# Expression, Circulation, and Excretion Profile of MicroRNA-21, -155, and -18a Following Acute Kidney Injury

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MicroRNAs (miRNAs) are endogenous noncoding RNA molecules that are involved in post-transcriptional gene silencing. Using global miRNA expression profiling, we found miR-21, -155, and 18a to be highly upregulated in rat kidneys following tubular injury induced by ischemia/reperfusion (I/R) or gentamicin administration. Mir-21 and -155 also showed decreased expression patterns in blood and urinary supernatants in both models of kidney injury. Furthermore, urinary levels of miR-21 increased 1.2-fold in patients with clinical diagnosis of acute kidney injury (AKI) (n = 22) as compared with healthy volunteers (n = 25) (p < 0.05), and miR-155 decreased 1.5-fold in patients with AKI (p < 0.01). We identified 29 messenger RNA core targets of these 3 miRNAs using the context likelihood of relatedness algorithm and found these predicted gene targets to be highly enriched for genes associated with apoptosis or cell proliferation. Taken together, these results suggest that miRNA-21 and -155 could potentially serve as translational biomarkers for detection of AKI and may play a critical role in the pathogenesis of kidney injury and tissue repair process.

*Key Words:* MicroRNAs; kidney; biomarker; ischemia/reperfusion injury; nephrotoxicity.

Acute kidney injury (AKI) is a complex disease accompanied by a number of clinical conditions that is defined by loss of kidney function as illustrated by the glomerular filtration rate (Singbartl and Kellum, 2012). AKI has been shown to be a strong independent risk factor in developing progressive chronic kidney disease (CKD) and end-stage renal disease, in addition to other nonrenal outcomes, such as cardiovascular diseases (Bucaloiu *et al.*, 2012; Coca *et al.*, 2012; Lo *et al.*, 2009). Although considerable progress has been made in the management of AKI in both clinical and outpatient scenarios, the incidence of AKI among hospitalized adults increased from 61 to 288 per 100,000 population in the past decade (Waikar *et al.*, 2006). AKI is associated consistently with higher risk and incidence of mortality even when taking into account comorbidities that may contribute to lung injury, liver injury, or other organ system failure (Chertow *et al.*, 2005). The primary causes of AKI are thought to be ischemic and toxic insults that compromise the glomerular and tubular function (Thadhani *et al.*, 1996).

MicroRNAs (miRNAs) are endogenous short noncoding RNA molecules of about 22 nucleotides in length and are involved in post-transcriptional gene regulation by targeting messenger RNAs (mRNAs) and inhibiting translation (Bartel, 2004). MiRNAs are associated with numerous functional roles in development and disease pathogenesis in multiple organ systems (Ambros, 2004; Erson and Petty, 2008; Stefani and Slack, 2008). The hallmark of gene regulation by these small molecules is that a given miRNA may regulate multiple target mRNAs because of the imprecise complementarity in base pairing and, conversely, a single gene may be governed by several regulatory miRNAs (Bartel and Chen, 2004). Lower complexity, no post processing modification, synthetic high-affinity "capture" reagent, tissue-specific expression, and amplifiable signals make circulating/soluble small RNAs ideal candidates as biomarkers to reflect various pathophysiological conditions and disease states (Chen et al., 2008; Hede, 2009; Rabinowits et al., 2009; Rosell et al., 2009; Sharma and Vogel, 2009; Wang et al., 2009). Exosomal and circulating miRNAs have recently shown great potential as biomarkers for detecting cancers such as prostate cancer (Mitchell et al., 2008), colorectal cancer (Ng et al., 2009), ovarian cancer (Resnick et al., 2009), and nonsmall

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cell lung cancer (Chen *et al.*, 2008; Rabinowits *et al.*, 2009), in addition to myocardial injury (Ji *et al.*, 2009) and liver damage (Laterza *et al.*, 2009; Wang *et al.*, 2009; Yang *et al.*, 2012). Apart from their diagnostic application, the potential therapeutic applications of miRNAs are also currently of interest because of their ability to target multiple pathways involving cell survival and differentiation in parallel (Iorio and Croce, 2012).

Most of the miRNA research in the kidney has focused on identifying unique miRNA expression patterns in kidney diseases including renal allograft (Anglicheau *et al.*, 2009; Sui *et al.*, 2008), renal cell carcinoma (Chow *et al.*, 2009; Nakada *et al.*, 2008; Siu *et al.*, 2009), and polycystic kidney disease (Lee *et al.*, 2008). Kidney cortex and medulla are known to have differential expression profiles of kidney-specific miRNAs (Tian *et al.*, 2008), and over the years, specific roles have emerged for key miRNAs in various renal diseases. However, relatively few reports have shown the use of soluble (cell-free) urinary miRNAs as biomarkers for early detection of kidney disease (Hanke *et al.*, 2010; Melkonyan *et al.*, 2008; Shekhtman *et al.*, 2009).

