MicroRNAs and drug-induced kidney injury

Mira Pavkovic a, b, Vishal S. Vaidya a, b, c, * 

a Laboratory of Systems Pharmacology, Harvard Medical School, Boston, MA, United States 
b Department of Medicine, Renal Division, Brigham and Women’s Hospital, Boston, MA, United States 
c Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, United States

Abstract

Drug-induced kidney injury (DIKI) is a severe complication in hospitalized patients associated with higher probabilities of developing progressive chronic kidney disease or end-stage renal diseases. Furthermore, DIKI is a problem during preclinical and clinical phases of drug development leading to high rates of project terminations. Understanding the molecular perturbations caused by DIKI would pave the way for a new class of therapeutics to mitigate the damage. Yet, another approach to ameliorate DIKI is identifying sensitive and specific translational biomarkers that outperform the current diagnostic analytes like serum creatinine and facilitate early diagnosis. MicroRNAs (miRNAs), a class of non-coding RNAs, are increasingly being recognized to have a two-pronged approach toward DIKI management: 1) miRNAs have a regulatory role in gene expression and signaling pathways thereby making them novel interventional targets and 2) miRNAs enable diagnosis and prognosis of DIKI because of their stable presence in biofluids. In this review, apart from summarizing the literature on miRNAs in DIKI, we report small RNA sequencing results showing miRNA expression profiles at baseline in normal kidney samples from mice and humans. Additionally, we also compared the miRNA expression in biopsies of normal human kidneys to patients with acute tubular necrosis, and found 76 miRNAs significantly downregulated and 47 miRNAs upregulated (FDR adjusted p < 0.05, +/− 2-fold change).

In summary, we highlight the transformative potential of miRNAs in therapeutics and translational medicine with a focus on drug-induced kidney damage.

© 2016 Elsevier Inc. All rights reserved.

Keywords: MicroRNA, Kidney, Kidney toxicity, Acute kidney injury, Biomarker, Therapeutic targets

1. Introduction

1.1. Drug-induced kidney injury

The high susceptibility of the kidney to toxicity is mainly due to its function of eliminating endogenous waste products as well as xenobiotics. These substances can induce toxic responses due to a high local concentration and/or transformation into reactive metabolites (Kahl et al., 2010). Commonly prescribed drugs (Table 1) are known to cause acute kidney injury (AKI) that is a severe condition associated with high probabilities of developing progressive chronic kidney disease.
evolutionary conserved small RNAs. MiRNAs were first discovered in 1993 (Lee et al., 1993; Wightman et al., 1993) followed by the recognition of their conservation in a wide range of species (Pasquinelli et al., 2000), leading to the current status of 788 known miRNAs in rats, 1899 in mice and 2585 in humans (miRBase, 2014).

In the cell, miRNAs regulate gene expression at the post-transcriptional level. As part of a ribonucleoprotein complex called miRISC (miRNA-induced silencing complex) they bind to complementary sequences in the 3′-untranslated regions of target mRNAs thus inhibiting miRNA translation. The process of miRNA maturation, miRISC incorporation and subsequent mRNA binding is relatively well explored and reviewed in detail in several review articles (Krol et al., 2010; Garcia-Lopez et al., 2013; Desvignes et al., 2015). The complementarity between miRNA and mRNA does not have to be perfect for translational inhibition, therefore one miRNA regulates several hundred mRNAs and likewise, one mRNA is regulated by several miRNAs (Filipowicz et al., 2001).

Kidney injury in humans is measured using functional biomarkers like blood urea nitrogen and/or serum creatinine. Although these biomarkers are considered to be the standard diagnostic analytes in routine care, they are known to be modified by nutrition, muscle mass, age, sex, muscle injury, and aggressive fluid resuscitation (Waikar et al., 2012). Furthermore, they increase only when the glomerular filtration rate decreases by more than 50% and they do not reflect dynamic changes in filtration rates (Uchino, 2010). Novel sensitive and specific biomarkers are urgently needed to provide for cost-effective and non-invasive methods of detecting and treating early stage kidney injury. Early diagnostic and predictive biomarkers would also allow for stratification of patients that may be susceptible to develop AKI thereby facilitating clinical trials. Currently, in the absence of any therapeutics for AKI, renal replacement therapy remains the only option (Bellomo et al., 2015) for severe AKI, leading to an indispensable need for improved kidney injury management, i.e. detection as well as improved therapy.

