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ORIGINAL RESEARCH - PRECLINICAL

Endothelial Overexpression of TGF-β-Induced Protein Impairs Venous Thrombus Resolution

Possible Role in CTEPH

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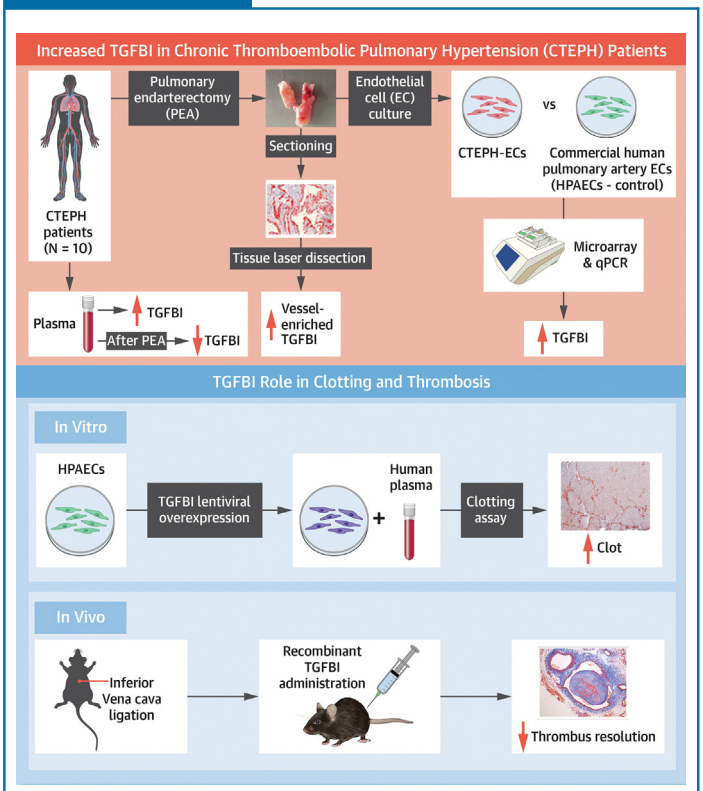
VISUAL ABSTRACT



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HIGHLIGHTS

- **Increased expression and elevated plasma levels of TGFBI were observed in endothelial cells and plasma of patients with CTEPH, and the latter significantly decreased on pulmonary endarterectomy and removal of thrombotic material from the pulmonary artery.**
- **Lentiviral overexpression of TGFBI in human pulmonary artery endothelial cells induced gene expression changes similar to those seen in primary ECs isolated from patients and altered human blood clot resolution.**
- **Delayed venous thrombus resolution was also observed in mice with elevated TGFBI plasma levels after induction of venous thrombosis.**

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SUMMARY

Endothelial cells play a critical role during venous thrombus remodeling, and unresolved, fibrotic thrombi with irregular vessels obstruct the pulmonary artery in patients with chronic thromboembolic pulmonary hypertension (CTEPH). This study sought to identify endothelial mediators of impaired venous thrombus resolution and to determine their role in the pathogenesis of the vascular obstructions in patients with CTEPH. Endothelial cells outgrown from pulmonary endarterectomy specimens (PEA) were processed for mRNA profiling, and nCounter gene expression and immunohistochemistry analysis of PEA tissue microarrays and immunoassays of plasma were used to validate the expression in CTEPH. Lentiviral overexpression in human pulmonary artery endothelial cells (HPAECs) and exogenous administration of the recombinant protein into C57BL/6J mice after inferior Vena cava ligation were employed to assess their role for venous thrombus resolution. RT2 PCR profiler analysis demonstrated the significant overexpression of factors downstream of transforming growth factor beta (TGFβ), that is TGFβ-Induced Protein (TGFBI or BIGH3) and transgelin (TAGLN), or involved in TGFβ signaling, that is follistatin-like 3 (FSTL3) and stanniocalcin-2 (STC2). Gene expression and immunohistochemistry analysis of tissue microarrays localized potential disease candidates to vessel-rich regions. Lentiviral overexpression of TGFBI in HPAECs increased fibrotic remodeling of human blood clots in vitro, and exogenous administration of recombinant TGFBI in mice delayed venous thrombus resolution. Significantly elevated plasma TGFBI levels were observed in patients with CTEPH and decreased after PEA. Our findings suggest that overexpression of TGFBI in endothelial promotes venous thrombus non-resolution and fibrosis and is causally involved in the pathophysiology of CTEPH. (J Am Coll Cardiol Basic Trans Science 2024;9:100–116) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ABBREVIATIONS AND ACRONYMS

ANGPT2 = angiopoietin 2

CD31 (PECAM1) = platelet endothelial cell adhesion molecule

CTEPH = chronic thromboembolic pulmonary hypertension

EC = endothelial cell

ELISA = enzyme-linked immunosorbent assay

EndMT = endothelial-to-mesenchymal transition

FSTL3 = follistatin-like 3

HAoAF = human aortic adventitial fibroblast

HPAEC = human pulmonary arterial endothelial cell

HUVEC = human umbilical vein endothelial cell

IPF = idiopathic pulmonary fibrosis

PAH = pulmonary arterial hypertension

PEA = pulmonary endarterectomy

STC2 = stanniocalcin 2

TAGLN = transgelin

TGF = transforming growth factor

TGFBI = transforming growth factor-β-induced protein

VEGFR = vascular endothelial growth factor receptor

Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare, progressive pulmonary vascular disease leading to (right) heart failure and death if left untreated. The characteristic feature of CTEPH, which distinguishes it from other forms of pulmonary hypertension, is the obstruction of pulmonary arteries with thrombofibrotic material.[1,2] Histologic analysis of human pulmonary endarterectomy (PEA) material revealed the simultaneous presence of fresh thrombi next to different stages of thrombus organization and fibrosis, suggesting that fibrotic tissue accumulation in CTEPH may be the result of an inadequate thrombus resolution and misguided healing response.[3,4] However, the pathomechanisms underlying the failure of thrombi to resolve and the potential mediators involved in the accumulation of thrombofibrotic tissue in CTEPH remain largely unknown.

Endothelial cells (ECs) play a pivotal role not only in the prevention of thrombus formation, but also in thrombus resolution and remodeling.[5] Experimental disruption of endothelial angiogenic signaling by genetic deletion of vascular endothelial growth factor receptors (VEGFRs) or blocking of ligand-induced VEGFR phosphorylation was shown to delay venous thrombus resolution,[6,7] whereas adenovirus-mediated transfection of VEGF accelerated thrombus recanalization.[8] Elevated circulating levels of angiopoietin 2 (ANGPT2), a negative regulator of angiogenesis, were observed in CTEPH patients, and exogenous ANGPT2 administration or transgenic endothelial ANGPT2 overexpression in mice delayed venous thrombus resolution.[4] We could recently show that activated transforming growth factor (TGF)-β signaling in ECs delays venous thrombus resolution and promotes thrombofibrosis via mechanisms involving endothelial transdifferentiation and acquisition of mesenchymal markers.[9] Collectively, these findings highlight the contribution of EC alterations for the pathophysiology of CTEPH and suggest that ECs residing in pulmonary thrombofibrotic material display a unique expression profile and secrete distinct biomarkers.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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The aim of the present study was to determine gene expression changes in ECs outgrown from PEA specimens of patients with CTEPH and to histologically localize and validate potential disease candidates mediating the endothelial-dependent nonresolution of thrombi and fibrosis, with the use of cells, mice, and patient biomaterial.

METHODS

A detailed description of the methods is provided in Supplemental Methods.

PATIENTS WITH CTEPH. Patients ≥18 years of age, diagnosed with CTEPH according to current guideline recommendations,[2] were referred for PEA to the Department of Thoracic Surgery at the Kerckhoff Clinic, Bad Nauheim, Germany, a national referral center for PEA surgery. These patients were included in the CTEPH Registry Bad Nauheim (CEPRA). Out of more than 100 PEA tissue specimens included in CEPRA, representative (based on their size and complexity of tissue organization) samples from 4 donors (mean age 63 ± 7.1 years, 50% male) were used for cryoembedding and nCounter gene expression analysis, and samples from 6 donors (mean age 67 ± 12.5 years, 50% male) were used for paraffin embedding, histology, and immunohistochemistry analysis. Samples from 10 donors (mean age 60 ± 3.1 years, 40% male) were immediately processed for EC isolation and culture followed by gene expression profiling (microarray analysis and RT^2 Profiler polymerase chain reaction [PCR] arrays). Blood samples obtained from patients with confirmed CTEPH (n = 27), who were included in CEPRA or the Pulmonary Hypertension Registry of the University Medical Center Mainz (PHYREM; German Clinical Trials Registry DRKS00006245), were used for enzyme-linked immunosorbent assay (ELISA) analysis. The results were compared with those from age- and sex-matched patients with pulmonary arterial hypertension (PAH; n = 23) included in the same registry, or to 6 apparently healthy control individuals (mean age 43 ± 5.1 years, 33% male). The clinical characteristics of the CTEPH patients examined in this study are presented in Supplemental Table 1. Out of 27 patients with CTEPH, 4 received aspirin and 2 received clopidogrel. Out of 23 patients with PAH, 10 received aspirin and 5 received clopidogrel. Both CEPRA and PHYREM are ongoing registries conducted in accordance with the amended Declaration of Helsinki and have been approved by the local ethics committees. All patients provided written informed consent for the analysis of their data, tissue, or blood samples.

