ORIGINAL ARTICLE

Increased Extracellular Vesicles Mediate WNT5A Signaling in Idiopathic Pulmonary Fibrosis

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Abstract

Rationale: Idiopathic pulmonary fibrosis (IPF) is a lethal lung disease characterized by lung epithelial cell injury, increased (myo)fibroblast activation, and extracellular matrix deposition. Extracellular vesicles (EVs) regulate intercellular communication by carrying a variety of signaling mediators, including WNT (wingless/integrated) proteins. The relevance of EVs in pulmonary fibrosis and their potential contribution to disease pathogenesis, however, remain unexplored.

Objectives: To characterize EVs and study the role of EV-bound WNT signaling in IPF.

Methods: We isolated EVs from BAL fluid (BALF) from experimental lung fibrosis as well as samples from IPF, non-IPF interstitial lung disease (ILD), non-ILD, and healthy volunteers from two independent cohorts. EVs were characterized by transmission electron microscopy, nanoparticle tracking analysis, and Western blotting. Primary human lung fibroblasts (phLFs) were used for EV isolation and analyzed by metabolic activity assays, cell counting, quantitative PCR, and Western blotting upon WNT gain- and loss-of-function studies.

Measurements and Main Results: We found increased EVs, particularly exosomes, in BALF from experimental lung fibrosis as well as from patients with IPF. WNT5A was secreted on EVs in lung fibrosis and induced by transforming growth factor-β in primary human lung fibroblasts. The phLF-derived EVs induced phLF proliferation, which was attenuated by WNT5A silencing and antibody-mediated inhibition and required intact EV structure. Similarly, EVs from IPF BALF induced phLF proliferation, which was mediated by WNT5A.

Conclusions: Increased EVs function as carriers for signaling mediators, such as WNT5A, in IPF and thus contribute to disease pathogenesis. Characterization of EV secretion and composition may lead to novel approaches to diagnose and develop treatments for pulmonary fibrosis.

Keywords: lung fibrosis; exosomes; lung fibroblasts; proliferation; WNT5A

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At a Glance Commentary

Scientific Knowledge on the Subject: Extracellular vesicles (EVs) are potent mediators of intercellular communication and have recently been implicated in chronic lung diseases. However, the relevance of EVs in pulmonary fibrosis and their potential contribution to pathogenesis remain unexplored.

What This Study Adds to the Field: We report for the first time that EVs are increased in experimental and human pulmonary fibrosis and lead to altered fibroblast function in disease. We show that the WNT (wingless/integrated) protein WNT5A is secreted on EVs and can be found in BAL fluid from patients with idiopathic pulmonary fibrosis (IPF). WNT5A on EVs isolated from IPF BAL fluid led to increased fibroblast proliferation, thus highlighting a pathophysiological role of EVs in IPF.

Idiopathic pulmonary fibrosis (IPF) is a lethal interstitial lung disease (ILD) of yet unknown etiology and limited therapeutic options. Current evidence suggests that IPF is a result of ongoing lung epithelial cell injury and aberrant wound healing, which impairs epithelial-to-mesenchymal cross-talk and subsequently leads to myofibroblast activation and increased deposition of extracellular matrix components (1, 2). Extracellular vesicles (EVs) are membranous-like vesicles with a diameter between 30 and 2,000 nm capable of transporting proteins, lipids, and nucleic acids (3) and are mediators of intercellular communication under both physiological and disease conditions (4). Recent studies have highlighted the potential contribution of EVs to chronic lung diseases and have investigated the role of serum-derived EVs as potential biomarkers (5–7). The expression and function of EVs in the local lung environment in the context of lung fibrosis and remodeling, however, remain largely unexplored.

Alterations in the WNT (wingless/integrated) signaling pathways are known to contribute to cellular (dys)functions in pulmonary fibrosis (8–10), and, more recently, it has been demonstrated that secreted WNT proteins can be transported by EVs to exert their intercellular communication (11). The vast majority of research has focused on the role of the WNT/β-catenin pathway in pulmonary fibrosis, which has been linked to disturbed lung epithelial cell function and impaired repair (8–10, 12). β-Catenin–independent WNT signaling in lung fibrosis is much less studied. The WNT protein WNT5A is largely known to exert its effects β-catenin independently and has been found upregulated in IPF fibroblasts (13). However, its potential involvement in EV-mediated signaling has not been investigated. In this study we sought to characterize the EV secretion profile in both experimental and human pulmonary fibrosis, and to investigate the secretion of WNT proteins on EVs. We have characterized EVs in BAL fluid (BALF) from IPF compared with non-IPF ILD/non-ILD patients as well as healthy volunteers in two independent cohorts. IPF-derived EVs carry WNT5A, and we identified lung fibroblasts as a major source of EV-bound WNT5A. IPF-derived EVs drive fibroblast proliferation, which was largely dependent on WNT5A. Thus, this study highlights EVs as potential mediators of disturbed cellular function and communication in IPF. Some of the results have been previously reported in the form of an abstract (14).

Methods

Isolation and Characterization of EVs

EVs were isolated from murine and human BALF samples, primary human cell cultures, and mouse lung tissue using ultracentrifugation (Thermo Fisher Scientific, Sorvall, rotors: fixed-angle T635.5 and TFT80) following state-of-the-art protocols (15). For some EV isolations an ExoQuick (Systems Biosciences) was used as indicated in the general text and figure legends. For all EV preparations, the EV-free supernatant was stored at -80°C, and the pellet containing EVs was resuspended in 30 to 100 μl of sterile phosphate-buffered saline (PBS) and directly used or stored at -80°C. Characterization was performed using several methods as recommended in Thery and colleagues (15) and outlined in the online supplement.

Detailed description of further methods is provided in the online supplement.

Results

EV Secretion Is Upregulated in Experimental and Human Lung Fibrosis

First, we asked the question whether the amount of EV protein and EV number is altered in lung fibrosis. To this end, we isolated and characterized EVs from BALF from experimental and human lung fibrosis samples and controls (Figure 1A). BALF was collected from mice 14 days after intratracheal bleomycin or PBS administration, or from IPF, non-IPF ILD, and non-ILD patients (Munich cohort, Table 1) as well as patients with IPF and healthy volunteers (University of California San Francisco [UCSF] cohort, Table 2). Morphological assessment of EVs by transmission electron microscopy revealed the presence of *1*) large amounts of exosomes (smaller concave vesicles between 30 and 200 nm; Figure 1B and Figure E1A in the online supplement, arrows), and *2*) a smaller fraction of microvesicles (irregular membranous vesicles between 200 and 1,000 nm; Figure 1B and Figure E1A, arrowheads). We further found enriched expression of the endosomal sorting complex required for transport component tumor susceptibility gene 101 (TSG101), a protein commonly used to identify EVs (16) (Figure E1B). TSG101 was increased in BALF EVs from fibrotic lungs compared with BALF EVs from control (Figure E1B), suggesting a potential increase in EVs under fibrotic conditions. Moreover, we found a significantly increased amount of protein content in EVs from fibrotic compared with healthy mice (Figure 1C, EV total micrograms of protein/ml: PBS, 51.3 ± 25.32; bleomycin, 266.2 ± 114.8; $P = 0.0001$). Next, we quantified EV numbers and determined the size distribution by nanoparticle tracking analysis (NTA) (Figure 1D) or by dynamic light scattering (Figure E1C). By NTA, we found increased numbers of EVs in the BALF from fibrotic mouse lungs compared with controls, in particular exosomes, indicating a change in number and size distribution of EVs upon fibrosis development (Figure 1D, exosome particles/ml: PBS, $1.93 \times 10^8 \pm 6 \times 10^6$; bleomycin, $4.3 \times 10^8 \pm 1.9 \times 10^8$; $P = 0.049$). We next aimed to translate these findings into the human disease. We explored whether EVs can be found in human BALF from non-ILD, non-IPF ILD, and IPF patients (Table 1, Munich cohort), as well as from BALF from patients with IPF and healthy volunteers (Table 2, UCSF cohort). EVs