The objective of this study was to characterize the miRNA expression, circulation, and excretion profile following the induction of AKI in rodents and humans. Accordingly, we performed a global miRNA expression for rat kidney cortices following 30-min renal bilateral ischemia/reperfusion (I/R) injury from which we selected three miRNAs (miR-21, -155, and -18a) as candidate renal injury markers. We investigated their expression profile in tissue, blood, and urine after 30-min I/R injury and gentamicin-induced nephrotoxicity (0, 200, and 300 mg/ kg, subcutaneous [sc]). We established the renal specificity of the expression profile using a model of galactosamine-induced hepatotoxicity (1.1 g/kg, intraperitoneal [ip]). The ability of urinary miR-21 and -155 to distinguish patients with or without AKI was also evaluated in comparison to a well-established kidney injury biomarker kidney injury molecule-1 (KIM-1) (Vaidya et al., 2008). The gene targets for the candidate mi-RNAs were predicted using a context likelihood of relatedness (CLR) algorithm, and 29 targets were found to be common to all three miRNAs and were enriched for genes that control cellular functions such as cell proliferation and apoptosis.

### MATERIALS AND METHODS

Animals. Rats were maintained in a central animal facility over hardwood chips free of any known chemical contaminants under conditions of  $21^{\circ}C \pm 1^{\circ}C$  and 50–80% relative humidity at all times in an alternating 12-h light–dark cycle except during surgery, toxicant injection, and blood and urine collection. Rats were fed with commercial rodent chow, given water ad lib, and were acclimated for 1 week prior to use. The I/R injury studies were performed on Wistar rats in our laboratory, and the gentamicin and galactosamine studies were performed on Sprague Dawley rats by collaborators from Center for Devices and Radiological Health, Food and Drug Administration. The different strains and methods of euthanasia were used in regulation with the protocols of the respective institutional guidelines. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory animals as adopted and promulgated by the National Institutes of Health and were approved by the Harvard Medical School Animal Care and Use Committees (IACUC).

**Renal bilateral I/R injury.** A total of 16 male Wistar rats (280-320 g) purchased from Harlan Laboratories (Indianapolis, IN) were subjected to 30 min of renal bilateral I/R injury as previously described (Vaidya *et al.*, 2010), and of the 16 rats, 4 rats underwent sham surgery simulating I/R injury. Under anesthesia (30 mg/kg ip sodium pentobarbital), both renal arteries were clamped at the pedicle for 30 min, and the animal was maintained at 37°C and sacrificed using an overdose of pentobarbital (180 mg/kg) 24, 72, and 120h following reperfusion. Sham rats underwent a laparotomy under anesthesia and were sacrificed 24h later. (n = 4/ttime point).

*Gentamicin-induced nephrotoxicity.* Male Sprague Dawley rats purchased from Harlan Laboratories received a sc injection of either 200 or 300 mg/kg of gentamicin or water (n = 6) daily for 3 days and were sacrificed 24h after the last dose by isoflurane anesthesia.

*Galactosamine-induced hepatotoxicity.* To establish the specificity of miRNA upregulation to kidney damage, we used a well-established model of liver toxicity caused by galactosamine administration. Male Sprague Dawley rats (Harlan Laboratories) were given a single ip dose of either 1.1 g/kg of galactosamine or 0.9% saline (n = 3) and were sacrificed by isoflurane anesthesia after 24 h.

*Urine and blood collection.* Urine was collected from I/R injured animals 4h before sacrifice, and from gentamicin- and galactosamine-treated animals 16h before sacrifice by placing them in metabolic cages. During the course of urine collection, 1 mL of RNAlater (Ambion) was added to the collection tubes beforehand to preserve the RNA. Samples for RNA extraction were centrifuged at 10,000 rpm for 15 min, and the supernatant was aliquoted and stored at  $-80^{\circ}$ C. Blood was collected during necropsy of which 100 µL was used for analyses of liver enzymes in the galactosamine-treatment model, and 2.5 mL was aliquoted in PAXgene Blood RNA tubes (PreAnalytiX) to preserve intracellular RNA. The rest of the blood samples were stored in heparinized tubes and centrifuged at 10,000 rpm for 10 min at 4°C to isolate the plasma that was then aliquoted and stored at  $-80^{\circ}$ C.

**Blood analyses.** The collected plasma was used for the measurement of parameters of kidney injury: Serum Creatinine (SCr) and Blood Urea Nitrogen (BUN) SCr concentrations were measured using a Creatinine Analyzer II (Beckman Coulter). BUN was measured spectrophotometrically at 340nm using a commercially available kit (ThermoFisher Scientific) as described previously (Krishnamoorthy *et al.*, 2010). Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) levels were measured in the whole blood using test strips for the Reflotron Analyzer.

*Urine analyses.* Urinary Kim-1 (Kim-1 in rodents and KIM-1 in humans) was measured using previously established Luminex-based assays (Vaidya *et al.*, 2008; Vaidya *et al.*, 2010). Urinary creatinine concentration (Cayman Chemical) was used to normalize protein biomarker measurements to account for urinary volume differences.