HUMAN PEA TISSUE. PEA tissue samples were classified by the surgeons to 4 levels of pulmonary occlusive disease related to organized thrombus, according to Kim et al[10] and Madani et al[11] and immediately placed in sterile Dulbecco Modified Eagle Medium containing high glucose (Gibco) and transferred to the laboratory on ice. If necessary, PEA tissue specimens were cut into small pieces (~1.0 × 0.5 cm) and either incubated in 4% paraformaldehyde (Sigma-Aldrich) for 2 hours followed by embedding in Tissue-Tek OCT compound (Sakura Finetek) for cryopreservation or fixed overnight in 4% zinc formalin and embedded in paraffin wax (Leica), or processed for cell culture studies.

Paraffin-embedded tissues from patients with PAH (n = 3), patients with idiopathic pulmonary fibrosis (IPF) (n = 3), and control patients (lung transplant donors; n = 3) were provided by the Kerckhoff Clinic as previously described,[3] and the MVZ Institute for Pathology, Cytology, and Molecular Pathology, Wetzlar, Germany.

ENZYME-LINKED IMMUNOASSAYS. To determine circulating levels of potential disease candidates, plasma samples were obtained from patients with CTEPH (n = 27) and compared with those from age- and sex-matched patients with PAH (n = 23) or healthy control individuals (n = 6), and examined with the use of ELISA. To determine levels of potential disease candidates in patients with CTEPH before and after PEA, plasma samples from 18 CTEPH patients were analyzed. Venous blood was collected on the day of surgery and at 12-month follow-up. The baseline characteristics of patients examined by ELISA are included in Supplemental Table 1. Plasma levels of human transforming growth factor-β-induced protein (TGFBI) (OriGene EA100707; detection limit: <10 pg/mL), follistatin-like 3 (FSTL3) (OriGene EA100552; detection limit: <10 pg/mL), stanniocalcin 2 (STC2) (Abcam ab222880; calculated minimal detectable dose: 0.95 ng/mL), and transgelin (TAGLN) (LS Biosciences LS-F22522; detection limit: <3.75 ng/mL) were determined with the use of specific ELISAs.

HUMAN EC CULTURE. To isolate ECs from CTEPH PEA specimens (CTEPH-ECs), PEA specimens were cut into small pieces, placed in 10-cm Petri dishes precoated with 0.2% gelatin, and cultured in Endothelial Cell Growth Medium MV2 Kit (PromoCell) until confluency (up to 7 days) at 37 °C and 5% CO_2 (Supplemental Figure 1A), similarly to published protocols.[12-15] CTEPH-ECs migrated most frequently out of PEA specimens surgically classified as level I (39% of tissue samples) and level II (32%), followed by

level III lesions (25%), whereas cell outgrowth was rarely observed from level IV lesions (4%). For most of the experiments, CTEPH-ECs from level I and level II were used. CTEPH-ECs (passage 3) were compared with human pulmonary artery endothelial cells (HPAECs) (PromoCell and ATCC), cultivated in Endothelial Cell Growth Medium MV2 Kit (PromoCell). HPAECs used in this study were isolated from 3 donors (an 84-year old Caucasian man, a 51-year old Caucasian woman, and a 20-year-old Caucasian man).

ENDOTHELIAL OVEREXPRESSION OF TGFBI WITH THE USE OF LENTIVIRUS PARTICLES. HPAECs were transduced with lentiviral particles (OriGene; control particles pLenti-C-mGFP, PS100071; or TGFBI Lentiviral Particles, RC200411L2V), following the instructions of the manufacturer. Forty-eight hours later, cells were either fixed in 4% paraformaldehyde for immunofluorescence staining with primary antibody (polyclonal TGFBI antibody ab99562; Abcam) or lysed in Trizol for mRNA isolation.

IN VITRO ENDOTHELIAL CLOT RESOLUTION ASSAY. HPAECs were transduced with human TGFBI ORF tagged with green fluorescent protein (GFP) (OriGene RC200411L2V) or stimulated with recombinant TGFBI (50 ng/μL; Biozol USB-157133) mixed with human venous whole blood and allowed to clot. Human aortic adventitial fibroblasts (HAoAFs) (PromoCell) were used as control. Clotted whole blood containing HPAECs or HAoAFs was placed in nonsuspension 96-well plates (Corning), and 100 μL endothelial MV2 medium or Fibroblast Growth Medium (PromoCell) was added. Blood clots were incubated for 2 weeks at 37 °C in 5% CO_2, before being fixed in 4% zinc-formalin and embedded in paraffin wax. Five-μm-thick serial sections were cut from clots containing HPAECs, dewaxed, and stained with the use of immunohistochemical protocols.

INFERIOR VENA CAVA LIGATION AND OSMOTIC PUMP IMPLANTATION. To exclude any effects of steroid hormones or differences in vein anatomy on the response to inferior Vena cava ligation,[16] male C57BL/6J mice were subjected to inferior Vena cava ligation, as previously published.[3,9] Two days after surgery, vascular ultrasound was performed, and only mice that developed a venous thrombus >3 mm^2 (10 out of 16 mice, 62.5%) were implanted with osmotic pumps (Model 2002, Alzet) containing either recombinant TGFBI (Biozol USB-157133) or vehicle (NaCl; control). Two weeks later, the animals were killed and anticoagulated whole blood was collected to determine plasma levels of endogenous and recombinant TGFBI (ThermoFisher Scientific EMTGFBI). Thrombi were fixed in 4% zinc-formalin, paraffin embedded, and stained with the use of immunohistochemical protocols. All experiments involving animals had been a priori approved by the Translational Animal Research Committee of the University of Mainz and the Landesuntersuchungsamt Rheinland-Pfalz (animal permit G19-1-092) and complied with national guidelines for the care and use of laboratory animals. Animal experiments are reported in accordance with the ARRIVE guidelines.[17]

