Solid organ transplantation requires treatment with immunosuppressants to prevent graft rejection. However, the most common class of immunosuppressants, calcineurin inhibitors such as cyclosporine and tacrolimus, lead to nephrotoxicity and limit graft survival. To better understand the molecular mechanisms underlying the pathology of cyclosporine-induced nephrotoxicity, we identified the microRNA:mRNA interactions governing gene expression changes in human proximal tubule epithelial cells treated with cyclosporine.

Ago-PAR-CLIP Defines Targetome and Role of MicroRNAs in Cyclosporine-induced Nephrotoxicity

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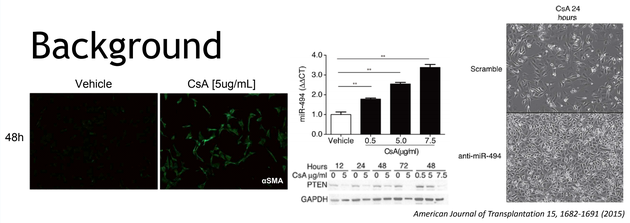
Motivation

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 (Yuan et al., 2014). Other labs have also demonstrated that miR-21 contributes to this pathway (Chen et al., 2014).

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 cross-linking and immunoprecipitation (PAR-CLIP) on endogenous Argonaute, the essential catalytic component of the RNA-induced silencing complex (RISC), in HK-2 cells treated with cyclosporine A.

Background



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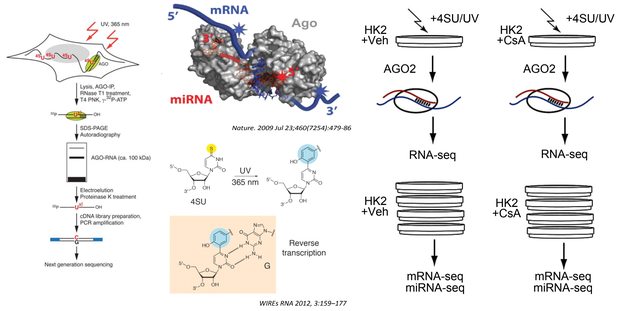
CsA treatment induces EMT in HK-2 cells (left). CsA induces increased expression of miR-494, which targets PTEN directly (middle). Treatment with anti-miR-494 rescues HK-2 cells from EMT phenotype after CsA treatment (right).

CsA → ⇧miR-494 ⇧miR-21 → ⇩PTEN → ⇧pAKT → ⇧ EMT markers: VIM, α-SMA

Further studies (Chen et al., 2014) have shown that inhibition of PTEN by miRNAs in cyclosporine-induced nephropathy activates AKT signaling and induces expression of EMT genes such as vimentin (VIM) and alpha smooth muscle actin (α-SMA).

Methods

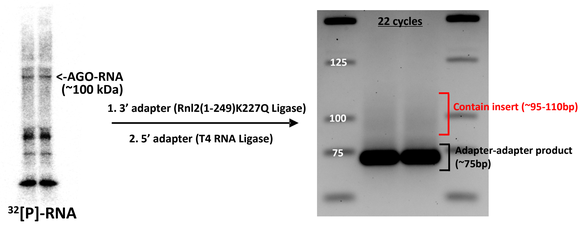
HK-2, human kidney proximal tubule epithelial cells, were cultured and expanded in serum-free media containing BPE and EGF. At approximately 80 percent confluency, cells were treated with 100 uM 4-thiouridine and incubated for 24h. CsA [5ug/mL] or vehicle, 0.1% ethanol, was added to samples at time of 4SU treatment. Irradiation with 365 nm UV light was conducted to induce irreversible cross-linking between RNA and RNA binding proteins. Using a monoclonal antibody against Ago2 (clone C34C6, Cell Signaling), RISC complexes were immunoprecipitated, RNA labeled with gamma-32P, and resolved by SDS-PAGE. Isolation of the cross-linked 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. Bioinformatic tools were used to identify T>C mutations caused by irreversible cross-linking to Ago2.



