Role of microRNAs in Renal Parenchymal Diseases – A New Dimension

Saeed Kamran Shaffi, M.D., M.S.1, *, David Galas, Ph.D.2 Alton Etheridge, Ph.D.3, Christos Argyropoulos, M.D., Ph.D.1

1 Affiliation 1: Department of Internal Medicine, Division of Nephrology, University of New Mexico, Albuquerque, NM, USA
2 Affiliation 2: Pacific Northwest Research Institute, Seattle, WA, USA
* Correspondence: kshaffi@salud.unm.edu

Abstract: Since their discovery in 1993, numerous microRNAs (miRNAs) have been identified in humans and other eukaryotic organisms, and their role as key regulators of gene expression is still being elucidated. It is now known that miRNAs not only play a central role in the processes that ensure normal development and physiology, but they are often dysregulated in various diseases. In this review, we present an overview of the role of miRNAs in normal renal development and physiology, in maladaptive renal repair after injury, and in the pathogenesis of renal parenchymal diseases. In addition, we describe methods used for their detection and their potential as therapeutic targets. Continued research on renal miRNAs will undoubtedly improve our understanding of diseases affecting the kidneys and may also lead to new therapeutic agents.

Keywords: miRNA; micro RNA; renal parenchymal diseases; miRNA in renal parenchymal diseases; miRNA detection; miRNA-based therapeutics
1. Introduction

Ribonucleic Acids (RNAs) are a ubiquitous class of unbranched polymeric molecules that serve as intermediates responsible for decoding genetic information from DNA to ribosomes in the form of messenger RNA (mRNAs), transfer of amino acids to ribosomes by transfer RNA (tRNA), and in protein synthesis itself in the form of ribosomal RNA (rRNA). In 1993, a new class of non-coding RNA molecule, microRNAs (miRNAs), was identified. These small molecules play pivotal roles in cell-growth cycle regulation, differentiation and survival by modulating mRNA stability and translational efficiency. Over the last two decades, the role of miRNAs in various diseases, as well as their role in maladaptive repair, has been elucidated. In addition, miRNAs have been studied for their potential use in disease diagnosis and prognostication, and as therapeutic targets.

In this review, we describe the role of miRNAs in renal physiology and pathology and their putative roles in various renal parenchymal diseases. We also discuss methods of their measurement as well as various strategies for using miRNAs as therapeutic agents.

2. miRNA discovery

Before 1993, it was thought that mRNAs, transcribed from the coding regions of DNA, are translated by ribosomes into proteins with no other non-coding regions of DNA playing a significant role in the processes of protein synthesis or regulation of gene expression. The discovery of the importance of non-coding regions of DNA in these processes was made by the Ambros and Ruvkun laboratories. Caenorhabditis elegans, a nematode worm, has been used extensively to study the role of genes in cell division and development. A loss-of-function mutation of the *lin-4* gene resulted in nematodes with morphologically and functionally abnormal sex organs [1]. Interestingly, Ferguson et al. found that in nematodes with *lin-4* mutations, suppressing the *lin-14* gene resulted in amelioration of abnormalities [2]. Next, two seminal discoveries improved our understanding of this phenomenon. Firstly, it was shown that the *lin-4* gene did not encode any protein, but made two small transcripts [2,3]. Secondly, it was found that *lin-4* transcripts are complementary to *lin-14* gene [3]. These elegant experiments showed that *lin-4* transcripts can down-regulate expression of the *lin-14* gene and *lin-4* was the first miRNA identified. At the time of writing this review, more than 28,000 miRNAs have been identified, out of which 2,588 are human. A central repository of known miRNA sequences has been established (miRBase) [4]. miRNAs have been investigated as potential biomarkers, specifically in extracellular compartments, and therapeutic roles for miRNAs are being actively explored for a number of human diseases [5].

3. miRNA biogenesis

The genes for miRNAs are found in the non-protein-coding segments of the genome. Mature miRNAs can be produced by either of the pathways described below.

3.1. Canonical pathway for miRNA biogenesis: RNA polymerase II generates a long, capped transcript molecule, which is called a primary miRNA (pri-miRNA). The ribonuclease III-like enzyme Drosha and its co-factor DGCR8 (DiGeorge Syndrome Critical Region Gene 8) [together called the microprocessor complex], bind to pri-miRNAs, and process them to form 60-110 nucleotide long precursor miRNAs (pre-miRNAs) which are then exported from the nucleus to the cytoplasm by the Exportin 5/GTP binding nuclear protein. Dicer1, another RNase III enzyme, then cleaves the pre-miRNA to form a double-stranded mature miRNA duplex comprised of “guide and passenger” strands (miRNA: miRNA*). This duplex then forms a complex with Argonaute (AGO) proteins and the passenger strand is released. The guide miRNA, approximately 20 nucleotides in length, and the associated Argonaute protein (particularly AGO2) form the RNA-induced silencing complex (RISC). This complex targets the
3' untranslated region (3' UTR) of mRNAs through partial complementarity with the miRNA sequence and leads to suppression of translation, and in many cases also to degradation of the target mRNA [6]. The regulatory effect of the RISC complex is to reduce the expression of proteins from the transcribed mRNA, either by translation suppression or by reduction of the mRNA half-life.

3.2. Non-Canonical pathway for miRNA biogenesis: Other miRNAs are located in pre-miRNA sized introns (mirtrons) within coding genes throughout the genome, and are processed by spliceosomes and debranching enzymes to directly produce pre-miRNAs [7,8]. This pathway is different from the canonical pathway in the sense that Drosha and DGCR8 are not necessary for pre-miRNA genesis. From there on, the non-canonical and canonical pathways share similar steps, however.

4. Role of miRNAs in renal development

Animal models have been developed to better understand the role of miRNAs in renal development. Removal of the enzyme Dicer in mice, and consequent loss of virtually all mature miRNAs, resulted in kidney atrophy, suggesting the importance of miRNAs in normal kidney development. Histologically, there was a marked reduction in nephron progenitors, which resulted in low nephron numbers, cyst formation, and disruption of ciliogenesis [9–11].