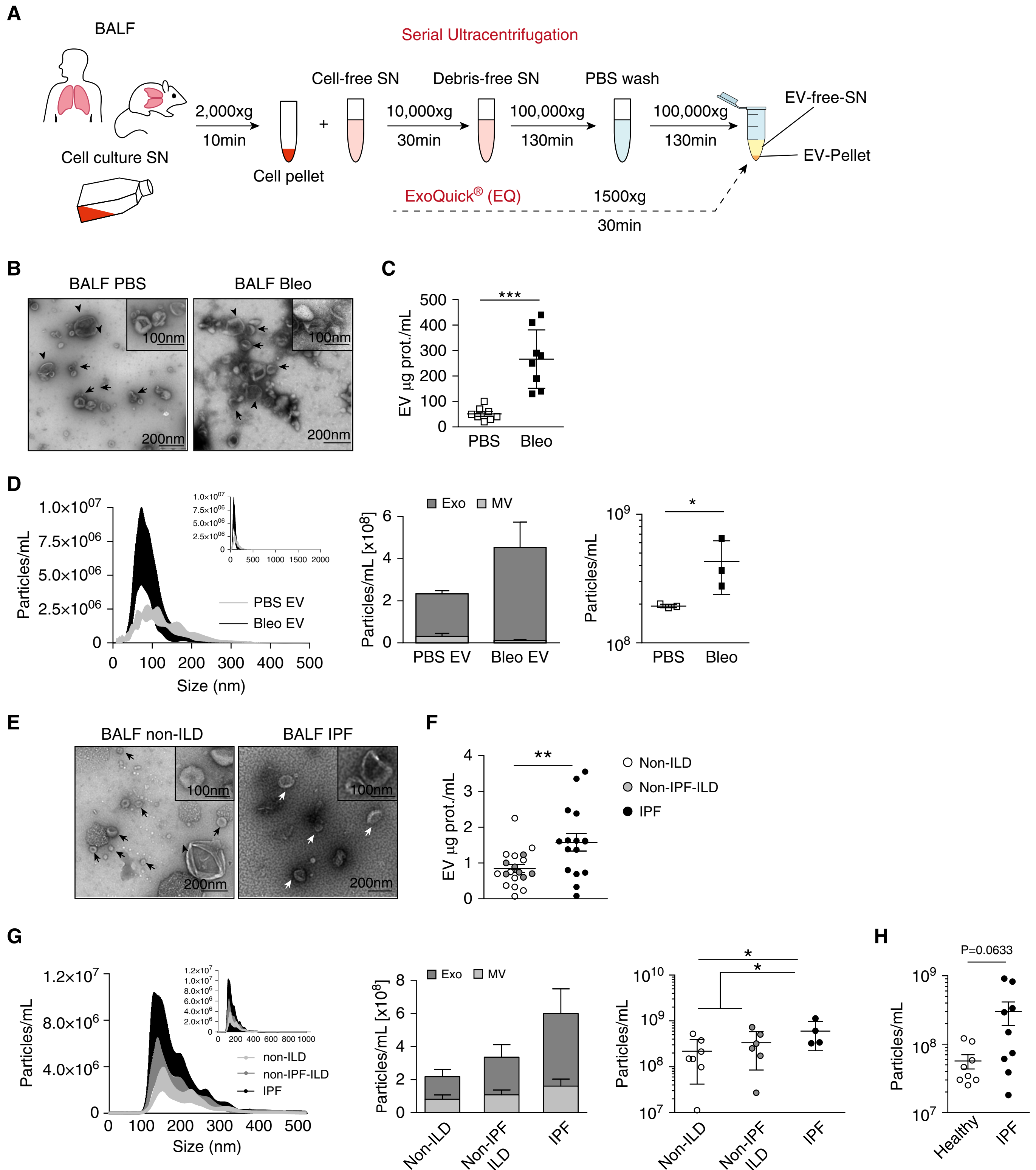

Figure 1. Extracellular vesicle (EV) secretion is increased in experimental and human pulmonary fibrosis. (*A*) Scheme of the protocols used for the isolation of EVs in BAL fluid (BALF) and cell culture (supernatant). (*B*) Representative transmission electron microscopy image of EVs isolated from BALF of mice treated with phosphate-buffered saline (PBS; vehicle) or bleomycin (BALF EV PBS and BALF EV Bleo, respectively). BALF was collected at Day 14 after instillation. Arrows indicate exosomes; arrowheads indicate microvesicles. (*C*) Total protein quantification in EVs isolated from BALF from PBS- and bleomycin-treated mice ($n = 8$ per group). (*D*) Histogram showing the results of nanoparticle tracking analysis performed on same samples as in *B*

Table 1. Characteristics of Patients Included in the Munich Cohort

Characteristics	Control, Non-ILD*	COP	HP	IPF	All
n	12	2	5	16	35
Male sex, *n* (%)	3 (25%)	1 (50%)	3 (60%)	10 (63%)	17 (49%)
Age, yr, mean ± SD	57.4 ± 14.3	73 ± 1.5	55.6 ± 5.9	68.7 ± 11.0	63.8 ± 12.3
FVC, L, mean ± SD	4.9 ± 0.4	5.0 ± 0.8	4.2 ± 0.7	3.4 ± 0.7	3.8 ± 0.8
$D_{L_{CO}}$, % predicted, mean ± SD	77.1 ± 10.7	82 ± 11	43.9 ± 7.16	54.3 ± 12.6	60.3 ± 18.5

Definition of abbreviations: COP = cryptogenic organizing pneumonia; HP = hypersensitivity pneumonitis; ILD = interstitial lung disease; IPF = idiopathic pulmonary fibrosis.
*For the control, non-ILD group: diagnostic evaluation of unclear cough (*n* = 10), previous breast cancer metastasis (*n* = 1), and post-transplantation (*n* = 1), all with no signs of ILD.

were characterized by transmission electron microscopy (Figure 1E), TSG101 expression, and the absence of calreticulin (an endoplasmic reticulum marker absent in EVs) (Figure E1D). We observed a significant upregulation of EV protein content in BALF from patients with IPF compared with non-ILD/non-IPF ILD (Figure 1F; EV total micrograms of protein/ml: non-ILD/non-IPF-ILD [*n* = 12/7], 251.6 ± 166.6; IPF [*n* = 16], 552.3 ± 427.3; *P* = 0.0212). Furthermore, using NTA, we found a significant increase in EVs, mainly corresponding to exosomes (Figure 1G, left and middle panels), in BALF from patients with IPF in comparison to non-ILD/non-IPF ILD (Figure 1G, right panel; particles/ml of initial sample: non-ILD [*n* = 7], $2.2 \times 10^8 \pm 1.8 \times 10^8$; non-IPF ILD [*n* = 6], $3.3 \times 10^8 \pm 2.5 \times 10^8$; and IPF [*n* = 4], $6.0 \times 10^8 \pm 3.8 \times 10^8$; non-ILD vs. IPF, *P* = 0.0438; and for combined non-IPF groups vs. IPF, *P* = 0.0387). Importantly, we confirmed an increase in EVs in IPF in a second independent cohort of patients with IPF and healthy volunteers, although this analysis did not reach statistical significance (Figure 1H; particles/ml of initial sample: healthy [*n* = 8], $5.7 \times 10^7 \pm 2.5 \times 10^7$; IPF [*n* = 9], $3.0 \times 10^8 \pm 3.4 \times 10^8$; *P* = 0.0633). Further analysis of this cohort suggests that EV numbers correlate with lung function (Figure E2A). Importantly, when combining both cohorts, EVs were significantly increased in IPF compared with non-IPF (Fig. E2B for combined analysis, *P* = 0.0428). Altogether, these results strongly support the notion of enhanced secretion of EVs into the BALF, in both experimental lung fibrosis and human IPF.

