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Application of Small RNA Sequencing to Identify MicroRNAs in Acute Kidney Injury and Fibrosis

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Abstract

Establishing a microRNA (miRNA) expression profile in affected tissues provides an important foundation for the discovery of miRNAs involved in the development or progression of pathologic conditions. We conducted small RNA sequencing to generate a temporal profile of miRNA expression in the kidneys using a mouse model of folic acid-induced (250 mg/kg i.p.) kidney injury and fibrosis. From the 103 miRNAs that were differentially expressed over the time course (>2-fold, $p < 0.05$), we chose to further investigate miR-18a-5p, which is expressed during the acute stage of the injury; miR-132-3p, which is upregulated during transition between acute and fibrotic injury; and miR-146b-5p, which is highly expressed at the peak of fibrosis. Using qRT-PCR, we confirmed the increased expression of these candidate miRNAs in the folic acid model as well as in other established mouse models of acute injury (ischemia/reperfusion injury) and fibrosis (unilateral ureteral obstruction). *In situ* hybridization confirmed high expression of miR-18a-5p, miR-132-3p and miR-146b-5p throughout the kidney cortex in mice and humans with severe kidney injury or fibrosis. When primary human proximal tubular epithelial cells were treated with model nephrotoxicants such as cadmium chloride (CdCl_2), arsenic trioxide, aristolochic acid

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Conflict of interest

The authors have no competing interests to declare.

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(AA), potassium dichromate ($K_2Cr_2O_7$) and cisplatin, miRNA-132-3p was upregulated 4.3-fold after AA treatment and 1.5-fold after $K_2Cr_2O_7$ and $CdCl_2$ treatment. These results demonstrate the application of temporal small RNA sequencing to identify miR-18a, miR-132 and miR-146b as differentially expressed miRNAs during distinct phases of kidney injury and fibrosis progression.

Keywords

Small RNA sequencing; miRNAs; Kidney toxicity; Kidney fibrosis; Folic acid

Introduction

MicroRNAs (miRNAs) are short, non-coding RNAs that post-transcriptionally regulate gene expression by preventing protein translation or inducing mRNA degradation and have demonstrated roles in the regulation of diverse cellular processes (Kim *et al.*, 2008; Bartel, 2009). MicroRNAs have also been identified to have various roles in mediating the metabolism and toxicity of xenobiotic compounds and their deregulation has been shown to lead to the pathogenesis of various diseases (Bolleyn *et al.*, 2014). Establishing miRNA expression changes that occur during pathologic conditions such as kidney disease allows for the identification of potential biomarkers and therapeutic targets. For example, miR-21 is highly upregulated during kidney injuries of diverse origin, such as ischemia, toxicant exposure, hypertension, and fibrosis (Denby *et al.*, 2011; Ben-Dov *et al.*, 2012; Chandrasekaran *et al.*, 2012; Saikumar *et al.*, 2012). Additionally, increased expression of circulating miR-21 is observed in the urine and blood of patients with acute kidney injury (AKI) or interstitial fibrosis (Du *et al.*, 2013; Glowacki *et al.*, 2013; Ramachandran *et al.*, 2013). The therapeutic potential of miR-21-specific antagomirs has also been demonstrated in mouse models of kidney fibrosis, where mice treated with these compounds had a reduced occurrence of fibrosis (Zarjou *et al.*, 2011; Chau *et al.*, 2012).

Small RNA sequencing offers a number of advantages over probe-based microarrays or qRT-PCR plate arrays in building miRNA expression profiles. For example, this technique allows for the detection of miRNAs even when they have undergone differential processing, offers the possibility of discovering novel miRNAs, and is very cost-effective when multiplexing barcoded samples (Ben-Dov *et al.*, 2012). In the current study, we used small RNA sequencing to profile miRNAs expressed during injury and fibrotic phases of kidney damage. Specifically, we found that miR-18a-5p is expressed during the acute stage of the injury, miR-132-3p is upregulated during transition between acute and fibrotic injury, and miR-146b-5p is highly expressed at the peak of fibrosis. We confirmed the expression of these candidate miRNAs in two different mouse models of kidney injury and fibrosis as well as in human kidney biopsy specimens obtained from patients with either AKI or kidney fibrosis. Furthermore, we also found significant changes in the expression of these candidate miRNAs *in vitro* in human primary proximal tubular epithelial cells following exposure to environmental toxicants or nephrotoxic drugs.

Materials and Methods

Mice

Male BALB/c mice at 25–30 g of body weight were purchased from Charles River Laboratories and maintained under standard housing conditions in accordance with NIH guidelines for the care and use of laboratory animals. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committees (IACUC) of Harvard Medical School.