RNA extraction and quantitative Polymerase Chain Reaction (qPCR). Total RNA isolation from rat tissue was carried out using TRIzol (Invitrogen) reagent according to manufacturer's instructions. Total RNA was isolated from 2.5 mL of blood collected during sacrifice using the PAXgene Blood RNA system according to manufacturer's instructions. About 300 µL of rat and human urine was used to extract total RNA using TRIzol reagent according to the manufacturer's instructions. Total RNA concentration was measured at 260nm using a NanoDrop 2000c spectrophotometer (ThermoFisher). Small RNA concentration was measured for urine RNA samples on an Agilent Bioanalyzer (Agilent). Quantitative miRNA analysis was performed using TaqMan miRNA assays from Applied Biosystems. The concentration measured using the NanoDrop was used to calculate 10 ng of total RNA in tissue, blood, and urine, which was then reverse transcribed into complementary DNA (cDNA) using specific stem loop primers for each of miR-21, -155, and -18a (16°C for 30 min; 42°C for 30 min; 85°C for 5 min). Quantitative PCR was performed using TaqMan primers and probes in a CFX96 RTPCR instrument (Bio-Rad) with the following temperature profile: 95°C for 10min followed by 44 cycles of 95°C for 15 s and 60°C for 1 min. U87 was used as an internal normalization control for tissue.

Global miRNA expression analysis and construction of functional network. A TaqMan Low Density Array (TLDA) was used to screen for 349 miRNAs rat kidney samples following 30-min renal bilateral I/R injury over time at 24, 72, and 120h. Using a cut off cycle threshold (Ct) value of 35, we were able to obtain a set of candidate miRNAs that were upregulated > 2-fold as compared with sham. A heatmap was generated to show the expression level of 349 miRNAs across the different time points after I/R injury. Log2 values of fold changes were calculated as (Ctcontrol – Ctexp) for each miRNA with Ctcontrol being calculated from the averages of the sham group. Hierarchical clustering was performed using 1 – Pearson correlation as a distance with average linkage.

To construct functional network between miRNAs and mRNAs, we used a CLR algorithm (Faith *et al.*, 2007). The normalized miRNA–mRNA matched expression profile was obtained from a literature search (Lu *et al.*, 2005). The profile contained 217 miRNAs and ~16,000 mRNA expression values measured across 89 human cancer and normal samples that include 5 tumor and 3 normal kidneys. We calculated Pearson correlation for possible miRNA–mRNA pairs and applied an adaptive background correction in which the correlation of miRNA–mRNA pair is compared with the background distribution of all correlation values that include either the miRNA or mRNA in the pair (Faith *et al.*, 2007). In brief, the *Z*-score was calculated for each miRNA–mRNA pair as  $Z_{ij} = (z_i + z_j)/\sqrt{2}$  in which  $z_i$  and  $z_j$  represent the *z* score of  $PCC_{ij}$  given  $f(cdf(PCC_i))$  and  $f(cdf(PCC_j))$  for miRNA *i* and mRNA *j*, respectively.

We considered the top five percentile of miRNA–mRNA anticorrelation pairs ( $Z \le 1.43$ ) as potential functional pairs from which 1018, 783, and 592 mRNA targets of miR-21, -155, and -18a were selected. The mRNA targets were measured for the significance of enrichment for Gene Ontology (GO) categories using Fisher's exact test. GO category gene sets were downloaded from MSigDB (c5 categories) (Subramanian *et al.*, 2005). As a result, 155, 100, and 17 GO categories were significantly enriched with false discovery rate (FDR) < 0.05, to mRNA targets of miR-21, -155, and -18a, respectively. We used Cytoscape (Shannon *et al.*, 2003) to construct the network of 62 GO categories significantly enriched for at least two out of these three miRNAs.

*Human studies.* Urine samples from humans with ischemic or septic AKI were obtained from critically ill patients admitted to the intensive care unit (ICU) with a rise in SCr of at least 100% over baseline values and from patients undergoing native or transplant kidney biopsy with histological confirmation of acute tubular injury. Urine samples from healthy volunteers were obtained from individuals participating in the Pheno Genetics study, a large prospective observational cohort examining the genetics of the immune system in healthy individuals conducted at Brigham and Women's Hospital, Boston, MA. Inclusion criteria for healthy individuals specified males or females aged 18–50 years; exclusion criteria were the presence of inflammatory diseases, autoimmune diseases, chronic metabolic diseases, and chronic infections. The institutional review board of Brigham and Women's Hospital approved all human studies. Urine samples were spun at 4000 rpm for 10 min, and the supernatant was aliquoted and stored at  $-80^{\circ}$ C as described previously (Vaidya *et al.*, 2008).

*Statistical analyses.* Data are expressed as average  $\pm$  standard error. Statistical difference (p < 0.05) was calculated by one-way ANOVA or Student's *t*-test. p < 0.05 is considered significant and is represented by a single asterisk (\*) where applicable. All graphs were generated by Prism software (GraphPad Software).

### RESULTS

## *I/R Injury Results in Significant Upregulation of miR-21,* -155, and -18a in the Kidney Tissue

In order to characterize the miRNA expression profile that regulates genes modulating early injury and repair processes following AKI, we used a well-established model of 30-min bilateral renal I/R injury. Animals sacrificed 24 h following reperfusion displayed maximum kidney dysfunction as assessed by serum creatinine (SCr) and blood urea nitrogen (BUN) and proximal tubular damage measured by Kim-1 (Fig. 1A). Histopathological findings revealed extensive tubulointerstitial damage particularly of the S3 segments of the straight portion of proximal tubules in the outer medulla and corticomedullary junction (Supplementary fig. S1). The individual tubules revealed extensive degenerative changes and necrosis of the epithelial cells, with occlusion of their lumens by desquamated cells and cellular debris. After 120h, the tubules were separated by mild chronic interstitial inflammatory infiltrate and a small amount of cellular necrotic debris was still noted in the lumens of some tubules (fig. S1).