In the last two decades, due to significant advances in understanding the molecular pathogenesis of AKI using state-of-the-art genome sequencing technologies, microRNAs (miRNAs) have emerged as novel therapeutic targets as well as biomarker candidates for AKI.

### 1.2. MicroRNA biogenesis, function and extracellular features

MiRNAs are approximately 20–25 nucleotides long, non-coding and evolutionarily conserved small RNAs. MiRNAs were first discovered in Caenorhabditis elegans (Lee et al., 1993; Wightman et al., 1993) followed by the recognition of their conservation in a wide range of species (Pasquinelli et al., 2000), leading to the current status of 788 known miRNAs in rats, 1899 in mice and 2585 in humans (miRBase, 2014).

In the cell, miRNAs regulate gene expression at the post-transcriptional level. As part of a ribonucleoprotein complex called miRISC (miRNA-induced silencing complex) they bind to complementary sequences in the 3′-untranslated regions of target mRNAs thus inhibiting miRNA translation. The process of miRNA maturation, miRISC incorporation and subsequent mRNA binding is relatively well explored and reviewed in detail in several review articles (Krol et al., 2010; Garcia-Lopez et al., 2013; Desvignes et al., 2015). The complementarity between miRNA and mRNA does not have to be perfect for translational inhibition, therefore one miRNA regulates several hundred mRNAs and likewise, one mRNA is regulated by several miRNAs (Filipowicz et al., 2008). In fact, it is estimated that over 50% of all protein-coding genes are regulated by miRNAs in mammals (Krol et al., 2010) revealing their overall involvement in diverse physiological as well as pathological processes (Wiemer, 2007; Visone & Croce, 2009; Wang & Lee, 2009; Ceman & Saugstad, 2011; T. Li et al., 2011; Szabo & Bala, 2013). Many miRNAs are found to be highly enriched in particular organs or at a particular stage of development or disease progression in human body (Landgraf et al., 2007; Kriel et al., 2013)—for instance the liver specific miR-122 (Lagos-Quintana et al., 2002), kidney cortex enriched miR-192 (Tian et al., 2008), skeletal muscle enriched miR-133a and -b (Sempere et al., 2004), or the cardiomyocyte specific miR-208a (van Rooij et al., 2007). The expression of the miR-17~92 cluster, consisting of miR-17, -18a, -19a, -20a, -19b1 and -92a1, seems to be essential for normal nephrogenesis since ablation of the cluster in a mouse model resulted in reduced numbers of nephrons (Marrone et al., 2014). Furthermore, miR-21 and miR-150 were found highly enriched in kidney cysts of patients with polycystic kidney disease and kidney biopsies from patients with lupus nephritis, respectively (Zhou et al., 2013; Lakhia et al., 2015).

Outside the cell, miRNAs were discovered for the first time in serum/plasma from cancer patients (Chen et al., 2008; Mitchell et al., 2008) and afterward in other body fluids like urine, breast milk, saliva and cerebral fluid (Weber et al., 2010). Extracellular miRNAs are very stable and
resistant to degradation even with long-time storage at room temperature, pH variability and multiple freeze–thaw cycles (Mitchell et al., 2008; Mráz et al., 2009; McDonald et al., 2011; Y. Li et al., 2011). Their stability is probably due to an association with RNA-binding proteins or being packed into vesicles (K. Wang et al., 2010; Arroyo et al., 2011; Vickers et al., 2011; Vickers & Remaley, 2012; Xu et al., 2013). The function of extracellular miRNAs is not yet understood and some studies suggest that they might be active signaling molecules (Zhang et al., 2010; Melo et al., 2014). However, unique features of extracellular miRNAs have made them promising biomarker candidates. Consequently, due to their intracellular function and their stable presence in biofluids, miRNAs could significantly contribute to improved kidney injury management as both potential biomarkers and interventional targets (Fig. 1).