WNT5A Is Upregulated in BALF EVs from Experimental and Human Lung Fibrosis

EVs exert their function by transporting a variety of mediators, and we wondered whether WNT proteins upregulated in IPF (13, 17) are present on EVs. We found an increase in both WNT5A mRNA and protein expression in lung homogenates from bleomycin-treated compared with PBS-treated mice (Figure 2A and 2B) and upregulated WNT5A protein in lung homogenates from IPF compared with donor tissue specimens (Figure 2C), expanding on a previous report (13). To investigate whether WNT secretion is increased in lung fibrosis, we analyzed the expression of the shuttle protein GPR177 (G protein–coupled receptor 177) required for WNT secretion on EVs (18) and found significant increased levels in lung homogenate and BALF from bleomycin-treated mice compared with PBS-treated controls (Figure 2D and 2E, respectively). Upregulation of GPR177 was further confirmed in lung homogenates from patients with IPF compared with donors (Figure 2F; GPR177 protein: donors, 0.35 ± 0.25; IPF, 0.48 ± 0.16; *P* = 0.0262).

To study whether WNT5A is indeed secreted on EVs, we looked at WNT5A in EVs and EV-free supernatants and found enriched WNT5A in EVs from BALF of fibrotic mouse lungs compared with controls (Figure 3A). We further found WNT5A enriched in EVs from supernatants from fibrotic compared with healthy three-dimensional lung tissue cultures, suggesting that these EVs carry WNT5A under fibrotic conditions and can originate from distal areas of lung tissue (Figure 3B, right panel; WNT5A protein: PBS, 0.12 ± 0.12; bleomycin, 1.34 ± 0.33; *P* = 0.0004). These results strongly indicate that WNT5A is transported by EVs in experimental lung fibrosis.

Next, we aimed to elucidate the potential clinical relevance of EV-mediated WNT5A signaling in IPF. EVs isolated from BALF of non-ILD, non-IPF ILD, or IPF patients were investigated for the expression of WNT5A along with the EV-enriched proteins TSG101 and CD81, with the latter being a tetraspanin involved in EV biogenesis (19). Analyzing the same amount of EV protein, we found increased levels of WNT5A in EVs from patients with IPF when compared with non-IPF EVs (Figure 3C; WNT5A expression: non-IPF [*n* = 5], 24.98 ± 9.51; IPF [*n* = 7], 72.09 ± 43.56; *P* = 0.0408). Notably, WNT5A correlated with CD81 (Figure 3D; $r^2 = 0.4586$, *P* = 0.0156), which is described to be highly expressed on the surface of

Figure 1. (Continued). (measurements in triplicates; *n* = 3 per group) (left), graph of the vesicles grouped in exosomes (30–200 nm) or microvesicles (200–2,000 nm) (middle), and statistics for the exosomal fraction (right). Data are represented as particles/ml of EV fraction. (*E*) Representative transmission electron microscopy images of EVs isolated from BALF from non–interstitial lung disease (ILD) or idiopathic pulmonary fibrosis (IPF) patients. (*F*) Total protein quantification in EV pellets isolated from BALF from non-ILD/non-IPF ILD (*n* = 12/7) and IPF (*n* = 16) patients. (*G*) Nanoparticle tracking analysis determining the size and number of the vesicles isolated from BALF from non-ILD (*n* = 7), non-IPF ILD (*n* = 6), and IPF (*n* = 4) patients. Measurements were done in five replicates (right), with the graph representing the results in exosomes (30–200 nm) versus microvesicles (200–1,000 nm) (middle) and statistics representing the amount of EVs in the three groups (left). Data are represented as particles/ml related to the initial sample. (*H*) Quantification of EVs isolated from BALF of healthy volunteers or patients with IPF from a second cohort of patients (Table 2). Data are represented as particles/ml related to the initial sample. All EVs were isolated by ultracentrifugation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student’s *t* test. Bleo = bleomycin; Exo = exosomes; MV = microvesicles; SN = supernatant.

Table 2. Characteristics of Patients Included in the UCSF Cohort

Characteristics	Healthy Volunteers	IPF	All
n	8	9	17
Male sex, *n* (%)	4 (50%)	9 (100%)	13 (76%)
Age, yr, mean ± SD	57.5 ± 6.7	71.1 ± 3.3	64.7 ± 8.6
FVC, L, mean ± SD	4.3 ± 1.0	3.6 ± 1.2	4.0 ± 1.1
D_{LCO}, % predicted, mean ± SD	No data	47.9 ± 12.9	—

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; UCSF = University of California San Francisco.

myofibroblasts (20) and as a marker of a specific EV subpopulation (21), as well as TSG101 (Fig. E3; $r^2 = 0.3762$, $P = 0.0339$). Collectively, these results suggest that WNT5A expression is increased in EVs in IPF and highlight a potential role for EV-bound WNT5A in intercellular communication during fibrogenesis.

EVs from Primary Human Lung Fibroblasts Transport WNT5A and Induce Fibroblast Proliferation

WNT5A has been recently found to be upregulated in lung fibroblasts in chronic lung disease (22). We thus investigated whether lung fibroblasts secrete EV-associated WNT5A in the distal lung and isolated EVs from cell culture supernatants from primary human lung fibroblasts (phLFs) and alveolar epithelial type II cells (Figure 4A and 4B and Figure E4). We found an enrichment of WNT5A in EVs from phLFs compared with EVs from primary human alveolar epithelial type II cells (Figure 4C),

