ONLINE SUPPLEMENTARY INFORMATION

Macrophage-derived exosomes attenuate fibrosis in airway epithelial cells through delivery of anti-fibrotic miR-142-3p

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Supplementary Methods

Patient cohorts.

The diagnosis of (definite) IPF was made according to the international recommendations of the ATS/ERS[1–3] using the pulmonary function test, HRCT scan (probable UIP pattern), bronchoalveolar lavage (when available), as well as the clinical history of the patient. All other causes of interstitial lung disease (such pneumoconiosis, hypersensitivity pneumonitis, pneumonia associated with connective tissue disease or drug induced interstitial lung disease) were excluded. HS showed any symptoms of a respiratory disease and all had normal spirometric values with predicted FEV1 >80% and FEV1/FVC ratio >70%.

Isolation of exosomes from biofluids (sputum, plasma) or culture medium

First, the biofluids (sputum (3 ml), plasma (3 ml)) were resuspended in PBS (25 ml) and precleared by centrifugation at 400 g for 5 minutes, then 2,000g for 20 minutes at 4°C, followed by centrifugation at 20,000 g for 120 minutes at 4°C. Then, the supernatants were passed through a 0.22-µm filter (Millipore). To isolate exosomes, the precleared supernatants of sputum or plasma were ultracentrifuged at 110,000g for 120 minutes at 4°C, followed by washing of the exosome pellet with PBS at 110,000g for 120 minutes at 4°C (Optima XPN-80 Ultracentrifuge, Beckman Coulter, SW32 rotor). The supernatant was discarded and the exosome pellet was resuspended in PBS or lysed with Qiazol and stored at -80°C. THP1 macrophages-derived exosomes were isolated using the same protocol. The protein levels of the exosome preparations were measured using the BCA Protein Assay kit (Pierce) following the manufacturer's instructions. Exosomes were characterized by Dynamic light scattering and western blotting.

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Dynamic light scattering

Exosomes were suspended in PBS at a concentration of 50 µg/ml, and analyses were performed with a Zetasizer Nano ZS (Malvern Instruments, Ltd.). Intensity, volume and distribution data for each sample were collected on a continuous basis for 4 minutes in sets of 3.

Western blotting

For exosomes, the lysis of the samples was performed using exosome lysis buffer (10% Triton, 1% SDS) and incubation at 60°C for 60 minutes. Samples were denatured by boiling at 95°C for 7 minutes in 1x loading buffer. Equal amounts of protein lysates (5 µg) were electrophoresed on 12% SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride membrane using a wet transfer system. The blots were then blocked with either 5% BSA or commercial powdered milk at 8% for 1 h and then incubated overnight at 4°C with the primary antibody CD63 (#106228D, Invitrogen), CD81 (#10630D, Invitrogen), cytochrome c (#556433, BD pharmingen), TGFβ-R1 (#Ab31013, Abcam), α-tubulin (#Ab6046, Abcam). After 3 washes with TBS/0.1% Tween-20, membranes were incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (anti-rabbit (#7074S, Cell Signaling), anti-mouse (#7076S, Cell Signaling) before being revealed with enhanced chemiluminescence (ECL) substrate (Pierce Biotechnology). The intensity of the bands was quantified using ImageJ

Quantitative RT-PCR (qRT-PCR)

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For quantification of miR expression, 50 ng RNA was reverse transcribed into cDNA using qScript miRNA cDNA Synthesis kit (Quanta Biosciences), and qRT-PCR was conducted in triplicate using Perfecta SYBR Green Super Mix (Quanta Biosciences). Thermal cycling was performed on an Applied Biosystems 7900 HT detection system (Applied Biosystems). In exosomes, the relative miR levels were normalized to 3 internal controls selected previously *via* Normfinder software, miR-222-3p, miR-191-5p and miR-16-5p, using the Delta-Delta Ct method. For cells, data was normalized to miR-16-5p and U6 small nuclear RNA.

For quantification of mRNA expression, 500 ng RNA was transcribed into cDNA using the iScript cDNA Synthesis (Bio-Rad), and qRT-PCR was conducted in triplicate using Takyon MasterMix (Eurogentec). Data was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the Delta-Delta Ct method. All qRT-PCR primers are listed in Supplementary Table S1.

