Online data supplement

Inhibition and role of Let-7d in Idiopathic Pulmonary Fibrosis

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Materials and Methods

microRNA promoter analysis: Genomic coordinates of all differentially expressed microRNAs between IPF and control lungs and the coordinates of their murine orthologues were obtained from the UCSC Genome Browser (E1). The 1kb sequence upstream of the intergenic microRNAs and the 1kb sequence upstream of the host gene for the intronic microRNAs were collected. The host gene promoter sequence was used for intronic microRNAs because previous reports have shown that the microRNA and host gene are co-transcribed and share the same promoter region (E2).

Quantitative RT-PCR: The quantity of the RNA was determined by optical density, measured at 260nm by Nanodrop spectrophotometer. RNA quality was measured using the RNA 6000 Nano kit and the small RNA kit on the Agilent Bioanalyzer 2100. For RT reactions, 50 ng of total RNA was used in each 15 µl reaction. The conditions for the RT reaction were: 16 °C for 30 min; 42 °C for 30 min; 85 °C for 5 min; and then held on 4 °C. The cDNA was diluted 1:14 and 1.33 µl of the diluted cDNA was used with the TaqMan primers in the PCR reaction. The conditions for the PCR were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in the ABI 7300 real-time PCR system. The results were analyzed by the ΔΔCt method using RNU43 control RNA for normalizing human microRNAs and snoRNA55 for mouse microRNAs. Fold change was calculated taking the mean of the controls as the baseline. TaqMan gene expression assays (ABI, Foster City, CA) were used to determine the relative expression levels of HMGA2, CDH2, VIM, ACTA2, CDH1, ZO1 and COL1A. 500 ng of RNA was reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen,

Carlsbad, CA) in a total reaction volume of 20 µl, the cDNA diluted 1:5 and 3 µl of this cDNA was used in a total volume of 31 µl for the PCR. PCR conditions were as follows: 12 min at 95°C, followed by 40 cycles with 15 s at 95°C and 1 min at 60°C in the ABI 7300 real-time PCR system. The results were analyzed by the ΔΔCt method and GUSB was used for normalization. Fold change was calculated taking the mean of the controls as the baseline.

Chromatin Immunoprecipitation (ChIP): A549 cells were grown to 5×10^7 – 1×10^8 cells per analysis condition. Cells were either untreated (control) or stimulated with 2 ng/mL TGF-β for 30 minutes. Chromatin cross-linking was performed by adding 1/10 volume of freshly prepared 11% formaldehyde solution for 15 minutes at room temperature. The cross-linking reaction was then quenched by adding 1/20 volume of 2.5M glycine. Cells were rinsed twice with PBS, collected with a silicon scraper, flash frozen in liquid nitrogen, and stored at −80°C until use. Upon thawing, cells were resuspended in a lysis buffer and sonicated at 4°C to solubilize cellular components and shear crosslinked chromatin. The cell lysate was incubated overnight at 4°C with 100 µl of Dynal Protein G magnetic beads that had been preincubated with 10 µg of either anti-flag (mock IP) or anti-SMAD3 antibodies (Millipore, Billerica, MA). Protein G magnetic beads were washed five times with RIPA buffer and one time with TE buffer containing 50 mM NaCl. Cross-linked promoter fragment/transcription factor complexes were eluted from the beads by heating at 65°C with vortexing at 2 minute intervals for 15 minutes. Crosslinking was reversed by incubation at 65°C overnight. Recovered promoter fragments were treated with RNaseA, proteinase K digestion, and purified by

phenol:chloroform:isoamyl alcohol extraction/ethanol precipitation. Gene-specific PCR was performed on a portion of the purified recovered nucleic acid (35 cycles) to verify the presence of the upstream sequence of pre-hsa-let-7d. The primers used for gene-specific PCR are: *let-7d forward*: 5' - CAC TTA AAC CCA GGA GGC AGA GGT T - 3' and *let-7d reverse*: 5' - ACC ACG TAT TAC TGG AGT CGC TGA - 3';