Human tissues

The Institutional Review Board approved the protocol for use of de-identified biospecimens from human patients. De-identified human kidney tissue samples from patients with AKI due to allograft rejection or from patients with severe kidney fibrosis were obtained from the Department of Pathology at Brigham and Women's Hospital.

Cell culture and toxicant exposure studies

Primary human proximal tubular epithelial cells (HPTECs) purchased from Biopredic International (Rennes, France) were cultured in supplemented DMEM/Hams-F12 with GlutaMAX™ medium. Medium was supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 1% insulin-transferrin-selenium and 4 pg/ml triiodothyronine on collagen coated tissue culture plates (6 µg/cm²) at 37°C in a humidified 5% CO₂ incubator. The medium was replaced one day after seeding and then every second day. Cells were treated in passage four in 6-well plates with test compounds dissolved in 0.5% DMSO. The five test compounds (100µM aristolochic acid, 25µM cadmium chloride, 50µM arsenic trioxide, 10µM potassium dichromate and 40µM cisplatin) were purchased from Sigma-Aldrich (St. Louis, USA) and used at half the LD₅₀ concentrations determined for HPTECs after 24 hours of treatment in previous dose-response studies (Adler *et al.*, 2015).

Folic acid administration

Folic acid was prepared at 25 mg/ml in 0.3 M sodium bicarbonate and male BALB/c mice were given a 250 mg/kg dose via intraperitoneal injection as described previously (Craciun *et al.*, 2014). The mice were sacrificed at 1, 2, 3, 7, 14, and 28 days following the administration of folic acid. An untreated group of mice that were matched for age, sex, strain and weight were used as controls.

Unilateral ureteral obstruction

Male BALB/c mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight), the left kidney was exposed via a flank incision and 3.0 silk suture thread was used to tie off the ureter at the lower pole as described previously (Craciun *et al.*, 2014). Following surgery, the mice received a subcutaneous injection of buprenorphine (0.05 mg/kg) every 12 hours following surgery for two days and then sacrificed at 3, 7 and 14 days post-surgery. The contralateral kidneys of these mice were

used as controls for each time point, and all results were also compared to an untreated group of age, sex, strain and weight matched mice that served as normal tissue controls.

Bilateral renal ischemia reperfusion injury

Male BALB/c mice were subjected to bilateral renal IRI by clamping both renal arteries for a period of 30 minutes as described previously (Ajay *et al.*, 2012). Age, sex, strain and weight matched animals that received sham surgery underwent anesthesia and laparotomy only and were sacrificed after 1 day to be used as controls. All mice received a subcutaneous injection of buprenorphine (0.05 mg/kg) every 12 hours for the first two days following surgery and were sacrificed at 1, 5, or 14 days post-surgery.

Tissue collection

At the time of sacrifice, blood was transferred into heparinized tubes and centrifuged at 10000g for 10 minutes at 4°C to separate the plasma, which was collected and stored at -80°C for further analysis. The kidneys collected for histology were cut along the frontal plane and fixed in 10% neutral buffered formalin overnight before being transferred to 70% ethanol. After being processed and embedded in paraffin, 6 µm sections were prepared. The remaining kidney tissue was snap frozen in liquid nitrogen for RNA and protein analysis.

Clinical chemistry and histology scoring

SCr and BUN were measured as described previously (Krishnamoorthy *et al.*, 2011; Hoffmann *et al.*, 2012). An independent pathologist evaluated Periodic acid-Schiff (PAS) or Masson's trichrome (MTS) stained sections under a light microscope.

RNA isolation and qRT-PCR

For the experiments using mouse kidney tissue, RNA was isolated using Trizol Reagent (Life Technologies), with the cDNA preparation and real time PCR carried out as described previously (Krishnamoorthy *et al.*, 2011). The miRNA from kidney tissue was analyzed using miRCURY LNA Universal RT microRNA PCR reagents with miRCURY LNA microRNA primer sets for miR-18a-5p, miR-132-3p, miR-146b-5p and let-7f-5p (Exiqon). For the cell culture experiments, RNA was isolated using QIAzol Lysis Reagent and miRNeasy Mini Kit and analyzed using miScript II RT and SYBR Green PCR kits with miScript primer assays for miR-18a-5p, miR-132-3p, miR-146b-5p and let-7f-5p (Qiagen).

In situ hybridization (ISH)

To visualize miRNA expression, ISH was performed on paraffin-embedded mice kidney tissue sections obtained at day 0, 2 and 14 after folic acid injection, as well as from biopsy sections obtained from patients with AKI or kidney fibrosis. Double digoxigenin (DIG)-labeled miRCURY LNA miRNA detection probes were designed by Exiqon to target miR-18a-5p, miR-132-3p and miR-146b-5p. ISH was performed using manufacturer's instruction manual of miRCURY LNA microRNA ISH Optimization Kit (Exiqon) with two modifications. First, the proteinase-K incubation was done at 15µg/mL for 25 min instead of 10 min. Second, the miRNA probes were all used in 60 nM concentrations. The U6 probe

was included as a positive technical control and a scrambled probe was used as a negative control.