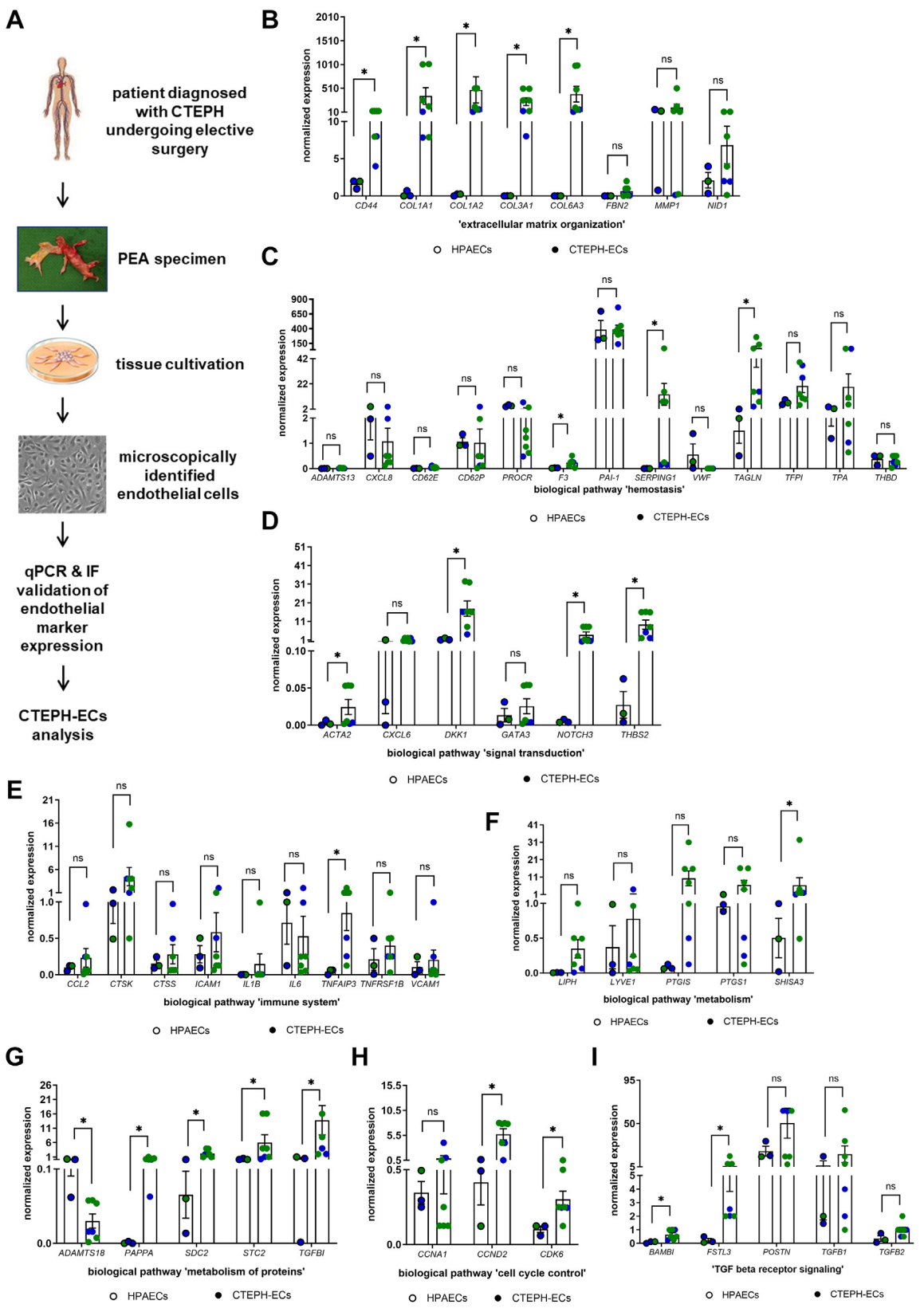
STATISTICAL ANALYSIS. Quantitative data are presented as mean ± SEM unless otherwise noted. Normal distribution was examined with the use of the Shapiro-Wilk test. If 2 groups with normal distribution were compared, Student's *t*-test was used, or Mann-Whitney *U*-test was used if data were not normally distributed. If more than 2 groups were compared, 1-way or 2-way analysis of variance followed by a Bonferroni, Dunnett, or Tukey post hoc test as appropriate was performed for normally distributed values, or Kruskal-Wallis followed by Dunn's post hoc test for multiple pairwise comparisons if not normally distributed. Findings in plasma of the same patients before and after surgical intervention were compared by means of paired *t*-test for normally distributed values or Wilcoxon signed-rank test if normal distribution was not present. All analyses were performed with the use of GraphPad Prism, version 9.0.1 for Windows (GraphPad Prism Software). Differences were considered to be statistically significant for 2-sided $P < 0.05$.

DATA AVAILABILITY STATEMENT. The data that support the findings of this study are available from the corresponding author on reasonable request.

RESULTS

CHARACTERIZATION OF ECs OUTGROWN FROM PEA SPECIMENS. To study the gene expression signature of ECs within CTEPH pulmonary lesions, freshly obtained PEA specimens from patients diagnosed with CTEPH undergoing PEA surgery (n = 10) were cultivated in endothelial growth medium to allow the outgrow of cells (**Figure 1A**). Preliminary studies were performed to optimize the isolation protocol; the results are shown in Supplemental Figure 1A. Brightfield microscopy confirmed the cobblestone appearance typical for ECs (Supplemental Figure 1B, upper panels), and confocal microscopy the expression of the endothelial adherence junction protein VE-cadherin (CDH5) (Supplemental Figure 1B, lower panels) in CTEPH-ECs outgrown from level I and II disease PEA material. Flow cytometry analysis of CD31-positive cells

FIGURE 1 Validation of Selected Genes With the Use of RT^2 Profiler PCR Array and qPCR Analysis



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(Supplemental Figure 1C) and immunofluorescence staining for CD31 and CDH5 (Supplemental Figure 1D) confirmed the endothelial phenotype of CTEPH cells, with HPAECs as positive control. Immunostaining for the proliferation marker Ki67 also did not reveal differences between CTEPH-ECs and HPAECs (Supplemental Figure 1D).

CTEPH-ECs DIFFERENTIALLY EXPRESS GENES IN ADDITIONAL BIOLOGICAL PATHWAYS. CTEPH-ECs (3 biological repeats) were subjected to exploratory microarray analysis, revealing alterations in biological pathways involved in extracellular matrix organization or degradation or in the formation, assembly, or degradation of collagens (data not presented). In addition, the expression of genes with functions potentially also relevant for the pathophysiology of CTEPH was found to be more than 2-fold up-regulated. From those, 48 genes were selected for further validation in CTEPH-ECs isolated from additional PEA specimens (7 biological repeats) using a custom-designed RT^2 Profiler PCR array (**Figure 1A**). Real-time quantitative polymerase chain reaction (qPCR) analysis was used to examine additional genes (n = 6) encoding known regulators of endothelial function. In addition to genes involved in extracellular matrix organization (*CD44*, *COL1A1*, *COL1A2*, *COL3A1*, *COL6A3*, *FBN2*, *MMP1*, and *NID1*) (**Figure 1B**), genes belonging to biological pathways such as hemostasis (*ADAMTS13*, *CXCL8*, *CD62E*, *CD62P*, *PROCR*, *F3*, *PAI-1*, *SERPING1*, *VWF*, *TAGLN*, *TFPI*, *TPA*, and *THBD*) (**Figure 1C**), signal transduction (*ACTA2*, *CXCL6*, *DKK1*, *GATA3*, *NOTCH3*, and *THBS2*) (**Figure 1D**), immune system (*CCL2*, *CTSK*, *CTSS*, *ICAM1*, *IL1B*, *IL6*, *TNFAIP3*, *TNFRSF1B*, and *VCAM1*) (**Figure 1E**), metabolism (*LIPH*, *LYVE1*, *PTGIS*, *PTGS1*, and *SHISA3*) (**Figure 1F**), metabolism of proteins (*ADAMTS18*, *PAPPA*, *SDC2*, *STC2*, and *TGFBI*) (**Figure 1G**), and cell cycle control (*CCNA1*, *CCND2*, and *CDK6*) (**Figure 1H**) were chosen. In addition, 5 highly, albeit not significantly, overexpressed genes involved in TGF-β signaling (*BAMBI*, *FSTL3*, *POSTN*, *TGFB1*, and *TGFB2*) were selected (**Figure 1I**). RT^2 Profiler and qPCR analysis confirmed significant misregulation of expression in 23 out of the 54 chosen genes compared with HPAECs (**Figures 1B-1I**, Supplemental Figure 2A), and 17 of them were significantly misregulated also compared to HUVECs (Supplemental Figure 2B).

POTENTIAL DISEASE CANDIDATES ARE HIGHLY EXPRESSED IN VESSEL-RICH REGIONS WITHIN PEA SPECIMENS. Out of the 23 genes expressed at significantly higher levels in CTEPH-ECs compared with HPAECs, 13 were selected to localize their expression to cells in PEA tissue microarrays with the use of laser microdissection followed by nCounter gene expression analysis (**Figure 2A**). Out of those, only *TGFBI* (also known as *BIGH3*) (**Figure 2B**), *COL1A1* (Supplemental Figure 3A), *FSTL3* (**Figure 2C**), *STC2* (**Figure 2D**), and *TAGLN* (**Figure 2E**) were expressed at significantly higher levels in vessel-rich areas (ie,