2. Mechanistic role of microRNAs in drug-induced kidney injury

2.1. MicroRNA expression in the kidney

By functioning as regulators of gene expression miRNAs play a crucial role in a variety of molecular processes in multiple organs including the kidney. To gain insight into the kidney miRNA expression at baseline, we conducted small RNA sequencing in normal human and mouse kidney samples. After ranking the normalized read counts, the top expressed miRNAs were compared between human and mouse kidneys (Fig. 2A). 60% of the top 20 miRNAs were overlapping in human and mouse kidney, of which for example miR-10b was in the top five of both. This confirms the high conservation between species and leads to the assumption that these kidney-enriched miRNAs are involved in normal kidney homeostasis. Less is known about miR-10b in kidney physiology, but deregulation was reported in the context of clear cell renal cell carcinoma and acute allograft rejection (Fritz et al., 2014; X. Liu et al., 2015). However, the role of other kidney-enriched miRNAs like the miR-192 or the miR-30 family are better elucidated. MiR-192 targets the p53 subunit of the Na+/K+ ATPase and by inhibiting the expression of this subunit it negatively affects the enzyme activity thereby contributing to renal handling of fluid balance, whereas the miR-30 family is involved in the nephron development and glomerular integrity (Agrawal et al., 2009; Mladinov et al., 2013; Wu et al., 2014).

Overall, several studies have demonstrated miRNA involvement in kidney physiology and pathophysiology (Bhatt et al., 2016). When we conducted small RNA sequencing comparing the miRNA expression in biopsies of normal human kidneys to patients with acute tubular necrosis (ATN), we found 76 miRNAs significantly downregulated and 47 miRNAs upregulated (FDR adjusted p < 0.05, +/−2-fold change; Fig. 2B and Table 2). Using target prediction software, the miRNA expression profile revealed affected pathways in the ATN as well as associated kidney disease states that highlight the importance of miRNA analysis for understanding the pathogenesis of DIKI.

In line with these findings as well as based on the results from other disciplines, miRNAs have emerged as promising therapeutic targets either by restoring or more commonly by inhibiting their function with synthetic miRNA mimics and anti-miRs, respectively.

Wei et al. (2010) showed for the first time the essential involvement of miRNAs in AKI by using a proximal tubule specific Dicer knockout (a miRNA processing enzyme). They reported a differential miRNA expression profile at baseline (80% and 16% of miRNAs were decreased and increased, respectively) due to the knockout which resulted in a significant protection against ischemic AKI (Wei et al., 2010). The majority of published studies have focused on understanding the regulation of miRNAs during ischemia/reperfusion (I/R)-induced AKI. For example, miR-24 was shown to be increased in mouse kidneys after I/R injury in human allograft biopsies as well as in primary human proximal tubule cells after anoxia/hypoxia (Lorenzen et al., 2014). Further, heme oxygenase 1 and H2A histone family, member X were verified as miR-24 targets and silencing miR-24 resulted in decreased injury in cells and in mouse kidneys probably via apoptosis inhibition. Similarly, miR-687 was found to be up-regulated in mouse kidneys after ischemic injury and again anti-miR-687 treatment led to significantly less injury (Bhatt et al., 2015). PTEN was identified as a direct target and thus the blocking of miR-687 preserved the PTEN expression and attenuated cell cycle activation and apoptosis.

The potential use of miRNAs for treating kidney injury is an extremely exciting area of ongoing investigation and since there are distinct as well as common mechanisms between different kinds of AKI, the results for I/R injury also provide new insights and innovative targets for DIKI.

2.2. MicroRNAs in kidney toxicity

The involvement of specific miRNAs in DIKI is overall less well explored. The currently known miRNAs with the respective DIKI models are summarized in Table 3. One of the earliest reports showed miR-34a, a p53 target, to be increased in mouse proximal tubular epithelial cells after cisplatin treatment (Bhatt et al., 2010). Silencing of miR-34a increased cisplatin toxicity leading to the conclusion that miR-34a has a protective role during kidney injury. Although miR-34a was also increased in an I/R mouse model, its inhibition was associated with decreased autophagy and thus aggravated injury (X. J. Liu et al., 2015). However, miR-155 was shown to be significantly upregulated in rats with kidney injury either induced by gentamicin administration or following I/R and, subsequently, miR-155 KO-mice exhibited significantly enhanced kidney toxicity in response to cisplatin administration (Saikumar et al., 2012; Pellegrini et al., 2014). This finding suggests not only a protective role of miR-155 during DIKI but also an involvement in a common mechanism between I/R and drug-induced AKI. A comparable protective effect was also seen for miR-125b, which was increased in vivo and in vitro models of cisplatin-induced kidney injury and was suggested to be part of the Nrf2 pathway (Joo et al., 2013).