Small RNA library preparation and sequencing

Small RNA sequencing libraries were prepared using the TruSeq Small RNA Sample Preparation Kit (Illumina) according to manufacturer instructions. Briefly, 1 µg of total RNA was ligated to 3' and 5' Adapters before being reverse transcribed, and amplified for 15 cycles. The product was separated and visualized by 6% TBE-PAGE, and the fragment corresponding to labeled microRNA was excised from the gel. The cDNA was eluted from the gel, concentrated by ethanol precipitation, and resuspended in 10 µl of 10 mM Tris-HCl (pH 8.5). The quality and concentration of the recovered cDNA was measured with a High Sensitivity DNA Chip using a 2100 Bioanalyzer (Agilent). For all samples, we confirmed the presence of a 140–150 bp fragment at sufficient concentration for sequencing. The concentration of each library was determined by qPCR before multiplexing all 21 samples into a single lane of a flowcell on the HiSeq2000 platform. Samples were sequenced by the Biopolymers Facility at Harvard Medical School to produce 50 cycle, single-end reads. The full dataset is available in the NCBI GEO database with the accession number GSE61328.

Analysis of sequence data

The fastq files were assessed using FastQC both before and after adapter trimming to ensure the quality of the sequence data. The sequence data was processed using miRDeep2 (Friedlander *et al.*, 2012). Initially, the fastq files were processed using the mapper.pl script to remove adapter sequence, retain reads with a minimum of 18 nucleotides, and collapse the reads into a fasta file. Additionally, the reads were mapped to the mm9 mouse genome build and this data was collected into arf files. Next, using the mirdeep2.pl script, we annotated the reads using miRBase v19. Although the data was also assessed for the presence of novel miRNAs using the mirdeep2.pl script, none were identified in this data set. Differentially expressed miRNAs were identified using edgeR (Robinson *et al.*, 2010; Robinson and Oshlack, 2010), with data normalized as counts/million reads (cpm) and filtered to remove miRNAs that were not present at a level of 5 cpm in at least three samples. We found that the use of a higher cut-off (10 cpm) did not appreciably change the number of genes identified as differentially expressed, while the use of a lower cut-off (1 cpm) increased the presence of genes with highly variable expression within each group.

Statistical analyses

Results are expressed as mean ± SEM. Statistical analyses were performed using ANOVA and Dunnett's multiple comparisons test (all samples compared to the control group) with a statistical significance of $p < 0.05$ (GraphPad Prism software).

Results

Induction of acute tubular injury and fibrosis in the kidneys of mice treated with folic acid

We utilized small RNA sequencing to profile miRNA expression changes in the kidney over the course of acute and fibrotic kidney injury induced by folic acid (Bechtel *et al.*, 2010; Bielez *et al.*, 2010). Significant increases in blood urea nitrogen (BUN) were observed at 1,

2, and 3 days following the administration of 250 mg/kg folic acid, with peak expression of 93.1 mg/dL observed at day 2 (Figure 1A). Additionally, expression of Kim-1 mRNA in the kidneys was increased by 83.6-fold at day 1 post-injection (Figure 1B). The expression of fibrosis-associated genes such as collagen 1A1 and fibronectin was increased during later time points. Peak expression for both of these genes occurred at day 7, with 20.4- and 7.4-fold increases observed for collagen 1A1 and fibronectin, respectively (Figure 1C and 1D). Acute tubular epithelial damage following the administration of folic acid was most apparent in the superficial renal cortex. Injured tubules had decreased epithelial lining, distension of the tubular lumens, intraluminal cell debris accumulation, and degenerative changes of epithelial cells with cytoplasmic vacuolization and single cell necrosis observed. This injury becomes evident at day 1, with more obvious acute damage and early regenerative changes occurring over day 3 to 7, and early interstitial fibrosis associated with tubular atrophy and mild nonspecific interstitial inflammation observed by day 14 (Figure 1E).

Preparation of libraries for small RNA sequencing and analysis of sequence data

Small RNA libraries were prepared from total RNA extracted from the kidneys of folic acid treated mice (n = 3 mice/group, seven groups) with each sample labeled with a different RNA PCR Primer Index (barcode sequence). This barcoding procedure made it possible for us to sequence all 21 samples in a single lane of a flow cell and collect between 6.3 and 10.7 million reads for each sample. The sequence data was processed using miRDeep2 software to quantify the expression of each miRNA (Friedlander *et al.*, 2012). Adapter sequences were removed and reads with at least 18 nucleotides were retained (90.3% of total reads). Of the reads that mapped to the mouse genome (mm9 build), 83.0% were identified as miRNAs according to miRBase v.19 annotations (Figure 2A). The data was normalized and filtered to retain miRNAs with at least 5 counts per million in three or more samples, and differentially expressed miRNAs were identified using edgeR (Robinson *et al.*, 2010; Robinson and Oshlack, 2010).