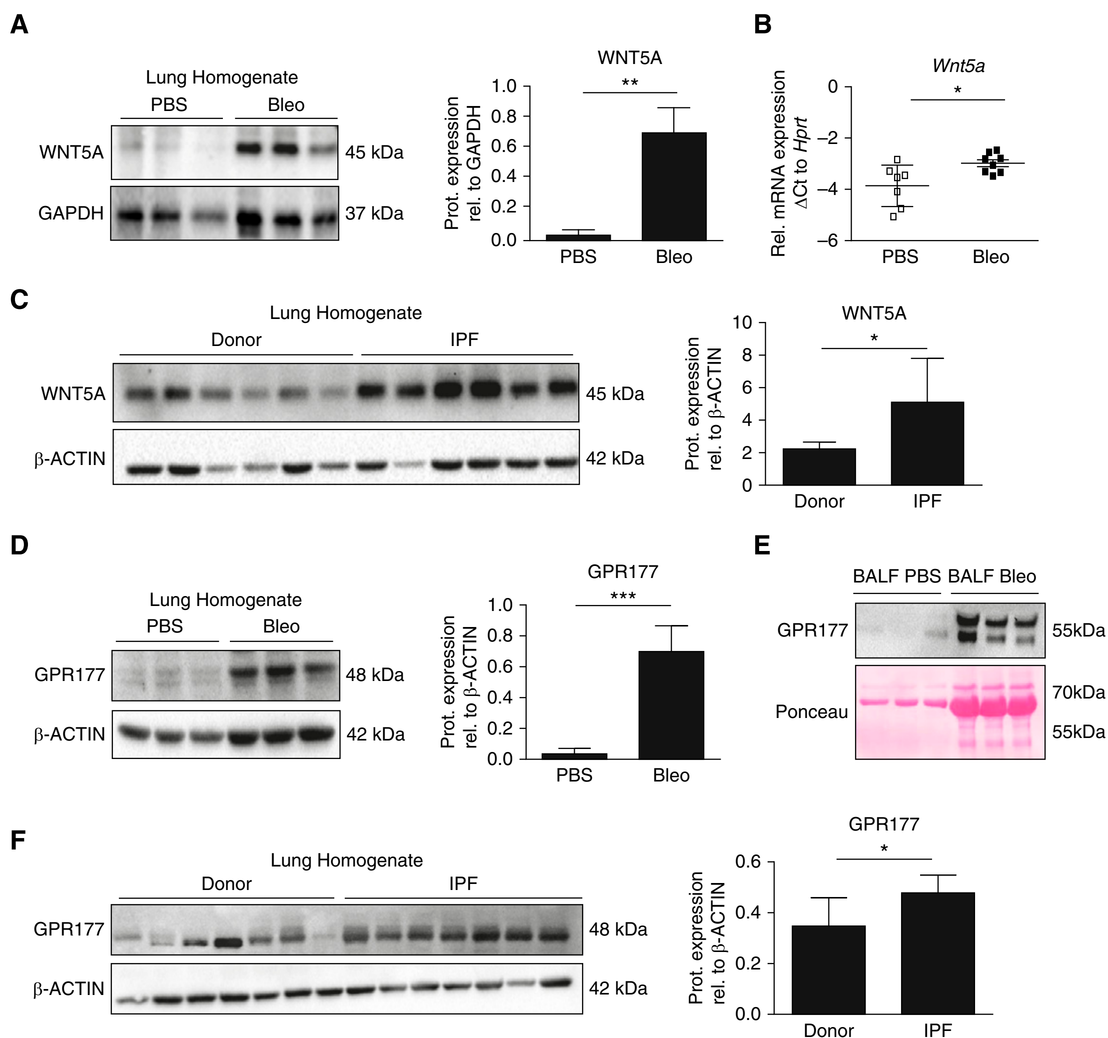

Figure 2. WNT5A is upregulated in lung homogenates from experimental and human pulmonary fibrosis. (*A* and *B*) Expression levels of WNT5A protein (*A*) and mRNA (*B*) in lung homogenates from PBS- or bleomycin-treated mice ($n = 3$–8 mice per group). (*C*) Protein analysis of WNT5A expression in lung homogenates from donors and patients with IPF and subsequent densitometry analysis ($n = 6$ per group). (*D* and *E*) Expression of the WNT shuttle protein GPR177 in lung homogenates (*D*) and BALF (*E*) from PBS- or bleomycin-treated mice ($n = 3$ per group). Ponceau S staining was used as loading confirmation. (*F*) Protein expression of GPR177 in lung homogenates from donors ($n = 7$) and patients with IPF ($n = 7$) and subsequent densitometry. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test. BALF = BAL fluid; Bleo = bleomycin; GPR177 = G protein–coupled receptor 177; *Hprt* = hypoxanthine phosphoribosyltransferase; IPF = idiopathic pulmonary fibrosis; PBS = phosphate-buffered saline; Prot. = protein; rel. = relative.

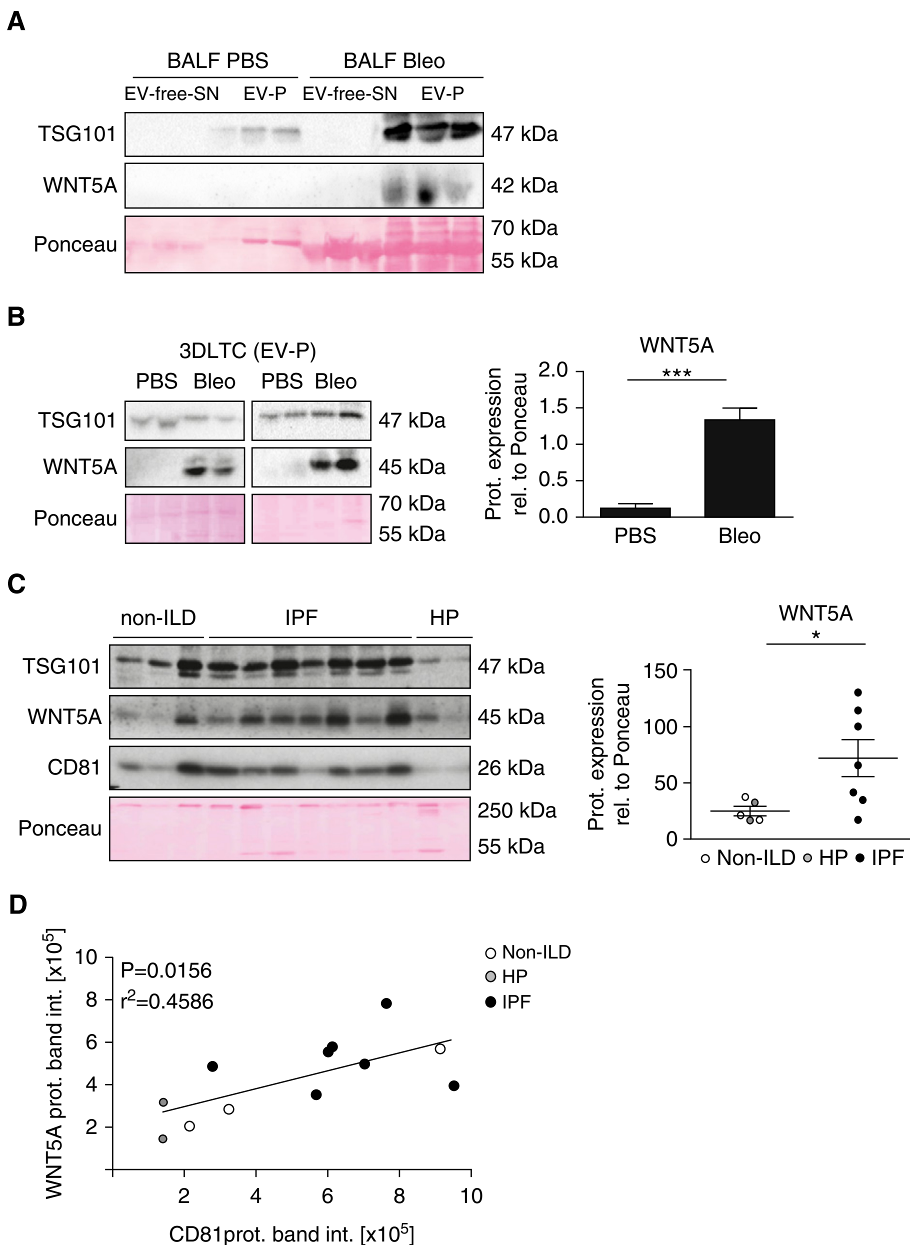

Figure 3. Increased WNT5A is carried on extracellular vesicles (EVs) in pulmonary fibrosis. (*A* and *B*) Analysis of TSG101 and WNT5A in whole EV pellets from BALF ($n = 3$ per group) (*A*) and EV pellets isolated from three-dimensional lung tissue culture supernatants (*B*) from PBS- and bleomycin-treated mice ($n = 4$ per group). EVs were isolated by ExoQuick. An equal amount of EV pellets was loaded (10 μg). (*B*, right panel) Densitometry analysis: WNT5A expression relative to Ponceau S. (*C*) Protein analysis of EV-enriched proteins TSG101 and CD81, as well as WNT5A in EV pellets isolated by ultracentrifugation in BALF from non-ILD ($n = 3$), IPF ($n = 7$), and hypersensitivity pneumonitis ($n = 2$), and corresponding densitometry of WNT5A relative to Ponceau S staining. (*D*) Correlation between WNT5A and CD81 expression (circles represent single values; linear regression test). Ponceau S staining was used for loading confirmation. $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t* test. 3DLTC = three-dimensional lung tissue culture; BALF = BAL fluid; Bleo = bleomycin; EV-P = EV pellets; HP = hypersensitivity pneumonitis; ILD = interstitial lung disease; int. = intensity; IPF = idiopathic pulmonary fibrosis; PBS = phosphate-buffered saline; Prot. = protein; rel. = relative; SN = supernatant; TSG101 = tumor susceptibility gene 101.

suggesting that phLFs are a major source of WNT5A secretion via EVs in the distal lung.