Although these observations highlight the relevance of miRNA regulation for renal development, they lack specificity about the identity of the miRNAs that are implicated and the tissue/cellular compartments they are expressed in. The first question was probed in targeted deletion experiments of the miR-17~92 cluster. Deletion of this family of miRNAs, which has essential roles in development and has been implicated in cancer, resulted in the preservation of the nephron progenitor population but impaired their proliferation and thus reduced nephron number. The mice phenotype was one of an early development of albuminuria by 6 weeks, and focal podocyte effacement and glomerulosclerosis by 3 months [12]. Many groups have reported compartmental specific deletion experiments. In one study, ablation of Dicer from maturing renal tubular epithelial cells reduced miR-200 cluster expression levels and upregulated the polycystic kidney disease 1 (PKD1) gene. Predictably, enhanced PKD1 is associated with inhibition of tubulogenesis and cyst formation [13]. In the same study, it was shown that the PKD1 gene was downregulated by miR-200b/c/429 using a variety of bio-informatics and in-vitro approaches. Note that the miR-200 cluster plays a regulatory role in the epithelial-to-mesenchymal transition (EMT) process which is central to fibrotic pathogenesis, suggesting that developmentally relevant miRNAs may also play important roles in the initiation and progression of kidney disease in the adult life. This concept was validated in another series of experiments, which examined the effects of selective inactivation of Dicer in mouse podocytes early in life. This highly specific genetic lesion caused proteinuria and death with foot process effacement, collapsing glomerulopathy, podocyte vacuolization, hypertrophy, and apoptosis. These histological features are also observed in severe forms of the nephrotic syndrome in adult animals and humans (e.g. due to focal segmental glomerulosclerosis). In this particular study mutant podocytes had lost the ability to generate miR-30a [14,15]. Specifically inactivating Drosha in podocytes led to collapsing glomerulopathy similar to Dicer knock-out mice [16]. Collectively, these data suggest that the establishment of renal structure and maintenance of kidney architecture is highly dependent on the normal expression of multiple miRNAs through the Dicer pathway expressed at different cellular compartments within the kidney.

5. Role of miRNAs in renal physiology

miRNAs play a diverse role in normal renal function, as demonstrated by the elimination of specific miRNAs and/or miRNA-processing enzymes in mouse models. For example, conditional deletion of Dicer in renin-expressing cells in mice resulted in reduced juxtaglomerular cell population,
decreased expression of Ren1 and Ren2 genes leading to decreased renin concentration, hypotension, abnormal renal function, renal vascular abnormalities, and strip fibrosis [17]. Hence, while deletion of Dicer in podocytes not only affects normal renal development when this protein is interrupted in podocytes, but it also leads to both structural and functional aberrations in renal function after nephrogenesis as well. A major physiological derangement in progressive renal impairment is the inability to fine tune the balance between the excretion of sodium and conservation of potassium. Such alterations underlie the sodium and potassium retention seen in progressive kidney disease. In that regards, it has been shown that specific miRNAs have been involved in fluid and electrolyte handling. A mouse model with selective miR-192 knock-out in the proximal convoluted tubule, the site of the fine regulation of sodium balance in the kidney exhibited upregulation of Na+/K+ ATPase β-1 subunit. These animals were unable to increase urine output when fed a high sodium diet [18]. MicroRNAs are also involved in the tight co-regulation of sodium excretion by the kidney by the feed-forward (FF) inhibitory control loops of the with No Lysine kinase system (WNK). This system is of emerging importance for understanding the development of systemic, volume sensitive hypertension. Control of the system of miRNAs exemplifies the integration between FF kinase and epigenetic regulatory loops and thus will be examined at some length here. In the normal state, this system ensures renal switching of roles from inter-meal sodium retention to post-meal sodium (natriuresis) and potassium (kaluresis) excretory states. WNK3 upregulates expression of the NaCl cotransporter (NCC) in the distal convoluted tubule of the nephron resulting in sodium retention. On the other hand, natriuresis is mediated by WNK4, which antagonizes WNK3 and decreases NCC expression. WNK4 also increases the expression of renal outer medullary potassium (ROMK) channels in the distal convoluted tubules, thus promoting kaluresis. WNK1 exerts a major regulatory role in switching between the phenotypes of sodium retention and natriuresis by cleaving WNK4, which in turn removes the antagonism on WNK3 mediated sodium retention. It has been shown that miR-192 negatively regulates WNK1, as sodium depletion, aldosterone infusion, and potassium load led to significant kidney-specific WNK1 mRNA expression and reduction in miR-192 expression [19]. Hence a single miRNA (miR-192) appears to play a major regulatory role in one of the most tightly controlled kinase systems in the kidney. Renal potassium handling may be directly controlled by miRNAs independently of effects on the WNK system. High-potassium diet increased miR-802 transcription in the cortical collecting duct in mice, which in turn decreased caveolin-1 expression—a protein which suppresses ROMK activity [20]. miR-9 and miR-374 suppress Claudin-14—a calcium-binding protein expressed in the thick ascending limb of loop of Henle, a major site of sodium, potassium and calcium exchange in the kidney. Extracellular calcium levels also directly regulate miR-9 and miR-374 levels [21].

It is evident that miRNAs provide an extra level of complexity and integration of the different systems that maintain the electroneutrality of urine on the one hand and maintaining homeostasis on the other.

6. Role of miRNAs in renal fibrosis and maladaptive repair

Renal fibrosis is the final common pathway of various forms of progressive renal disease. TGF-β signaling plays a central role in renal fibrosis. Renal parenchymal cells synthesize TGF-β1 and its isoforms (β2 and β3). Experimental models and human studies have shown that TGF-β1 is upregulated in diseased and fibrotic kidneys [22]. Various stressors inducing stimuli such as hyperglycemia [23], angiotensin II [24], and reactive oxygen species [25] increase TGF-β production. It is then activated and exerts its effects in autocrine and paracrine fashion via Smad-dependent and/or Smad-independent pathways [26]. TGF-β1 initiates Smad2 and Smad3 complex formation with Smad4, leading to its activation, translocation to the nucleus, and ultimately transcription of its targets. It is important to note that Smads2 and 3 can also be activated by mediators other than TGF-β [27]. Noting the role of TGF-β in renal fibrosis, we will now discuss the interplay between TGF-β and various miRNAs.
miRNAs regulate TGF-β activity by modulating expression of various components of the TGF-β signaling pathway. In particular, miR-774 has been shown to post-transcriptionally inhibit expression of the TGF-β1 ligand [28]. Similarly, miR-200a has been shown to repress expression of TGF-β2, which prevents renal fibrogenesis [29].