Cell culture and treatments

Human alveolar basal epithelial cell line (A549) and human lung fibroblast cell line (MRC5) were cultured in Eagle's Minimum Essential Medium (EMEM) (#21090-055, Gibco) with 10% FBS (Invitrogen), 2 mM L-glutamine (#A2916801, ThermoFischer Scientific), 1% Non Essential Amino Acid (#11140-035, Gibco) and 5 mM sodium pyruvate (#11360-039, Gibco) for A549 only. Primary lung fibroblasts (HLF) were cultured in EMEM with 10% FBS (Gibco). THP-1 monocytes (ATCC) were cultured in RPMI1640 with L-glutamine and 10% FBS (Invitrogen).

To induce the differentiation of THP-1 monocytes into macrophage-like cells (THP-1 macrophages), THP-1 monocytes were incubated with phorbol 12-myristate 13-

acetate (PMA) (#P1585, Sigma) (100 ng/ml) for 2 days followed by 3 days of rest. A549 and MRC5 were stimulated with 5 ng/ml of TGF-β for 4 h.

To generate macrophage-derived exosomes, THP-1 macrophages were culture for 72 h in exosome-depleted medium (exosomes were depleted from the serum by overnight centrifugation at 110,000 g and 4ºC) Then, culture medium was collected and macrophage-derived exosomes were isolated using standard ultracentrifugation protocol. For macrophage-derived exosomes experiments, A549 and MRC-5 cells were treated with 5 µg/ml of exosomes for 24 h. To silence the expression of miR-142-3p, THP-1 macrophages were transfected with inhibitor control or miR-142-3p inhibitor (20 nM). After 24h, the culture medium was refreshed and miR-142-3p depleted macrophage exosomes were isolated after additional 72h.

Transfection of cells

Transfection of miR Mimics: 200,000 cells were transfected in 6-well plates with pre-miR control or human pre-miR-142-3p (12.5 nM) using DharmaFECT 4 transfection reagent (T-2004-03, Thermo Scientific) and analyzed after 48h.

Transfection of miR inhibitors: 250,000 cells/ml were transfected with inhibitor control or miR-142-3p inhibitor (20 nM) using DharmaFECT 4 transfection reagent (T-2004-03, Thermo Scientific) and analyzed after 24h.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay

Proliferation was determined using the BrdU assay. A total of 5000 cells/well were seeded into 48-well plate and transfected the next day. 48 h following transfection, BrdU incorporation was assayed by incubating the cells with BrdU using BrdU Cell

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Proliferation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

miR target prediction and pathway analysis

In order to identify biological pathway associated to the IPF-related miRs, *in silico* analysis was performed with *mirPath v.3* using *Tarbase v.7* database. The Database for Annotation, Visualization and Integrated Discovery (*DAVID v.6.8*) was used to visualized the direct targets belonging to TGF-β signaling pathway.

References

1 Raghu G, Remy-Jardin M, Myers JL, *et al.* Diagnosis of Idiopathic Pulmonary Fibrosis. An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline. *Am J Respir Crit Care Med* 2018;**198**:e44–68. doi:10.1164/rccm.201807-1255ST

2 Fidler L, Shapera S. Diagnostic criteria for idiopathic pulmonary fibrosis. 2018. doi:10.1016/S2213-2600(18)30020-1

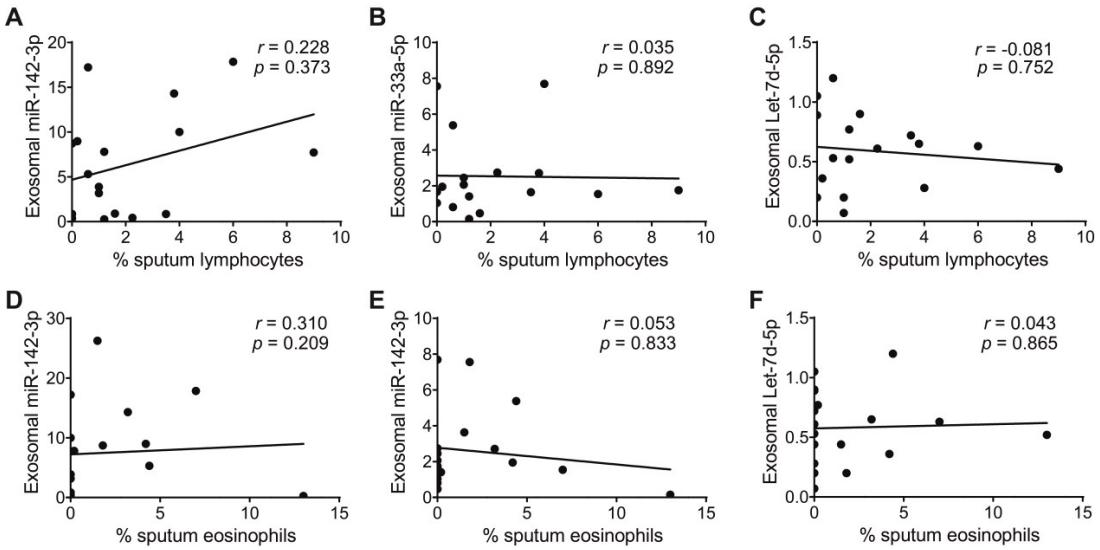
3 Richeldi L, Wilson KC, Raghu G, *et al.* Diagnosing idiopathic pulmonary fibrosis in 2018: bridging recommendations made by experts serving different societies. doi:10.1183/13993003.01485-2018