FIGURE 1 Continued

(A) Schematic representation of the experimental flow, including the cultivation of chronic thromboembolic pulmonary hypertension (CTEPH) endothelial cells (ECs) from pulmonary endarterectomy (PEA) specimens and preparation for further analysis. (B to I) Real-time qPCR analysis (using custom-designed RT^2 Profiler PCR assay or qPCR with custom oligonucleotides) of CTEPH-ECs (7 biological repeats) compared with human pulmonary arterial endothelial cells (HPAECs) (3 biological repeats) for genes (B) involved in extracellular matrix organization (ie, CD44 molecule [*CD44*], collagen type 1 alpha 1 chain-(*COL1A1*), collagen type 1 alpha 2 chain [*COL1A2*], collagen type 3 alpha 1 chain [*COL3A1*], collagen type 6 alpha 3 chain [*COL6A3*], fibrillin 2 [*FBN2*], matrix metalloproteinase 1 [*MMP1*], and nidogen 1 [*NID1*]) or belonging to the biological pathways (C) hemostasis (ie, ADAM metallopeptidase with thrombospondin type 1 motif 13 [*ADAMTS13*], C-X-C motif chemokine ligand 8 [*CXCL8* or *IL8*], selectin E [*CD62E*], selectin P [*CD62P*], endothelial protein C receptor [*PROCR*], coagulation factor III [*F3*], plasminogen activator inhibitor-1 [*PAI-1*], serpin family G member 1 [*SERPING1*], von Willebrand factor [*VWF*], transgelin [*TAGLN*], tissue factor pathway inhibitor [*TFPI*], tissue-type plasminogen activator [*TPA*], and thrombomodulin [*THBD*]), (D) signal transduction (ie, smooth muscle α-actin [*ACTA2*], chemokine C-X-C motif ligand 6 [*CXCL6*], Dickkopf-related protein 1 [*DKK1*], Gata-binding protein 3 [*GATA3*], neurogenic locus Notch homolog protein 3 [*NOTCH3*], and thrombospondin [*THBS2*]), (E) immune system (ie, C-C motif chemokine ligand 2 [*CCL2*], cathepsin K [*CTSK*], cathepsin S [*CTSS*], intercellular adhesion molecule-1 [*ICAM1*], interleukin-1β [*IL1B*], *IL6*, tumor necrosis factor-α-induced protein 3 [*TNFAIP3*], TNF receptor superfamily member 1B [*TNFRSF1B*], vascular cell adhesion molecule-1 [*VCAM1*]), (F) metabolism (ie, lipase H [*LIPH*], lymphatic vessel endothelial hyaluronan receptor 1 [*LYVE1*], prostaglandin 2 [*PTGIS*], prostaglandin endoperoxide synthase 1 [*PTGS1*], and Shisa family member 3 [*SHISA3*]), (G) metabolism of proteins (ie, ADAM metallopeptidase with thrombospondin type 1 motif 18 [*ADAMTS18*], pappalysin 1 [*PAPPA*], syndecan 2 [*SDC2*], stanniocalcin 2 [*STC2*], and transforming growth factor-β-induced [*TGFBI*]), and (H) cell cycle control (ie, cyclin A1 [*CCNA1*], cyclin D2 [*CCND2*], and cyclin-dependent kinase 6 [*CDK6*]), as well as (I) playing a role in TGFβ signaling (ie, bone morphogenetic protein and activin membrane bound inhibitor [*BAMBI*], follistatin like protein 3 [*FSTL3*], periostin [*POSTN*], transforming growth factor-β1 [*TGFB1*], and *TGFB2*). Quantitative data are presented as mean ± SEM. *P* values were determined with the use of Student's *t*-test for normally distributed values (*ACTA2*, *ADAMTS13*, *DKK1*, *F3*, *FBN2*, *LIPH*, *NID1*, *PAI-1*, *PTGIS*, *PTGS1*, *POSTN*, *SDC2*, *THBS2*, *TFPI*, *TNFRSF1B*, and *VWF*) and Mann-Whitney *U*-test for values that did not pass the normality test (*ADAMTS18*, *BAMBI*, *BIGH3*, *CCL2*, *CCNA1*, *CCND2*, *CD44*, *CD62E*, *CD62P*, *CDK6*, *COL1A1*, *COL1A2*, *COL3A1*, *COL6A3*, *CTSK*, *CTSS*, *CXCL6*, *CXCL8*, *FSTL3*, *GATA3*, *ICAM1*, *IL1B*, *IL6*, *LYVE1*, *MMP1*, *NOTCH3*, *PAPPA*, *PROCR*, *SERPING1*, *SHISA3*, *STC2*, *TAGLN*, *TGFB1*, *TGFB2*, *THBD*, *TNFAIP3*, *TPA*, and *VCAM1*) and are shown within the graph: *$P < 0.05$; ns = nonsignificant. Data points in blue are from male patients, green from female patients.

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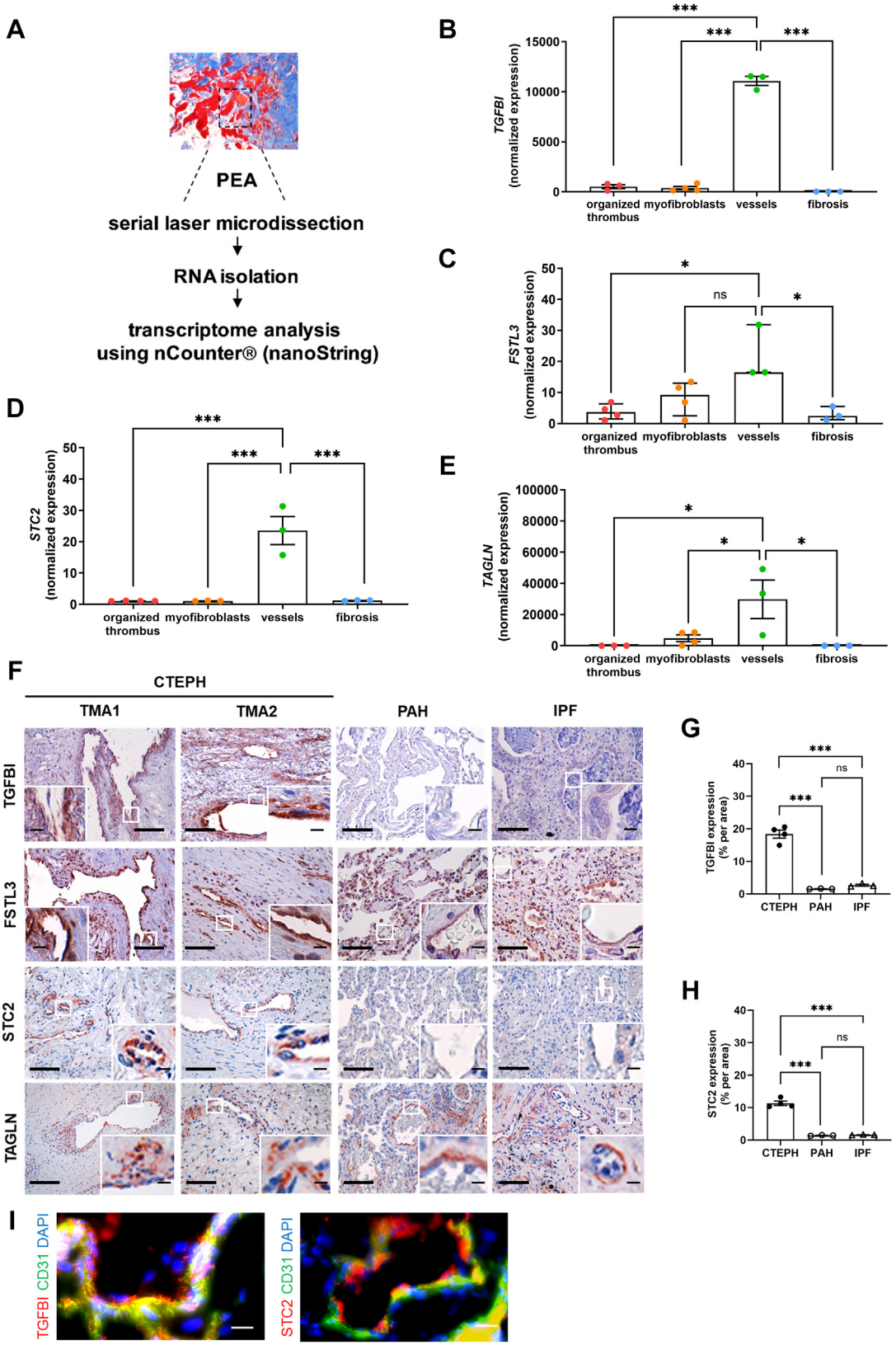
FIGURE 2 Histologic Localization of Selected Genes in PEA Tissue Microarrays



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areas containing the highest number of endothelial-lined structures with a lumen, compared to areas histologically classified as organized thrombus, myofibroblasts, or fibrosis, according to Bochenek et al[3]). In addition, *COL3A1* (Supplemental Figure 3B), *NOTCH3* (Supplemental Figure 3C), and *SERPING1* (Supplemental Figure 3D) were expressed significantly higher in vessel-rich area compared with organized thrombus, whereas *COL1A2* (Supplemental Figure 3E), *COL6A3* (not shown), *CD44* (Supplemental Figure 3F), TGFBI (Supplemental Figure 3G), and *THBS2* (Supplemental Figure 3H) were expressed at similar levels in all of the above areas. qPCR analysis of HPAECs at 2 different passage numbers showed that mRNA levels of *TGFBI*, *FSTL3*, *STC2*, and *TAGLN* do not depend on length of the cell culture (Supplemental Figure 4). Immunohistochemistry confirmed strong expression of TGFBI, FSTL3, STC2, and TAGLN in cells lining vessels within tissue microarrays from CTEPH PEA specimens (**Figure 2F**); additional findings, also in lungs from transplant donors, are shown in Supplemental Figures 5A and 5B). Importantly, only TGFBI (**Figure 2G**) and STC2 (**Figure 2H**) immunosignals were specifically increased in CTEPH and also significantly higher compared with pulmonary lesions of patients with PAH or IPF, whereas FSTL3 and TAGLN were also observed in cells lining vascular structures in PAH and IPF (**Figure 2F**). However, confocal fluorescence microscopy showed TGFBI expression in CD31-immunopositive cells, whereas STC2 expression did not directly overlap with CD31 immunosignals (**Figure 2I**), suggesting endothelial-specific expression only of TGFBI.