Nature. 2009 Jul 23;460(7254):479-86

WIREs RNA 2012, 3:159–177

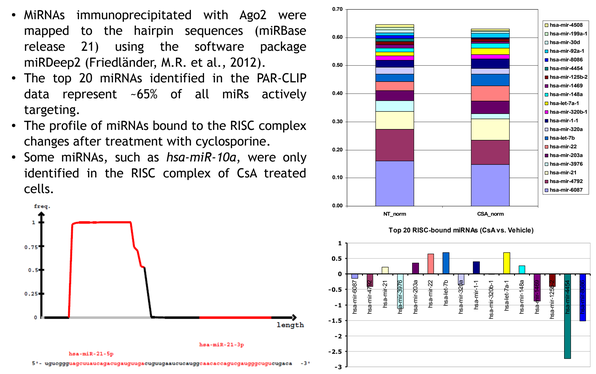
Results



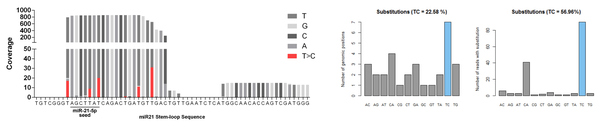
RNA immunoprecipitation, isolation and cDNA library construction for deep sequencing

Identifying Active miRNAs and mRNA Targets in the RISC Complex

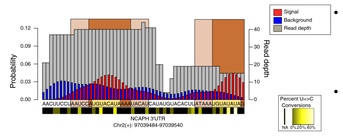
- MiRNAs immunoprecipitated with Ago2 were mapped to the hairpin sequences (miRBase release 21) using the software package miRDeep2 (Friedländer, M.R. et al., 2012).
- The top 20 miRNAs identified in the PAR-CLIP data represent ~65% of all miRs actively targeting.
- The profile of miRNAs bound to the RISC complex changes after treatment with cyclosporine.
- Some miRNAs, such as *hsa-miR-10a*, were only identified in the RISC complex of CsA treated cells.



MiRNAs identified in the PAR-CLIP data exhibit T>C mutations, evidence of direct cross-linking to Ago2:

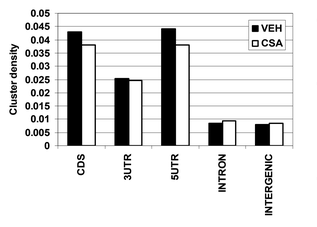


T>C mutations define cross-linking sites in messenger RNAs and miRNA targets



- PARalyzer (Corcoran et at., 2011) and PIPE-CLIP (Chen et al., 2014) identify cross-linking regions in target mRNAs based on read depth and number of T>C conversions.
- Putative target regions are analyzed for n-mer sequences matching the seed regions of the previously identified miRNAs.

Genomic distribution of Ago2 PAR-CLIP clusters



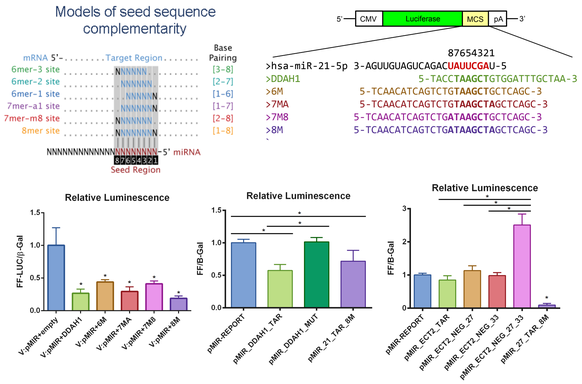
- Over 32,000 target regions were identified in each condition.
- Ago2-PAR-CLIP clusters were identified in all genomic regions, but were enriched in the 5'UTR, CDS, and 3'UTR of mRNAs.
- The level of enrichment in 5'UTR and CDS regions was higher than previously reported (Hafner et al., 2012) but non-canonical binding by Ago is not unprecedented (Helwak et al., 2013).
- Based on available tools and knowledge we focused primarily on clusters mapped to 3'UTRs.