Electrophoretic Mobility Shift Assay (EMSA): The supernatant was reserved and snap frozen in liquid nitrogen as the nuclear protein fraction. Nuclear extracts and recombinant full length SMAD3 protein (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with 5'-end Cyanine-5 labeled probe and/or non-labeled competitor oligonucleotide for 20 minutes at room temperature in a binding buffer consisting of 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 25 mM DTT, 200 mM NaCl, 50 mM Tris HCl pH 7.6, and 0.25 mg/mL poly(dI-dC). The complementary oligonucleotides (5' - GATAATTAAATGTTAAAAGTCAGC - 3', 5' - GCTGACTTTTAACATTTAATTATC - 3') were synthesized by Integrated DNA Technologies (Coralville, IA), and consisted of a sequence upstream of the predicted SMAD3/let7d binding site (GGCTGAGTA). Additionally, a supershift assay was performed by incubating nuclear extract with different amounts of polyclonal antibody to SMAD3 (Abcam, Cambridge, MA) prior to incubating with the target oligonucleotide. The protein/DNA complexes were run on a 6% native polyacrylamide gel and visualized on a Typhoon imaging and documentation system using Cyanine-5 dye excitation and fluorescence settings.

Tissue microarray construction: Tissues were snap-frozen and stored at -70^{0}C. Specimens were fixed in cold-ethanol for 16 h and then embedded in paraffin. Hematoxylin and eosin (H&E) -stained sections were made from each block to define representative fibrotic and inflammatory lesion regions. Areas of interest were identified in H&E stained slides by a conventional microscope (Olympus BX-50). Tissue cylinders with a diameter of 1.5 mm were punched from selected areas of each "donor" block using a thin-wall stainless tube from a precision instrument (TMA-100, Chemicon, USA) and were transferred by a solid stainless stylet into defined array coordinates in a 45 * 20 mm new recipient paraffin block. The tissue microarray blocks were constructed in three copies (each containing one sample from a different region of all lesions). One sample was taken from the center and two samples from different peripheral areas. Ultimately, we constructed three tissue microarray blocks comprising of 80 tissue elements each. Each tissue element in the array was 1.5 mm in diameter and spacing between two adjacent elements was 0.1mm. After the TMA construction 5 μm sections for *in situ* hybridization analysis were cut from the "donor" blocks and were transferred to glass slides using an adhesive-coated tap sectioning system.

In situ hybridization: Lung tissues were obtained from the tissue bank of two different pathology centers (University Hospital of Alexandroupolis, Greece and the Veterans Administration Hospital, N.M.T.S, Athens, Greece). The tissues were fixed in 10% formalin, paraffin-embedded and after the TMA construction samples were cut into 5 μm thick serial sections and were transferred to glass slides using an adhesive-coated tap sectioning system. The paraffin sections were dewaxed in xylene for 2 x 5 min, soaked in

100% ethanol. Then the paraffin sections were soaked in 75% ethanol and after in wash buffer solution so the tissue can retain its initial pH. The sections were then treated with proteolytic solution supplied in the kit for 20 min at 37°C. Excess proteolytic solution was discarded and the slides were dehydrated in 75%, 95% and 100% ethanol for 1 min each and then air-dried. Denaturation and hybridization was done overnight at 37°C with 20nM 5`-digoxigenin-labeled miRCURY LNA detection probe (Exiqon, Denmark) diluted in hybridization buffer (50% Formamide, 5xSSC, 0.1%Tween, 9.2mM citric acid for adjustment to pH6, 50ug/ml heparin, 500ug/ml yeast RNA). The slides were washed in TBS buffer for 3 x 1 min. Slides were transferred onto a 37°C heating block or slide warmer and 2-3 drops of alkaline phosphatase conjugate were applied to each specimen. Slides were then incubated for 30 minutes at 37°C. Excess detection reagent was tapped off and slides were washed in TBS buffer. Slides were then soaked in three changes of TBS buffer for 1 minute each and then transferred into a container with distilled or deionized water and soaked for 1 additional minute. They were then taken out; excess of water was wiped off and dried around the edges using a lint-free cloth. Sections were then transferred onto a 37°C heating block and 2-3 drops of NBT/BCIP substrate were applied to each specimen. Then slides were incubated in the dark for 5-15 minutes at 37°C (color development was examined every 5 minutes with a light microscope) and removed from the heating block. They were then washed three times for 1 minute in changes of distilled or deionized water. The slides were counterstained using Nuclear Fast Red. 2-3 drops of counterstain were applied to each slide (with hematoxylin for 10 sec) and rinsed in distilled water for 3 min. Slides were then incubated for 15 sec, excess of counterstain was tapped off and then they were washed briefly in distilled or deionized