Identification of highly abundant and differentially expressed miRNAs in folic acid-induced kidney damage

The majority of mouse miRNAs included in miRBase v.19 were undetectable or present at low levels, but there were 17 miRNAs present at over 10000 cpm (which is equivalent to 1% of the total miRNA reads; Figure 2B). To investigate these highly abundant miRNAs further, we calculated the expression of each miRNA as a percentage of total miRNA reads and identified six miRNAs highly expressed in the kidney. Together these six miRNAs accounted for over 60% of all miRNA sequences detected in the kidneys of untreated mice, with the two most abundant miRNAs, miR-10a-5p and miR-10b-5p, comprising almost 25% of the reads (Figure 2C). A two-dimensional scaling of the 100 most differentially expressed miRNAs demonstrated strong associations amongst samples collected from each time point based on their miRNA expression profiles, and indicated three distinct groupings: untreated mice, day 1 and 2 – during the acute response, and day 3, 7, 14, and 28 – during the development and progression of fibrosis (Figure 2D).

Of the 368 miRNAs that met our expression criteria and were included in our analysis, 103 were differentially expressed with at least a 2-fold change during one or more of the time

points investigated (Figure 3). The magnitude and significance of the fold change was larger for miRNAs that showed upregulation, so we chose to focus on miRNAs that were increased during the folic acid-induced kidney injury (Table 1). We found mmu-miR-21-3p and -5p to be highly upregulated from day 1 to 14, with peak expression occurring at day 2 (8.7- and 5.2-fold increase, respectively), consistent with previous observations of increased miR-21 expression in both acute and fibrotic injury (Denby *et al.*, 2011; Zarjou *et al.*, 2011; Chau *et al.*, 2012; Saikumar *et al.*, 2012) (Table 1). The miRNAs encoded on the miR-17/92 cluster were highly expressed during the early time points, with the most differentially expressed miRNA of this cluster, mmu-miR-18a-5p, increased by 4.4- and 4.9-fold on day 2 and 3, respectively (Figure 3). Another highly expressed cluster was the miR-132/212 cluster, which encodes mmu-miR-132 and mmu-miR-212 (Figure 3). While peak expression of mmu-miR-212-3p occurred on day 3 (6.8-fold increase), peak expression of mmu-miR-132-3p was observed on day 7 (6.0-fold; Table 1). Of the miRNAs that were observed to have peak expression during later time points (specifically day 14), mmu-miR-146b-5p expression was increased by 6.7-fold, and the expression of mmu-miR-199a/b-5p and -3p was increased by 3.3- and 3.0-fold, respectively (Figure 3, Table 1).

Confirmation of miR-18a-5p, miR-132-3p, and miR-146b-5p expression by qRT-PCR

For the purpose of this study, we decided to further investigate the temporal expression patterns of miRNAs not previously described in the context of kidney disease. Specifically, we selected individual miRNAs from the sequencing results that had peak expression during one of three distinct phases of injury: acute injury (up to 3 days after FA), the transition from acute injury to fibrosis (7 days after FA), and fibrosis (14 days after FA). We chose miR-18a-5p as it was highly expressed during the acute stage of the injury, our previous work had shown it to be important in AKI (Saikumar *et al.*, 2012), and it had not yet been studied extensively in the context of fibrotic kidney disease, which was the case for the more highly expressed miRNA, miR-21. Our selection of miR-132-3p and miR-146b-5p was based on their significant upregulation during either the transition between acute and fibrotic injury (miR-132-3p) or the peak expression of fibrosis-associated genes (miR-146b-5p). We also performed NormFinder analysis on the small RNA sequencing data to identify mmu-let-7f-5p as a normalizer for qRT-PCR (Andersen *et al.*, 2004).

Initially, expression of the three target miRNAs was measured in a larger set of samples from the folic acid treated mice (n = 5 mice/group). The expression profiles for each miRNA in these samples corresponded well with the sequencing data (Figure 4A-4C). Expression of mmu-miR-18a-5p was highest at day 2 (7.2-fold) and decreased over the remaining time points (Figure 4A). The expression of mmu-miR-132-3p was between 3.0- to 5.0-fold higher than the untreated mice at all time points, but the peak expression occurred at day 3 (5.2-fold; Figure 4B). In contrast, miR-146b-5p expression was over 4.0-fold higher in samples collected from day 3 onwards, but the peak expression of 5.3-fold occurred on day 14 (Figure 4C).

