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Ago-PAR-CLIP Defines MicroRNA Targets in Human Proximal Tubule Epithelial Kidney Cells

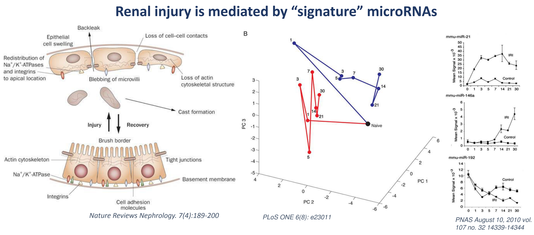
Christopher J. Benway, John Iacomini

Tufts University School of Medicine, Department of Developmental, Molecular and Chemical Biology, Genetics Program

Background

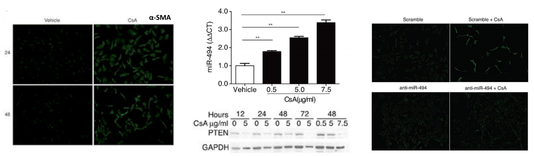
Ischemia reperfusion injury (IRI) is a leading cause of acute kidney injury (AKI), a common clinical event associated with significant morbidity and mortality. Our lab has previously examined the role of microRNAs in renal IRI via expression profiling and other conventional methods. This examination identified a miRNA "signature" in damaged kidney cells and demonstrated an intrinsic protective role of miR-21 in injured tubular epithelial cells by regulating PTEN and increasing proliferation.

Renal injury is mediated by "signature" microRNAs



Despite the immediate immunosuppressive effects of cyclosporine (CsA) preventing allograft rejection, drug induced nephrotoxicity limits the long term use of CsA in the clinic. Currently, the mechanisms leading to chronic CsA nephropathy are not completely understood. Processes that lead to apoptosis of renal tubular cells, the generation of reactive oxygen species, and epithelial-mesenchymal transition (EMT) have been shown to contribute to injury. Our lab has identified miR-494 as a functional negative regulator of PTEN in CsA-treated tubular epithelial cells, leading to increased EMT. Other labs have also demonstrated that miR-21 contributes to this pathway.

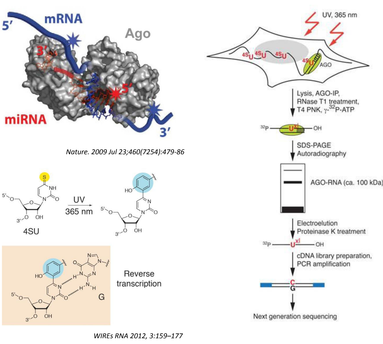
In order to more completely characterize the genome-wide function of microRNAs in normal and injured renal tubular epithelial cells we have performed photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) on endogenous Argonaute, the essential catalytic component of the RNA-induced silencing complex (RISC).



CsA treatment induces EMT in HK-2 cells (left). CsA induces increased expression of miR-494, which targets PTEN directly (middle). Treatment with anti-miR-494 rescues HK-2 cells from EMT phenotype after CsA treatment (right).

Methods

HK-2, human kidney proximal tubule epithelial cells, were cultured and expanded in serum-free media containing BPE and EGF. At approximately 80 percent confluency, cells were treated with 100 uM 4-thiouridine and incubated for 16-24h. hours. 5 ug/mL CsA or ethanol (vehicle) was added to samples at time of 4SU treatment. Irradiation with 365 nm UV light was conducted to induce irreversible cross-linking between RNA and RNA binding proteins. Using a pan-Argonaute monoclonal antibody, RISC complexes were immunoprecipitated, RNA labeled with gamma-32P, and resolved by SDS-PAGE. Isolation of the crosslinked RNAs was followed by a small-RNA cDNA library protocol which utilizes a pre-adenylated 3' DNA adapter and a modified T4 ligase. Library amplification and agarose gel electrophoresis confirmed the presence of expected inserts and removed unwanted adapter-adapter products.