The transcript levels of 349 miRNAs were measured in the kidney cortex for three time points after I/R injury by TaqMan Low Density Array (TLDA; Supplementary tables 1 and 2, Fig. 1B). As primary candidates, we selected miRNAs that showed > 2-fold changes upregulation (compared with sham) in all of the three time points measured. Among them, we selected three miRNAs (miR-21, -155, and -18a) for further experimental verification (indicated by arrows in Fig. 1B).

We further confirmed the expression analysis by quantitative real-time PCR (qPCR) for all three miRNAs in kidney cortex and medulla samples. The raw Ct values for miR-21 in the sham animals were ~23 in the cortex as well as in the medulla, indicating a high constitutive expression in the kidney. The fold change expression showed a modest elevation in the cortex of ~2.5-fold, but the most significant upregulation was observed in the medulla at 120h (~13-fold) (Fig. 2A). Constitutive miR-155 Ct values in the cortex were about 31, whereas they were decreased by a cycle in the medulla, indicating a 2-fold change between the two kidney zones. The expression of miR-155 was increased by 24h in the cortex to ~6-fold, and the levels lowered to 3-fold by 120h. The medulla on the other hand showed a gradual increase over time, peaking to 5-fold at 120h (Fig. 2A). Sham Ct values of miR-18a were ~31 in the cortex and ~29 in the medulla. The fold change of miR-18a was the highest in the cortex at 72h (8-fold) and remained constant in the medulla over all three time points (Fig. 2A). We further evaluated the expression profile of all three miRNAs in heart, liver, lung, and spleen and observed no change between sham and 24 h following renal I/R injury (Supplementary fig. S2).

# Circulating Levels of miR-21, -155, and -18a in Blood Significantly Decrease Following Kidney I/R Injury

To investigate the possibility that certain miRNAs could act as sensitive and specific markers of AKI, we assessed the levels of miR-21, -155, and -18a in whole blood and urinary supernatant. Ct values of miR-21, -155, and -18a in the sham group were 28, 32, and 30, respectively, in whole blood. All three miRNAs showed a similar pattern of decrease at both 24 (3-fold for miR-21 and 6-fold for miR-155 and -18a) as well as 120h (7-fold for miR-21, 5-fold for miR-155, and 10-fold for miR-18a) (Fig. 2B).

In the urinary supernatants, miR-21 Ct values of sham rats were ~31 and miR-155 values were slightly higher ~33, whereas



# Hours post Bilateral I/R

FIG. 1. miRNA expression profile indicates that miR-21, -155, and -18a are amongst the significantly upregulated miRNAs in rat kidney following renal I/R injury compared with sham surgery.

Male Wistar rats were subjected to 30-min renal bilateral I/R injury and were sacrificed 24, 72, and 120h following reperfusion. (A) Biochemical parameters of SCr, BUN, and Kim-1 as measures of kidney function. (B) A heatmap (left) shows the relative expression (log2 of fold change) of 204 miRNAs at 3 time points after I/R injury relative to averaged values of the control group. Red and blue represent the relative up- and downregulation, respectively. A total of 33 miRNAs showing constitutive upregulation after I/R injury are selected and the initial candidates miR-21, -155, and -18a are marked by arrows (right).

miR-18a levels were undetectable by qPCR and usually not reproducible with Ct values of around 35 or higher. As expected, miRNA levels are lesser in urine than kidney tissue, but they are comparable to expression levels in whole blood. The levels of miR-21 were modestly elevated to about 2-fold within 72 h and remained high until 5 days following reperfusion. The miR-155 expression remained unchanged for the first 3 days and the level slightly decreased, but nonsignificantly at 120 h (Fig. 2C).

## Gentamicin-Induced Nephrotoxicity Results in Marked Increase in miR-21 and -155 Corresponding to Histological Damage

In order to evaluate the reproducibility of the expression profile of miR-21, -155, and -18a in kidney tissue, whole blood, and urinary supernatant, we used a rat model of gentamicininduced kidney toxicity. There was a modest increase in SCr and BUN with a marked increase in urinary Kim-1 in a dosedependent manner (Fig. 3A). This correlated with histopathological findings that revealed mild tubular distension and subtle degenerative changes of the epithelium, including vacuolization and coarse granulation of the cytoplasm for a dosage of 200 mg/kg. With a higher dose, more prominent injury, with more pronounced distension and epithelial cell necrosis of the proximal convoluted tubules, with karyopyknosis and accumulation of cellular debris in their lumens was noted (fig. S3). The raw Ct numbers of miR-21, -155, and -18a in the kidney, blood, and urine from control group were similar to the sham group in I/R injury, indicating the robustness and reproducibility of their baseline expression pattern across two different strains of rats. MiR-21 showed ~2-fold upregulation in both the medulla and cortex that was dose dependent and maximum for rats treated with 300 mg/kg. The fold change of expression of miR-155 was higher in the cortex (~6-fold) than in the medulla (~2-fold), but the expression did not seem to be dose dependent. MiR-18a did not show any significant change across the treatment groups or between cortex and medulla (Fig. 3B).