water. Images were acquired by using the high-resolution DUET, BioView scanning system for CISH, morphology applications.

CISH semi-quantitative image analysis: The number of let-7d positive alveolar epithelial cells (AECs)/mm^2 in 5 fields per case was counted by two independent pathologists - observers using the high-resolution DUET, BioView scanning system for CISH morphology and immunocytochemistry applications, at x100 magnification. Independent t-test and Mann-Whitney test were used to compare let-7d positive AECs/mm^2 between IPF and control lung samples.

Immunohistochemistry: Anti-HMGA2 rabbit polyclonal antibody (4 μg/ml) (Abcam) was applied and samples were incubated at 4°C overnight. A secondary biotinylated anti-immunoglobulin followed by horseradish peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) was used according to manufacturer's instructions. 3-amino-9-ethyl-carbazole (BioGenex) in acetate buffer containing 0.05% H_2O_2 was used as substrate. The sections were counterstained with hematoxylin. The primary antibody was replaced by non-immune serum for negative control slides. Mouse lung tissue was fixed and inflated with PROTOCOL SafeFix (Fisher Scientific, Waltham, MA). Tissue sections were deparaffinized and rehydrated using xylene and sequential ethanol rinses, endogenous peroxidase activity blocked with methanol and hydrogen peroxide, antigen retrieval done by heating the slides in sodium citrate buffer pH 6.0 at 95 °C for 30 min followed by blocking in 5% goat serum for 30 min. The sections were incubated with an antibody to ACTA2 (ab21027, Abcam) for 1 hour at room temperature. Following two 5

min washes in TBS, the sections were incubated in the secondary antibody for 30 min, washed twice for 5 min in TBS, incubated for 30 min with RTU Vectastain Elite ABC Reagent (Vector Laboratories), washed in TBS stained with DAB Substrate kit (Vector Laboratories) according to the manufacturer's instructions, counterstained with hematoxylin and mounted in Vectashield hardset mounting medium (Vector Laboratories). The number of ACTA2 positive alveolar epithelial cells (AECs) in 10 fields per slide was counted by two independent pathologists at x40 magnification. T-test was used to compare ACTA2 positive AECs between saline and antagomir-treated lung sections.

Immunofluorescence: A549 cells were plated on cover slips. Cells were starved for 24 hours by the removal of serum and transfected with 50 nM anti-let7d for 48 hours. Cover slips were removed, and fixed in 2% paraformaldehyde (Sigma) for 40 minutes. Permeabilization of cells was carried out by using 0.1% Triton X in PBS for 40 minutes, with three washes in PBS each for five minutes followed by blocking with 0.3% BSA and 5% goat serum in PBS for 60 minutes, and incubated with anti-cytokeratin, anti-vimentin, anti-N-cadherin, or anti-alpha-smooth muscle actin (all from Abcam Inc., Cambridge, MA) in 0.5% BSA in PBS for 60 minutes. Following three washes with 0.5% BSA in PBS (5 minutes each), coverslips were incubated with the appropriate secondary antibody (Invitrogen, Carlsbad, CA) for one hour at 37°C. After staining, cover slips were washed with 0.1% Triton in PBS for 2 times 5 minutes each followed by three washes with PBS 5 minutes each. Coverslips were inverted onto slides and mounted in Vectashield anti-fade medium that contained DAPI for nuclei staining (Vector Laboratories, Burlingame, CA)