Diverse models of kidney injury and fibrosis also show differential expression of miR-18a-5p, miR-132-3p, and miR-146b-5p

We also sought to determine the expression of these miRNAs in contrasting models of kidney injury and fibrosis. The unilateral ureteral obstruction (UUO) model is characterized by distension of the pelvicaliceal structures and hydronephrosis, with consequent compression of the medulla and thinning of the cortex, distension of the tubules, extravasation of the Tamm-Horsfall protein, tubular atrophy, and diffuse interstitial fibrosis with non-specific interstitial inflammation (Chevalier *et al.*, 2009). In contrast, bilateral renal ischemia/reperfusion injury (IRI) results in profound injury to the corticomedullary junction with extensive necrosis and sloughing of the epithelium, particularly in S3 segments of the proximal tubules. Affected tubules may reveal regenerative changes, with frequent mitotic activity and nuclear enlargement (Duffield *et al.*, 2005). In this model, collecting ducts and distal tubules in the medulla may contain extensive accumulations of cellular debris, but hydronephrosis is not a feature of this type of injury. Additionally, the superficial cortex is relatively spared in the IRI-type of injury, in contrast to typical toxic insults such as the folic acid model. While some interstitial fibrosis was observed on day 14 in the IRI model, the severity and frequency of fibrosis was much higher in the UUO model (Supplementary Figure S1).

When we investigated the expression of mmu-miR-18a-5p, mmu-miR-132-3p, and mmu-miR-146b-5p in these models, we found the expression profiles to be similar to that observed in the folic acid model (Figure 4D–4I). In both the UUO and IRI models, peak expression of mmu-miR-18a-5p occurred at the earliest time point investigated, with expression levels decreasing at later time points (UUO – day 3, 4.5-fold; IRI – day 1, 4.6-fold; Figure 4D and 4G). In the IRI model, the expression profile of mmu-miR-132-3p was similar to that of mmu-miR-18a-5p, with peak expression at day 1 (3.2-fold; Figure 4H), whereas the expression of mmu-miR-132-3p increased over the time course in mice subjected to UUO with the highest level of expression occurring at day 14 (5.9-fold; Figure 4E). There was increased expression of mmu-miR-146b-5p over the time course in both models, with peak expression observed at day 14 in both models. However, the highest level of expression of mmu-miR-146b-5p was 3.4-fold in the IRI model, while it was much higher in the UUO group (8.8-fold; Figure 4F and 4I).

Localization of miR-18a-5p, miR-132-3p and miR-146b-5p in the kidneys of folic acid-treated mice

To visualize miR-18a-5p, miR-132-3p and miR-146b-5p expression, *in situ* hybridization (ISH) was performed on paraffin-embedded kidney sections from folic acid-treated mice. In general, the expression patterns of each miRNA determined by ISH corresponded well to the expression profiles measured using qRT-PCR (Figure 4). High expression of miR-18a-5p was observed during the acute stage of injury (day 2) throughout the cytosol of cells in the cortex, including the glomeruli and tubules, but was greatly reduced in the fibrotic stage (day 14) although it was still detectable in the injured tubules (Figure 5A–5C). Expression of miR-132-3p was most pronounced throughout the cytosol of cells in the cortex in the acute stage of injury and remained strongly expressed in the cytosol of damaged tubular cells in the fibrotic stage of injury (Figure 5D–5F). Interestingly, 14 days after folic acid treatment,

miR-132-3p expression was specifically reduced in normal tubules and glomeruli (Figure 5F). Lastly, miR-146b-5p was present at very low amounts in the cytosol of tubular cells during acute injury and in the damaged tubules in the fibrotic phase of injury (Figure 5G–5I).

Tissues from patients with chronic kidney disease demonstrate high expression of miR-132-3p and moderate expression of miR-18a-5p and miR-146b-5p

To investigate whether miR-18a-5p, miR-132-3p and miR-146b-5p induction during kidney injury also occurs in humans with kidney disease, ISH was performed on human kidney biopsies of AKI and chronic kidney disease (CKD). The human AKI biopsies were taken from patients with allograft rejection, but none of the investigated miRNAs were observed to increase in this specific form of AKI (Figure 6B, 6E and 6H). The CKD biopsies were taken from patients with severe kidney fibrosis. The expression of miR-18a-5p was only slightly elevated in the cytosol near the injured tubules, as determined by the presence of hyaline casts and tubular distention (Figure 6C), while moderate induction of miR-146b-5p was observed in the cytosol near the injured tubules (Figure 6I). Expression of miR-132-3p was very strong throughout the cytosol of cells in the tubules and glomeruli (Figure 6F).

