**Niehof et al. miRNA profiling in human precision-cut lung slices (PCLS)**

**Additional file 1. Methods.**

**PCLS preparation, cultivation, and storage**

Human lungs were filled ex situ and PCLS were prepared as previously described [1–3]. Briefly, the trachea was cannulated and the lungs were filled up with 37 °C-warm, 2 % low-gelling agarose medium solution (Sigma-Aldrich, Munich, Germany). After polymerization of agarose, lung lobes were cut into 200- to 300-µm-thick slices using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) filled with 4 °C-cold EBSS (Sigma-Aldrich, Munich, Germany). Subsequently, precision-cut lung slices were incubated in DMEM (Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 Ham (DMEM, pH 7.2-7.4) with L-glutamine and 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) without phenol red and fetal bovine serum supplied from Gibco™ (Life Technologies/Thermo Fisher Scientific, Dreieich, Germany). The culture medium was supplemented with 100 units/mL penicillin and streptomycin (Lonza, Verviers, Belgium). PCLS were cultured for 72 h under standard cell culture conditions (37 °C, 5 % CO2, 100 % humidity). Two PCLS were cultured together in 500 µL DMEM as described previously [2]. Two slices were pooled and immediately transferred into liquid nitrogen and subsequently stored at -80 °C.

**Isolation of total RNA including miRNA and quality assessment**

RLT lysis buffer (Qiagen, Hilden, Germany) was added and PCLS were disrupted and homogenized using an Ultra-Turrax® (T10basic, IKA, Staufen, Germany) for 20 s. The homogenate was transferred to 1 volume of phenol/chloroform, carefully shaken for 30 s, and centrifuged for 5 min at 12,000xg. Subsequently, 1 volume of chloroform/isoamyl alcohol was added, again carefully shaken for 30 s, and centrifuged for 5 min at 12,000xg. The aqueous phase was transferred and RNA was cleaned up with RNA binding beads from the Mag MAX mirVana Total RNA Isolation Kit (ThermoFisher Scientific, Dreieich, Germany). The clean up procedure including a DNase treatment was performed according to the manufacturer’s recommendation. Total RNA was eluted in RNase-free water, pooled from two samples (corresponding to four PCLS in total) and stored at -80 °C. RNA concentration (A260) and purity (A260/A280 ratio) were measured by spectrophotometry (NanoDrop™ 2000 Spectrophotometer, software version 1.6.198, ThermoFisher Scientific, Dreieich, Germany). The RNA 600 Nano assay (Agilent Technologies, Ratingen, Germany) was used for integrity assessment of RNA samples and the Small RNA assay (Agilent Technologies, Ratingen, Germany) was used to visualize small RNAs between 6 and 150 nucleotides. Both assays were analyzed on the Agilent 2100 Bioanalyzer® (Agilent Technologies, Ratingen, Germany).

**Quantitative real time RT-PCR analysis (RTqPCR)**

RTqPCR for miR-15a was performed using the miScript PCR system (Qiagen, Hilden, Germany). The miScript system covers all the steps of conversion of the RNA into cDNA to detection of miRNA in SYBR® Green-based real-time PCR. cDNA was prepared in a reverse transcription reaction using miScript HiSpec buffer. RTqPCR was performed using a human miR-15a-specific miScript primer assay (forward primer) and the miScript universal primer (reverse primer) with QuantiTect SYBR® Green PCR master mix. qPCR reactions were performed using an ABI PRISM 7500 real-time PCR detection system (Applied Biosystems) with the following conditions: 15 min 95 °C; 15 s 94 °C/30 s 55 °C/30 s 70 °C, for 40 cycles; and 15 s 95 °C/1 min 60 °C/ 30 s 95 °C for the melting curve. At the end of each extension phase, fluorescence was recorded and at the end of a run quantification cycles (Cq) were determined for each sample. Serial dilutions of RT reactions were prepared in triplicate (from 2 ng to 62.5 pg) and samples were analyzed by qPCR to measure Cq values. A plot of Cq values versus logarithm of target concentrations resulted in a standard curve, which was used for efficiency calculation (10(-1/slope) – 1, corresponding to 100%) [4–6].

**Data analysis**

Quality control of microarray analysis and visualization of the miRNA data were undertaken using metrics and methods contained in Transcriptome Analysis Console Software (TAC 4.0, Thermo Fisher Scientific, Dreieich, Germany). Quality control metrics include hybridization and RNA spike-in controls, as well as visualization methods of gene expression data such as signal box plots, principal component analysis (PCA), and hierarchical clustering. The hybridization controls are composed of a mixture of biotinylated and fragmented cRNA of bioB, bioC, and bioD from E. coli and cre from P1 bacteriophage in staggered concentrations. The hybridization controls are high-quality controls used for monitoring array hybridization, washing, and staining for reproducible results. The hybridization controls are spiked into the hybridization cocktail, independent of RNA sample preparation, and are thus used to evaluate sample hybridization efficiency on eukaryotic gene expression arrays. The RNA spike-in controls consist of five oligos to confirm poly(A) tailing, ligation, and lack of RNAses in the RNA sample. Oligos 2, 23, and 29 are RNA, and confirm poly(A) tailing and ligation. Oligo 31 is poly(A) RNA, and confirms ligation. Oligo 36 is poly(dA) DNA, and confirms ligation and lack of RNAses in the RNA sample. Normalization and summarization of the microarray data were performed using the Robust Multi-array Average (RMA) method. Furthermore, we also included a treatment group to enable characterization of the miRNA profiles of control donors using methods for gene expression arrays in the TAC software. Differential gene expression was obtained with the default filter criteria Fold Change: > 2 or < -2; and P-val: < 0.05.

**Data comparison with published data from human lung**

To compare the miRNA profiles of control donors with those from published or publicly available datasets from human lungs, we created a list of miRNAs using a cut-off filter of average log 2 signal intensity > 6. We then searched NCBI PubMed and the Gene Expression Omnibus (GEO) repository for miRNA profiles from human lungs, and determined which of the identified miRNAs in the PCLS control samples were also expressed in those human lungs. For instance, for comparison, we downloaded Raw.CEL files from GSE81293 (Expression of miRNA from lung tissue from Systemic Sclerosis patients with interstitial lung disease (SSc-ILD) and healthy controls [7]. We re-analyzed the CEL files using TAC Software and likewise created a list of miRNAs from the healthy controls. On the basis of average log 2 signal intensities obtained in PCLS control samples and healthy controls (GSE81293), a heatmap was generated manually on a list of lung-disease associated miRNAs using GraphPad Prism 8.3.1.

References

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