to prevent photobleaching. Slides were examined using a Leica TCS-SP2 laser scanning confocal microscope equipped with appropriate lasers for simultaneous imaging of up to four fluorophores. Digital data was archived to compact disk or DVD and prepared for publication using Adobe Photoshop software (Adobe Systems Inc., MountainView, CA). Lung tissue from mice was frozen into OCT blocks and sectioned. The frozen sections were fixed in ice cold acetone for 15 min. After washing the sections twice for 2 min each in PBS, avidin-biotin blocking was performed using the Avidin/Biotin Blocking kit (Vector Laboratories) according to the manufacturer's instructions. The sections were blocked for 30 min in a blocking buffer consisting of 5% goat serum and 3% bovine serum albumin in PBS. The sections were then incubated in the desired primary antibody (ab27957 (FSP1) and ab32575 (ACTA2), Abcam) for 1h, washed for 5 min in PBS and incubated in the appropriate secondary antibody for 30 min. After washing for 5 min in PBS, the sections were incubated in Fluorescein Avidin DCS (Abcam) for 10 min followed by another wash of 5 min in PBS. This procedure was repeated for the second antibody (ab40879 (SPC), Abcam) but incubated with Texas Red Avidin DCS (Abcam) for 10 min. The nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI) for 1 min, washed in PBS for 5 min and mounted using VECTASHIELD HardSet Mounting medium (Vector Laboratories). Slides were examined using an Olympus Fluoview 1000 confocal microscope equipped with appropriate lasers. Colocalization was quantified using MetaMorph software (Molecular Devices, CA).

Immunoblotting: Immunoblotting was used to detect the protein expression in RLE-6TN cells grown in 6-well plates were transfected either with the scrambled oligonucleotide or

let-7d inhibitor. The cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed at 4º C with a solution containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM PMSF, 1mM EDTA, 5µg/ml aprotinin, 5µg/ml leupeptin, 1% Triton x-100, 1% sodium deoxycholate and 0.1% SDS. The soluble cell lysate was centrifuged at 16000 x g for 15 min and the supernatant was transferred to a clean microcentrifuge tube. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). 10 µg of the lysate was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P membrane (Millipore Corporation, Billerica, MA). After blocking with 3% bovine serum albumin for 1h, the membrane was incubated overnight with the desired antibody at 4º C. Following three 10 min washes with tris-buffered saline Tween-20 (TBST), the membranes were incubated with the appropriate secondary antibody for 1h at room temperature. Target proteins were observed using the Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer Life Sciences, Boston, MA) according to the manufacturer's instructions. Equal protein loading was assessed by stripping the membranes with 0.1M glycine, pH 2.0 and reprobing with antibodies against β-actin (Sigma) or laminin A/C. Densitometric analysis was performed using the ImageJ software (National Institute of Health).

MicroRNA microarrays: 100 ng of total RNA was dephosphorylated using calf intestine alkaline phosphatase (GE Healthcare, Piscataway, NJ), denatured with DMSO, and labeled with pCp-Cy3 using T4 RNA ligase (New England Biolabs, Ipswich, MA) at 16°C for 2h. The labeled RNA was purified using Micro Bio-spin 6 columns and hybridized onto the Agilent microRNA microarrays at 55°C for 20h. The arrays were

washed with Gene Expression Wash Buffers 1 and 2 (Agilent) and scanned using the Agilent Microarray Scanner. The scanned images were processed by Agilent's Feature Extraction software version 9.5.3.

Bleomycin administration: Mice were anesthetized by placing them in a chamber having paper towels soaked with 40% isoflurane solution. 0.0375 U of bleomycin (Hospira, IL) was administered intratracheally in 50 µl of 0.9% saline. Mice were sacrificed after 14 days and RNA isolated from the lungs.

Synthesis of antagomir: The single-stranded RNA 5` -aacuaugcaaccuacuaccucu- 3` was custom synthesized from Dharmacon, Lafayette, CO. The sequence of the oligonucleotide was complementary to that of mmu-let-7d. All bases had 2'-O-methyl modifications, the first two bases and the last four bases had phoshorothioate linkages and a cholesterol molecule was conjugated at the 3' end. The scrambled control had the sequence 5` -aaccauguaaacuacuacaucu- 3` with the same modifications as the antagomir.

