Supplemental data to article

miRNA Profiling Discriminates Types of Rejection and Injury in Human Renal Allografts

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Table of Contents

Workflow of comparative pathway analysis

After miRNA profiling and statistical work-up comparing control and study groups, target genes of the identified differentially regulated miRNAs were determined utilizing various prediction models as well as experimentally verified targets, complemented by further functional characterization on the molecular process level. For allowing comparative analysis on the molecular process and pathway level the same bioinformatics procedures were applied for differentially regulated mRNAs derived from patients developing acute rejection or delayed graft function. Pathways identified as affected on both, direct mRNA and miRNA target level were used for functional interpretation.

mRNA datasets for comparative analysis

A gene expression dataset of post-transplant biopsies is publicly available for download in the Gene Expression Omnibus (GEO) at NCBI with the identification GSE1563 as published by Flechner et al. . The dataset covers 23 gene expression profiles from post-transplant biopsies for the following groups: Samples from patients with well-functioning transplants with no clinical evidence of rejection (PBx, n = 10), samples from transplant patients with kidneys undergoing acute rejection (combining AREJ and ABMR, n=7), and samples from transplant patients with renal allograft dysfunction without rejection (n=5; 2 CNI toxicity, 2 ATN, 1 focal segmental glomerulosclerosis). We re-analyzed the Affymetrix raw datafiles by utilizing the Bioconductor package SimpleAffy to assess data quality , followed by preprocessing, normalization, and annotation using the robust multi-average (RMA) method and quantile normalization as implemented in the Bioconductor packages affy, gcrma and annaffy . Significance Analysis of Microarray (SAM) was used to evaluate differences between control and study groups. Genes with a fold change of 2 or higher and a delta value of 3.5 was considered as differentially regulated between groups and used for further analysis.

Saint-Mezard et al. published an analysis of independent microarray datasets of renal allograft biopsies revealing an acute rejection transcript set(ARTS) consisting of 70 unique genes , subsequently also used as reference mRNA data set.

The Edmonton data set (Müller et al.) covering gene expression profiles of 28 biopsies for T-cell-mediated rejection, 8 biopsies characterized by antibody mediated rejection, and 72 biopsies without sign of rejection were additionally included in our comparative analysis. Microarray raw data files are available at http://transplants.med.ualberta.ca. We used the same bioinformatics workflow for identification of differentially regulated genes as applied for the Cleveland dataset (Flechner et al.). Significance Analysis of Microarray (SAM) was used to evaluate differences between control and study groups. Genes with a fold change of 2 or higher and a delta value of 2 were considered as differentially regulated between groups. This dataset allowed us to separately analyse acute cellular rejection (AREJ) and antibody mediated rejection (ABMR) as also derived on the level of miRNA profiles.

Table S1. Number of predicted targets (DIANAmT, miRanda and Targetscan), number of experimentally validated targets (miRTarBase) of significantly differentially regulated miRNAs. The number in bracket indicates the no. of unique genes.

	miRNA	No. of predicted targets	No. of experimentally verified targets
DGF versus PBx			
up-regulated	hsa-miR-182	2305	9
	hsa-miR-106b	2976	16
	hsa-miR-20a	2960	26
	hsa-miR-21*	0	0
	hsa-miR-18a	1402	16
	hsa-miR-17	2588	39
	hsa-miR-106a	2664	10
delayed graft function		**14895 (5320)**	**116 (67)**
AREJ versus PBx			
up-regulated	hsa-miR-150	3133	5
	hsa-miR-155	1600	161
	hsa-miR-663a	1145	2
	hsa-miR-638	295	0
acute cellular rejection (up-regulated miRNAs)		6173 (4960)	168 (167)
down-regulated	hsa-miR-138	1634	11
	hsa-miR-125a	2354	16
	hsa-miR-455	1473	0
	hsa-miR-30c-2*	0	0
	hsa-miR-574-3p	261	0
	hsa-miR-502-3p	968	0
	hsa-miR-181b	2339	12
	hsa-miR-99b	105	1
	hsa-miR-139-5p	1710	0
	hsa-miR-27b	2843	8
	hsa-miR-424*	0	1
	hsa-miR-193b	1199	5
	hsa-miR-99b*	0	0
	hsa-let-7b	1570	146
	hsa-miR-181a	2340	14
	hsa-miR-23b	2280	4
	hsa-miR-361-5p	1629	1
	hsa-miR-125b-2*	0	0
acute cellular rejection (down-regulated miRNAs)		22705 (8765)	219 (208)
acute cellular rejection		**28878 (9833)**	**387 (369)**
ABMR versus PBx			
up-regulated	hsa-miR-663	1145	2
	hsa-miR-146b-5p	1855	8
	hsa-miR-1228	0	0
	hsa-let-7i	1569	1
	hsa-miR-21*	0	0
	hsa-miR-182	2305	9
humoral rejection		**6874 (5119)**	**20 (19)**
acute rejection (total)		**35752 (10251)**	**407 (384)**

Table S2. The number of differentially regulated transcripts between control group and study groups. Raw data were obtained from the Cleveland study (Flechner et al.) and the Edmonton study (Müller et al.).