Conversely, TGF-β signaling also influences miRNA expression. TGF-β administration increases expression of miR-192 in human, mouse, and rat, tubular epithelial, mouse mesangial, and rat proximal tubular epithelial cells, respectively [30–32]. Low miR-192 is associated with interstitial fibrosis and tubular atrophy [33]. TGF-β is well known to regulate the expression of the miR-200 family of miRNAs, and administration of TGF-β does indeed lead to decreased expression of the miR-200 family in kidney and rat proximal tubular epithelial cells [29].

The final common pathway of TGF-β signaling is the production of extracellular matrix (ECM) proteins and their deposition into the interstitium. Several lines of evidence demonstrate that this process is under miRNA control. In systemic sclerosis - a disease characterized by widespread fibrosis - expression of the miR-29 family is decreased. miR-29a suppresses expression of collagen type I and III [34]. Furthermore, TGF-β suppressed miR-29, and down-regulation of miR-29 results in upregulation of TGF-β. A mouse model of bleomycin-induced skin fibrosis was associated with decreased miR-29, which was reversed by tyrosine-kinase inhibitor imatinib [34], a potent inhibitor of the TGF-β pathway. The role of miR-29 appears not to be limited to systemic sclerosis, since a mouse model of miR-29 inhibition demonstrated protection against salt-induced hypertensive renal sclerosis. There was up-regulation of various genes involved with laying of ECM when miR-29 was silenced in the kidneys of these animals [35]. miR-337 was shown to be involved in diabetic nephro sclerosis. It has recently been shown that miR-337 was upregulated when cultured human and mouse mesangial cells were exposed to high glucose and TGF-β to imitate a diabetic milieu. Fibronectin – a key protein involved in fibrosis – was in fact directly induced by miR-377 [36]. Other animal models have been used to study the role of miRNAs in renal fibrosis: miRNA-449a/b expression was downregulated in hypoxic fibroblasts. Furthermore, miRNA-449a/b caused upregulation of profibrotic proteins (serine protease inhibitor protein -SERPINE1) [37]. These experiments show that profibrotic proteins are under the control of miRNAs.

7. miRNAs in select renal parenchymal diseases

miRNA expression profiles have been studied in many renal parenchymal diseases. Specific miRNA expression signatures have been identified for some diseases. We will briefly review some of these associations, since they provide the basis for detecting miRNAs as disease-specific biomarkers and potential therapeutic targets.

7.1. Diabetic nephropathy

miRNAs have been directly implicated in the pathogenesis of diabetic nephropathy. miR-29c expression – which is associated with podocyte apoptosis - is increased in both the glomeruli and microvascular endothelial cells in a mouse diabetic model [38]. In addition, miR-29c overexpression promoted activation of the Ras homolog gene family, member A (RhoA) – by suppressing the Spry1 gene - which has been shown to play a role in the pathogenesis of diabetic nephropathy [39]. Analysis of kidney biopsy samples from patients with diabetes revealed that miR-192 expression was inversely related to tubulointerstitial fibrosis and directly related to estimated glomerular filtration rate (eGFR) [33].

This association may be causal since the introduction of TGF-β to proximal convoluted tubule cells exposed to high glucose conditions leads to decreased miR-192 expression. Conversely, overexpression
of miR-192 ameliorated the TGF-β-mediated fibrosis [33]. Hence once TGF-β has been activated in high glucose conditions, the decreased expression of miR-192 brought about by TGF-β may further amplify tissue fibrosis.

Several miRNAs may be involved in the expression of the fibrotic renal phenotype. TGF-β increased miR-216a and collagen type I α1 expression in mouse mesangial models of diabetes [40]. A miRNA circuit has been shown to be directly involved in mediating the autoregulation of TGF-β and the production of ECM. TGF-β induced miR-192 inhibits the expression of the E-box repressors Zeb1/2 which in turn increases the expression of miR-200b and miR-200c. These miRNAs further inhibit Zeb1/2 leading to enhanced expression of TGF-β and the ECM components collagen type I α2 and collagen type IVα1. [41]. Hyperglycemia activates phosphatidylinositol (PI) – 3 kinases/Akt pathway leading to cell hypertrophy and increased matrix protein in mouse diabetic models [42]. miR-21 mediates this process by reducing tumor suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN). Overexpression of miR-21 is seen to inhibit PTEN expression with an increase in the PI3/Akt pathway, leading to renal cell hypertrophy and fibronectin expression [43]. Overall it is clear that the effect of the miRNAs on these functions and pathologies is significant and important.

Many of these miRNAs have been shown to be associated with features of the diabetes phenotype (insulin secretion or sensitivity) and the development of diabetic kidney disease in human studies [44, 45].

7.2. Hypertension

Hypertension is a major risk factor for developing coronary artery disease, congestive heart failure, sudden death [46], left ventricular hypertrophy [47], and stroke [48]. Coronary artery disease and stroke are the two major causes of death in the U.S. [49]. Hypertension is more prevalent in patients with chronic kidney disease (CKD) and is thought to be the second most common cause of end-stage renal disease in the U.S. [50, 51]. Genetic, environmental, hemodynamic, renal, and hormonal factors have been implicated in the pathogenesis of hypertension. miRNAs are involved in nearly all pathophysiological alterations that underline the development of hypertension and its cardiovascular and renal complications.