There are some published reports that show the contribution of miRNA deregulation in the onset and progression of injury. Treating the immortalized human proximal tubule epithelial cell line HK-2 with cisplatin increased the expression of miR-181a which subsequently inhibited its known target BCL-2 resulting in apoptosis (Chen et al., 2010; Zhu et al., 2012) and correspondingly less apoptosis was detected when cells were treated with anti-miR-181a. In mouse kidneys and in a rat kidney cell line, miR-122 was decreased after cisplatin, gentamicin and doxorubicin treatment (Lee et al., 2014). Since FOXO3 is a verified target of miR-122, FOXO3 stimulated downstream activation of p53 in the absence of miR-122 and resulted in progression of apoptosis and kidney injury. Similar findings were observed in a mouse toxicity model with doxorubicin where miR-133a was increased which in turn was found in other models to directly inhibit the multidrug resistance-associated protein 2 (MRP2; Loeser et al., 2015). MRP2 is one of the numerous
transporters in proximal tubule epithelial cells located on the apical membrane and is known to be decreased in injured proximal tubules and thereby further augmenting kidney toxicity (Wen et al., 2014).

Besides the kidney toxicity of cytostatic drugs including cisplatin or doxorubicin and aminoglycoside antibiotics like gentamicin, miRNAs have been studied in cyclosporine A toxicity. Cyclosporine A is an immunosuppressive agent commonly given to transplant or autoimmune disease patients but it is known for its long-term kidney toxicity characterized by severe renal tubulointerstitial fibrosis. Treating human proximal tubular epithelial cells in vitro with cyclosporine A deregulated 46 miRNAs (Chen et al., 2015). One of the few increased miRNAs was miR-21 (~5.5-fold), which is widely explored in the context of kidney disease and injury (Li et al., 2013; Zhou & Jiang, 2014). In the cyclosporine A model, miR-21 up-regulation was associated with AKT activation, PTEN decrease and the increase of several markers of epithelial–mesenchymal transition (EMT) including vimentin and α-smooth muscle actin. Epithelial to mesenchymal transition has been shown to play an important role in kidney fibrosis (Lovisa et al., 2015) and also in the context of cyclosporine A toxicity (Slattery et al., 2005). These results correspond with a therapeutic study on kidney fibrosis, demonstrating that the inhibition of miR-21 was protective against TGF-β-induced fibrogenesis in a mouse model of Alport nephropathy (Gomez et al., 2015). Further, Yuan et al. (2015) found another miRNA, miR-494, to be involved in cyclosporine A-induced EMT (Yuan et al., 2015). MiR-494 was increased approximately 2-fold in
Again a PTEN decrease was observed (~4-fold), which was identified in mouse kidneys as well as in HK-2 cells after cyclosporine A treatment. A direct target of miR-494. Counteracting a PTEN inhibition by using anti-miR-494 prevented cyclosporine A induced EMT. In summary, the further exploration of the miRNA role in the pathogenesis of DlKI could lead to the development of miRNA-based therapeutics and this seems promising based on the current findings and results seen in other related disciplines.