Altered expression of miR-18a-5p, miR-132-3p, and miR-146b-5p is observed in human proximal tubular epithelial cells (HPTECs) in response to environmental toxicants or nephrotoxic drugs

As the increased expression of miR-18a-5p, miR-132-3p and miR-146b-5p was localized to the tubules, we sought to determine whether these miRNAs could be induced *in vitro* in HPTECs after treatment with various nephrotoxicants. Environmental toxicants and nephrotoxic therapeutics like cadmium chloride (CdCl_2), arsenic trioxide (As_2O_3), aristolochic acid (AA), potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) and cisplatin (CDDP) were tested using one-half of the LD_{50} concentrations determined at 24 hours from our previous work (Adler *et al.*, 2015). Data was normalized to let-7f-5p, which we used earlier in the qPCR analysis of the mouse tissue and also demonstrated a coefficient of variation <2% for all HPTEC experiments. After 6 hours of treatment, the only miRNA that showed differential expression as compared to the 0.5% DMSO control was miR-132-3p, which demonstrated significant increases in both the AA (2.1-fold) and CdCl_2 (1.3-fold) treatment groups (Figure 7A). By 24 hours, differential expression was observed for all three miRNAs, with miR-18a-5p downregulated after treatment with CdCl_2 (–1.6-fold) and CDDP (–1.6-fold); miR-132-3p upregulated after treatment with AA (4.3-fold), $\text{K}_2\text{Cr}_2\text{O}_7$ (1.5-fold) and CdCl_2 (1.5-fold); and miR-146b-5p downregulated after treatment with $\text{K}_2\text{Cr}_2\text{O}_7$ (–1.7-fold), CdCl_2 (–3.3-fold), and CDDP (–2.3-fold) (Figure 7B).

Discussion

Using small RNA sequencing, we profiled global expression of miRNAs in 21 samples to build a time course of miRNA expression and identify miRNAs expressed at different stages of folic acid-induced injury. From this analysis, we selected three miRNAs for further study – miR-18a-5p, miR-132-3p, and miR-146b-5p – and found similar expression profiles for these miRNAs in two additional, but diverse, models of kidney damage. This suggests that

there may be similarities in the temporal expression of kidney miRNAs following injuries of distinct origins. In particular, miR-18a-5p was associated with the acute response to kidney injury, miR-132-3p had moderate to high expression throughout the entire response to injury, and the highest expression of miR-146b-5p was observed during the later stages of injury and fibrosis. Interestingly, some of the miRNAs found to be differentially expressed in our study have also been shown to be significantly increased in the urine of rats treated with cisplatin, including miR-18a, which was upregulated at early time points following injury (days 5 and 8), and miR-146b, which was upregulated weeks after initiation of injury (days 15 and 26) (Pavkovic *et al.*, 2014). This suggests that these miRNAs may have potential application as biomarkers in rodent models, although this would require additional investigation.

There were notable differences in miRNA expression for the human tissues and cells as compared to the mouse models. Although miR-18a-5p and miR-132-3p were significantly increased during acute injury in the mouse models, the human AKI samples showed no evidence of upregulation for any of the investigated miRNAs. This could be due to various reasons, including the differing mechanisms of AKI pathogenesis during allograft rejection, the immunosuppressants taken by these patients, the time after initiation of AKI that these samples were obtained and even the extent of damage (Cooper and Wiseman, 2013). In contrast, the tissues from CKD patients demonstrated a significant increase in miR-132-3p, although miR-18a-5p and miR-146b-5p were not highly expressed in these patients either. Our *in vitro* experiments with HPTECs also indicated differences in expression patterns for these miRNAs between mice and humans, as we only observed an upregulation of miR-132-3p in response to treatment with nephrotoxics while either no difference or a decrease in expression was observed for the other miRNAs studied. These differences may be due to the known limitations of *in vitro* proximal tubular cells, such as the lack of an immune system, other cell types, and the inherent lack of tubule architecture in 2D tissue culture (Akca *et al.*, 2009; Jang and Rabb, 2009; Jang *et al.*, 2013), but further studies in human tissues are required to determine whether HPTECs represent an appropriate model to study miRNA expression. The strong expression of miR-132-3p in human CKD and after the treatment of HPTECs with nephrotoxics indicates the potential relevance of miR-132-3p to human kidney disease and the ability to study it further in different model systems to elucidate the mechanisms of induction and action in kidney disease pathogenesis.