Oxidative stress due to inhibition of nitric oxide (NO) production and generation of reactive oxygen species could be the final common pathway for hypertension development [52]. Production of reactive oxygen species may be influenced by specific miRNAs. In experimental models of oxidative stress (reactive oxygen species [ROS] generation, hydrogen peroxide exposure) caused apoptosis of human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner with concomitant increase in miR-210 levels. Overexpression of miR-210 resulted in inhibition of apoptosis and decreased the concentration of reactive oxygen species. Thus, miR-210 may prevent the deleterious effects of ROS [53]. miR-155 was shown to directly inhibit endothelial nitric oxide synthase (eNOS) production by binding to the 3' UTR of its mRNA, leading to increased oxidative stress. Furthermore, simvastatin decreased miR-155 expression, thus restoring endothelium-dependent vasorelaxation, an effect that was independent of cholesterol levels. Inhibition of miR-155 may be a therapeutic target for improving endothelial dysfunction [54] and may even underline some of the non-cholesterol (pleiotropic) effects of statins.

miRNAs may also play a role in the development of hypertension, by their effects on vascular smooth muscle cells (VSMCs). Aberrant division of VSMCs leads to vascular luminal hypertrophy and luminal narrowing which causes and propagates hypertension. miR-143 and miR-145 ensure proper development and regulation of VSMCs. VSMCs deficient in miR-143 and miR-145 did not respond to vasocontractile
stimuli but had increased synthetic activity. These miRNAs played a critical role in class switching of VSCMs from a synthetic unit to a vasocontractile unit [55].

Activation of renin-angiotensin-aldosterone systems (RAS) plays a cardinal role in pathophysiology and maintenance of different forms of hypertension. Activation of the angiotensin 1 receptor (AT1R) by Angiotensin II (Ang II) increases blood pressure by vascular smooth muscle cell proliferation, vascular constriction, cardiac remodeling, aldosterone production, and sodium retention, which plays a central role in the pathogenesis of hypertension [56]. These angiotensin mediated processes are under miRNA control. miR-155 inhibits AT1R expression and VSMC proliferation [57]. miRs-29b, -129-3p, -132, -132* and -212 were upregulated by Ang II in human cell culture (HEK293N) [58]. miR-483-3P expression downregulated angiotensinogen and angiotensin-converting enzyme (ACE) and could be a novel therapeutic agent for hypertension management [59]. Inhibitors of the angiotensin-converting enzyme inhibitors (ACEi) have been shown to decrease renal disease progression in early diabetic nephropathy in type 1 and type 2 diabetes mellitus [46] and in preventing coronary artery disease and strokes [60]. Angiotensin-converting enzyme inhibitors have become a mainstay for the therapy of hypertension [61]. Some of the beneficial effects of ACEi could be mediated by suppression of miR-324-3p. In the Munich Wistar Fromter (MWF) rat model, which develops spontaneous progressive nephropathy, ACEi suppress miR-324-3p and attenuate the development of hypertensive nephropathy [62].

Sympathetic nervous system overactivity is one of the mechanisms for development and maintenance of hypertension. The role of the sympathetic nervous system (SNS) and RAS in the maintenance of hypertension was studied in mice which were genetically prone to develop hypertension (BPH/2J mice) [63]. Ganglion blocker use (SNS suppressor) in mice that were pre-treated with an ACEi (RAS suppressor) showed that hypertension in the BPH/2J was primarily mediated by the sympathetic nervous system during the active periods and RAS system during the inactive periods. During active periods, BPH/2J mice had higher renal Ren1 mRNA and lower miR-181a indicating SNS mediated release of renin. These findings suggest that miR-181a inversely regulates the Ren1 mRNA. The authors postulated that miR-181a suppression potentiates sympathetic nervous system-mediated increase in renin production in BPH/2J mice during the active periods [64]. These findings were confirmed by a human study of mRNA and miRNA expression profiles in renal biopsies of hypertensive patients that showed miRNA-181a inversely regulated the Ren1 mRNA [65].

Various animal models have been developed to study the effects of hypertension on kidneys. Dahl salt-sensitive (Dahl-SS) rats develop hypertension with medullary interstitial fibrosis when exposed to a high salt diet. Consomic SS-13BN rats are genetically modified Dahl-SS rats that have less pronounced blood pressure rise and medullary interstitial fibrosis when exposed to a high salt diet [66]. Liu et al. studied miRNA expression profiles in these two rat models and showed that a high salt diet resulted in upregulation of miR-29b in Consomic SS 13BN rats but not in Dahl-SS rats. Various collagen genes that promote fibrosis were upregulated in Dahl-SS rats but not in Consomic SS 13BN rats – a pattern opposite of miR-29b expression. Furthermore, a miR-29b knockdown Consomic SS 13BN rat model had upregulation of various collagen genes, suggesting that miR-29b expression protects rats from hypertension-associated renal medullary injury [67].

Kidney biopsies in patients with hypertension reveal glomerulosclerosis, tubular atrophy, interstitial fibrosis, and vascular smooth muscle cell hypertrophy. In a recent study, intrarenal miRNA expression profiles of 30 patients with hypertensive nephrosclerosis were compared to 20 normal controls. miR-200a, miR-200b, miR-141, miR-429, miR-205, and miR-192 were significantly increased in patients with hypertensive nephrosclerosis [68].

7.3. Glomerulonephritis
Glomerulonephritides (inflammation of glomeruli) are a group of diverse disorders that may present as proteinuria and/or hematuria with renal dysfunction. Kidney biopsy findings include podocyte injury, mesangial and endocapillary proliferation, and disruption of basement membranes leading to focal and segmental glomerulosclerosis, tubular atrophy and interstitial fibrosis. We will now discuss the role of miRNAs in some of the conditions that can cause glomerulonephritis.