3. MicroRNAs as biomarkers for drug-induced kidney injury

3.1. Biomarker candidates

With the 21st century advances in omics technologies, biomarker science has emerged as a very exciting multidisciplinary approach to understand and classify disease pathogenesis. However, the biomarker science pipeline that involves carrying the biomarker from discovery to confirmation, evaluation, qualification and validation steps requires a significant commitment of resources and time (Fig. 3). Based on the platform of choice for a biomarker discovery effort, starting with >10,000 candidates, one could identify a handful of successfully qualified and validated biomarkers that enables improved patient care as well as a more efficient drug development. Over a decade ago, this path was formed and successfully taken, culminating in 2008 with a scientific drug development. Over a decade ago, this path was formed and successfully taken, culminating in 2008 with a scientific drug development. Over a decade ago, this path was formed and successfully taken, culminating in 2008 with a scientific...
features miRNAs have shown great promise as non-invasive biomarkers. They were detected in almost all body fluids including the clinically most relevant blood and urine (Weber et al., 2010). Although high concentrations of RNA degrading enzymes are present in the extracellular space, miRNAs are found to be remarkably stable (McDonald et al., 2011), which is probably due to their packing into microvesicles and exosomes or their association with proteins and high-density lipoproteins (Arroyo et al., 2011; Turchinovich, Weiz, Langheinz, & Burwinkel, 2011; Hoy & Buck, 2012). Furthermore, miRNAs not only show organ specificity many times but are also highly conserved in sequence across species (Sun et al., 2004; Landgraf et al., 2007). Commonly, they are measured by real-time PCR which is a sensitive and well-established method for nucleic acids. To date, several hundred studies have evaluated the potential of miRNAs as biomarkers for various pathological conditions including cancers, cardiovascular and neurodegenerative diseases. For circulating miRNAs i.e. miRNAs from blood, only, 35 different clinical studies are currently registered assessing their performance as biomarkers for human diseases (clinicaltrials.gov, 2015); several of these are also associated with kidney diseases like autosomal dominant polycystic kidney disease, renal cell carcinoma and AKI after cardiac surgery.

In terms of kidney toxicity biomarkers, the work with miRNAs has thus far been focused on urine as it is non-invasive, directly derived from kidneys and has been shown to contain miRNAs. Our laboratory was among the first few laboratories to demonstrate the isolation of miRNAs from urinary supernatants and showed the differential expression of various miRNAs in healthy and diseased kidneys.

Table 3

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>DIKI models</th>
<th>In vitro models</th>
<th>In vivo models</th>
<th>Expression</th>
<th>Upstream regulator</th>
<th>Targets</th>
<th>Proposed effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-122</td>
<td>Cisplatin, gentamicin, doxorubicin</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Decrease</td>
<td>FOXO3</td>
<td>p53 activation</td>
<td>(Lee et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>miR-124</td>
<td>Cyclosporin A</td>
<td>HPTEC</td>
<td>Mouse</td>
<td>Increase</td>
<td>ET-1/ET-B receptor</td>
<td>MRP2</td>
<td>Increasing injury</td>
<td>(Chen et al., 2015; Loeser et al., 2015)</td>
</tr>
<tr>
<td>miR-133a</td>
<td>Doxorubicin</td>
<td>HPTEC</td>
<td>Mouse, human kidney biopsies</td>
<td>Increase</td>
<td>c-Fos</td>
<td>(Pellegrini et al., 2015; Saikumar et al., 2012; Pellegrini et al., 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-146b</td>
<td>Cisplatin</td>
<td>Mouse</td>
<td>Mouse, rat</td>
<td>Decrease</td>
<td></td>
<td></td>
<td>(Zhu et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>miR-155</td>
<td>Cisplatin, gentamicin</td>
<td>HPTEC</td>
<td>Mouse</td>
<td>Increase</td>
<td></td>
<td></td>
<td>(Chen et al., 2015; Saikumar et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>miR-181a</td>
<td>Cisplatin</td>
<td>HK-2</td>
<td>Mouse</td>
<td>Increase</td>
<td></td>
<td></td>
<td>(Jia et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>miR-143</td>
<td>Cisplatin</td>
<td>HPTEC</td>
<td>Mouse</td>
<td>Increase</td>
<td></td>
<td></td>
<td>(Chen et al., 2015; Saikumar et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>miR-21</td>
<td>Cyclosporin A</td>
<td>HPTEC</td>
<td>Mouse</td>
<td>Increase</td>
<td></td>
<td></td>
<td>(Sun et al., 2004; Landgraf et al., 2007; Bhatt et al., 2010; Pavkovic et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>miR-34a</td>
<td>Cisplatin</td>
<td>BUMPT-306</td>
<td>Mouse</td>
<td>Increase</td>
<td>p53</td>
<td>Cytoprotection</td>
<td>(Pellegrini et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>miR-494</td>
<td>Cisplatin, gentamicin, doxorubicin</td>
<td>HK-2</td>
<td>Mouse</td>
<td>Increase</td>
<td>PTEN</td>
<td>EMT</td>
<td>(Yuan et al., 2015)</td>
<td></td>
</tr>
</tbody>
</table>

EMT, epithelial–mesenchymal transition.