	No. of up-regulated transcripts	**No. of down-regulated transcripts**	**SAM delta value**	**fold change**
Cleveland study				
renal allograft dysfunction (n=5) versus controls (n=10)	67	579	3.5	>2
acute tubular necrosis (n=2) versus controls (n=10)	144	284	3.5	>2
acute rejection (n=7) versus controls (n=10)	47	389	3.5	>2
Edmonton study				
acute cellular rejection (n=28) versus no rejection (n=72)	430	0	2	>2
antibody-mediated rejection (n=8) versus no rejection (n=72)	105	0	2	>2

Table S3. Pathway enrichment analysis of the validation data set from Edmonton. Enriched pathways in the predicted and validated miRNA target lists in AREJ or ABMR and differentially regulated transcript lists are represented.

Acute cellular rejection	**predicted targets**	**exp. validated targets**	**regulated transcripts (SAM)**
Pathways	p-value	p-value	p-value
Inflammation mediated by chemokine and cytokine signaling pathway	<0.001	<0.001	<0.001
Apoptosis signaling pathway	<0.001	0.007	<0.001
Interleukin signaling pathway	<0.001	0.009	0.004
Nicotinic acetylcholine receptor signaling pathway	0.001	0.009	0.033
Cytoskeletal regulation by Rho GTPase	0.002	0.009	0.034
Antibody-mediated rejection			
Inflammation mediated by chemokine and cytokine signaling pathway	<0.001	<0.001	0.003
Interleukin signaling pathway	0.002	<0.001	0.018
Apoptosis signaling pathway	<0.001	0.006	0.005

qRT-PCR validation

Methods

The TaqMan® MicroRNA Reverse Transcription Kit were used to synthesize single stranded cDNA. Real-time PCR was performed using the TaqMan® Gene Expression Master Mix, TaqMan® miRNA expression assays (see table below) with the ABI 7300 Real-Time PCR System. All instruments and reagents were purchased from Applied Biosystems. Relative gene expression values were evaluated with the $2^{-\Delta\Delta Ct}$ method using U6 snRNA as control small RNA and Stratagene Universal human reference RNA (Stratagene, La Jolla, CA) as reference RNA. qRT-PCR conditions were set according to the manufacturer's recommendations: 10min 95°C, 40 cycles (15sec 95°C, 1min 60°C) with fluorescence reading during annealing step.

TaqMan miRNA assays

Assay Name	miRBase ID	Mature miRNA Sequence
hsa-miR-182	hsa-miR-182-5p	UUUGGCAAUGGUAGAACUCACACU
hsa-miR-21*	hsa-miR-21-3p	CAACACCAGUCGAUGGGCUGU
hsa-miR-155	hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-146b	hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU
hsa-miR-125a	hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA

TaqMan miRNA assay control

Assay Name	NCBI Accession	Control Sequence
U6 snRNA	NR_004394	GTGCTCGCTTCGGCAGCACATATACTAAAATT GGAACGATACAGAGAAGATTAGCATGGCCCC TGCGCAAGGATGACACGCAAATTCGTGAAGC GTTCCATATTTT