Score	Annotation	Gene Symbol	miR seed 2-8 (7mer)	miR seed 2-7 (6mer)
2119	3' UTR (NM_018098, exon 24 of 24)	ECT2	none	hsa-miR-33a-5p [1] 7 hsa-miR-27b-3p [1] 1 hsa-miR-27a-3p [1] 1
1697	3' UTR (NM_001134445, exon 7 of 7)	DDAH1	none	hsa-miR-21-5p [1] 5
938	3' UTR (NM_000684, exon 1 of 1)	ADRB1	none	hsa-miR-4746-5p [1] 9
915	3' UTR (NM_001104647, exon 7 of 7)	SLC25A36	none	none
549	3' UTR (NM_013988, exon 9 of 9)	PARK2	none	hsa-miR-1910-5p [1] 10 hsa-miR-449a [1] 12
369	3' UTR (NM_181787, exon 19 of 19)	DPY19L4	hsa-miR-27b-3p [1] 7 hsa-miR-27a-3p [1] 7	hsa-miR-27b-3p [1] 8 hsa-miR-27a-3p [1] 8

The enriched sequence clusters identified by PIPE-CLIP and PARalyzer contain sequences complementary to the seeds of miRNAs identified as bound to Ago2/RISC.

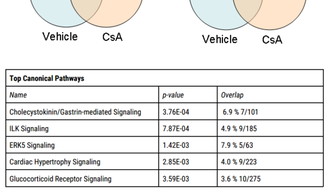
Left, a sequence in the 3'UTR of DDAH1 was identified to contain a miR-21 seed site. DDAH1 was determined to be targeted by miR-21 in Liu XI, et al., 2015.

PAR-CLIP target clusters are regulated my miRNAs *in vitro*



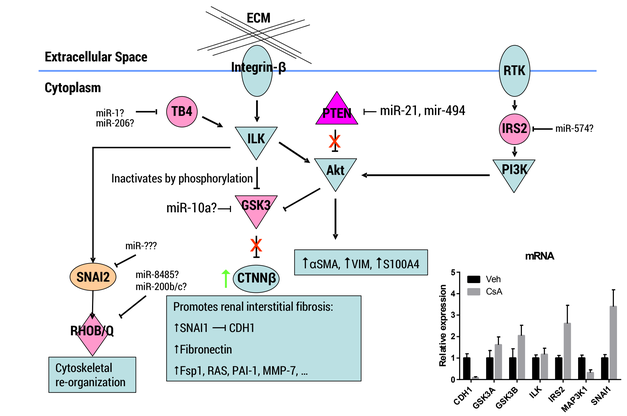
Luciferase reporter constructs containing 3'UTR target sequences identified by PAR-CLIP were transfected into 293T cells. Positive control constructs were designed to contain canonical seed complementarity to the putative miRNA regulator. Putative seeds within the target clusters were mutated by flipping the region of seed complementarity.

The integrin-linked kinase pathway is regulated by miRNAs in CsA treated HK2 cells

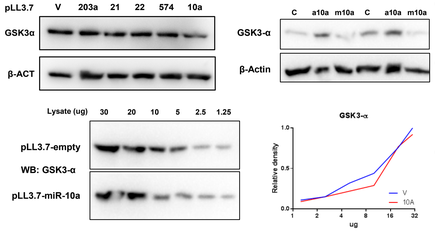


- Ingenuity Pathway Analysis (IPA) was used to identify the canonical pathways enriched in CsA-treated 3'UTR PAR-CLIP datasets.
- Several key members of the integrin-linked kinase pathway (GSK3A, IRS2, TB4, SNAI2, RHOB and RHOQ) were putative targets of miRNAs (below).
- RNA-seq and qPCR data has shown that GSK3A, IRS2, TB4, and SNAI2 are all up-regulated after CsA treatment.