*In the context of DIKI.

b No direct interaction shown in this study.

c Kidney injury model but not DIKI.

Fig. 3. Biomarker development pipeline. The overview is based on estimations and the experiences gained during the Critical Path’s Predictive Safety Testing Consortium’s work on protein biomarkers for kidney injury. *Dependent on platforms used as well as type of biospecimen.
expression of miR-21 and miR-155 in the urines of rats with AKI or gentamicin-induced AKI (Saikumar et al., 2012). These results were confirmed by a precompetitive consortium of pharmaceutical industries, ILSI Health and Environmental Sciences Institute. In spite of using a different cisplatin dose, fasting/feeding conditions and different rat strains, approximately 20 miRNA candidates were found to be the same in both studies (Kanki et al., 2014; Pavkovic et al., 2014). Increases of specific miRNAs were also measured in urine from rats treated with gentamicin or doxorubicin (Church et al., 2014; Nassirpour et al., 2014). All miRNA levels correlated with histopathological changes as well as the qualified protein biomarker Kim-1 and were increased before serum creatinine and blood urea nitrogen. Almost all these studies were conducted in rats, (Table 4), but for example, miR-21 was also found in urine from human AKI patients from the intensive care unit (Ramachandran et al., 2013) as well as in patients with acetaminophen or cisplatin induced AKI (Pavkovic et al., 2015b) thereby strengthening the translational potential of miRNAs in DIKI settings. A recent study evaluated miRNAs in plasma as biomarkers for contrast-induced kidney injury (Gutierrez-Escolano et al., 2015). First, the miR-30 family (miR-30a, -c, and -e) was found to be increased in rat plasma after the administration of a contrast agent and these results were validated in a patient cohort where especially miR-30a performed very well in differentiating patients with contrast-induced nephropathy from those without. Even though the number and diversity of miRNA biomarker studies specifically for DIKI are small, the data is promising and the results from other kidney diseases including immunoglobulin A nephropathy, ischemic AKI, glomerulonephritis or focal segmental glomerulosclerosis (Wang et al., 2011; Ichii et al., 2014; Wang et al., 2014; Zhang et al., 2014; Pavkovic et al., 2015a) support the value of further exploration.

In addition, extracellular miRNA biomarkers could have more advantages in drug development. Since they are conserved and ubiquitously expressed, they could not only be implemented in preclinical and clinical studies but also be implemented in vitro studies for screening of compounds with potential toxic effects. The presence of miRNAs in cell culture medium was mostly demonstrated while exploring their potential role in inter-cellular communication (McDonald et al., 2013; Rani, 2014; Zhou et al., 2014). However, assuming that miRNAs can be actively as well as passively released from injured kidney cells, cell culture media of treated and untreated cells could have different miRNA profiles. We had previously found miR-21, -200c and -423 to be increased in patients with AKI and here we show that when primary human proximal tubular epithelial cells are treated with the contrast agent sodium diatrizoate all three miRNAs, miR-21, -200c and -423 significantly increased in medium of cells (Fig. 4). Furthermore, due to their intracellular function, extracellular miRNAs could mirror the events taking place in the injured kidney, thus being indicative not only of the injury itself but also of the affected pathways. For instance, cisplatin induces miR-34a expression in rat kidneys which itself is involved in the p53-mediated apoptosis pathway and increased miR-34a levels were also measured in urine of the same cisplatin treated rats (Pavkovic et al., 2014).

All findings and hypotheses around miRNA biomarkers for kidney toxicity seem promising and valuable; nonetheless more replication and validation of the biomarkers are needed in large multi-centered cohorts to confirm the reproducibility and performance.

