the endothelium and within the subendothelial compartment in all SSc-associated ILD

samples (Figures 2A-2C). In contrast, samples from normal controls did not show any cells co-expressing the endothelial and mesenchymal cell markers. Furthermore, in contrast with control lungs (Figure 3A), in SSc-lungs the EC layer of some vessels displayed areas of detachment from the vessel wall (Figure 3B, arrow) and numerous vessels showed portions of vessel lumen denuded from endothelial lining (Figure 3B, arrowheads). The endothelial layer in the SSc tissues also displayed very weak or absent CD31 staining (Figures 3B, 3C, and 3D) compared with the very intense CD31 staining in the vessels in the control lungs (Figure 3A). Marked sub-endothelial accumulation of COL1 (Figure 3D) and COL3 (Figures 3B and 3C) was also observed throughout the sub-intimal tissue in the SSc lung vessels. The abundant deposition of sub-endothelial collagens was very consistent regardless of the vessel size and could be appreciated in various degrees of severity in all small and medium size arterioles.

**Gene expression analysis of CD31+/CD102+ lung EC.** The gene expression assessment of immunopurified CD31+/CD102+ EC obtained from lung tissues from two patients with SSc-associated ILD compared to the average gene expression of immunopurified CD31+/CD102+ EC from two control lungs is shown in Figure 4. The results demonstrated a very strong expression of COL1A1 and COL3A1 in the CD31+/CD102+ purified EC from lungs from SSc patients and these values were up to 21 times and 26 times higher, respectively, than the expression of the same collagen genes in CD31+/CD102+ EC purified from the normal control lungs. The expression of FN1 and ACTA2 ($\alpha$-SMA), other profibrotic genes such as TGFB1 and CTGF, and that of several EndoMT-related genes such as SNAI2 and TWIST was also substantially increased in the CD31+/CD102+ EC from the lungs of SSc patients.

**Indirect immunofluorescence and Western blots of CD31+/CD102+ lung EC.** Indirect

immunofluorescence analysis of the cultured CD31+/CD102+ EC from SSc lungs showed intense

staining for the EC-specific marker vWF as well as for the myofibroblast marker $\alpha$-SMA in the

same cells (not shown). Western blots of the culture media from CD31+/CD102+ EC isolated

from the two SSc lungs confirmed the gene expression results showing statistically significant

greater amounts of type I and type III collagens compared to the culture media from the

CD31+/CD102+ EC isolated from the two normal control lungs (Figure 5).

**DISCUSSION.**

The generation of activated myofibroblasts, which are distinguished from quiescent resident

fibroblasts by the initiation of expression of α-SMA and the increased production of fibrillar

type I and type III collagens (9-11), is a crucial mechanism in the development of tissue fibrosis

in SSc (7-9). There are numerous sources of activated myofibroblasts (12,13), including TGF-β-

induced fibroblast to myofibroblast phenotypic change of resident quiescent fibroblasts (14),

recruitment and activation of bone marrow-derived fibrocytes (15,16), and transition of

epithelial cells , pericytes, and Gli1-positive perivascular progenitor cells into mesenchymal cells

(17-19). Recently, EndoMT has been recognized as an important mechanism in the generation

of activated myofibroblasts involved in the development of several experimentally-induced

animal models of fibrotic diseases (20-23,26-29) and may also play a role in some fibrotic

human diseases (30-34). EndoMT is a complex biological process during which EC lose their

specific endothelial characteristics and the expression of EC-specific molecular markers such as

VE-cadherin/Cdh5, PECAM1/CD31, ICAM2/CD102, and vWF, and concomitantly acquire the

upregulated expression of α-SMA as well as that of mesenchymal cell-specific components such

as fibrillar type I and type III collagens. The detailed mechanisms involved in EndoMT have not been fully elucidated, although the crucial role of TGF-β in its initiation and maintenance, as well as the participation of several transcription factors involved in cellular transdifferentiation including SNAI1, SNAI2 or SLUG, and TWIST1 has been well documented (20-23, 42-44). Despite the extensive experimental evidence supporting a role of EndoMT in the pathogenesis of tissue fibrosis there have been some reports raising controversy as to whether EndoMT was indeed a source of activated myofibroblasts contributing to the development of cardiac fibrosis *in vivo* (45). However, a recent study using endothelial lineage tracing in transgenic mice with experimentally-induced renal fibrosis showed that from 10 to 20% of fibroblasts in the fibrotic kidneys arise from endothelial cells via EndoMT (28) *in vivo* and another study in cardiac fibrosis also showed that a population of cardiac interstitial fibroblasts were of EC origin (46).

Here, we provide immunohistopathological evidence of the presence of cells expressing EC molecular markers in the subendothelial neointima as well as in the perivascular regions and in the parenchyma of lung tissues from patients with SSc-associated ILD and fibroproliferative vasculopathy. Furthermore, we show confocal microscopy evidence of the presence of numerous cells simultaneously expressing EC and mesenchymal cell molecular markers in the SSc lungs. Finally, we show that immunopurified CD31+/CD102+ lung EC isolated and expanded *in vitro* from lung tissues from patients with SSc-associated pulmonary fibrosis exhibited marked up-regulation in the expression and production of interstitial type I and type III collagens, increased levels of transcripts for the potent profibrotic growth factors, TGF-β and CTGF, and for several other EndoMT related proteins, such as the transcription factors TWIST and SNAI2.