7.3.1. Focal Segmental Glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) is a pattern seen on kidney biopsy characterized by involvement of some of the glomeruli, with part of the involved glomerulus showing obliteration of the capillary lumen and increase in mesangial matrix. FSGS is usually caused by infections, medications, and conditions that cause chronic renal injury. At times, no cause of FSGS is found, and this is labeled as primary FSGS. A molecule that increases the permeability of glomerular basement membranes has been postulated in the pathogenesis of primary FSGS [69,70] but the exact nature of that molecule remains elusive. Podocyte injury is considered an inciting event in the development of FSGS. In a puromycin-induced FSGS rat model, researchers found diminished miR-30s. Replacement of miR-30s resulted in resolution of podocyte injury and proteinuria. Furthermore, cytoskeletal damage and apoptosis induced by puromycin or TGF-β treatment was ameliorated or exacerbated in human podocytes with miR-30a overexpression or knockdown, respectively. Glucocorticoid use caused sustained expression of miR-30a in podocytes, and miR-30a plays a role in podocyte health and maintenance of cytoskeletal integrity [71]. The histological findings in this model recapitulated the abnormal morphology in the miR-30a deficient Drosha and Dicer knockout podocyte models that were discussed previously [14–16].

miRNAs have also been used as biomarkers – both in serum and urine – to assess FSGS disease activity. In one study, researchers found elevated plasma miR-125b, miR-186 and miR-193a-3p in patients with FSGS with area under curve (AUC) of 0.88, 0.78, and 0.91, respectively. Patients in remission had lower miR-125b and miR-186 concentrations [72]. These miRNA levels remained unchanged in patients who did not achieve remission. miR-186 levels also correlated with proteinuria [72]. Patients with FSGS and minimal change disease had higher urinary miR-200c levels [73]. miR-196a, miR-30a-5p and are miR-490 were associated with FSGS disease activity. Urinary miR-30a-5p was a weak predictor of steroid responsiveness in patients with active FSGS [74].

7.3.2. IgA nephropathy

IgA nephropathy is the most prevalent primary glomerulonephritis in the world. Abnormal O-galactosylation of IgA causes the formation of IgA complexes against which IgG and IgA are formed with deposition in kidneys and activation of the complement system leading to kidney injury.

Genome-wide analysis has revealed various miRNAs which might play a role in IgA nephropathy. miRNA expression of 6 patients, each with biopsy-proven IgA nephropathy, compared to those with renal cell carcinoma revealed upregulation of 11 miRNA and downregulation of 74 miRNAs in the IgA nephropathy group [75]. Members of the miR-200 and miR-29 families which regulate EMT and development of tissue fibrosis showed prominent expression changes in patients with IgA nephropathy, tissue fibrosis, and proteinuria.

miRNA let-7b and miR-148b control N-acetylgalactosaminyltransferase 2 (GALT2) and 1 β 1,3 galactosyltransferase 1 (C1GALT1), respectively – these enzymes play a central role in aberrant IgA galactosylation. It has been shown that these enzymes are overexpressed in the peripheral blood mononuclear cells of IgA nephropathy patients [76,77].
7.3.3. **Lupus nephritis**

Systemic lupus nephritis (SLE) is a systemic disease due to dysregulated immune system activity. Kidney involvement by SLE often leads to chronic kidney disease and eventually kidney failure if left untreated, and it is the major cause of morbidity and mortality. Genetic factors have been implicated in SLE pathogenesis, but the underlying control mechanisms remain poorly defined.

Various lines of evidence point towards the role of miRNAs in SLE. miRNAs regulate 72 genes labeled as “autoimmune genes” that control various aspect of the immune system [78]. miR-181, miR-186, and miR-590-3p regulate more than 50% of the genes that are known to be differentially expressed in SLE patients. Epstein-Barr virus (EBV) infection could be one of the initiating agents responsible for dysregulated immune response in SLE. EBV affects SLE patients more commonly with an increased number of infected peripheral white cells than healthy controls [79]. The exact causal link between EBV infection and SLE is not known; however, molecular mimicry is suspected. EBV virus latent membrane protein 1 activates miR-155 transcription through the nuclear factor kappa beta (NF-κβ) pathway [80].

miR-155 is expressed in regulatory T cells [81] and macrophages and promotes the development of inflammatory T cells [82]. B6.MRLc1 mice exhibit an immune complex-mediated glomerulonephritis with proliferative lesions that progress to glomerulosclerosis, tubular atrophy, and interstitial fibrosis. These lesions showed expression of miR-146a which increased with age, suggesting that it plays a role in renal inflammation [83]. Kidney biopsy analysis of patients with lupus nephritis showed upregulation of miR-146a and miR-198 in the glomerular lesions and miR-638 in tubulointerstitial lesions [84]. In this study, the degree of interstitial miR-638 expression was significantly correlated with clinical markers of kidney damage (proteinuria) and the disease activity score. Conversely, glomerular miR-146a correlated with clinical markers of renal function (estimated glomerular filtration rate) and the disease activity score. Hence, these two miRNAs may play a pathogenic role in the development of clinical lupus nephritis.

7.3.4. **Anti-Neutrophilic Cytoplasmic Antibodies associated Vasculitis (ANCA)**

ANCA vasculitis is a small vessel vasculitis involving the kidneys as well as other organs and is characterized by the presence of either anti-Proteinase 3 (PR3) or anti-Myeloperoxidase (MPO) (components of neutrophils) antibodies. Currently, it is not known whether these antibodies are pathogenic and what are the inciting factors for production of these antibodies.

Pooled plasma samples from 40 patients who had active ANCA vasculitis or were in remission showed up-regulation of Let-7f, and miR-424 and downregulation of miR-106b, -9, -125a, and -15b. These miRNAs regulate various aspects of the immune system [85], suggesting a direct role in the development of clinical disease.

miR-155 is upregulated in patients with ANCA-associated crescentic GN. Nephrotoxic nephritis is a mouse model of ANCA vasculitis developed by injecting rats with rabbit or duck nephrotoxic sera [86] and has been found to closely correlate with human renal ANCA vasculitis [87]. A miR-155 knockout in this mouse model exhibited less severe lesions. It was noted that miR-155 mediates the TH17 immune response and thus may be a therapeutic option for ANCA associated crescentic GN [88].