### 3.2. Challenges of microRNAs as biomarkers

Despite all the promising results with miRNA biomarkers it is worth mentioning that the field is facing several basic challenges that hinder a smooth process of biomarker qualification and validation.

One of the biggest challenges in clinical miRNA biomarker studies is the comparison with serum creatinine (Scr) as a gold standard definition for acute kidney injury. Although in preclinical studies renal histopathological examination is the gold standard for DIKI diagnosis, in clinical assessments Scr remains widely used. In fact, moderate performances of new AKI biomarker candidates are frequently seen in clinical studies, where AKI is mostly defined based on increased Scr levels (Pavkovic et al., 2015b). A potential solution for DIKI studies

### Table 4

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>DIKI (model)</th>
<th>Species</th>
<th>Biofluid</th>
<th>Direction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7g</td>
<td>cisplatin</td>
<td>Rat</td>
<td>Urine</td>
<td>Up</td>
<td>(Kanki et al., 2014)</td>
</tr>
<tr>
<td>miR-15</td>
<td>cisplatin</td>
<td>Rat</td>
<td>Urine</td>
<td>Up</td>
<td>(Pavkovic et al., 2014)</td>
</tr>
<tr>
<td>miR-21</td>
<td>cisplatin, acetaminophen</td>
<td>Patients</td>
<td>Urine</td>
<td>Up</td>
<td>(Pavkovic et al., 2015)</td>
</tr>
<tr>
<td>miR-203a, let-7d</td>
<td>gentamicin</td>
<td>Rat</td>
<td>Urine</td>
<td>Down</td>
<td>(Nassirpour et al., 2014)</td>
</tr>
<tr>
<td>miR-21, miR-155</td>
<td>gentamicin</td>
<td>Rat</td>
<td>Urine</td>
<td>Down</td>
<td>(Saikumar et al., 2012)</td>
</tr>
<tr>
<td>miR-30a,c,e</td>
<td>Contrast agent*</td>
<td>Rat/patients</td>
<td>Plasma</td>
<td>Up</td>
<td>(Gutierrez-Escolano et al., 2015)</td>
</tr>
<tr>
<td>miR-320</td>
<td>gentamicin</td>
<td>Rat</td>
<td>Urine</td>
<td>Up</td>
<td>(Nassirpour et al., 2014)</td>
</tr>
<tr>
<td>miR-34c</td>
<td>doxorubicin</td>
<td>Rat</td>
<td>Urine</td>
<td>Up</td>
<td>(Church et al., 2014)</td>
</tr>
</tbody>
</table>

* Iohexol + furosemide + indomethacin were used for rats.

**Fig. 4.** MicroRNAs as biomarkers for in vitro toxicity testing. Normal cells have been shown to release miRNA-containing vesicles. Likewise, injured cells also release miRNAs actively as well as passively (apoptosis/necrosis) which then can be measured in different biofluids. In in vitro studies, this would correspond to miRNA profiles in the supernatant of untreated and treated cultured cells. Differential levels could enable an evaluation of these extracellular miRNAs as biomarker candidates for screening of potential kidney toxic agents. To test this hypothesis, primary human proximal tubular epithelial cells (HTPECs) were cultured and treated with the contrast agent sodium diatrizoate (200 mM) for 24 h. Total RNA was isolated from 200 μl medium and miR-21, -200c and -423 were measured by qRT-PCR as these miRNAs have been reported to increase following kidney injury in human urines (Ramachandran et al., 2013). All three miRNAs were found to be significantly increased in the medium of treated HTPECs as compared to untreated cells. Data is presented as mean fold changes with standard deviation (n = 4) relative to the untreated cells (2−ΔΔCt with internal reference). 1-way ANOVA with Dunnett’s test was used for p-value calculation: *p < 0.01 and ***p < 0.001.
could be to use the treatment with the nephrotoxic drug per se for comparison.