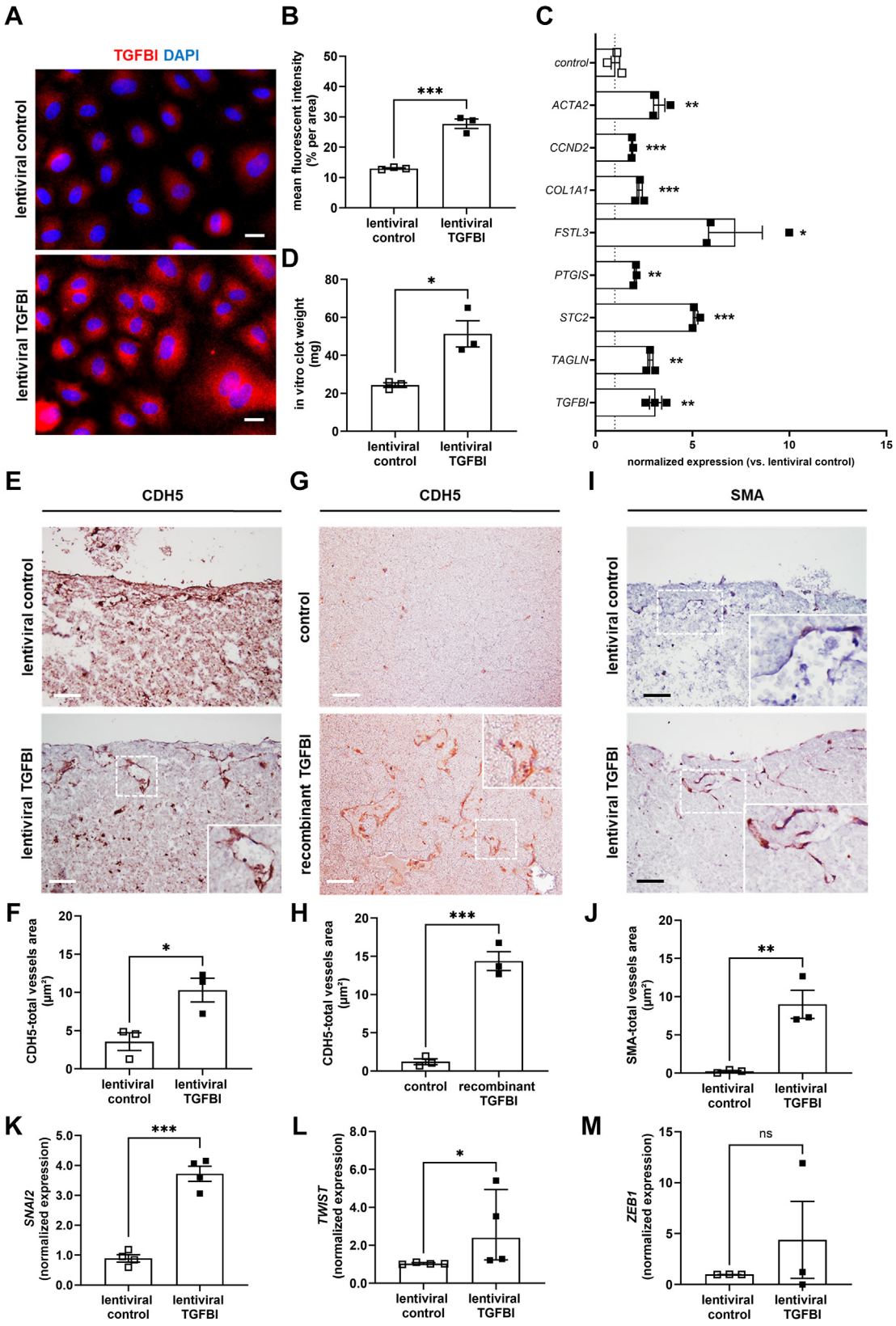
OVEREXPRESSION OF TGFBI IN HUMAN PULMONARY ARTERY ECs PARTLY PHENOCOPIES THE GENE EXPRESSION CHANGES SEEN IN CTEPH-ECs. We selected TGFBI as the most promising candidate to study its function in CTEPH-ECs. For this, gain-of-function experiments were performed, and HPAECs were transduced with the use of GFP-tagged lentiviral particles containing human TGFBI. Overexpression of TGFBI in HPAECs was confirmed with the use of immunofluorescence staining (**Figures 3A and 3B**); lentiviral GFP expression is shown in Supplemental Figure 6A. RT^2 Profiler PCR array analysis revealed significantly increased mRNA levels of several of the genes significantly up-regulated in CTEPH-ECs, that is, *ACTA2*, *CCND2*, *COL1A1*, *FSTL3*, *PTGIS*, *STC2*, and *TAGLN* (**Figure 3C**), thus positioning these changes downstream of TGFBI, whereas other genes differentially expressed in CTEPH-ECs were not altered (data not presented).

ENDOTHELIAL OVEREXPRESSION OF TGFBI RESULTS IN LARGER HUMAN BLOOD CLOTS. To study the role of endothelial TGFBI overexpression for thrombus resolution and remodeling in CTEPH, human whole blood was mixed with HPAECs overexpressing lentiviral TGFBI, allowed to clot, and incubated in endothelial culture medium for 2 weeks. In addition to significantly higher thrombus weights (**Figure 3D**), immunostaining of CDH5 to visualize ECs revealed larger, irregular vascular structures in clots containing HPAECs overexpressing TGFBI (**Figures 3E and 3F**). Similar findings were observed in HPAECs stimulated with recombinant TGFBI (**Figures 3G and 3H**). Interestingly, clots containing HAoAF also were significantly larger than clots containing just HPAECs, similarly to HPAECs treated with recombinant TGFBI (Supplemental Figure 6B), further supporting a role for a fibroblast-like phenotype acquisition in ECs on TGFBI treatment. Furthermore, vascular structures in clots mixed with TGFBI-overexpressing human ECs exhibited a more pronounced expression of the mesenchymal marker smooth muscle actin (**Figures 3I and 3J**). Real-time qPCR analysis revealed

FIGURE 2 Continued

(A) Flowchart showing consecutive steps to histologically localize selected genes to specific areas in CTEPH PEA specimens. Laser microdissection and nCounter analysis of mRNA expression levels of (B) *TGFBI*, (C) *FSTL3*, (D) *STC2*, and (E) *TAGLN* in PEA specimens isolated from 4 CTEPH patients. Quantitative data are presented as mean ± SEM, and *P* values were determined by comparing findings in vessels area with all other areas (3 comparisons) by means of 1-way ANOVA followed by Bonferroni's multiple comparisons test for normally distributed values (B, D, E). Values that did not pass the normality test are presented as median with IQR, and *P* values were determined by means of Kruskal-Wallis test (C). *$P < 0.05$; ***$P < 0.001$; ns = nonsignificant. (F) Representative images after immunohistochemical staining of TGFBI, FSTL3, STC2, and TAGLN on tissue microarrays from patients with CTEPH (PEA material: TMA1 and TMA2), pulmonary arterial hypertension (PAH; lung biopsies) or idiopathic pulmonary fibrosis (IPF; lung biopsies). Insets show higher magnification of selected areas of interest. Scale bars represent 100 μm; scale bars in insets represent 10 μm. Semiquantitative analysis of (G) TGFBI and (H) STC2 expression in tissue. Quantitative data are presented as mean ± SEM, and *P* values were determined by comparing the mean of each group with the mean of every other group (3 comparisons) by means of 1-way ANOVA followed by Bonferroni's multiple comparisons test. ***$P < 0.001$; ns = nonsignificant. (I) Representative confocal images of (left) CD31-positive cells (green) expressing TGFBI (red) and (right) STC2 (red) on cryopreserved CTEPH PEA tissue. Scale bars represent 50 μm. Abbreviations as in **Figure 1**.

FIGURE 3 Overexpression of TGFBI in HPAECs Promotes the Expression of Genes Similar to CTEPH-ECs and Results in Larger Human Blood Clots



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significantly increased levels of transcriptional factors regulating the expression of mesenchymal markers, such as *SNAI2* (Figure 3K) and *TWIST* (Figure 3L), whereas expression of *ZEB1* did not differ (Figure 3M).