7.3.5. **Systemic Sclerosis (Scleroderma)**

Systemic sclerosis is a condition associated with multiple organ fibrosis. It affects the kidney by causing thickening of the blood vessels, leading to hypertension, endothelial injury, and thrombotic microangiopathy. It has been shown that TGF-β [89] and miR-21 [90] are upregulated in systemic sclerosis. Furthermore, TGF-β regulates the expression of miR-21 and fibrosis-related genes, and miR-21
is inversely associated with Smad7 expression and may therefore be a therapeutic target for this condition [91].

7.3.6. **Autosomal Dominant Polycystic Kidney Disease (ADPKD)**

ADPKD is a disease characterized by impaired ciliary function leading to kidney and liver cyst formation and kidney failure in the vast majority of patients.

In the Sprague-Dawley rat model of ADPKD, 29 miRNAs were downregulated and only 1 miRNA (miR-21) was upregulated. Most of the dysregulated miRNAs control cell-to-cell interaction and crosstalk [92]. Global gene-expression studies in embryonic kidneys in an animal PKD model found differential expression of miRs-10a, 30a-5p, -96, -126-5p, -182, -200a, -204, -429 and -488 [93]. miR-21 expression has also been associated with cyst progression. Inhibition of miR-21 slows cyst growth in a mouse model of ADPKD [94]. miR-17 cluster of miRNAs is upregulated in mouse models of PKD, and deletion of miR-17 cluster results in resolution of cysts, and better renal and animal survival [94].

7.3.7. **Alport syndrome**

Alport syndrome is due to abnormalities in genes coding α3, α4 or α5 chains of collagen Type IV, resulting in abnormal basement membranes in the kidney, eyes, and inner ear. These changes in the kidney lead to abnormalities of glomerular basement membrane and progressive renal disease. miR-21 is preferentially expressed in the tubulointerstitium instead of glomeruli in normal mice; however, in the Col4α3-/- mice, miR-21 is expressed equally in both compartments [95]. As described previously, miR-21 has been associated with renal fibrosis. Introduction of anti-miR-21 oligonucleotides in Col4α3-/- mice resulted in the preservation of renal function, reduction in albuminuria, improved survival, reduced glomerulosclerosis, crescent formation, and tubular injury [95]. Currently, a Phase 1 clinical trial is being conducted to assess the safety, pharmacodynamics, and pharmacokinetics of a molecule that inhibits miR-21 [5].

8. **miRNA detection**

The preceding section highlights the role of specific miRNAs in normal renal development, physiology, but also the initiation and the progression of the interstitial fibrosis that underlines progressive forms of chronic kidney disease. It follows, that miRNAs detected in either plasma or urine, the two fluidic compartments directly affected by renal processing, may be mechanistically plausible, rational biomarkers for diverse forms of kidney diseases. Nevertheless, detection of miRNAs poses unique challenges because of their short size and the similarity of many sequences to one another. These biochemical features of miRNAs may directly impact the performance of the three methods most commonly used for miRNA detection: quantitative real-time PCR (qPCR), microarrays, and next-generation sequencing (NGS). Each of these approaches comes with its distinct advantages, but also limitations when used as the basis for the development of miRNA biomarker assays.

Of the methods listed above, qPCR has the highest sensitivity, with a theoretical limit of detection of just a few copies per sample [96]. Several commercial kits are available for detection of miRNAs by qPCR, and although the specifics of each kit differ, they generally involve addition of a known sequence to the 3′ end of the miRNA, followed by reverse transcription and PCR amplification using a miRNA-specific primer. Because each target of interest requires a separate PCR reaction and cannot be highly multiplexed, qPCR is less well-suited to high-throughput profiling than either microarrays or NGS. However, for targeted detection of a specific, small set of miRNAs, the cost of qPCR is comparatively low, and the hands-on time required is much less than that of other methods. Furthermore, the development of droplet digital PCR (ddPCR) more recently has improved the precision and
reproducibility of qPCR measurements, especially for samples with low target abundance or high contaminant concentrations, and made absolute quantitation more accessible [97].

Microarray-based methods allow for the simultaneous measurement of many miRNAs, making them a better choice than qPCR for profiling a large set of targets. Commercial products are available that cover all the mature miRNA sequences in miRBase on a single array. However, the amount of starting material required for microarray analysis is relatively high (~100ng per sample) and it remains difficult to design probes and hybridization conditions that can distinguish between closely related miRNA sequences. In addition, the dynamic range of microarrays tends to be lower than either qPCR or NGS.

Unlike qPCR and microarrays, NGS requires no prior knowledge of the target sequences in the sample, and so is ideal for discovery studies. In addition, NGS is not affected by the complications of designing primers or probes with the specificity needed to distinguish between short sequences with high sequence identity. Consequently, as costs for NGS library preparation and sequencing have dropped, small RNA sequencing (sRNA-seq) has become more widely used for miRNA profiling because of its ability to comprehensively interrogate the miRNAs (and other types of small non-coding RNAs) in a sample. Unlike the other methods described above, sRNA-seq allows the analysis of miRNAs with single nucleotide resolution, so not only can the canonical sequences be studied, but also variants arising from RNA editing or imprecise miRNA processing (isomiRs). sRNA-seq is not without drawbacks, however. Compared to qPCR and even microarray analysis, NGS is more expensive and more time intensive, both in sample preparation and data analysis. Most methods for preparing sRNA-seq libraries involve sequential ligation of adapters to the 3’ and 5’ ends of the miRNAs, followed by reverse transcription and PCR amplification to add indexes and other sequences needed for attachment to the flow cell and sequencing. The adapter ligation steps have previously been shown to introduce bias into small RNA libraries, which leads to decreased library diversity and prevents making quantitative comparisons of the expression level of different miRNAs in a sample [98–100]. Moreover, because the biases vary depending on the library preparation protocol, comparing data generated by two different sample prep methods is difficult [101,102]. Although attempts have been made to alleviate bias in sRNA-seq through modifications in library preparation methods [100,103,104] or to compensate for it during data analysis [105], it remains a significant issue that will need to be addressed in the future.