MiR-21 expression in blood was significantly reduced in the blood by 3-fold in both treatment groups. The expression level of miR-155 and -18a both showed a very slight (statistically insignificant) decrease for both doses (Fig. 3C). Interestingly, contrary to I/R injury, both miR-21 and -155 were both significantly decreased in the urine (2-fold) for both 200 as well as 300 mg/kg doses (Fig. 3D). MiR-18a was undetectable in the urine with Ct values > 35.



**FIG. 2.** Tissue, blood, and urinary profile of miR-21, -155, and -18a following 30-min renal bilateral I/R. qPCR analyses for miR-21, -155, and -18a in (A) kidney cortex and medulla samples, (B) whole blood, (C) urine supernatant, and fold change is determined over sham (n = 4/group). Tissue samples are normalized to an internal reference gene, U87. \*p < 0.05 as determined by Student's *t*-test.



FIG. 3. Gentamicin-induced nephrotoxicity results in increased expression of miR-21 and -155 in rats.

Male Sprague Dawley rats were treated with 0, 200, and 300 mg/kg gentamicin, sc for 3 days, and sacrificed 24 h after the last dose. (A) Biochemical parameters of SCr, BUN, and Kim-1 as parameters of renal injury. qPCR analyses in (B) kidney cortex and medulla samples, (C) whole blood, (D) urine supernatant for miR-21, -155, and -18a; tissue is normalized to an internal reference gene, and *U*87 and fold change are determined over the control group (n = 6/group). \*p < 0.05 as determined by Student's *t*-test.

# Galactosamine-Induced Liver Injury Does Not Result in Any Change in miR-21, -155, and -18a, Suggesting Their Kidney Injury-Specific Upregulation

To establish the specificity of the expression profile of miRNA upregulation to AKI, we used an established model of liver toxicity induced by galactosamine administration. With a dosage regimen of 1.1 g/kg, we observed significant loss of hepatic structure and function as illustrated by activity of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measured in whole blood at 24 h (Fig. 4A). On the other hand, the kidney showed no obvious damage as evidenced by SCr and BUN levels (Fig. 4A). An evaluation of the three miRNAs in kidneys showed no significant change in any of the levels either in the kidney (Fig. 4B) or in the blood (Fig. 4C) suggesting the specificity of increased expression of miR-21, -155, and -18a to renal injury. The expression of miR-21 and -155 showed a nonsignificant decrease in the urinary levels (Fig. 4D) and miR-18a levels were nondetectable.

## Urinary Levels of miR-21 and -155 Distinguish Patients With AKI as Compared With Patients Without AKI

We next investigated whether soluble miRNAs in urine could serve as effective biomarkers of AKI and/or other chronic forms of kidney disease. We measured urinary levels of miR-21 and -155 in 22 patients (Fig. 5A) admitted to the intensive care unit (ICU) of Brigham and Women's Hospital with an elevated SCr (Table 1) and elevated levels of urinary KIM-1 (Fig. 5B) and compared it to the levels in 25 healthy volunteers from the hospital. We found that miR-21 levels were constitutively higher in the urines of the healthy volunteers than miR-155 with miR-21 yielding Ct values of ~30, whereas miR-155 Ct values were close to 34. MiR-21 was increased by 1.2-fold in the AKI patients over the healthy volunteers (p < 0.05) and miR-155 was decreased by 1.5-fold in patients with AKI (p < 0.01) (Fig. 5A). Receiver operating characteristic curve analyses indicated that both miR-21 (AUC-ROC = 0.71, p = 0.01) and miR-155 (AUC-ROC = 0.7, p = 0.02) were able to differentiate between healthy and AKI patients (Table 2).

# Functional Roles of miR-21, -155, and -18a Inferred From mRNA–miRNA Coexpression Network

To identify the putative functions mediated by mRNA targets of miR-21, -155, and -18a, we first predicted expression-based mRNA targets of these miRNAs. We used the CLR algorithm to identify potential mRNA targets of miR-21 (n = 1018), -155 (n = 783), and -18a (n = 592) (Fig. 6A) whose expression showed significant anticorrelation against the expression of the corresponding miRNA across diverse human tissue samples (Lu *et al.*, 2005). MiR-21 and -155 shared more than 200 targets ( $p = 1.97 \times 10^{-119}$  Fisher's exact test) suggesting that they could possibly control several similar pathways. The other miRNA pairs also showed significant overlap of their predicted mRNA targets (miR-18a-vs.-miR-21,  $p = 8.38 \times 10^{-55}$ , miR-18a-vs.miR-155,  $p = 1.32 \times 10^{-10}$ ). Table 3 lists the 29 mRNA predicted targets of the three miRNAs. The genes include those with known



# Galactosamine (g/kg)

FIG. 4. Galactosamine-induced liver toxicity does not alter the expression profile of miR-21, -155, and -18a in rats.