To address the potential functional implications of EVs, we investigated lung fibroblast proliferation, which has been linked to lung fibrosis (13, 23). We collected EVs from phLF supernatant and treated phLFs in an autocrine fashion as outlined in detail in the METHODS section. Upon EV treatment, phLFs exhibited a significant increase in their metabolic activity compared with phLFs treated with EV-free medium (Figure 4D; percentage increase in metabolic activity to control: 0.01 μg of EVs, 19.7 ± 10.4, $P = 0.0064$; 0.5 μg of EVs, 17.6 ± 12.45, $P = 0.0199$). Similarly, we found a significant increase in the number of cells upon EV treatment (Figure 4E). Notably, the proliferative effect was significantly decreased in disrupted EVs treated with detergent prior to treatment when compared with intact EVs (Figure 4F). Notably, next to an effect on proliferation, we observed decreased gene expression of the myofibroblast markers *FN1*, *ACTA2*, *COL1A1*, and *TNC* upon EV treatment (Figure E5). These data indicate that EV-bound WNT5A promotes a proliferative and not a synthetic cellular phenotype of fibroblasts.

EV-induced Proliferation in Lung Fibroblasts Is Mediated by WNT5A

To elucidate whether the effects of EVs on lung fibroblast proliferation are mediated by WNT proteins, we used the inhibitor of WNT production molecule 2 (IWP2), which inhibits overall WNT (24). Pretreatment of phLFs with IWP2 efficiently decreased WNT5A secretion in phLF supernatants (Figure 5A) and, importantly, reduced the proliferative capability of EVs in the recipient phLFs when compared with EVs from vehicle-treated cells (Figure 5B; percentage increase in metabolic activity to control: DMSO [vehicle] EVs, 45.8 ± 26.6 vs. IWP2 EVs, 27.0 ± 12.3). To further confirm that the proliferative effect was mediated specifically by WNT5A, we performed siRNA-mediated silencing of WNT5A in phLFs prior to EV isolation, which efficiently inhibited WNT5A secretion while not modifying the expression of the EV-enriched proteins CD81 and TSG10 or the EV secretion profile (Figure 5C and Fig. E6A and E6B). Notably, WNT5A-depleted EVs exhibited significant reduced potential to induce

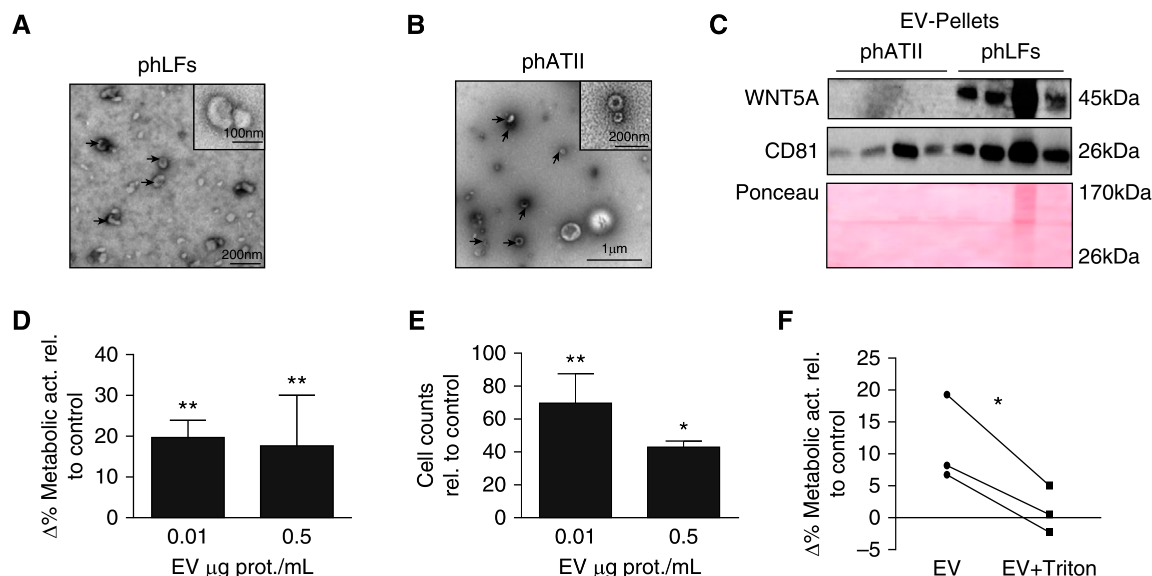

Figure 4. Human lung fibroblasts are a source of extracellular vesicles (EVs), which induce lung fibroblast proliferation. (*A* and *B*) Representative transmission electron microscopy images of EVs (arrows) isolated from phLFs (*A*) and primary human alveolar epithelial type II cell culture supernatants (*B*). (*C*) Comparison of the EV-enriched proteins tumor susceptibility gene 101 and CD81, as well as WNT5A, in equally loaded EV pellets isolated from phLFs or primary human alveolar epithelial type II cell culture supernatants. Ponceau S staining was used as loading control. (*D* and *E*) Assessment of proliferation by WST-1 assay (*D*) or cell counting (*E*) of phLFs stimulated with EVs at the indicated concentrations for 48 h ($n = 3$ and $n = 6$, respectively). *$P < 0.05$, **$P < 0.01$, one-way ANOVA, Dunnett *post hoc* test. (*F*) Proliferation analysis by WST-1 assay in phLFs stimulated with autocrine EVs alone or pretreated with detergent. $n = 3$ per group. *$P < 0.05$, paired Student's *t* test. act. = activity; phATII = primary human alveolar epithelial type II cells; phLF = primary human lung fibroblast; prot. = protein; rel. = relative.

proliferation (Figure 5D; percentage increase in metabolic activity: scramble siRNA-EV, 23.7 ± 15.5 vs. WNT5A siRNA-EV, 4.2 ± 9.1; $P = 0.0401$). These results are further supported by the finding that WNT5A silencing in fibroblasts decreased mRNA and protein expression of the cell cycle regulator cyclin D1 (Figure 5E and 5F, respectively). To further confirm these results and exclude off-target effects on EV composition by silencing WNT5A, we incubated EVs with a WNT5A neutralizing antibody (WNT5A-AB) prior to phLF stimulation, which also decreased the potential of EVs to induce proliferation (Figure 5G; percentage increased metabolic activity: EV plus IgG, 23.8 ± 10.3 vs. EV plus WNT5A-AB, 10 ± 14.6; $P = 0.0449$). In summary, these results provide evidence that WNT5A mediates the pro-proliferative effect of EVs on lung fibroblasts.