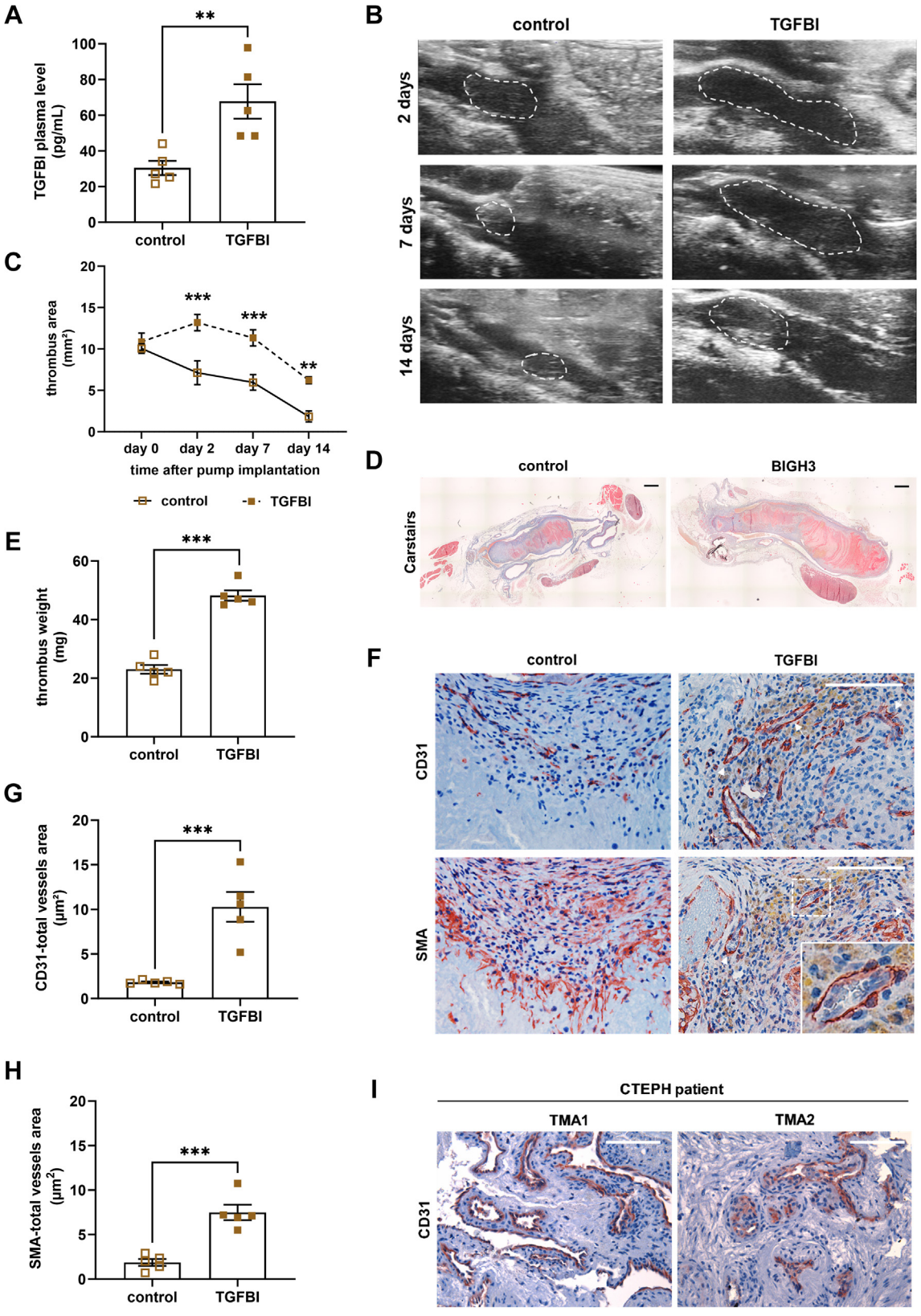
ADMINISTRATION OF RECOMBINANT TGFBI DELAYS THROMBUS RESOLUTION IN MICE. Next, and to examine the role of increased circulating TGFBI levels in venous thrombus resolution in vivo, male C57BL/6J mice were subjected to IVC ligation. On day 2 after surgery, osmotic minipumps filled with recombinant murine TGFBI or vehicle alone (NaCl; control), were implanted on the backs of mice that had developed a venous thrombus >3 mm^2 (10 out of 16 mice, 62.5%), as confirmed by vascular ultrasound. ELISA analysis of plasma from those mice 2 weeks after surgery showed that endogenous and recombinant TGFBI plasma concentrations were elevated 3-fold compared with control-treated mice (Figure 4A). Importantly, ultrasound analysis revealed that both the absolute (Figures 4B and 4C) and the relative (expressed as % of thrombus size at the time of pump implantation; not shown) thrombus sizes were significantly increased in mice receiving recombinant TGFBI compared with the control treatment, beginning at day 2 after pump implantation and lasting until day 14. Histochemical staining of longitudinal sections (Figure 4D) as well as measuring the thrombus weight at the time of tissue harvest (Figure 4E) confirmed larger venous thrombi in mice treated with TGFBI. Similarly to our in vitro observations, histochemical analysis revealed that the area of vessels immunopositive for endothelial (CD31) or mesenchymal (smooth muscle actin and platelet-derived growth factor receptor α) markers was significantly larger in venous thrombi of TGFBI-treated mice compared with the control-treated mice (Figures 4G and 4H, Supplemental Figures 7A and 7B). Importantly, the vessels within the murine TGFBI-exposed venous thrombi were similar to the vascular structures seen in CTEPH PEA specimens (Figure 4I). On the other hand, the area immunopositive for the macrophage marker Mac-2 did not significantly differ (Supplemental Figures 7C and 7D).

CIRCULATING LEVELS OF TGFBI ARE ELEVATED IN PATIENTS WITH CTEPH AND DECREASE AFTER PEA. To translate our findings to human disease, plasma concentrations of TGFBI were measured in patients with CTEPH (baseline characteristics presented in Supplemental Table 1) and compared with those in plasma of age- and sex-matched patients with PAH (Figure 5A). ELISA analysis revealed that circulating levels of TGFBI (Figure 5B) and STC2 (Figure 5C) were significantly higher in patients with CTEPH than in those with PAH, and only TGFBI plasma levels were also significantly higher compared with healthy control subjects. On the other hand, plasma levels of FSTL3 (Figure 5D) and TAGLN (Figure 5E) were significantly elevated compared with healthy control subjects, but did not differ from those in patients with PAH. Importantly, analysis of plasma levels before and at 12-month follow-up after PEA revealed a significant reduction of plasma levels of TGFBI (Figure 5F), whereas plasma levels of FSTL3 (Figure 5G) and TAGLN (Figure 5H) did not change,

FIGURE 3 Continued

(A) Representative immunofluorescence images and (B) quantitative analysis of the mean fluorescent intensity of HPAECs in culture after treatment with control lentiviral particles (lentiviral control) or lentiviral particles containing human TGFBI (TGFBI lentiviral particles) and immunostaining for TGFBI (red) and DAPI (blue). Quantitative data are presented as mean ± SEM. ***$P < 0.001$ (Student's *t*-test). Scale bars represent 10 μm. (C) Quantitative RT^2 Profiler PCR array analysis confirmed significantly increased expression of TGFBI and also revealed significant changes of genes belonging to the biological pathways signal transduction (*ACTA2*), metabolism of proteins (*STC2*), cell cycle control (*CCND2*), hemostasi' (*TAGLN*), and metabolism (*PTGIS*) and genes involved in TGFβ signaling (*FSTL3*) and extracellular matrix organization (*COL1A1*) in HPAECs overexpressing TGFBI (n = 3 experimental repeats; female donor). Data are expressed as fold change vs lentiviral control cells. Quantitative data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test). (D) Weight of blood clots generated in vitro followed by addition of HPAECs expressing lentiviral TGFBI or control and incubation for 14 days (n = 3 biological repeats and 2 experimental repeats). Quantitative data are presented as mean ± SEM. *$P < 0.05$ (Student's *t*-test). (E and G) Representative immunohistochemical images of human blood clots after immunostaining of the endothelial marker VE-cadherin (CDH5) and the results of the quantitative analysis of the total vessel area after addition of HPAECs (F) overexpressing TGFBI or (H) stimulated with recombinant TGFBI. Scale bars represent 10 μm. Quantitative data are presented as mean ± SEM. *$P < 0.05$; ***$P < 0.001$ (Student's *t*-test). (I) Representative immunohistochemical images and (J) results of the quantitative analysis of human blood clots mixed with human ECs overexpressing lentiviral TGFBI or control after immunostaining of the mesenchymal marker smooth muscle actin (SMA). Scale bars represent 10 μm. Quantitative data are presented as mean ± SEM. **$P < 0.01$ (Student's *t*-test). Real-time qPCR analysis to detect the transcription factors (K) *SNAI2*, (L) *TWIST*, and (M) *ZEB1* in HPAECs overexpressing TGFBI or in lentiviral control cells. Data were normalized to the housekeeping gene HPRT1. Quantitative data are presented as mean ± SEM and compared by means of Student's *t*-test (K and M), or as median with IQR and compared by means of Mann-Whitney *U*-test (L). *$P < 0.05$; ***$P < 0.001$; ns = nonsignificant. Abbreviations as in Figure 1.