Male Sprague Dawley rats were treated with 0.9% saline or 1.1 g/kg galactosamine, ip, and sacrificed 24h later. (A) ALT and AST were measured in whole blood establishing liver injury, and SCr and BUN were measured as indicators of kidney function. qPCR analyses in (B) kidney cortex and medulla samples, (C) whole blood, (D) urine supernatant for miR-21, -155, and -18a; tissue is normalized to an internal reference gene, and *U*87 and fold change are determined over the control group (n = 3/group). \*p < 0.05 as determined by Student's *t*-test.



**FIG. 5.** Differences in urinary excretion patterns of miR-21 and -155 in patients with AKI as compared with patients without AKI. (A) qPCR analyses for miR-21 (\*p < 0.05) and -155 (\*p < 0.01) in cell-free urine supernatant of healthy and AKI patients. \*p as determined by Student's *t*-test. (B) Urinary KIM-1 as a biomarker of acute tubular injury differentiating healthy and AKI patients (\*p < 0.0001).

	TABLI	E 1			
<b>Clinical Characteristics of Human Subjects</b>					
	Healthy volunteer	AKI			
Number of subjects	25	22			
Mean age, y (SD)	35.6 (10.7)	53.5 (16.7)			
Female	68%	41%			
White	80%	73%			
SCr (mg/dL)	a	$3.7 \pm 2.8$			
Cause of AKI	N/A	Acute tubular injury identified on			
(Number of		transplant kidney biopsy			
subjects)		(9 patients); acute tubular injury			
-		identified on native kidney biopsy			
		(3 patients); shock from sepsis			
		and/or ischemia (10 patients)			

TABLE 2
Comparative Diagnostic Performance Characteristics of miRNAs
Compared With an Established Protein Biomarker Using
<b>Receiver Operating Characteristics Curve</b>

Biomarker	AUC	95% CI	<i>p</i> Value
miR-21	0.715	0.557-0.873	0.012
miR-155	0.699	0.544-0.855	0.022
KIM-1	0.965	0.923-1.01	< 0.0001

<sup>*a*</sup>Healthy volunteers were excluded if they reported a diagnosis of chronic kidney disease. SCr was not measured.

roles in apoptosis/proliferation (*ARG2*, *BECN1 CFDP1*, *COQ6*, *ERC1*, *FAM134B*, *FKBK1B*, and *PTGS2*), chromatin remodeling (*BAZ2A*), differentiation (*SOX3*), nucleotide metabolism (CTPS2), transport (*MCFD2*, *RAB21*, and SEC31), and transcription/ translation (*EIF4E*, *TCEA2*, and *ZBTB47*). To examine the potential molecular functions associated with mRNA targets

of each miRNA, we performed gene set enrichment analysis using Gene Ontology (GO) functional categories (MSigDB; c5 categories) (Subramanian *et al.*, 2005). MiR-21, -155, and -18a showed significant enrichment with False Discovery Rate (FDR) < 0.05 for 153, 98, and 17 GO categories (Fig. 6B). The miRNA pairs also showed significant overlap of enriched functions. We selected 62 GO categories that were enriched with more than one out of three miRNAs for network visualization (Fig. 6C). Among them, 13 functional categories associated with all three miRNAs are largely enriched to kinase activity and cell proliferation (4 and 3 out of 13 categories, respectively).



FIG. 6. Functional analysis of predicted mRNA targets of miR-21, -155, and -18a.

(A) A Venn diagram shows the number of mRNA targets of miR-21, -155, and -18a predicted by CLR algorithm. The significance of mRNA target overlap between two miRNAs is calculated by Fisher's exact test. The full list of 29 core mRNAs is shown in Table 3. (B) The overlap between GO categories significantly (FDR < 0.05) enriched to miR-21, -155, and -18a. (C) 62 GO categories shared by more than one miRNA are shown as nodes in a network. Edge represents the significant (FDR < 0.05) enrichment between mRNA targets and GO categories. Red and pink nodes are mRNA targets of the corresponding miRNA and GO categories shared by all three miRNAs, respectively.

### DISCUSSION

Prerenal and intrinsic renal failure caused by nephrotoxins and ischemic injuries are responsible for 35 and 50% of episodes of acute renal failure, respectively (Thadhani *et al.*, 1996). The primary aim of this study was to characterize the expression, circulatory, and excretory profile of miRNAs following AKI due to ischemic or toxic insult. In this study, we show (1) miR-21, -155, and -18a were among the highest upregulated miRNAs in the kidney following I/R injury (compared with sham); (2) the upregulation of miR-21, -155, and -18a in the kidney was observed in multiple models of AKI but was not observed following liver damage demonstrating the robustness, reproducibility, and specificity of the miRNA response; (3) the excretory profile of miR-21 and -155 in urine could successfully distinguish patients with and without AKI; (4) a novel approach that identified 29 mRNA core target genes of the three miRNAs associated with apoptosis or cell proliferation following kidney injury.