In conclusion, from our small RNA sequencing studies described here, we have identified miR-18a-5p, miR-132-3p and miR-146b-5p to be highly expressed at different stages in a mouse model of folic acid-induced kidney injury and fibrosis. This robust approach has advanced our understanding of miRNAs involved in kidney disease of diverse etiology and could easily be applied to profile miRNA expression in other pathologies as well. The differential expression of miRNAs has been demonstrated in response to various environmental toxicants and drugs, and may contribute to the development of toxicity (Bolleyn *et al.*, 2014). Further studies to understand how these miRNAs act to suppress specific downstream targets may provide important information about the mechanism of action for these compounds, and possibly indicate novel approaches to reduce the development of toxicity or improve the efficacy of pharmacological treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We used small RNA sequencing to identify differentially expressed miRNAs in kidney
- Distinct patterns were found for acute injury and fibrotic stages in the kidney
- Upregulation of miR-18a, -132 and -146b was confirmed in mice and human kidneys

Figure 1.

Kidney phenotype of mice injected with folic acid. The levels of blood urea nitrogen (BUN; A) were measured at 0, 1, 2, 3, 7, 14, and 28 days following the administration of folic acid. Kim-1, collagen, and fibronectin mRNA from kidney lysates was assessed by qRT-PCR, normalized to Gapdh, and are shown as fold change relative to the untreated group (B, C, and D, respectively). Data is represented as mean $\pm$ SEM and * $p < 0.05$ in comparison to the untreated group ($n = 5$ mice/group). Histological features of the kidneys were assessed in Periodic acid-Schiff (PAS) or Masson's Trichrome (MTS) stained sections, and representative images from each time point are shown (E; PAS scale bar = 50 μm ; MTS scale bar = 100 μm).

Figure 2. Small RNA sequencing of folic acid treated kidneys. Using miRDeep2, reads were trimmed, mapped to the genome (mm9), and annotated. The number of reads passing through each stage of the analysis is shown (A; the range for all samples shown in parentheses). The data for each sample was normalized to the total number of reads for that sample and expressed as counts/million. Each miRNA was assigned to one of the indicated count ranges based on the average counts/million from all samples, and the number of miRNAs within each count range (per million reads) is shown (B). Using the data from the untreated mice, the percentage of reads attributable to each miRNA was determined, and the miRNAs that comprised more than 5% of the total are represented (C). A two dimensional scaling of the 21 samples based on the 100 most heterogeneous miRNAs with expression of at least 5 counts/million was prepared using the edgeR function ‘plotMDS’ (D).

Figure 3. Differentially expressed miRNAs during folic acid injury and fibrosis. Using edgeR, the small RNA sequencing data was analyzed to identify miRNAs that demonstrated a significant change in expression during at least one time point following folic acid administration. The expression of these miRNAs is represented as the average fold change in comparison to the untreated group (n = 3 mice/group).

Figure 4.

Expression of miR-18a-5p, miR-132-3p, and miR-146b-5p in various models of kidney injury. Three miRNAs were chosen from the small RNA sequencing data to represent the acute (18a-5p), intermediate (132-3p), and fibrotic (146b-5p) stages of injury. The expression of these miRNAs was assessed in the folic acid model (FA; A–C), as well as unilateral ureteral obstruction (UVO; D–F), which causes a severe fibrotic injury, and bilateral ischemia reperfusion injury (IRI; G–I), which induces acute tubular injury. Data was normalized to mmu-let-7f-5p and is represented as mean $\pm$ SEM. * $p < 0.05$ in comparison to the untreated control group for each experiment ($n = 5–6$ mice/group). The data for the CoK group was derived from the contralateral kidney, which was left untouched during the UVO surgery.

Figure 5.

In situ hybridization analysis of miR-18a-5p, miR-132-3p and miR-146b-5p in the kidneys of folic acid-treated mice. Expression of miR-18a-5p (A–C), miR-132-3p (D–F) and miR-146b-5p (G–I) was investigated in mice at day 0 (Normal), as well as 2 and 14 days following the administration of 250 mg/kg folic acid (FA). A scrambled (Scr; J–L) and U6 probe (M–O) were used as negative and positive controls, respectively. The development of purple staining indicates detection of the ISH probe. Black arrows represent injured tubules and blue arrows represent normal tubules and glomeruli. Representative images from each group are shown (scale bar = 50 μm; n = 3 mice/group).

Figure 6.

In situ hybridization analysis of miR-18a-5p, miR-132-3p and miR-146b-5p in human kidney disease. The expression of miR-18a-5p (A–C), miR-132-3p (D–F) and miR-146b-5p expression (G–I) was investigated in the kidneys of patients with no evidence of kidney disease, patients with AKI due to renal allograft rejection, or patients with severe kidney fibrosis due to CKD. A scrambled (J–L) and U6 probe (M–O) were used as negative and positive controls, respectively. The development of purple staining indicates detection of the ISH probe. Asterisks represent tubular distension or hyaline casts. Representative images from each group are shown (scale bar = 50 μm; n = 5 samples/group).

Figure 7.