Transforming Growth Factor-β Leads to Increased WNT5A Secretion on EVs and Exaggerated Proliferation of phLFs

TGF-β (transforming growth factor-β) is a key profibrotic cytokine (25) and has recently been reported to induce WNT5A expression in lung fibroblasts (23). TGF-β stimulation of phLF led to an increased secretion of GPR177 when compared with control EVs (Figure 6A), and it further increased WNT5A in EVs (Figure 6B; WNT5A protein in EVs: control, 0.52 ± 0.33; TGF-β, 1.68 ± 0.66; $P = 0.0262$). Of note, WNT5A was particularly enriched in the EV fraction compared with the EV-free supernatant (Figure 6B). Accordingly, we further observed that TGF-β–derived EVs induced a dose-dependent increase in proliferation (Figure 6C). This effect was reduced when EVs were incubated with WNT5A antibody before treatments on EV recipient cells (Figure 6D).

EVs from BALF of IPF Patients Increase Lung Fibroblast Proliferation in a WNT5A-Dependent Manner

Finally, we wondered whether EV-associated WNT5A in BALF from patients with IPF was able to induce fibroblast proliferation. We isolated EVs from different IPF BALFs and all of them significantly increased phLF proliferation in a dose-dependent manner as measured by total metabolic activity (WST-1 assay), cell counting, as well as by a DNA synthesis–based method (5-bromo-2′-deoxyuridine assay) (Figure 7A, 7B, and 7C, respectively). An intact EV structure was required for the effect on proliferation (Figure 7D). Importantly, the induction of proliferation was significantly decreased when IPF EVs were preincubated with a WNT5A-AB (Figure 7E; percentage increased metabolic activity: EV plus IgG, 19.3 ± 10 vs. EV plus W5A-AB, 8 ± 13.9; $P = 0.0284$).

Discussion

EVs, including exosomes, are secreted membranous vesicles known to drive biological processes by transporting a variety of cargos depending on the cellular source and context (5). In recent years, EVs have emerged as essential vehicles of both physiological and pathological processes by harboring specific mediators of a (diseased) cell and facilitating communication with other cellular compartments and tissues. Within the lung, our understanding of EV function in disease has just recently begun to grow, with studies highlighting a

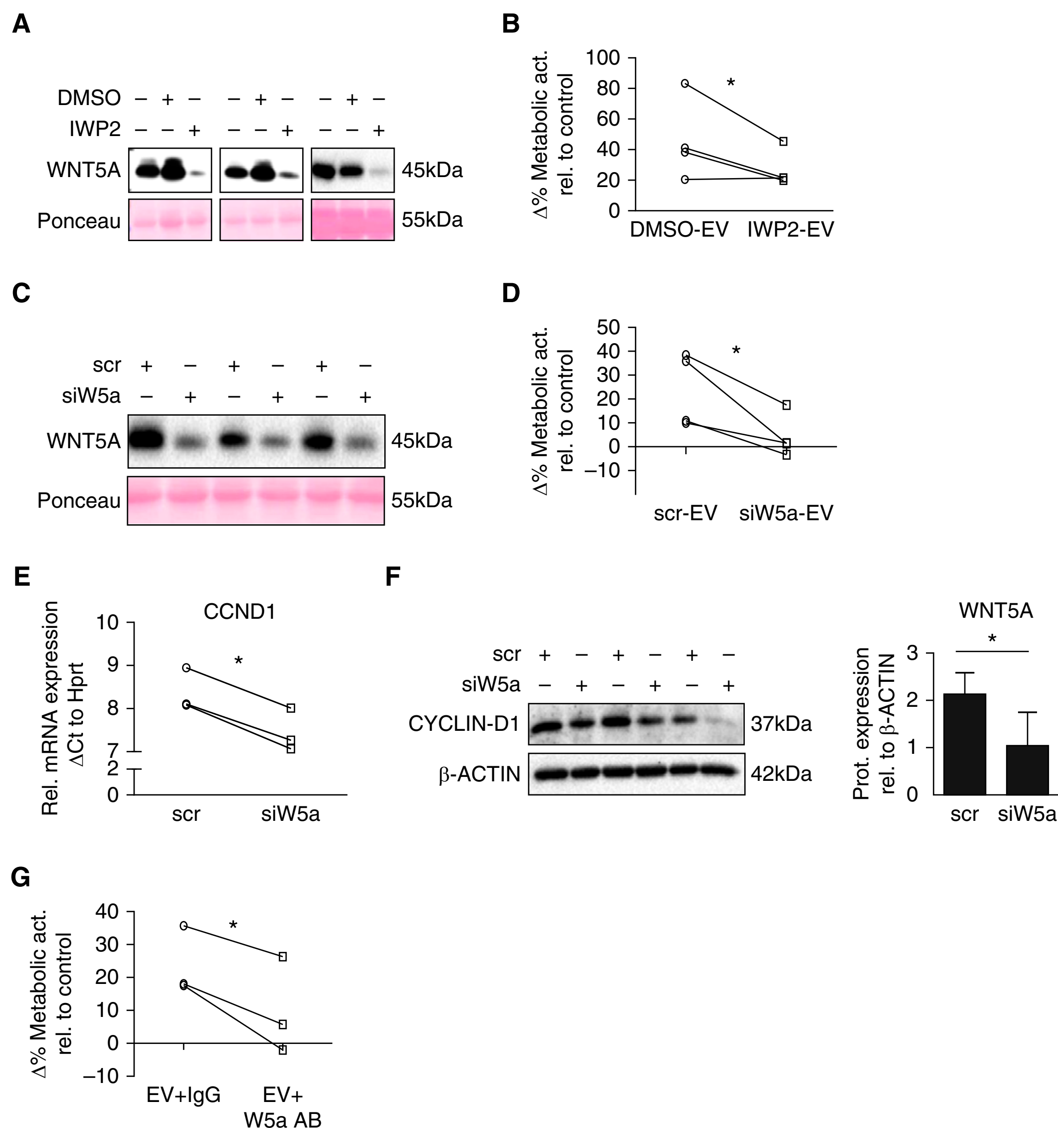

Figure 5. Human lung fibroblast–derived extracellular vesicle (EV)-induced lung fibroblast proliferation is WNT5A dependent. (*A*) Detection of WNT5A protein in primary human lung fibroblast (phLF) supernatants after treatment with inhibitor of WNT protein secretion IWP2 or DMSO as control (data show $n = 3$ independent experiments). (*B*) Proliferation analysis by WST-1 assay after 48 h stimulation of phLFs with 0.5 μg of protein/ml of autocrine EVs isolated in phLFs following treatment with IWP2 (IWP2-EV) or DMSO control (DMSO-EV) ($n = 4$ per group). (*C*) Analysis of WNT5A protein in supernatants from phLFs treated with WNT5A siRNA (siW5A) or scrambled siRNA control (scr) for 24 h. Ponceau S staining was used for loading confirmation of the supernatants. (*D*) Proliferation assay after 48 h stimulation of phLFs with 0.5 μg of protein/ml of EVs isolated from phLFs after treatment with WNT5A siRNA (siW5A-EV) or scrambled (scr-EV) ($n = 3$ per group). (*E* and *F*) mRNA (*E*) and protein (*F*) expression levels of CCND1 (cyclin D1 gene) after 24 h stimulation of phLFs with siW5A or scr. $n = 3$ per group. (*G*) Proliferation assay after 48 h of stimulation of phLFs with 0.01 μg of protein/ml of autocrine EVs that have been incubated with 1 μg of WNT5A neutralizing antibody (EV + W5A AB) or IgG control (EV + IgG) ($n = 3$ per group). $^*P < 0.05$, paired Student's *t* test. act. = activity; *Hprt* = hypoxanthine phosphoribosyltransferase; IWP2 = inhibitor of WNT production molecule 2; Prot. = protein; rel. = relative.

potential role of EVs in lung cancer and inflammatory lung disease (5, 26, 27). However, besides an observational study detecting increased tissue factor activity in microparticles from BALF from patients with ILD (28), EV function in the context of fibrotic processes in the lung remains unknown. In this study, we demonstrate for the first time that *1*) EVs, in particular exosomes, are increased in experimental and human pulmonary fibrosis; *2*) WNT5A can be detected on EVs derived from lung biosamples; and *3*) EVs, in part via WNT5A, contribute to fibroblast function and thus disease pathology.