The rationale behind studying the miRNA expression profile over a time course of renal I/R injury was to identify key miRNAs that are involved in different stages of kidney injury, such as the damage and repair processes, as well as those that are important throughout. Ischemic-induced injury causes significant tubular damage in the first 24 h, with widespread congestion and dilation of the epithelium accompanied by luminal cast and debris accumulation in the S3 segments (Sabbahy and

#### SAIKUMAR ET AL.

TABLE 3Predicted mRNA Targets of miR-21, -155, and -18a

Symbol	Entrez ID	Ref Seq ID	Description
FAM134B	54463	NM_001034850	Family with sequence similarity 134, member B
EIF4E	1977	NM_001130678	Eukaryotic translation initiation factor 4E
COQ6	51004	NM_182476	Coenzyme Q6 homolog, monooxygenase (Saccharomyces cerevisiae)
WDR89	112840	NM_001008726	WD repeat domain 89
PXMP3	5828	NM_000318	Peroxisomal membrane protein 3, 35kDa
BAZ2A	11176	NM_013449	Bromo domain adjacent to zinc-finger domain, 2A
GPRASP2	114928	NM_001004051	G-protein-coupled receptor associated sorting protein 2
MRPL40	64976	NM_003776	Mitochondrial ribosomal protein L40
LOC100132969	100132969	NM_032508	Hypothetical protein LOC100132969/transmembrane protein 185A
RAB21	23011	NM_014999	RAB21, member RAS oncogene family
PDHX	8050	NM_001135024	Pyruvate dehydrogenase complex, component X
TCEA2	6919	NM_003195	Transcription elongation factor A (SII), 2
ARG2	384	NM_001172	Arginase, type II
LAMP2	3920	NM_001122606	Lysosomal-associated membrane protein 2
SOX3	6658	NM_005634	SRY (sex-determining region Y)-box 3
MCFD2	90411	NM_139279	Multiple coagulation factor deficiency 2
CTPS2	56474	NM_019857	CTP synthase II
CFDP1	10428	NM_006324	Craniofacial development protein 1
PTGS2	5743	NM_000963	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
BECN1	8678	NM_003766	Beclin 1, autophagy related
ZBTB47	92999	NM_145166	Zinc-finger and (for BR-C, ttk and bab) domain containing 47
PLA2G12A	81579	NM_030821	Phospholipase A2, group XIIA
FKBP1B	2281	NM_004116	FK506-binding protein 1B, 12.6 kDa
CTF8	54921	NM_001039690	Chromosome transmission fidelity factor 8 homolog (S. cerevisiae)
GSTT1	2952	NM_000853	Glutathione S-transferase $\theta$ 1
ERC1	23085	NM_015064	ELKS/RAB6-interacting/CAST family member 1
EPHX2	2053	NM_001979	Epoxide hydrolase 2, cytoplasmic
SEC31A	22872	NM_001077206	Protein-transport protein SEC31 variant mRNA, partial coding sequences; alternatively spliced
ISYNA1	51477	NM_016368	Inositol-3-phosphate synthase 1

Vaidya, 2011). Most nephrotoxicants including gentamicin also affect the S3 segments of the proximal tubule owing to the accumulation and metabolism that occurs in this region (Lash and Cummings, 2010).

Among the 349 miRNAs that were probed following I/R injury, 204 miRNAs met the cut-off in Ct values of < 35 out of which we found 16 that were significantly upregulated > 2-fold as compared with the average of sham over all three time points. Of the 16, we picked miR-21, -155, and -18a on the basis of what was known about their function and relevance to kidney injury from literature. Several groups have reported the regulation of miR-21 following both acute as well as chronic models of kidney injury (Chau et al., 2012; Shapiro et al., 2011). MiR-21 is known to regulate several processes in the kidney that are essential to and mediate I/R injury such as inflammation (Lindsay, 2008), apoptosis, proliferation (Godwin et al., 2010), and fibrosis (Zarjou et al., 2011). Studies have shown miR-21 to have antiapoptotic effects and promote tumorigenesis in several cancers (Asangani et al., 2008; Chan et al., 2005; Shibuya et al., 2010), whereas in the kidney, miR-21 is thought to promote proliferative responses by upregulating the survival factor Bcl-2 and downregulating the tumor suppressor gene-programmed cell death 4 (Godwin et al., 2010). Moreover, miR-21 knockouts have been shown recently to have significantly lesser renal interstitial fibrosis (Chau et al., 2012). Taking into account the established role miR-21 has to play in the kidney, we were interested in exploring the pattern of expression in circulation and excretion and any correlation it might have to the tissue expression.

The role of miR-155 has been well documented in the regulation of the inflammatory response in models of experimental autoimmune encephalomyelitis (Murugaiyan et al., 2011), graft versus host disease (Ranganathan et al., 2012), alcoholic liver disease (Bala et al., 2011), and rheumatoid arthritis (Leng et al., 2011). Considering the fact that I/R injury has a significant involvement of inflammatory components (Bonventre and Zuk, 2004) in its pathogenesis and that the contribution of miR-155 to it has not been studied in detail, we included miR-155 as a candidate in our studies. Because of the fact that we see an enormous upregulation in the cortex (~6-fold; Fig. 2A), we hypothesize that miR-155 may be produced endogenously by the various subtypes of kidney cells in response to the stimulation of inflammatory cytokines like Tumor Necrosis Factor-alpha (TNF- $\alpha$ ), Interleukin-6 (IL-6), Interleukin-1 $\beta$  (IL-1 $\beta$ ), and Transforming Growth Factor- $\beta$  (TGF- $\beta$ ). Additionally, it has been reported that miR-155 is expressed in a wide range of immune cells upon activation or maturation, such as macrophages (O'Connell et al., 2007), dendritic cells (Martinez-Nunez et al., 2009), and T cells (Rodriguez et al., 2007).