References

E1. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at ucsc. *Genome research* 2002;12:996-1006.

E2. Baskerville S, Bartel DP. Microarray profiling of microrna reveals frequent coexpression with neighboring mirnas and host genes. *RNA (New York, NY* 2005;11:241-247.

Table E1: Significantly upregulated microRNAs

Table E2: Gene symbols and their common designations

Figure E1: Down-regulated microRNAs have many overlapping targets

(A) Representation of the computationally predicted targets of a few down-regulated microRNAs color-coded by the number of sites in the 3'UTR as predicted by TargetScan. **(B)** Direct comparison of microarray gene expression between IPF and control. X axis – Average gene expression in control, Y axis - Average gene expression in IPF. Red circles represent the genes in (a) and significantly different between IPF and control lungs.

Figure E2: let-7d in mice treated with bleomycin

Expression of let-7d in control mice lungs and 14 days after bleomycin instillation. The results represent an average expression ± S.D. with 4 mice in each group.

Figure E3: Specificity of the let-7d inhibitor and effect of HMGA2 inhibition on expression of mesenchymal markers

Expression levels of **(A)** let-7d **(B)** let-7c and **(C)** miR-10a determined by qRT-PCR after transfecting A549 cells with a let-7d inhibitor. **(D)** inhibiton of HMGA2 does not completely ablate increase in CDH2, ACTA2 and VIM determined by qRT-PCR in A549 cells after 48h of let-7d inhibition alone and in combination with HMGA2 inhibition.

“Scr mir” is the negative control for let-7d inhibition and “scr scr” refers to the negative controls for let-7d inhibition and HMGA2 inhibition.

Figure E4: Efficacy of the let-7d antagomir

Expression levels of let-7d in mice lungs after 10 mg/kg antagomir treatment for 4 days and 5 mg/kg antagomir treatment for 18 days.

Figure E5: Minimal colocalization of ACTA2 and S100A4 with SFTPC in control tissues

Immunofluorescence imaging of mouse lungs treated with scrambled control. The red fluorescence represents SFTPC, an epithelial marker. The green fluorescence denotes the mesenchymal markers S100A4 and ACTA2. Nuclei were counterstained with DAPI.

Table E1:

microRNA	p- value
hsa-miR-409-3p	3.78199E-10
hsa-miR-92b	0.004010043
hsa-miR-376a	0.001474672
hsa-miR-205	0.004607749
hsa-miR-31	0.037779374
hsa-miR-765	0.047904179
hsa-miR-199b	0.007501247
hsa-miR-198	0.027274441
hsa-miR-622	0.047421911
hsa-miR-330	0.046221628
hsa-miR-379	0.019656786
hsa-miR-659	0.033551884
hsa-miR-182	0.006544536
hsa-miR-487b	0.008208623
hsa-miR-299-5p	2.82814E-05
hsa-miR-127	1.84057E-07
hsa-miR-296	0.017690471
hsa-miR-509	0.0309535
hsa-miR-557	0.039409913
hsa-miR-134	0.016639634
hsa-miR-491	0.040645633
hsa-miR-132	0.000826688
hsa-miR-155	0.043100133
hsa-miR-99a	0.012154322
hsa-miR-324-3p	0.016792331
hsa-miR-214	0.000936264
hsa-miR-199a	0.000574496
hsa-miR-320	0.03124245

Table E2:

Entrez Gene ID	Gene Symbol	Other Designations	Marker
59	ACTA2	α-smooth muscle actin	mesenchymal
7431	VIM	vimentin	mesenchymal
1000	CDH2	N-cadherin	mesenchymal
7082	TJP1	zona-occludens 1	epithelial
999	CDH1	E-cadherin	epithelial
6275	S100A4	fibroblast-specific protein 1	mesenchymal
6440	SFTPC	surfactant protein C	epithelial