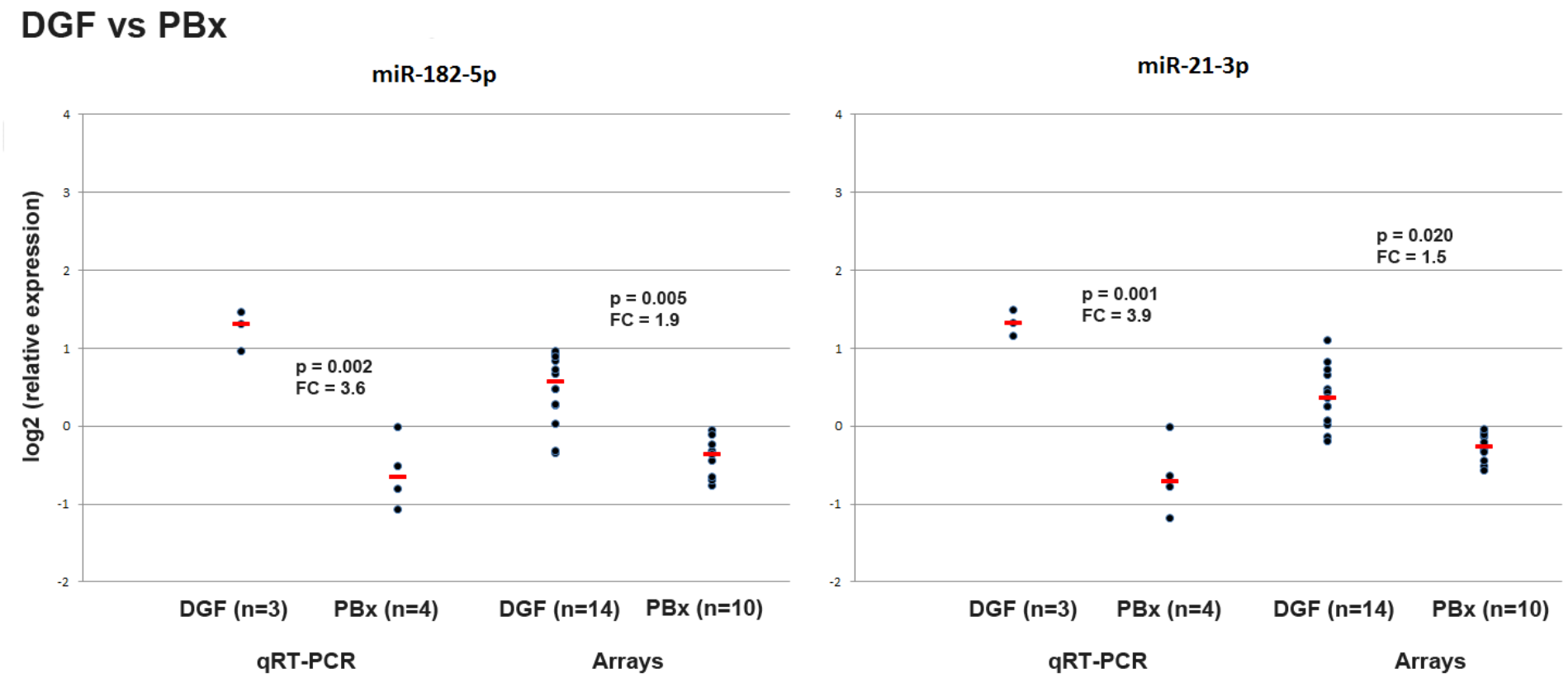

Figure S1. qRT-PCR validation of the most significantly differentially regulated miRNAs between DGF and control group (PBx). Log_2 (relative expression) values are shown for the qRT-PCR and the array experiment. The red line indicates the median.