FIGURE 4 Exogenous Administration of TGFBI to Male C57BL/6J Mice After Inferior Vena Cava Ligation Delays Venous Thrombus Resolution and Promotes Irregularly Vascularized, Fibrotic Thrombi



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further strengthening the potential of TGFBI as circulating marker of thrombofibrosis in CTEPH.

DISCUSSION

The central role of ECs in the pathophysiology of CTEPH is supported by the results of a number of experimental studies in which the role of specific endothelial factors during thrombus remodeling was examined.[4-8,18] However, comprehensive gene expression analyses and studies on their spatial expression patterns and functions in chronic thrombotic disease are sparse. Using RT^2 Profiler PCR analysis, we show that CTEPH-ECs from PEA specimens overexpress components and regulators of the TGFβ signaling pathway, such as TGFBI, FSTL3, STC2, and TAGLN. nCounter gene expression analysis of laser-microdissected PEA material and immunohistochemical analysis of tissue microarrays localized these differentially expressed genes to vessel-rich regions. Importantly, TGFBI and STC2 were detected in vascular structures in CTEPH but not in other pulmonary vasculopathies such as in PAH or IPF. Gain-of-function experiments in human pulmonary artery ECs demonstrated that endothelial overexpression of TGFBI induced the transcription of STC2, as well as of FSTL3, TAGLN, and others, thus positioning TGFBI upstream of the gene expression changes seen in CTEPH-ECs, and was associated with larger blood clots formed in vitro. Proof-of-concept studies showed that exogenous administration of TGFBI to C57BL/6J mice after venous thrombus formation delayed thrombus resolution and resulted in larger thrombi containing irregular vascular structures lined by ECs expressing mesenchymal markers. Underscoring the translational importance of our findings, TGFBI plasma levels were significantly higher in patients with CTEPH compared with those with other, nonthrombotic forms of pulmonary hypertension and decreased after surgical removal of the obstructive thrombofibrotic material.

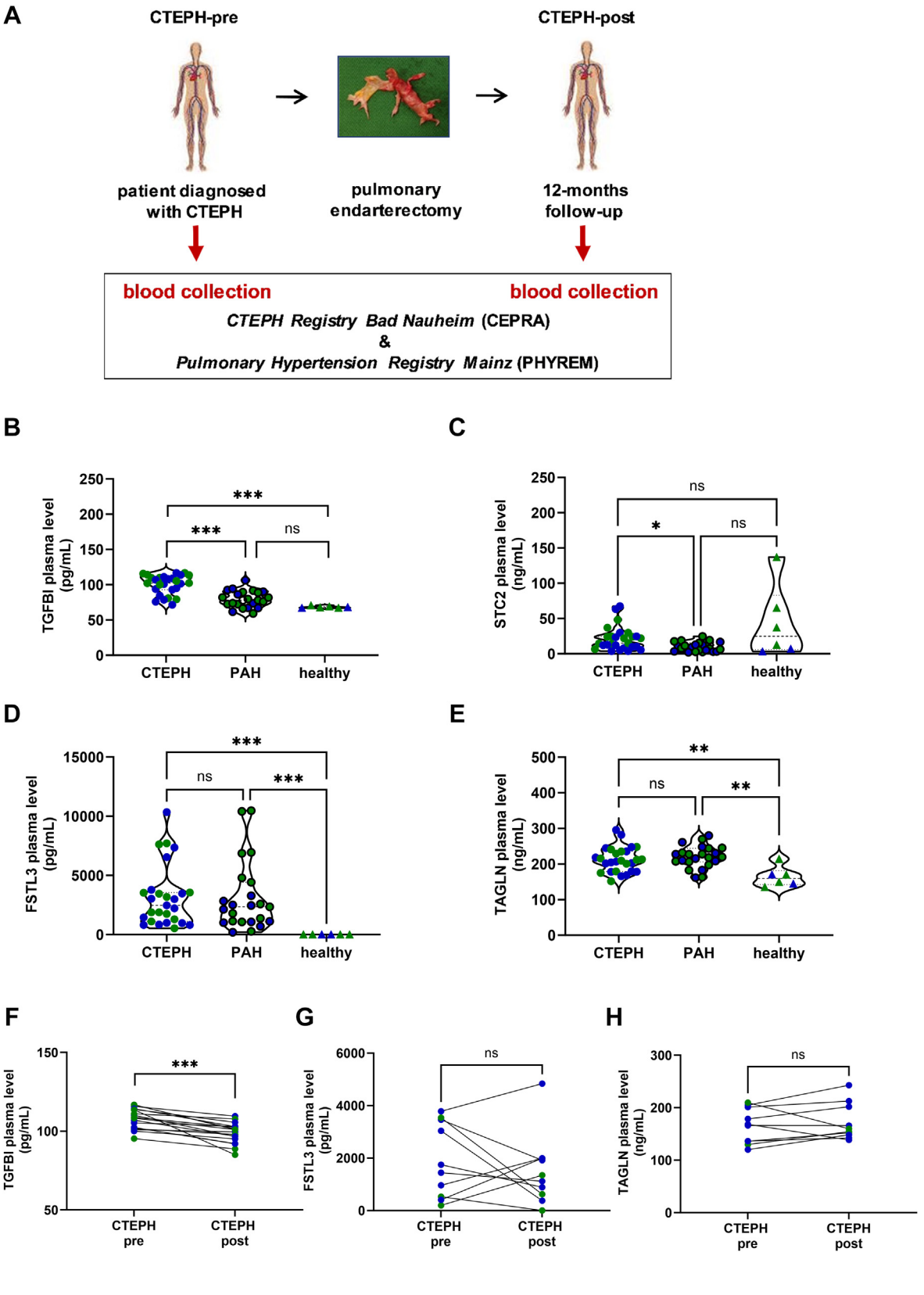
Although general EC markers, such as CDH5 and CD31, were not found to be altered, specific intrinsic differences in CTEPH-ECs are suggested by findings that ECs within PEA tissue specimens exhibit highly increased expression of markers of the mesenchymal lineage, such as *ACTA2* and *TAGLN*, in line with their reported higher potential to undergo endothelial-to-mesenchymal transition (EndMT).[9,19] EndMT is induced by TGFβ and involved in fibrotic processes in lung, heart, and liver[20,21] as well as venous thrombofibrosis,[9,22] and EndMT transcription factors were also found to be up-regulated during angiogenic sprout formation.[23] We have recently shown that overactivated TGFβ signaling in ECs promotes endothelial phenotype conversion and the acquisition of mesenchymal features via mechanisms involving endothelin 1.[9] Although TGFβ signaling pathways were found to be differentially regulated in previous studies examining alterations in gene expression patterns in CTEPH,[24,25] the functional relevance and translational implications of such findings had not been examined. Moreover, and in contrast to those previous studies, our analyses focused on ECs and particularly those with the potential to grow and migrate out of PEA tissue specimens, similarly to human pulmonary artery ECs examined in parallel.

Endothelial extracellular matrix proteins and the local microenvironment support key functions in vascular remodeling and stabilization processes.[26] Our analyses identified the secreted extracellular matrix protein TGFBI as one potential candidate acting to promote thrombofibrosis in CTEPH. TGFBI (or BIGH3) is also known as kerato-epithelin, and a point mutation in the TGFBI gene located on

FIGURE 4 Continued

(A) Results of the quantitative analysis using enzyme-linked immunosorbent assay (n = 5 biological repeats per group) of plasma levels of total (endogenous and recombinant) TGFBI in C57BL/6J mice implanted with osmotic pumps containing recombinant TGFBI or vehicle (NaCl; control). Quantitative data are presented as mean ± SEM. $^{**}P < 0.01$ (Student's *t*-test). (B) Representative ultrasound images of venous thrombi in mice subjected to inferior Vena cava ligation followed by implantation of osmotic pumps containing either recombinant TGFBI or vehicle alone (NaCl; control). (C) Quantitative analysis of the absolute thrombus size (in mm^2; 5 biological repeats; at day 2 after implantation 2 mice from the control group and 1 mouse from the TGFBI-treated group were not examined. Quantitative data are presented as mean ± SEM, *P* values were determined by comparing findings in TGFBI-treated mice with those in control-treated mice at the same time point by means of 2-way ANOVA followed by Sidak's multiple comparisons test (4 comparisons). $^{**}P < 0.01$; $^{***}P < 0.001$. (D) Representative images following Carstairs staining of longitudinal sections through isolated IVC segments. Scale bars represent 100 μm. (E) Summary of thrombus weights 2 weeks after pump implantation. Quantitative data are presented as mean ± SEM. $^{***}P < 0.001$ (Student's *t*-test). (F) Representative immunohistochemical images showing CD31-positive endothelial cells and smooth muscle actin (SMA)–positive mesenchymal cells on neighboring sections. Scale bars represent 100 μm. Quantitative analysis of the total vessel area, defined as a lumen (G) covered by CD31-positive ECs or (H) surrounded by SMA-positive mesenchymal cells. Quantitative data are presented as mean ± SEM. $^{***}P < 0.001$ (Student's *t*-test). (I) Representative immunohistochemical images of 2 examples of CTEPH tissue microarrays (TMA1 and TMA2) showing CD31-positive endothelial cells. Scale bars represent 10 μm. Abbreviations as in **Figure 1**.

