ONLINE DATA SUPPLEMENT

# MIR-29 IS A MAJOR REGULATOR OF GENES ASSOCIATED WITH PULMONARY FIBROSIS

Leah Cushing[1], Ping Ping Kuang[1], Jun Qian[1], Fengzhi Shao[1], Junjie Wu[1], Frederic Little[1], Victor J. Thannickal[2], Wellington V. Cardoso[1], Jining Lü [1*]

## EXPERIMENTAL PROCEDURES

*Northern Blot Analysis:* 5-10 ug of total RNA was separated on 15% Novex TBE Urea Gels (Invitrogen) and electrotransferred to Bright Star-Plus nylon membranes (Ambion), followed by UV cross-linking and Pre-hybridization using ULTRAhyb-Oligo Buffer (Ambion), then subjected to overnight hybridization with 0.1 nm 5' DIG-labeled LNA probes (Exiqon) at $40^0$C. After stringent washes three times at $40^0$C in pre-made Northern Max Low Stringency Wash Solution #1 (Ambion), blots were rinsed in washing buffer (0.1 maleic acid, 0.15 M NaCl, 0.1% Tween, pH 7.5) and blocked for 30 minutes at room temperature (Roche). Blots were subsequently incubated for 1 hour at room temperature with anti-DIG-AP antibody (Roche) in blocking buffer, washed twice for 15 minutes in washing buffer, and equilibrated in detection buffer (0.1 M Tris pH 9.5, 0.1 M NaCl) for 5 minutes. Signal was detected using CDP-star chemiluminescent substrate (Roche).

*miRNA in situ hybridization on frozen sections:* We performed in situ hybridization on frozen sections using 5' DIG-labeled LNA probes (Exiqon) according to Obernosterer's protocol (Alenius, et al., 2007). Briefly, tissue slides were first washed with DEPC-treated PBS (3 X 3min), followed by acetylating in triethanolamine buffer plus acetic anhydride for 10 min, permeabilized in PBST (PBS plus 0.1% Triton X-100 in DEPC-treated water) for 30 min, and washed 3X5min at RT. After pre-hybridization at RT for 2 hrs, hybridization was carried out at $50^oC$ overnight in the same pre-hybridization buffer (50% formamide, 5x SSC, 1x Denhardt's solution, 500ug/mL salmon sperm DNA, 5% dextran sulfate) containing 25nM of miR-29b LNA probe. Then, slides were sequentially washed with 0.2xSSC at $50^oC$ and with 0.2 X SSC and PBS for 5 min at RT. Slides were then incubated in blocking solution (TTBS, 0.05M Tris, pH 7.5, 0.15M NaCl, 0.1% Tween-20, plus 5% sheep serum) and incubated with anti-digoxigenin-AP antibody (1:2500, Roche) overnight at $4^oC$. After washing in TTBS for 3X10 min, signals were developed using BM purple (Roche).

*Native PAGE and Western Analysis:* Anti-collagen a1(I) antibody (1: 500, Rockland) and reagents from Invitrogen were used for Native PAGE Western Analysis of COL1A1 following the manufacture's instruction. IMR-90 cells were seeded in 6-well plates and grown to 60% confluence. 24 hrs after transfection with different oligos, cells were washed with PBS and maintained in serum-reduced media (0.4%) for additional 48hrs before lysing with Native PAGE Sample Buffer containing 1% n-dodecyl-b-D-maltoside(DDM) (Invitrogen). Equal amounts of proteins

(5 ug) were resolved using Native PAGE Novex 4-16% Bis-Tris Gel system, and transferred to PDVF membranes using the iBlot Dry Blotting System. Membranes were washed with TBST and blocking with 7% nonfat dairy milk in TBST at RT for 1 hr, and then incubated with at 4$^{o}$C overnight. Immuno-Star was used for detection (Bio-Rad).

***mRNA array analysis**:* Mouse Gene 1.0 ST Whole Genome Array (Affymetrix) was used for mRNA expression profiling according to the manufacturer's protocol. Experiments were performed in triplicate for each condition. The expression array .cel files were imported using PGS Gene Expression Workflow tool (subject to RNA normalization and log2 transformation). A two-sample t-test was performed in order to identify differential gene expression between comparative groups ($P<0.05$ considered significant). To explore the functional significance of differentially expressed transcripts, DAVID (http://david.abcc.ncifcrf.gov/) and Targetscan(http://www.targetscan.org/> ) databases were used for data analysis. The array data has been deposited into the GEO database in NCBI (GSE18651). Other GEO array database GSE17518 (TGF β 1 treated IMR-90 cells) and GSE2640 (bleomycin treated C57/Bl6 mouse lungs) were used to examine the expression of miR-29 targets in respond to TGFβ1 and in bleomycin-treated lungs.

**miRNA Microarray Hybridization and Data Analysis**. 300ng of total RNA were labeled by using the FlashTag labeling kit (Genisphere, Inc.). In brief, miRNAs were first poly(A) tailed and then directly ligated to the biotinylated signal molecule (Biotin labeled 3DNA dendrimer). We followed Affymetrix GeneChip miRNA Array Procedure for the hybridization and staining. Affymetrix Command Console Software was used for scanning and data acquisition. We used the miRNA QC Tool software (Affymetrix) for data summarization, normalization, and quality control. miRNAs that can be reliably detected at least in one condition were included for further analysis. A two-sample t-test was performed to identify differential miRNA expression between comparative groups ($P<0.05$ considered significant).