Yet, another challenge is the lack of standardization not only in miRNA isolation and measurement approaches but also in sample collection, handling and storage conditions. The most common methods of miRNA isolation are phenol/chloroform-based techniques including silica column purification, but these have also been shown to vary greatly depending on the vendor (El-Khoury et al., 2016; Martinez-Fernandez et al., 2016). In terms of miRNA measurement, qRT-PCR is widely used especially from biospecimens having overall low RNA yields like serum or urine. The issue here is the absence of generally agreed endogenous control miRNAs. The reported normalization strategies differ greatly, including normalization with 1) synthetic miRNAs that were spiked in during RNA isolation (J. F. Wang et al., 2010; Argyropoulos et al., 2013), 2) small RNAs like RNU48 or U6 (G. Wang et al., 2012; N. Wang et al., 2012) or 3) invariant miRNAs identified within the specific study (Hanke et al., 2010; Yang et al., 2012). All three approaches have disadvantages: normalization to spike-ins does not account for a true biological variability but rather accounts for a technical variation; normalizing to small RNAs could bias the results because the small RNA itself could have been differentially expressed during injury or the isolation and transcription efficiency is different for small RNAs vs. miRNAs (e.g. U6 ~106 nucleotides vs. miRNAs 20–25 nucleotides); finally the use of invariant miRNAs for normalization, although popular, seems not to be universal and only limited to specific studies or even specific datasets. Furthermore, urine as a biospecimen has its own challenges regarding normalization, which is necessary to account for variations in urine flow rate/concentrations due to hydration or diuresis. Yet, urine is very interesting since it is directly associated with the kidneys and thereby serves as a relevant and most-proximal non-invasive matrix to perform miRNA biomarker discovery for AKI. Normalizing biomarker levels with urinary creatinine as is usually done for protein biomarkers was suggested in a recent rat study (Pavkovic et al., 2014). This approach, however, has several limitations due to potential changes of urinary creatinine induced by factors like variations in diurnal production, physical activity, diet, and muscle mass (Greenblatt et al., 1976; Heymsfield et al., 1983; Waiker et al., 2010). Furthermore, simulations on creatinine kinetics revealed that protein biomarker performance is actually affected by this way of normalization (Waiker et al., 2010) which could also be the case for miRNAs.

In general the harmonization of miRNA biomarker evaluation is important to enable the establishment of reference ranges accounting for potential differences in strain, species, fasting/feeding conditions in pre-clinical studies and age, sex, ethnicity, and comorbid conditions in clinical studies. The expression analyses in kidneys during the life span of F344 rats have shown that miRNA levels were sex and age dependent (Kwek et al., 2015), therefore also miRNA levels in biofluids could be affected by these factors. Thus, a systematic effort toward standardization could accelerate qualification of these miRNAs and improve the interpretation of miRNA biomarker candidates from different studies.

4. Conclusion

DIKI is still a large problem in clinical as well as non-clinical settings. The detection of DIKI is hindered by poorly performing standard diagnostic analyses and once AKI is diagnosed therapeutic approaches are nearly nonexistent. On the one hand, miRNAs regulate over 50% of all protein-coding genes at the post-transcriptional level. Thus miRNAs are involved in almost all physiological as well as pathological processes, which make them interesting therapeutic targets. On the other hand, extracellular miRNAs are remarkably stable and found to be valuable biomarker candidates in diverse disease settings. Therefore, due to the intracellular function of miRNAs and their presence in biofluids, miRNAs could significantly contribute to improved management of DIKI not only as interventional targets but also as potential biomarkers. Positive results from other disciplines and the limited yet promising data in the field of kidney medicine hold great promise for a successful application of miRNA-based interventions in the context of DIKI.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

MP is a recipient of a research fellowship from the Deutsche Forschungsgemeinschaft (DFG). We thank Cory Gerlach for scientific and linguistic support. Work in the Vaidya laboratory was supported by Outstanding New Environmental Sciences (ONES) award from NIH/ NIEHS (ES017543), Innovation in Regulatory Science Award from Burroughs Wellcome Fund (BWF–1012518) and a collaborative research agreement with Biogen (AZ4378).

References


To, P., Teng, J., Zou, J., Fang, Y., Xing, H., Bao, J., ... Ding, X. (2013). miR-21 contributes to xeronen-converted amelioration of renal ischemia-reperfusion injury in mice. *Anesthesiology*.