Supplementary Tables

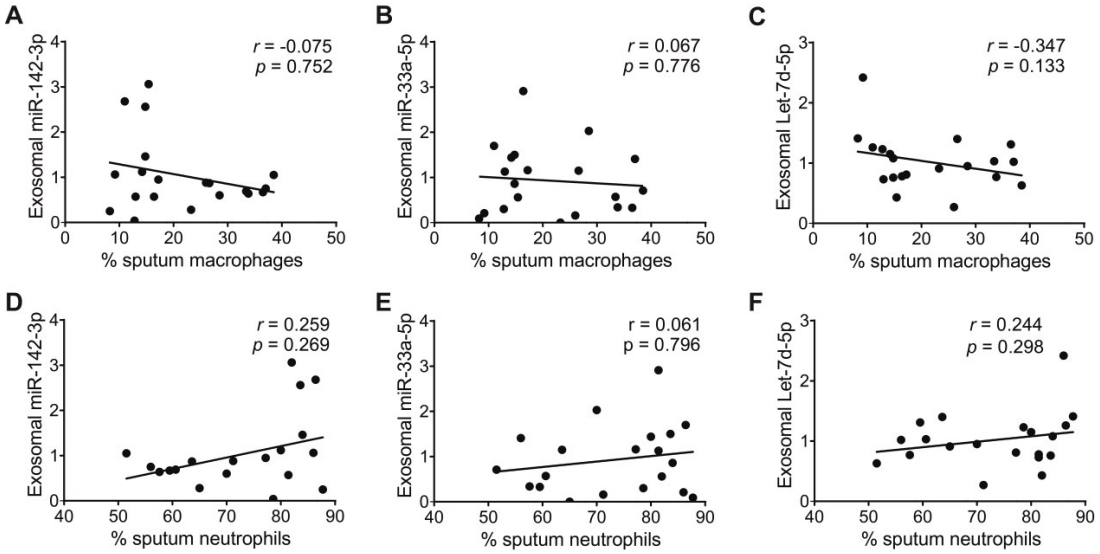
Supplementary Table S1: Primers used for qRT-PCR

Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')
hsa TGFβ-R1	CCTCTTCAAAAACTGGGTCTG	ACCTTTGCCAATGCTTTCTT
hsa COL1A1	TGTTCAGCTTTGTGGACCTC	CGTTCTGTACGCAGGTGATT
hsa COL3A1	TGTGAATCATGCCCTACTGG	ATAGCCTGCGAGTCCTCCTA
hsa TGF-β1	ACACATCAGAGCTCCGAGAA	GAGGTATCGCCAGGAATTGT
hsa miR-142-3p	CGCCATGTAGTGTTTCCTACTTT	Universal reverse primer
hsa miR-33a-5p	CGCGTGCATTGTAGTTGCATT	Universal reverse primer
hsa let-7d-5p	GGGACGAGAGGTAGTAGGTTGC	Universal reverse primer
hsa miR-21-5p	GCTAGCTTATCAGACTGATGTTGAAA	Universal reverse primer
hsa miR-26a-5p	TTCAAGTAATCCAGGATAGGCTAAA	Universal reverse primer
hsa miR-200c-5p	CCCAGCAGTGTTTGGAAAA	Universal reverse primer
hsa miR-222-3p	GCTACATCTGGCTACTGGGTAAA	Universal reverse primer
hsa miR-191-5p	CAACGGAATCCCAAAAGCAGCTG	Universal reverse primer
hsa miR-16	TAGCAGCACGTAAATATTGGCG	Universal reverse primer
hsa U6 snRNA	CACCACGTTTATACGCCGGTG	Universal reverse primer