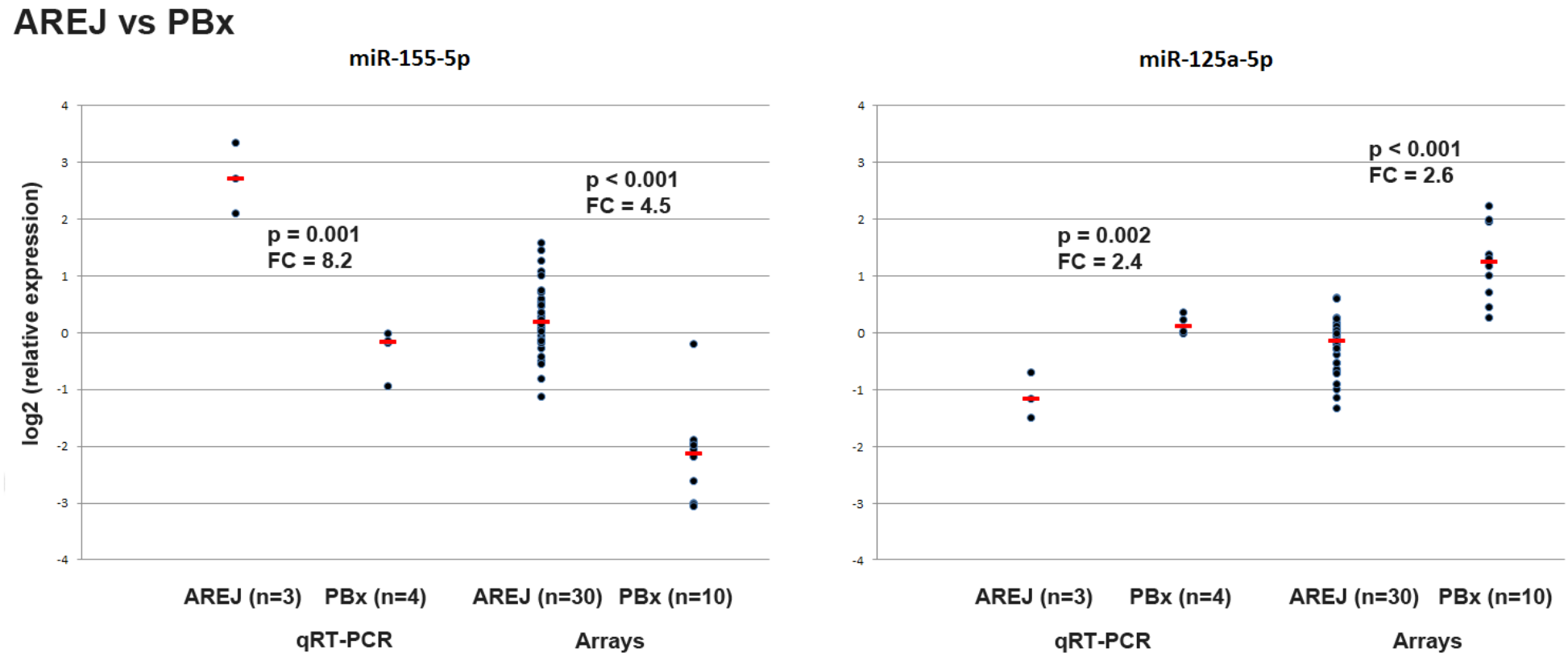

Figure S2. qRT-PCR validation of the most significantly differentially regulated miRNAs between AREJ and control group (PBx). Log_2 (relative expression) values are shown for the qRT-PCR and the array experiment. The red line indicates the median.

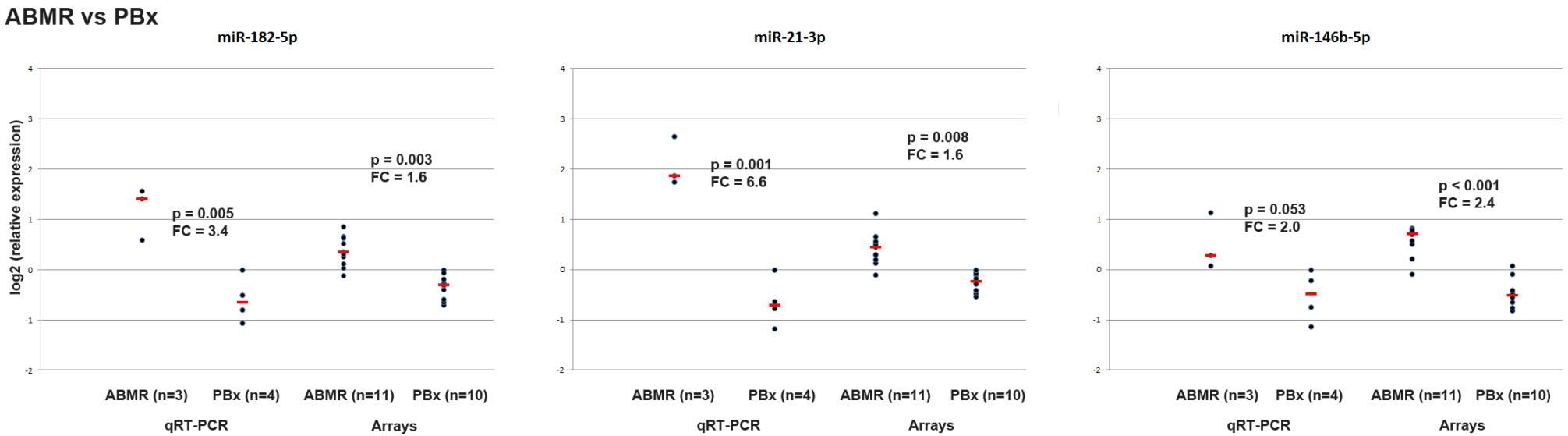

Figure S3. qRT-PCR validation of the most significantly differentially regulated miRNAs between ABMR and control group (PBx) or of miRNAs being also differentially regulated in other groups. Log_2 (relative expression) values are shown for the qRT-PCR and the array experiment. The red line indicates the median.

References

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