FIGURE 5 Plasma Levels of TGFBI, STC2, FSTL3, and TAGLN in Patients With CTEPH, Patients With PAH, and Healthy Control Subjects



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chromosome 5q31 locus is the cause of a hereditary corneal dystrophy.[27] In addition, a large number of studies have documented a role for TGFBI in cancer, where it may act as tumor suppressor or promoter[28] or promote aberrant angiogenesis.[29] TGFBI has been identified as a major TGFβ-responsive gene residing in the extracellular matrix, where it interacts with collagen, fibronectin, laminin, and glycosaminoglycans[30] and functions as a link between extracellular matrix proteins and integrins.[31] Regarding the vascular abnormalities seen in patients with CTEPH, binding of TGFBI via its RGD-binding motif to integrin αvβ3 or αvβ5 may alter VEGFR2 signaling, affecting endothelial proliferation and migration during vessel formation.[32] Indeed, TGFBI was found to be highly expressed in myofibroblasts of the developing lung and to promote nuclear factor κB activation, resulting in pulmonary EC migration and angiogenesis.[33] The importance of changes in TGFBI in the context of pulmonary vasculopathy associated with fibrosis is supported by the findings of the present study, both in vitro and in vivo. Importantly, and as previously reported for circulating C-reactive protein and D-dimer levels,[34] we found that plasma TGFBI levels decreased on removal of the thrombofibrotic material from the pulmonary arterial tree. Changes in TGFBI expression in fibrotic lung disease have been reported by others, for example, in rats following bleomycin-induced pulmonary fibrosis[35] and in patients with idiopathic pulmonary fibrosis,[36] and its profibrotic activities were found to involve induction of the transcriptional repressor SNAIL[35] or down-regulation of matrix metalloproteinase-14 and changes in collagen turnover.[37] It is also worth mentioning that TGFBI is expressed in human platelets as well, and its release from activated platelets may promote thrombogenesis,[38] but may also explain the profibrotic effects of platelet releasates.[39] Tumor-associated macrophages also have been shown to secrete TGFBI.[40] Of note, we did not observe differences in the macrophage content in venous thrombi of mice treated with recombinant TGFBI.

In addition to TGFBI, we observed an up-regulation of other members of the TGFβ signaling pathway, such as FSTL3, a secreted glycoprotein that binds and neutralizes TGFβ ligands, including activins.[41] We found local expression and circulating levels of FSTL3 to be increased in patients with CTEPH, suggesting a possible role of this protein in pulmonary fibrotic tissue accumulation. Overexpression of FSTL3 in ECs derived from pluripotent stem cells was shown to enhance endothelial angiogenic features via induction of endothelin-1.[42] However, and in contrast to TGFBI, we observed no significant differences in FSTL3 plasma levels between patients with CTEPH and those with PAH, suggesting a role in pulmonary remodeling independent from thrombosis. Of note, lentiviral overexpression experiments suggested that changes in the expression of FSTL3 and other genes may have occurred secondary to TGFBI. Another TGF-β-inducible gene found to be significantly up-regulated in CTEPH-ECs is TAGLN, an actin-cross-linking protein also known as SM22α. It has been shown that TAGLN is up-regulated by activated TGF-β1 signaling[43] and that its expression is further enhanced by inflammation[44] or in response to hypoxia inducible factor (HIF)-2α.[43] TAGLN is an early marker of smooth muscle cell differentiation,[45] and its expression is increased during EndMT, as shown after bone morphogenetic protein 9 stimulation of lung ECs.[46] The expression of STC1 and its paralog STC2, another potential disease candidate selected for further validation in the present study, is also regulated by hypoxia and HIF1α,[47] and previous work has shown that overexpression of STC2 in HUVECs promotes proliferation, migration and tube formation.[48] Interestingly, mesenchymal stem cells (MSCs) secrete large amounts of STC1 in response to TGF-β1, whereas STC1 plasmid-transfected mesenchymal stem cells exert antifibrotic effects.[49] As a result, STC1 molecular therapy has been suggested to target increased TGF-β signaling and epithelial-mesenchymal transition in IPF.[50]

FIGURE 5 Continued

(A) Flowchart showing steps of blood analyses in patients with CTEPH enrolled in the CTEPH Registry Bad Nauheim, before and at 12-month follow-up after PEA and in patients with PAH enrolled in the Pulmonary Hypertension Registry Mainz. Results of the quantitative analysis using specific enzyme-linked immunosorbent assay (ELISA) of plasma levels of (B) TGFBI, (C) STC2, (D) FSTL3, and (E) TAGLN in patients with CTEPH (n = 27) compared with patients with PAH (n = 23) or healthy control individuals (n = 6). Quantitative data are presented as violin plot (showing all points). *P* values were determined by comparing the mean of each group with the mean of every other group (3 comparisons) by means of 1-way ANOVA followed by Bonferroni's multiple comparisons test for normally distributed values (E) or Kruskal-Wallis test followed by Dunn's multiple comparisons test for values which did not pass the normality test (B, C, D). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns = nonsignificant. Results of the quantitative analysis using ELISA of plasma levels of (F) TGFBI (n = 18), (G) FSTL3 (n = 10), and (H) TAGLN (n = 10) in patients with CTEPH, before (CTEPH pre) and at 12-month follow-up after PEA surgery (CTEPH post). Quantitative data are presented as symbols and lines. *P* values were determined with the use of paired Student's *t*-test (F) or Wilcoxon matched-pairs signed rank test (G, H). ***$P < 0.001$; ns = nonsignificant. Data points in blue are for male patients, in green for female.

STUDY LIMITATIONS. A possible caveat when interpreting the findings of the present study is that the observed changes in extracellular matrix remodeling may be due to the analysis of tissue samples from patients with symptomatic late-stage disease undergoing PEA surgery. However, we have previously shown that PEA samples from any given patient contain different stages of thrombus formation and organization.[3] Another limitation of the present study is that the comparative analysis of plasma samples from patients with CTEPH vs PAH was performed within a cohort (PHYREM) other than that from which the tissue specimens and the pre- vs post-PEA blood probes were obtained (CEPRA). However, both registries were performed in large centers in which the diagnosis of CTEPH (and PAH, where applicable) is standardized on the basis of current guideline recommendations.[2] It should also be noted that selections for specific genes for further validation had to be made at different points during the study and that our findings do not preclude a role for additional endothelial factors.

CONCLUSIONS

The results of the present study strengthen the importance of EC phenotype alterations and induction of TGF-β signaling during thrombus nonresolution and fibrosis, and identify TGFBI as a specifically up-regulated endothelial marker and a possible causal factor in the pathogenesis of CTEPH. Although the potential diagnostic value of TGFBI needs to be further studied and validated in independent cohorts, measurement of specifically up-regulated markers may be more suitable for the detection and monitoring of CTEPH progression compared with general biochemical markers reflecting systemic inflammation or thrombosis. Regarding the latter, direct comparisons of plasma TGFBI levels and circulating markers of endothelial activation or D-dimer levels could not be performed in the present study.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: CTEPH is characterized by the obstruction of pulmonary arteries by organized thrombi leading to elevated pulmonary arterial pressure and eventually right heart failure. PEA and anticoagulant therapy is the treatment of choice for eligible patients with CTEPH. However, timely diagnosis and treatment are often hampered by nonspecific symptoms and signs. The findings of this study suggest that plasma levels of TGFBI are specifically elevated in plasma of patients with CTEPH, but not in those with other, nonthrombotic forms of PAH, and that circulating TGFBI levels decrease on removal of thrombotic material, suggesting a causal role of TGFBI in this disease.

TRANSLATIONAL OUTLOOK: The results of our analyses, using biomaterial from patients with CTEPH and other forms of pulmonary hypertension, human primary ECs in culture, and a mouse model of venous thrombosis, strongly suggest a role for endothelial TGFBI overexpression and induction of downstream gene expression changes in venous thrombus vascularization and remodeling that could be exploited to improve not only the diagnosis and monitoring of the disease, but also EC-mediated thrombus resolution.

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APPENDIX For supplemental methods, figures, and tables, please see the online version of this paper.