Top Canonical Pathways		
Name	*p*-value	Overlap
Cholecystokinin/Gastrin-mediated Signaling	3.76E-04	6.9 % 7/101
ILK Signaling	7.87E-04	4.9 % 9/185
ERK5 Signaling	1.42E-03	7.9 % 5/63
Cardiac Hypertrophy Signaling	2.85E-03	4.0 % 9/223
Glucocorticoid Receptor Signaling	3.59E-03	3.6 % 10/275

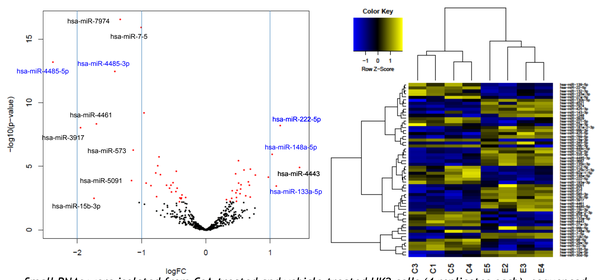


miR-10a targets GSK3-alpha in HK2 cells

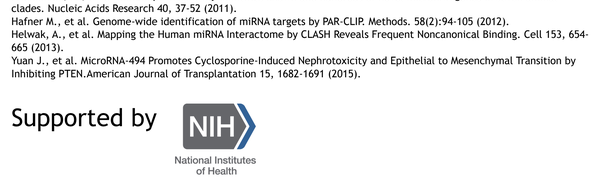


- A miR-10a expressing lentiviral construct specifically repressed GSK3A expression in HK2 cells (top, left).
- Transfection of a miR-10a antagomiR oligo increased expression of GSK3A whereas transfection of a miR-10a mimic oligo reduced expression (top, right).
- Since subtle expression changes are difficult to quantify by western blot, a serial dilution of the cell lysate indicates that miR-10a repressed GSK3A protein expression ~30%.

MiRNA-seq and RNA-seq identify transcriptome-wide gene expression changes in cyclosporine-treated HK2 cells



Small RNAs were isolated from CsA-treated and vehicle-treated HK2 cells (4 replicates each), sequenced and differential expression analysis performed with EdgeR. 57 miRNAs are differentially expressed (adj pVal <0.05): 26 up-regulated, 31 down-regulated. Only 13 miRNAs have [log2-fold change]>1. MiRNAs in blue were also detected in the RISC complex by PAR-CLIP



mRNAs were isolated by polyA magnetic beads from CsA and vehicle-treated HK2 cells (4 replicates each), sequenced and differential expression analysis was performed with DESeq2. Left,1706 genes are differentially expressed at the transcript level (adjP <0.05, [log2fc] >1). 176 genes were differentially expressed with [log2fc] > 2. Right, top: The 5 canonical pathways enriched in 3'UTR PAR-CLIP clusters are largely dis-regulated after CsA treatment. Right, bottom: The expression of these 3'UTR targets are largely upregulated after CsA treatment suggesting that miRNAs are act to 'tune' expression of new transcripts.

Conclusions and Future Directions

- Ago-PAR-CLIP identified thousands of novel miRNA target sites genome-wide in human proximal tubule epithelium cells (HK2 cell line) after treatment with cyclosporine A.
- These target sequences are functional in *in vitro* luciferase assays.
- Identification of miRNAs crosslinked to Ago2/RISC provides insight into the physiological relevant molecules involved in the EMT phenotype observed in these cells. We will further evaluate their role in promoting/preventing EMT in HK2 cells.
- Several canonical pathways, including the Integrin-linked Kinase pathway, are regulated at many levels by miRNAs. As 'tuning' regulators of expression these miRNAs may serve as potential therapeutic targets. We will evaluate by mimic and antagomiR administration, the effects on EMT progression.
- Differential expression analysis of miRNA-seq and RNA-seq libraries identified the key genes involved in the the pathogenesis of cyclosporine-induced nephrotoxicity in our cell model.

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