Several important issues affect all methods commonly used for measuring miRNAs. For example, whereas several housekeeping genes have been widely used for normalization in mRNA expression profiling studies, there is less consensus on similar invariant transcripts suitable for miRNA expression analysis. Other options include normalization based on sample input (input mass when practical or input volume when the amount of RNA in the sample is too low to reliably measure) or based on the addition of spike-in oligonucleotides added during sample processing. Normalization based on relative read counts (e.g., reads per million total miRNA reads or reads per million genome-mapped reads) is also frequently used to report sRNA-seq data, but this method can yield inaccurate results if samples have different proportions of small RNA species, either because of biological differences or technical differences such as small variations in the size selection step during sRNA-seq library preparation.

Another issue affecting miRNA measurement is the lack of correspondence between data generated by different methods. It is not an uncommon practice to identify changes in miRNA expression using high-throughput methods such as microarrays or NGS and subsequently validate those changes by qPCR. However, it has been known for some time that changes in miRNA expression detected by one method are often difficult to corroborate across methods [106]. High among the problems here is that different methods exhibit different sequence-specific biases. Even within a given method, however, results may not be comparable if the data is generated using kits from different vendors, as mentioned
above for sRNA-seq, and as previously shown for microarray and qPCR [107,108]. For all these reasons, measurement of miRNAs requires careful planning, care in precisely executing protocols, and repeated measurements when possible.

Measurement of extracellular miRNAs is further complicated by low concentration and inhibition by other macromolecules in the sample. Although there is great interest in profiling RNAs present in biofluids such as blood, urine, and saliva to identify disease biomarkers, obtaining accurate and reproducible results from these sources remains challenging. The primary reason that measuring miRNA in biofluids is difficult is that the RNA concentration is much lower than in cells or tissues. Typical RNA isolation methods recover only about 10-50ng of total RNA per milliliter of cell-free plasma, with even lower yields from fluids such as urine and saliva. In addition, the presence of inhibitors, derived either from the sample itself or during the collection procedure and co-purified with the RNA, can be problematic for the enzymatic steps in library preparation. Salts present in urine samples and heparin used as an anticoagulant during plasma collection are two examples of inhibitors commonly encountered with biofluid samples. Furthermore, the handling and storage methods of the samples before RNA extraction can have a significant impact on the results. Lysis of cells during collection (hemolysis in plasma, for example) or incomplete removal of intact cells before RNA isolation can also significantly distort the miRNA profile from biofluids since cellular RNA content is much higher than that of the cell-free fluid. Thus, sample collection, RNA isolation and library protocols are all critical for accurate profiling.

9. Micro RNAs as a therapeutic option in renal diseases

In previous sections, we discussed the roles of miRNAs in normal kidney function as well as kidney diseases associated with miRNA dysregulation. There is significant interest in the use of miRNAs as therapeutic agents since they modulate the activity of numerous genes. miRNA-based therapeutics are based on either inhibiting a deleterious miRNA or replacing a deficient beneficial miRNA. miRNA antagonists – also called antagonimirs or antmiRs– are single-stranded molecules that are designed to bind directly to a mature miRNA and to block its action. Deficient miRNAs can be replaced by either making small interfering miRNAs (siRNAs), which are small double-stranded RNA molecules encapsulated in nanoparticles and delivered to the target site [109] or by viral vectors that express the desired miRNA.

9.1. miRNA delivery

Designing an effective miRNA mimic and delivering it to its intended target organ without degradation or causing unintended effects has been the subject of intense research. Designing miRNA mimics that effectively block their targets without affecting any unintended transcripts has proven problematic [110]. Therefore, these molecules must be thoroughly tested to fully understand all their intended and unintended effects.

The process of delivering a miRNA molecule to its intended target is fraught with difficulties. Naked miRNAs are unstable in the blood and are rapidly degraded by the mononuclear macrophage system and removed from circulation by the kidneys and liver. Therefore, effective delivery methods that prevent miRNA degradation must be devised. These delivery vehicles should be non-toxic, have low immunogenicity, and should be able to deliver a large proportion of the miRNAs to their intended target [111]. Some of the techniques used to deliver miRNAs are shown in Table 1.

### Viral Vectors

Pathogenic genes are removed from the virus and are replaced by the miRNA gene. These modified viruses make a double-stranded miRNA mimic which associates with Ago proteins and forms the miRNA silencing complex. Adenovirus, adeno-associated virus, retrovirus, and lentivirus have been
used as miRNA vectors. This approach is limited by low vector titers, high immunogenicity, the ability to work only in dividing cells, and clinical safety issues [112].

**Nano-particles**

Polylactic-co-glycolic acid (PLGA) particles are small polymers that have been used to deliver siRNAs, miRNAs and viral vectors [113]. They are non-toxic and have been used in clinical medicine for a long time. There is often poor loading of siRNAs and miRNAs although techniques are being developed to solve this problem [113].

**Natural lipid emulsion**

Natural lipid emulsions have been used to replace tumor suppressor genes in lung cancer. These particles are uncharged, do not make aggregates in the liver and are not scavenged by macrophages [114]. Questionable delivery of the siRNAs to the target site is an issue with this technique.

**Cationic Lipid-based nano-liposomes**

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) nano-liposomes have been found to be highly effective in delivering miRNAs [115].

**Bacterial mini-cells**

Bacterial mini-cells that are produced by inactivating genes involved in bacterial growth have been used to deliver chemotherapeutic agents [116]. A phase 1 study is currently ongoing to deliver miR-16 family miRNAs, which suppress tumor growth in malignant pleural mesothelioma and non-small cell lung cancer, using this technique [117].

**Cationic polymers**

Low molecular weight with a branched structure polyethyleneimine has been used for siRNA delivery [118].

**Polyamidoamines**

Initially designed for delivery of plasmids, polyamidoamines polymers have been used for siRNA delivery. These molecules can be designed precisely to the desired sizes and molecular weights [119].

**Collagen-based molecules**

Atelocollagen is a calf dermis derived type 1 collagen which has been used to deliver siRNA locally [120] as well as systematically [121].

**Cyclodextrin polycation**

siRNA, when complexed with cyclodextrin polycation delivery system, was shown to effectively silence the intended oncogene [122].