Surprisingly, little is known about miR-18a, and no previous work has been done with regard to its involvement with the kidney. MiR-18a is thought to impair DNA damage repair through regulation of ataxia-telangiectasia mutated kinase in breast cancer models (Song *et al.*, 2011). Given that there was significant upregulation in the kidney following I/R injury (> 3.5-fold in all three time points for cortex and medulla), we wanted to further characterize its expression and excretion profile following kidney damage.

Though there have been innumerable reports of detection of miRNAs in serum or plasma in a variety of cardiovascular diseases (Creemers et al., 2012), much work remains to be done regarding circulating miRNAs in kidney disease. Our studies showing a decline in miRNA levels in blood are consistent with other reports of lowered circulating levels in plasma. Neal et al. (2011) showed a decrease of total and specific miRNA levels in plasma samples of patients suffering from severe CKD, whereas Lorenzen et al. (2011) reported a dysregulation in plasma levels of 13 miRNAs out of which 5 were induced and 8 were repressed in patients with AKI. However, these studies examined human miRNA levels only in extracellular fluid and did not have a correlation of circulating levels with miRNA expressed in the diseased tissue. The molecular mechanisms underlying the dysregulation of circulating miRNAs have not been addressed in this study, which makes it impossible to discern their origin. Based on our observations of contrasting expressions in tissue and blood, it is possible to broadly speculate that lowered circulating levels could indicate alterations in the degradation of miRNAs in circulation because of the increased amounts of RNA degrading enzymes in circulation following injury, as has been suggested before (Neal et al., 2011; Weickmann et al., 1984).

The most important finding of the human study was that soluble miRNAs could be isolated from as little as 300 µL of urine supernatant and still be present in detectable levels. The raw Ct values from both rats and humans indicate that there is an appreciable amount of soluble miRNAs in the urine supernatant, which makes the idea of using miRNAs as biomarkers of kidney injury quite feasible and attractive. MiRNAs, unlike proteins or mRNAs, are extremely stable and resistant to degradation in clinical plasma and urine samples. Studies have shown the stability of circulating miRNAs in the plasma (Mitchell et al., 2008), most commonly explained by their packaging into exosomes or associations with RNA-binding molecules. The idea that circulating miRNAs in plasma can be used as biomarkers has been present for a while now, and several groups have reported the use of plasma miRNAs as sensitive and specific indicators of various cancers (Mitchell et al., 2008), cardiovascular diseases (Creemers et al., 2012), and liver, muscle, and brain damage (Laterza et al., 2009). However, the domain of excreted urinary miRNAs is just beginning to be explored and already they fulfill several requisites of good biomarkers; nonetheless, further investigation and qualification are necessary before they can be preclinically and clinically applicable.

One of the challenges of using miRNAs as biomarkers is the lack of an established normalization control that remains consistent across disease conditions as well as tissue types. The battery of usual normalization controls used in tissue was either undetectable or largely variant in each sample. A spiked in exogenous control is a good way to normalize for extraction variability and is thus a good technical control, but it does not in any way account for biological inter-individual variability as a biological control. A comprehensive analysis of normalization methods proved that spiked in controls did not improve the inter-assay variability (McDonald et al., 2011). Traditionally, urinary creatinine is used to normalize for the dilution state of samples for proteins, but we feel that it does not perform as well for miRNA analyses mainly because the technique of isolation and detection is not as volume dependent as it is in protein studies. The RNA isolation process that we used was carried out with equal volumes of urine sample (300µL); also, equal amounts of the extracted total RNA (10 ng) was converted to complementary DNA and used to run the PCR, thus eliminating the influence of urinary volume differences between samples.

The most commonly used tools of *in silico* miRNA target prediction such as TargetScan, which work on the basis of sequence-based prediction to pair miRNA with target genes, are often challenging because even imprecise complementarity of miRNA to the target sequence can lead to gene regulation, (Ambros, 2004) opening up a very large pool of potential targets, many of which have little functional relevance. To avoid this problem, we used a CLR algorithm that infers regulatory interactions based on established gene expression profiles. The 29 mRNA core targets of the three miRNAs are highly enriched for genes associated with apoptosis or cell proliferation, and those mRNAs may play a role as direct or indirect mediators of the miRNAs in kidney I/R injury and regeneration.

In summary, our study characterizes the expression, circulatory, and excretory profile of miR-21, -155, and -18a following kidney damage and identifies miR-21 and -155 as potential translational, urinary biomarkers for detection of kidney injury. The regulatory role of these miRNAs alone, or in combination, in the pathogenesis of kidney injury remains to be investigated.

### SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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### NOTE

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