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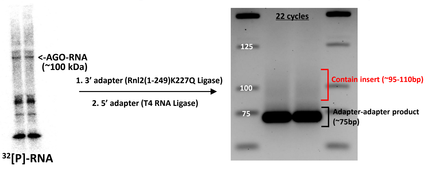
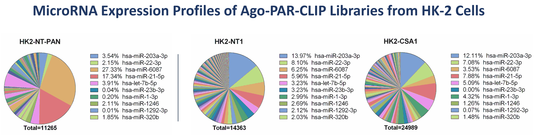
Size Fractionation of Ago Bound RNA and cDNA Library Prep



Single-end Sequencing was performed using the Illumina HiSeq 2500 platform. Removal of the 3' adapter sequence was achieved by FASTX_clipper (-a TCGTATGCCGTCTTCTGCTTG) and resulting reads below 20 bp were discarded prior to alignment. Alignment to the human reference genome (hg19) was performed using Bowtie (v -2 –m 10 –best – strata). Two mismatches were allowed in order to retain T>C crosslinking mutations for downstream analysis. Various bioinformatic pipelines (PARalyzer, PIPE-CLIP, wavClusteR, CLIPZ) were then used to assess the frequency of crosslinking mutations and to generate clusters of overlapping reads containing crosslinking sites, i.e. the RNA recognition elements (RREs).

Results

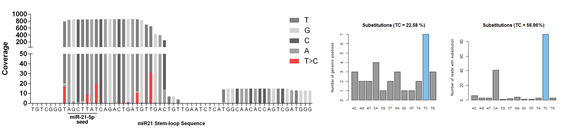
MicroRNA Expression Profiles of Ago-PAR-CLIP Libraries from HK-2 Cells



The pan-Ago library displayed a distinct microRNA composition compared with subsequent Ago2 libraries, although this is not a robust means to evaluate expression per se.

Results

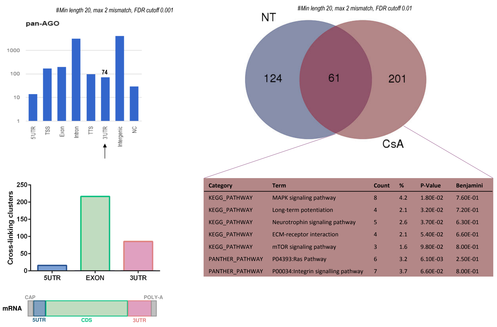
Sequences Contain Characteristic T>C Mutations Induced by Cross-linking



The highly expressed miR-21-5p demonstrates increased T>C mutations, indicative of cross-linking. It has previously been reported that these mutations are less frequent within the mature miRNA seed sequence.

T>C Mutations Define Crosslinking Sites and miRNA Targets

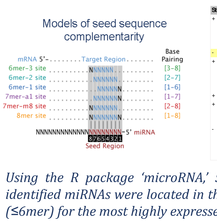
Two different platforms were used to identify clusters of sequences that feature enriched T>C mutations. **PARalyzer** utilizes this substitution in a kernal density estimate classifier to determine protein-RNA interaction sites. **PIPE-CLIP** identifies enriched sequence and mutation clusters separately using statistical methods.



Category	Term	Count	%	P-Value	Benjamini
KEGG_PATHWAY	MAPK signaling pathway	8	4.2	1.80E-02	7.60E-01
KEGG_PATHWAY	Long-term potentiation	4	2.1	3.20E-02	7.20E-01
KEGG_PATHWAY	Neurotrophin signaling pathway	5	2.6	3.70E-02	6.30E-01
KEGG_PATHWAY	ECM-receptor interaction	4	2.1	5.40E-02	6.60E-01
KEGG_PATHWAY	mTOR signaling pathway	3	1.6	9.80E-02	8.00E-01
PANTHER_PATHWAY	P04393:Ras Pathway	6	3.2	6.10E-03	2.50E-01
PANTHER_PATHWAY	P00034:Integrin signalling pathway	7	3.7	6.60E-02	8.00E-01

With stringent parameters PIPE-CLIP revealed 74 high-confidence cross-linking sites within the 3'UTR of genes from pan-Ago sequences (top left). Despite many clusters mapping to intron or intergenic sequences, a relatively high number of clusters mapping to transcripts mapped to the 3'UTR (bottom left). Analysis of untreated versus CsA treated HK-2 cells by PIPE-CLIP identified 3'UTR target genes that were unique or identical (top right) suggesting different recognition elements are occupied and targeted in the presence of the drug. Interestingly, functional annotation with DAVID revealed MAPK, mTOR, Ras, and Integrin pathways as enriched.