Research in EV biology is highly dependent on standardized protocols for the isolation (15) and proper characterization (29) of EVs. In this study, we largely used serial ultracentrifugation to isolate EVs, which is a state-of-the-art method that does not modify the original sample. We used a variety of methods to characterize EV

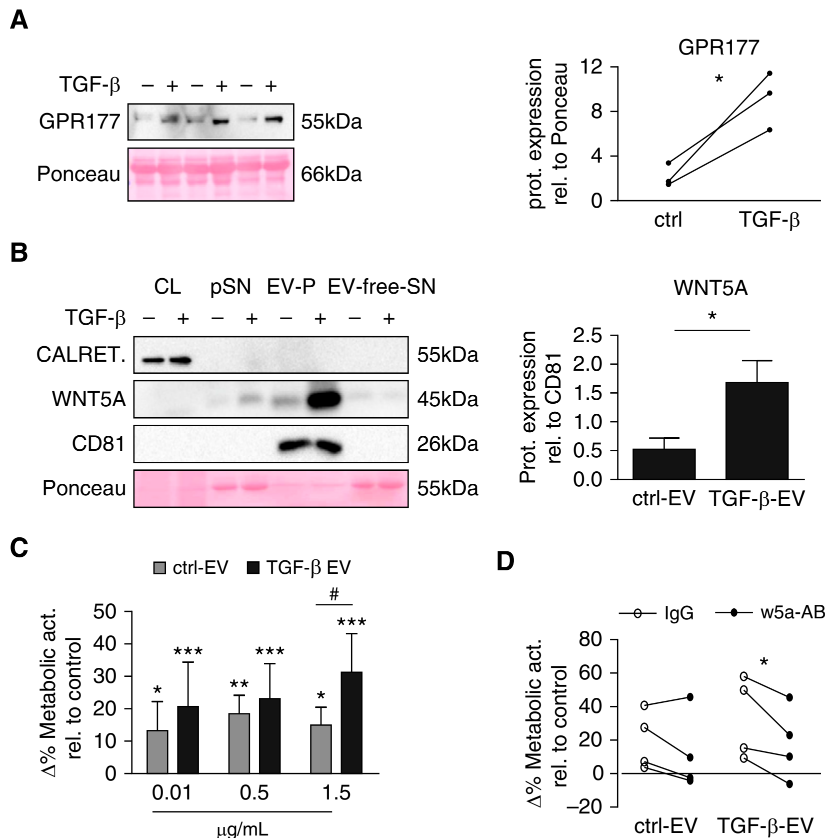

Figure 6. TGF-β (transforming growth factor-β) induces WNT5A expression in primary human lung fibroblast (phLF) extracellular vesicles (EVs). (*A*) Protein levels of secreted GPR177 in the supernatants of phLFs after stimulation with TGF-β and subsequent densitometry relative to Ponceau S. $n = 3$ per group. $^{*}P < 0.05$, paired Student's *t* test. (*B*) Protein expression analysis of endoplasmic reticulum protein calreticulin (CALRET.), the EV-enriched protein CD81, and WNT5A in cell lysates (CL), pure supernatants (pSN), EV pellets (EV-P), and EV-free supernatants (EV-free-SN) from phLFs treated with TGF-β (2 ng/ml) for 48 h, and respective densitometry analysis of WNT5A relative to CD81 (graph represents three independent experiments). $^{*}P < 0.05$, paired Student's *t* test. (*C* and *D*) Assessment of proliferation by WST-1 assay of phLFs stimulated for 48 h with (*C*) EVs from TGF-β–stimulated phLFs at the indicated concentrations ($n = 4$) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA, Dunnett *post hoc* test; $^{\#}P < 0.05$, Bonferroni *post hoc* test), and (*D*) the same EVs incubated with WNT5A antibody ($^{*}P < 0.05$, paired Student's *t* test). act. = activity; ctrl = control; GPR177 = G protein–coupled receptor 177; Prot. = protein; rel. = relative.

populations based on morphology, number, size, and expression of EV-enriched proteins in murine and human biosamples. These analyses resulted in the first description of intact exosome-enriched vesicles from BALF of fibrotic murine and human lungs that exhibit functional properties.

EVs are prime mediators for intercellular communication. In IPF, altered cellular cross-talk and communication is a key feature with aberrant epithelial wound healing and fibroblast activation and proliferation (1). In this study, we describe that lung fibroblasts are a source of EVs and demonstrated autocrine effects of EVs on fibroblast proliferation, which was enhanced by TGF-β. Similarly, mesenchymal stem cell (MSC)–derived exosomes were found to induce dermal fibroblast proliferation (30). In addition to lung fibroblast proliferation, myofibroblast differentiation occurs during lung remodeling (25). It is thought that fibroblasts exert different phenotypes within a fibrotic lung based on their spatiotemporal distribution. Notably, we did not observe that fibroblast-derived EVs promote myofibroblast differentiation, but rather they decrease mRNA levels of myofibroblast markers and as such might promote a proliferative rather than a synthetic phenotype. Similarly, MSC-derived exosomes have also been reported to suppress myofibroblast differentiation (31).

We found that the effect of EVs on fibroblasts was to a large extent mediated by WNT5A. Disturbed WNT signaling has been implicated in the pathogenesis of several chronic lung diseases, including IPF (12, 17). WNT5A has been found in IPF fibroblasts (13, 23), and, more recently, we found upregulated WNT5A in early fibrotic-like changes in human three-dimensional lung tissue cultures (32). Thus far, most studies have focused on the WNT5A/β-catenin pathway in IPF, whereas β-catenin–independent WNT signaling is much less explored. Although WNT5A has been largely reported to act β-catenin independently, also β-catenin–dependent function can be observed. Nabhan and colleagues (33) recently reported that WNT5A-expressing fibroblasts might induce WNT/β-catenin signaling in ATII cells; however, whether this effect is mediated by EVs has not been investigated. Importantly, Gross and colleagues (11) discovered that WNT proteins are transported on EVs, thus urging us to investigate the role of EVs, and in particular EVs carrying WNT proteins, in lung fibrosis. WNT transport on EVs has important implications with respect to the signaling range of WNT proteins, which is thought to be rather short and limited to close neighboring cells. EV-mediated transport can contribute to a larger signaling range of WNT proteins and thus determine the signaling outcome on other cells. WNT5A has also been reported to promote processes as fibroblast adhesion (34) or invasion (35), as well as epithelial–mesenchymal transition (36), all of which need to be further studied in the context of EV-associated WNT5A. In this study, we demonstrate that WNT5A on EVs promotes fibroblast proliferation, and, importantly, this effect could not only be attenuated by siRNA-mediated WNT5A knockdown, but further by antibody-mediated neutralization of WNT5A on EVs or upon destruction of EV structure. These data corroborate that indeed WNT5A transported by EVs is responsible for the observed effects.