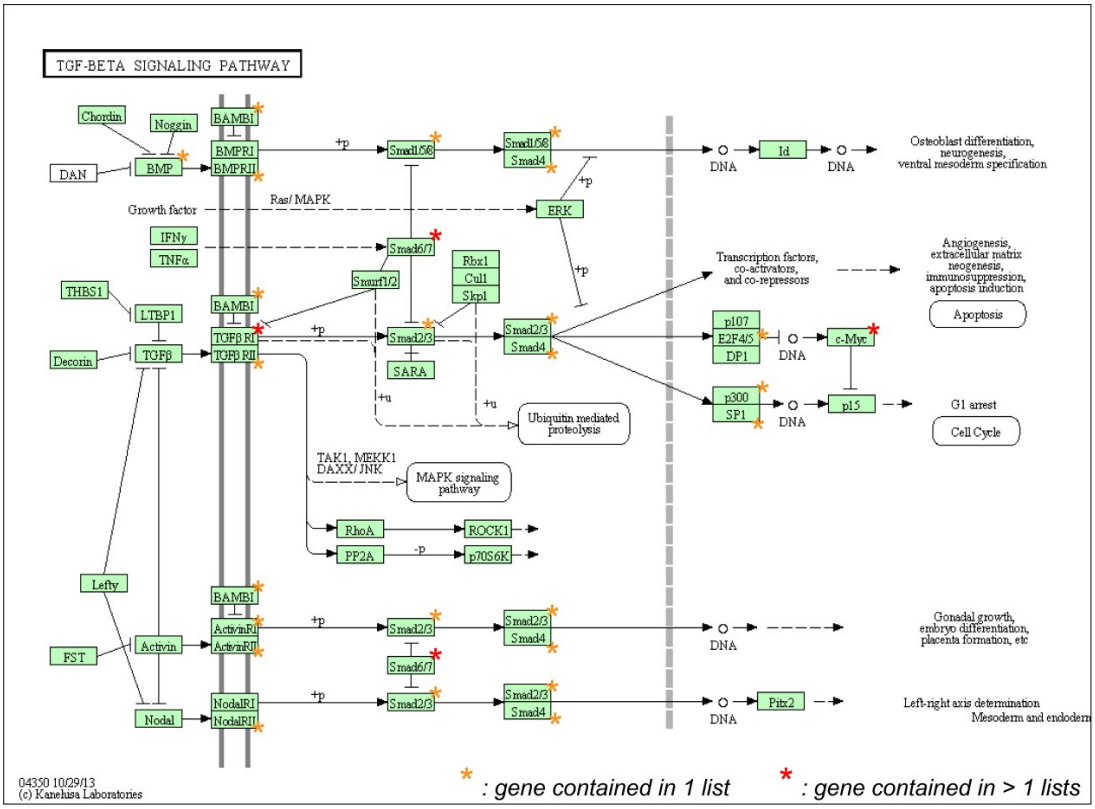
Supplementary Figures



Supplementary Figure S1. Correlation between the percentage of sputum lymphocytes/eosinophils and expression of exosomal miRs in IPF context. (A-C) Spearman correlation between the percentage of sputum lymphocytes and expression level of (A) exosomal miR-142-3p, (B) exosomal miR-33a-5p and (C) exosomal Let-7d-5p from sputum of IPF patients. (D-F) Spearman correlation between the percentage of sputum eosinophils and expression level of (D) exosomal miR-142-3p, (E) exosomal miR-33a-5p and (F) exosomal Let-7d-5p from sputum of IPF patients. Panels A-F: data are non-normally distributed and analyzed using Spearman correlation.