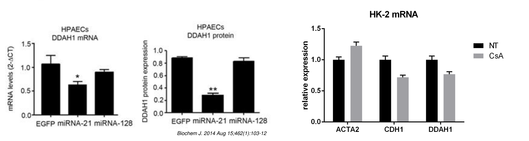
Clusters Contain Seed Sequences for Present miRNAs



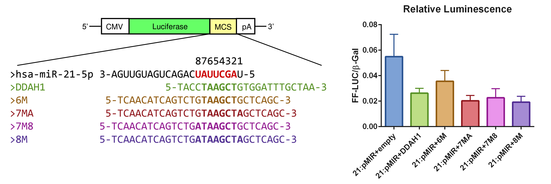
Strand	Score	Annotation	Gene Symbol	miR-seed2-7 (6mer)	miR-seed2-8 (7mer)	miR-seed2-8 (8mer)
+	2119	3' UTR (NM_018096, exon 24 of 24)	EC72	none	hsa-miR-33a-5p [1] 7	hsa-miR-33a-5p [1] 8
					hsa-miR-27b-3p [1] 1	hsa-miR-128-3p [1] 2
					hsa-miR-27a-3p [1] 1	hsa-miR-27b-3p [1] 2
						hsa-miR-27a-3p [1] 2
-	1697	3' UTR (NM_001134465, exon 7 of 7)	DDAH1	none	hsa-miR-21-5p [1] 5	hsa-miR-21-5p [1] 6
+	938	3' UTR (NM_000684, exon 1 of 1)	ADRB1	none	hsa-miR-4746-5p [1] 9	hsa-miR-4497 [1] 12
						hsa-miR-3605-3p [1] 13
						hsa-miR-5100 [1] 2
						hsa-miR-4746-5p [1] 10
+	915	3' UTR (NM_001104647, exon 7 of 7)	SLC25A36	none	none	hsa-miR-149a-5p [1] 16
+	549	3' UTR (NM_013988, exon 9 of 9)	PAIP2	none	hsa-miR-1910-5p [1] 10	hsa-miR-1910-5p [1] 11
					hsa-miR-449a [1] 12	hsa-miR-3190-5p [1] 15
						hsa-miR-449a [1] 13
-	369	3' UTR (NM_181787, exon 19 of 19)	DPY19L4	hsa-miR-27b-3p [1] 7	hsa-miR-27b-3p [1] 8	hsa-miR-1910-5p [1] 6
				hsa-miR-27a-3p [1] 7	hsa-miR-27a-3p [1] 8	hsa-miR-128-3p [1] 9
						hsa-miR-27b-3p [1] 9
						hsa-miR-27a-3p [1] 9

Using the R package 'microRNA,' 5-, 6-, and 7-mer seed matches from the previously identified miRNAs were located in the 3'UTR clusters. Most clusters contain seed sequences (≤6mer) for the most highly expressed mature miRNAs.

DDAH1 is a Known Target of MiR-21 and Responds to CsA Treatment



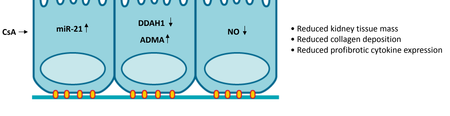
DDAH1, dimethylarginine dimethylaminohydrolase-1, is regulated by miR-21 in other cell types and may contribute to adverse injury responses. 48h CsA (5 ug/mL) induced expression of EMT marker ACTA2 and decreases expression of epithelial marker e-cadherin as well as DDAH1. miR-21 expression is increased by CsA treatment (not shown).



The identified cluster in target gene DDAH1 was cloned into the multiple cloning site of a luciferase reporter (pMIR-REPORT). Controls for the possible canonical miR-21 matches were also constructed and evaluated by transfection in 293T cells.

Conclusions

Ago-PAR-CLIP and subsequent analysis performed on endogenous Argonaute levels in human kidney tubule epithelial cells identified many high-confidence putative mRNA targets of biologically active miRNAs. Further, we have identified the miRNAs and mRNA targets unique to tubule epithelial cells after insult with Cyclosporine A. To confirm the results of the sequencing experiment in vitro, we validated the identified target cluster from DDAH1. We hypothesize that miR-21 induction after CsA treatment may regulate DDAH1 expression levels in vivo, contributing to asymmetric dimethylarginine (ADMA) metabolism and downstream nitric oxide (NO) synthesis. Recent studies using *Ddah1* knockout mice specific to the proximal tubule cells lead us to hypothesize that the cell-intrinsic role of miR-21 is largely protective after renal injury.



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