Expression of miR-18a-5p, miR-132-3p, and miR-146b-5p in vitro following injury with various nephrotoxicants. The expression of these miRNAs was measured in human primary proximal tubular epithelial cells at 6h (A) and 24h (B) following treatment with 100μM aristolochic acid (AA), 50μM arsenic trioxide (As₂O₃), 10μM potassium dichromate (K₂Cr₂O₇), 25μM cadmium chloride (CdCl₂) and 40μM cisplatin (CDDP). Data was normalized to let-7f-5p and is represented as log₂ of mean ± SEM. *p < 0.05 in comparison to DMSO-treated cells for each experiment (n = 3 replicates/treatment).

Table 1

The most significantly changed miRNAs following folic acid-induced kidney injury and fibrosis.

	UPREGULATED			DOWNREGULATED		
	MicroRNA	Fold change	FDR	MicroRNA	Fold change	FDR
DAY 1	mmu-miR-21-3p	5.98	2.44E-34	mmu-miR-101a-5p	-1.98	4.11E-06
	mmu-miR-21-5p	3.89	4.75E-27	mmu-miR-30e-3p	-1.60	8.72E-05
	mmu-miR-132-3p	4.58	3.90E-22	mmu-miR-151-5p	-1.57	1.38E-04
	mmu-miR-212-3p	5.79	6.17E-19	mmu-miR-720	-2.17	1.71E-04
	mmu-miR-19a-3p	3.55	2.89E-13	mmu-miR-1981-3p	-1.84	3.06E-04
DAY 2	mmu-miR-21-3p	8.67	2.00E-48	mmu-miR-30e-5p	-2.17	1.13E-09
	mmu-miR-21-5p	5.15	1.61E-38	mmu-miR-203-3p	-1.95	4.71E-09
	mmu-miR-132-3p	5.19	6.37E-26	mmu-miR-101a-5p	-2.10	7.84E-08
	mmu-miR-212-3p	6.72	4.84E-23	mmu-miR-30e-3p	-1.81	1.09E-07
	mmu-miR-223-3p	4.09	1.46E-18	mmu-miR-874-3p	-2.19	2.53E-07
DAY 3	mmu-miR-21-3p	6.26	5.32E-36	mmu-miR-30e-3p	-2.03	1.27E-10
	mmu-miR-21-5p	4.83	8.38E-36	mmu-miR-3073b-3p	-2.94	2.77E-09
	mmu-miR-146b-5p	5.99	1.24E-30	mmu-miR-203-3p	-1.94	6.83E-09
	mmu-miR-298-5p	12.45	1.24E-30	mmu-miR-30e-5p	-2.00	5.51E-08
	mmu-miR-132-3p	5.34	6.03E-27	mmu-miR-101c	-1.93	5.59E-08
DAY 7	mmu-miR-21-5p	5.05	2.09E-37	mmu-miR-3073b-3p	-4.88	2.06E-17
	mmu-miR-146b-5p	6.39	1.69E-32	mmu-miR-3073b-5p	-3.91	5.19E-15
	mmu-miR-132-3p	5.96	4.10E-30	mmu-miR-203-3p	-2.04	1.93E-10
	mmu-miR-298-5p	11.85	1.67E-29	mmu-miR-107-3p	-2.07	4.06E-10
	mmu-miR-21-3p	4.64	1.85E-26	mmu-miR-193-3p	-2.40	2.90E-09
DAY 14	mmu-miR-146b-5p	6.65	1.87E-33	mmu-miR-3073b-3p	-4.10	1.42E-13
	mmu-miR-132-3p	4.70	9.04E-23	mmu-miR-29c-3p	-2.02	6.59E-09
	mmu-miR-199a/b-5p	3.25	5.61E-20	mmu-miR-193-3p	-2.38	6.66E-09
	mmu-miR-199a/b-3p	2.99	7.62E-20	mmu-miR-192-5p	-1.87	3.10E-08
	mmu-miR-21-5p	2.89	2.94E-17	mmu-miR-107-3p	-1.90	6.29E-08
DAY 28	mmu-miR-146b-5p	3.01	1.70E-11	mmu-miR-187-5p	-2.00	4.40E-04

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	UPREGULATED			DOWNREGULATED		
	MicroRNA	Fold change	FDR	MicroRNA	Fold change	FDR
	mmu-miR-199b-3p	2.18	7.81E-10	mmu-miR-33-5p	-1.80	6.25E-04
	mmu-miR-142-5p	2.19	7.81E-10	mmu-miR-193-3p	-1.73	1.18E-03
	mmu-miR-31-5p	2.57	8.83E-10	mmu-miR-101a-5p	-1.65	1.32E-03
	mmu-miR-199a/b-5p	2.21	3.35E-09	mmu-miR-101c	-1.52	2.30E-03

FDR = False Discovery Rate