The novel observations described here provide strong evidence indicative of the participation of EndoMT in the development of the severe fibroproliferative vasculopathy and progressive parenchymal fibrosis which are the hallmarks of the pulmonary involvement in SSc and are largely responsible for the high mortality and poor therapeutic response of SSc-associated interstitial lung disease (47-50). These results also suggest that greater understanding of the molecular mechanisms involved in EndoMT and its pharmacological modulation may represent a novel therapeutic approach for the fibroproliferative vasculopathy of SSc-associated tissue fibrosis including ILD (35). The feasibility of such an approach has recently been supported by the demonstration that pharmacologic modulation of EndoMT pathways was a feasible and highly effective approach *in vitro* and may be applicable as a potential therapeutic intervention (33). Finally, our observations may be relevant to other disorders with clinical manifestations caused by tissue fibrosis and a fibroproliferative vasculopathy such as PAH or idiopathic pulmonary fibrosis.

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**LEGENDS TO FIGURES.**

**Figure 1. Histopathology and immunohistology of SSc-associated ILD lung tissues**. A small artery in the lung of a patient with SSc-associated ILD stained with hematoxylin-eosin (A). Note the severe narrowing of the vessel lumen as a result of the accumulation of elongated mesenchymal cells and large amounts of fibrous tissue in the subendothelial intimal space. Immunohistochemical staining of the same tissue for the endothelial cell specific antibody marker CD31 (B, C, D). Note the presence of CD31 positive cells in the subendothelial space (B) besides their expected endothelial location. Two cells bearing the EC-specific CD31 marker are embedded within the neointimal tissue removed from the endothelium (C). CD31-positive cell clusters within the fibrotic lung parenchyma (D).

**Figure 2. Confocal microscopy staining for vWF  and α-SMA of a medium-sized arteriole in the lung of a patient with SSc-associated ILD**. Staining for vWF is shown in green (A) and staining for α-SMA is shown in red (B). Cells co-expressing vWF and α-SMA (shown in yellow in the merged image;C) are present within the endothelium and in the subendothelial tissue.

**Figure 3. Confocal microscopy staining of lung tissues from SSc-associated ILD for either COL3 or COL1 and CD31**. CD31 is stained green (A-D). COL3 (B,C) or COL1 (D) are stained red. Comparison of normal (A) and SSc (B-D) lung parenchymal arterioles and medium size arteries. In the normal lung vessels (A), there is a very strong endothelial CD31 staining and the endothelium appears well attached to the vessel architecture. COL3 staining is very faint and mostly restricted to structures beyond the elastic lamina (A). In SSc vessels (B) the endothelial

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lining is often detached from the vessel wall (arrow) and some areas of denuded endothelium are noticeable (arrowheads). CD31 staining is noticeably weaker in the endothelial layer in SSc vessels compared to normal vessels (compare Figure A with Figures B-D). Intense and abundant sub-endothelial staining of COL3 is noted in the SSc vessels (B and C) with a uniform distribution through the whole intimal and subintimal regions of the vessel wall. COL1 staining was also intense and displayed strong subendothelial distribution (D).

**Figure 4. Quantitative PCR assessment of expression levels of selected genes in CD31+/CD102+ lung EC obtained from two patients with SSc-associated ILD compared to the average of CD31+/CD102+ EC obtained from two normal lungs.** Two different preparations of CD31+/CD102+ EC isolated from the lungs of each SSc patient or from each of the normal lungs were analyzed. Each sample was analyzed in duplicate. Shown are measurements of transcripts corresponding to interstitial collagen genes (COL1 and COL3), fibronectin 1 (FN1), α-smooth muscle actin (SMA), EC-specific genes (COL4A1, VE-cadherin, vWF and VEGF), profibrotic genes (TGF-β1 and CTGF), and genes encoding EndoMT-related transcription factors (SNAI2, and TWIST1). The vertical scale shows expression levels as fold change in CD31+/CD102+ EC from each of the SSc lungs (SSc1 and SSc2) compared to the average levels of the CD31+/CD102+ EC from the normal lungs.

**Figure 5. Protein expression of CD31+/CD102+ EC from lung tissues from two patients with SSc-associated ILD compared with the average values obtained from CD31+/CD102+ EC from two normal lungs**. Western blot analysis for secreted type I and type III collagens in culture

media from duplicate cultures of CD31+/CD102+ EC from two normal and two SSc lungs. The

intensity of the bands was analyzed by fluorescence quantitation and is expressed in arbitrary

units of fluorescence. Representative Western blots for two different normal and two different

SSc cell lines are shown. *:p values for the difference between SSc and normal cells were <0.05.