We report that fibroblasts are a major source for WNT5A-bound EVs, and our data suggest that these contribute to EVs in BALF from patients with IPF. However, it is highly likely that other cells contribute to

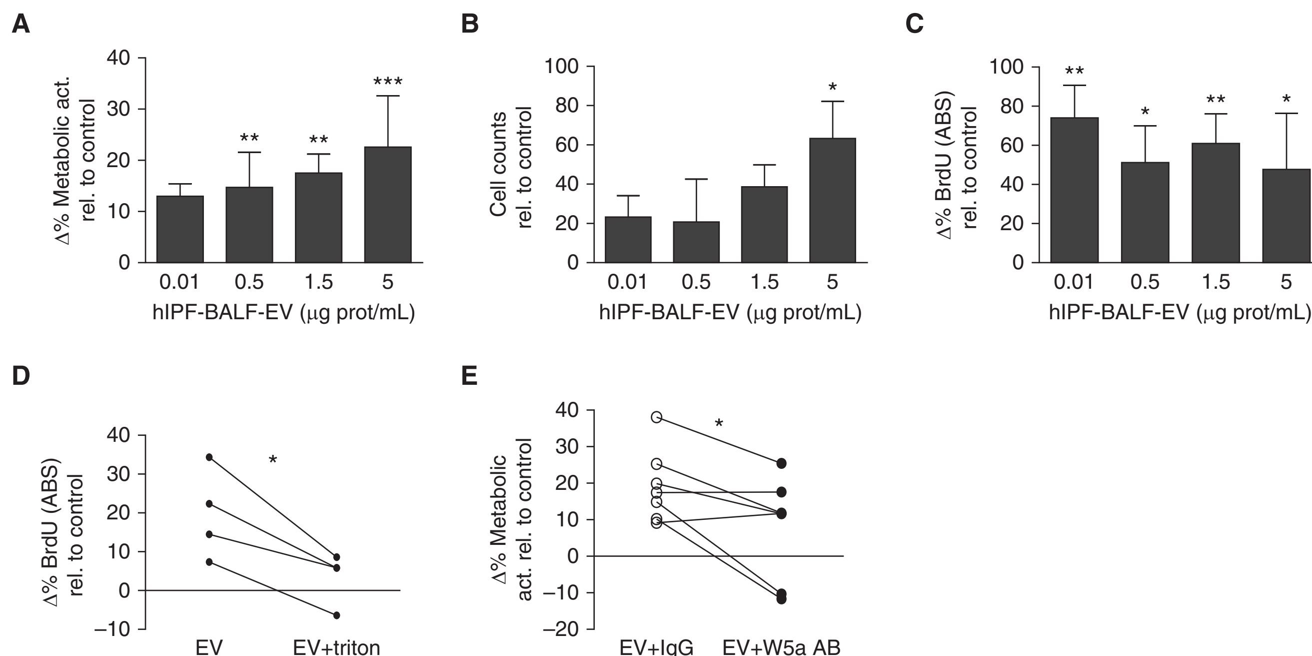

Figure 7. WNT5A on BAL fluid (BALF) extracellular vesicles (EVs) from patients with idiopathic pulmonary fibrosis (IPF) drives lung fibroblast proliferation. (*A*–*C*) Assessment of proliferation by WST-1 assay (*A*), cell counting (*B*), or 5-bromo-2′-deoxyuridine (BrdU) assay (*C*) of primary human lung fibroblasts (phLFs) stimulated with EVs isolated from human IPF BALF at the indicated concentrations for 48 h ($n = 7$, $n = 3$, and $n = 8$, respectively). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA, Dunnett *post hoc* test. (*D*) Proliferation analysis by BrdU assay in phLFs stimulated with IPF EVs alone (EV) or pretreated with detergent (EV + triton). $n = 4$ per group. $^{*}P < 0.05$, paired Student's *t* test. (*E*) Proliferation assay by WST-1 of donor phLFs after 48 h treatment with human IPF BALF EVs incubated with WNT5A antibody (W5a AB) or IgG control ($n = 7$ per group). $^{*}P < 0.05$, paired Student's *t* test. ABS = absorbance; act. = activity; hIPF = human IPF; prot = protein; rel. = relative.

the EV composition of the BALF in IPF, such as epithelial cell or immune cell subpopulations, which thus might alter the functional outcome. Therefore, it will be important to further investigate EV heterogeneity in the BALF of patients with IPF, EVs from different cellular sources, and their distinct effects on the fibrotic lung cell phenotypes. In this study, we provide evidence that WNT5A-bound EVs in IPF BALF contributes to the functional effects, thus suggesting that fibroblast-derived EVs can be found in IPF BALF. However, future studies are needed to decipher whether BALF EVs from other (fibrotic) lung disease also promote cellular proliferation and whether and how other disease-specific mediators are involved in that process. We recently reported that fibroblast-derived WNT5A affects alveolar epithelial cell function in chronic obstructive pulmonary disease (22), highlighting the need to further investigate EV-mediated mesenchymal–epithelial cross-talk. Interestingly, we found that WNT5A was able to inhibit WNT/β-catenin–driven repair in chronic obstructive pulmonary disease (22, 37). Nevertheless, in IPF, several lines of evidence suggest active WNT/β-catenin signaling in the lung epithelium (38). These probably disease-specific effects on cellular phenotype might be mediated by the distinct composition of EVs or a specific surface receptor profile on the recipient cell. It has been demonstrated that EVs are taken up by specific cell types due to a distinct integrin expression pattern (39). Another intriguing hypothesis is that EVs, depending on the microenvironment, harbor diverse components of a specific pathway, including specific signaling receptors that enable the recipient cell to exert novel functions and phenotypes.

Our work further raises the more general question of whether EVs promote lung fibrosis development or might have a protective role *in vivo*. Notably, several studies to date indicate that EVs play versatile roles depending on the (disease) specific microenvironment with MSC-derived EVs able to facilitate tissue repair (31, 40, 41), and modulating myofibroblast differentiation in IPF fibroblasts (42), whereas others have suggested proinflammatory roles (27, 43, 44). Moreover, EVs derived from lung cancer cells modulate the tumor microenvironment and can promote tumor metastasis (26, 45, 46). Thus, additional studies inhibiting EV secretion or altering EV composition *in vivo*, ideally in a cell-specific manner, are needed to elucidate the potential damaging versus resolving role of EVs in pulmonary fibrosis.

We investigated a limited amount of patient samples in this study to provide the first evidence that EVs are increased in IPF and carry important functional mediators, such as WNT5A. A comprehensive characterization of EVs requires a large amount of biosamples, which restricted the overall number of patient samples we could investigate in this study. Although both independent cohorts showed similar results, future investigations of larger cohorts will be essential to further confirm the potential correlation of EVs with clinical parameters, such as lung function. These studies will need to consider the heterogeneity of the BALF samples (such as differential cell counts), include different ILDs, and allow adjustment for parameters such as age and sex. Furthermore, it will be interesting to explore the potential role of

EVs as blood biomarkers for ILDs (47), and other EV components such as DNA, mRNA, or microRNAs that might contribute to disease (5, 26, 48).

In summary, our present study reports for the first time that EVs can be found in experimental and human pulmonary fibrosis, carry fibrotic mediator such as WNT5A, and contribute to fibrogenesis. Further investigations of EVs in this devastating disease to better understand the contribution to fibrotic pathomechanisms, as well as to elucidate their potential as therapeutic and biomarkers, are warranted. ■

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