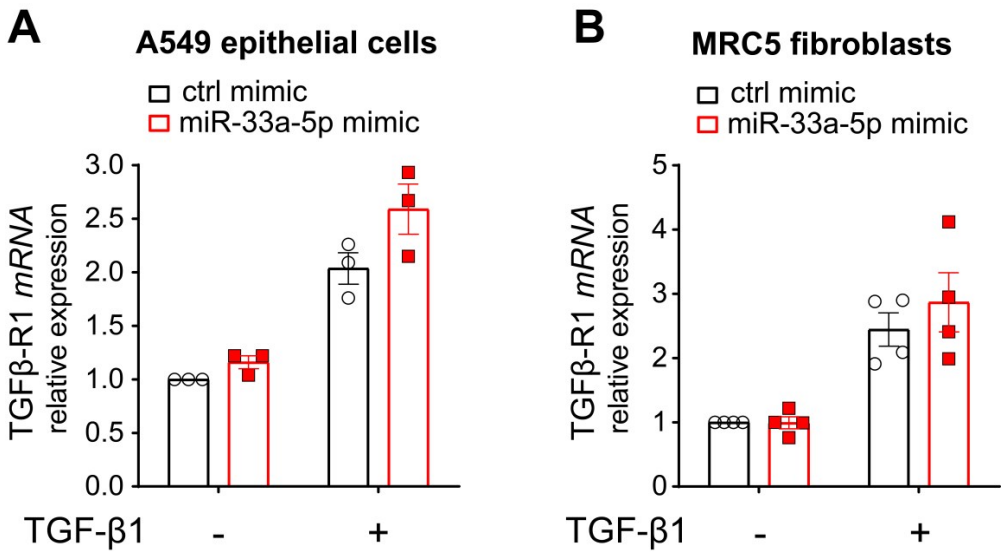


Supplementary Figure S2. Correlation between the percentage of sputum macrophages/neutrophils and expression of exosomal miRs in physiological context. (A-C) Spearman correlation between the percentage of sputum macrophages and expression level of (A) exosomal miR-142-3p, (B) exosomal miR-33a-5p and (C) exosomal Let-7d-5p from sputum of healthy subjects. (D-F) Spearman correlation between the percentage of sputum neutrophils and expression level of (D) exosomal miR-142-3p, (E) exosomal miR-33a-5p and (F) exosomal Let-7d-5p from sputum of healthy subjects. Panels A-F: data are non-normally distributed and analyzed using Spearman correlation.

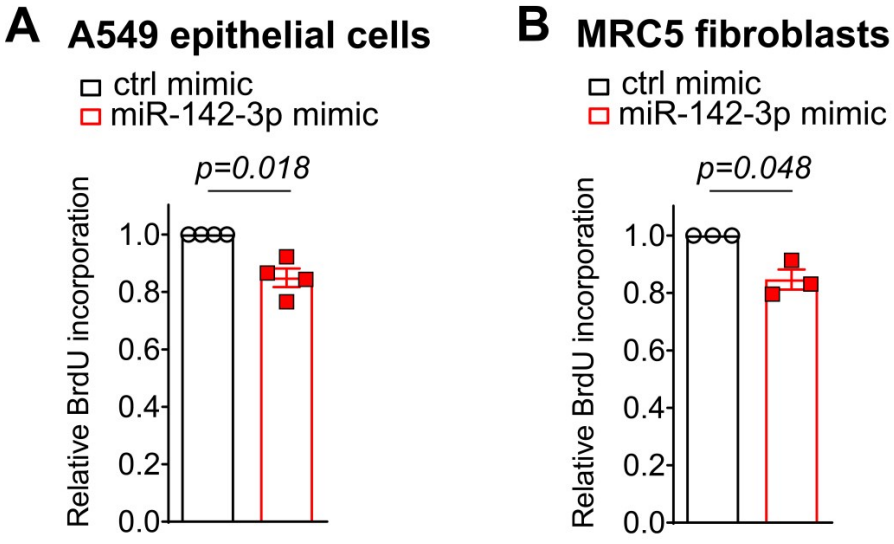


TGFβ-R1 is targeted by the 3 sputum-dysregulated miRs from IPF patients

Supplementary Figure S3. Visualization of the targeting of TGF-β signaling pathway by sputum IPF-related miRs. The Database for Annotation Visualization and Integrated Discovery (DAVID v.6.8) was used to visualize the direct targets of miR-142-3p, miR-33a-5p and Let-7d-5p belonging to TGF-β signaling pathway.



Supplementary Figure S4. MiR-33a-5p presents no effect on TGFβ-R1 expression. MiR-33a-5p overexpression did not modify the expression of TGFβ-R1 mRNA in (A) alveolar epithelial cell line (A549) (n=3) and (B) lung fibroblast cell line (MRC5) (n=4), as assessed by qRT-PCR. Cells were either unstimulated or treated with TGF-β1 (5ng/ml) for 4 hours. Data passed Shapiro-Wilk normality test and are expressed as mean (±SEM) and analyzed using paired two-tailed t-test.



Supplementary Figure S5. MiR-142-3p represses the proliferation of A549 and MRC5 cells. BrdU incorporation is decreased by miR-142-3p mimic in (A) A549 and (B) MRC5 cells. Data passed Shapiro-Wilk normality test and are expressed as mean (±SEM) and analyzed using paired two-tailed t-test.