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Therapeutic agent/ mode of action</th>
<th>Pharm* Company</th>
<th>Targeted disease</th>
<th>Trial Description</th>
<th>Trial results</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG-012</td>
<td>miR-21/ Inhibits</td>
<td>Regulus/ Genzyme</td>
<td>Alport Syndrome</td>
<td>Phase 1, open-label, multi-center study of the subjects with Alport syndrome, n=10</td>
<td>Ongoing, Estimated completion date December 2018</td>
</tr>
<tr>
<td>MRG-201</td>
<td>miR-29/ Promotes mirage</td>
<td>Scar tissue formation in skin, intended uses in Scleroderma, Diabetic nephropathy and pulmonary fibrosis</td>
<td>Phase 1, Double-Blind, Placebo-Controlled, Single and Multiple Dose-Escalation Study to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics activity of MRG-201 following local intradermal injection in normal healthy volunteers, n=54</td>
<td>Less induced fibrosis in humans who received MRG-201</td>
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<td>-------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>RG-125/AZD4076</td>
<td>miR-103/107 AstraZeneca/Regulus</td>
<td>Type 2 diabetes and non-alcoholic steatohepatitis</td>
<td>Phase I/IIa to investigate the effect on whole-body insulin sensitivity, liver fat content, safety, and tolerability</td>
<td>Discontinued – June 2017</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2: Trials involving miRNAs in renal parenchymal disease**

Regulus Therapeutics in collaboration with Genzyme has developed a single-stranded molecule RG-012 that inhibits miR-21. In a rat model of Alport syndrome, miR-21 inhibition by this molecule led to milder kidney disease and improved survival than control mice. There was less glomerulosclerosis and tubulointerstitial fibrosis in the treated mice with no adverse events [95]. A phase I randomized, double-blinded, placebo-controlled study is currently being conducted to study the safety and efficacy of RG-012 in male subjects with Alport syndrome [5]. miRagen Therapeutics is developing a molecule MRG-201 that promotes miR-29 activity and thus modulates fibrosis. This molecule has potential roles in preventing progression of CKD in diabetic nephropathy, IgA nephropathy, and scleroderma. A phase 1 study to evaluate the safety and tolerability of this agent has been completed [123].
10. Conclusions

It has been shown in practice over the past decade that extracellular miRNAs can provide informative biomarkers for multiple biological effects and pathologies. The value of understanding miRNA function, however, is much broader. In concert with the multiple factors regulating transcription, miRNAs provide an additional level of control of gene expression, largely at the post-transcriptional level. Their influence on various biological pathways is both widespread and complex and is often subtle. In the last 20 years, tremendous progress has been made in understanding their roles in renal physiology and pathology, and this is beginning to open several new lines of investigation. Research is currently underway to study and modulate miRNAs specifically to control maladaptive repair that leads to fibrosis in various renal diseases. miRNAs also provide us a novel opportunity to develop new ways of studying disease activity and to assess the efficacy of therapeutic agents. Since miRNAs can be targeted directly, although this is sometimes difficult in practice, they provide the opportunity to develop a new class of therapeutic agents. miRNA-based diagnostics and therapeutics, therefore, have the potential to lead medicine into a new era of effectiveness.

Acknowledgments: This work was supported by grants from the NIH Common Fund Extracellular RNA Communication Consortium (ERCC) to D.G. [U01HL126496 and U54DA036134], the National Institutes of Health, National Center for Advancing Translational Sciences [UL1TR001449 to C.A, in part] Dialysis Clinic Inc (DCI) [DCI #C-3765 and DCI RF#C-3861.201 to C.A] and by the Pacific Northwest Research Institute. Funding for open access charge: DCI [DCI #C-3765] We thank Elaine Skeffington for careful copy edits.

Author Contributions: All authors planned the manuscript together. A.E. and D.G. wrote the miRNA detection section. S.S wrote all the other sections. All the authors edited, critically reviewed the manuscript, and agreed on the final manuscript.

Conflicts of Interest: None

Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic acid</td>
</tr>
<tr>
<td>AGO2</td>
<td>Argonaute Proteins</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge Syndrome Critical Region Gene 8</td>
</tr>
<tr>
<td>RISC</td>
<td>Ribonucleic acid-induced silencing complex</td>
</tr>
<tr>
<td>Mir-trons</td>
<td>Pre-Micro Ribonucleic acid introns</td>
</tr>
<tr>
<td>PKD1</td>
<td>Polycystic Kidney Disease 1</td>
</tr>
<tr>
<td>WNK</td>
<td>With No Lysine kinase system</td>
</tr>
<tr>
<td>NCC</td>
<td>Sodium Chloride Co-transporter</td>
</tr>
<tr>
<td>ROMK</td>
<td>Renal Outer Medullary Potassium Channel</td>
</tr>
<tr>
<td>TGF-ß</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time Polymerase Chain Reaction</td>
</tr>
</tbody>
</table>
NGS     Next-Generation Sequencing
ddPCR    Droplet Digital Polymerase Chain Reaction
sRNA-seq Small RNA Sequencing
isomiRS  Imprecise miRNA Processing
RhoA     Ras Homolog Gene Family, Member A
eGFR     Estimated Glomerular Filtration Rate
PI-3/Akt Phosphatidylinositol–3 kinases/Akt pathway
PTEN     Tensin Homolog Deleted on Chromosome 10
ESRD     End-stage Renal Disease
NO       Nitric Oxide
HUVECs   human umbilical vein endothelial cells
ROS      Reactive Oxygen Species
eNOS     Endothelial Nitric Oxide Synthase
3' UTR   3' Untranslated Region
VSCM     Vascular Smooth Muscle Cell
ACE      Angiotensin-converting Enzyme
AT1R     Angiotensin 1 Receptor
Ang II   Angiotensin 2
ACEi     Angiotensin-converting Enzyme Inhibitors
MWF      Munich Wistar Fromter (MWF) Rat Model
Dahl-SS  Dahl salt-sensitive
Consomic SS-13BN Consomic Salt Sensitive Rats
FSGS     Focal segmental glomerulosclerosis
GALNT2   N-acetylgalactosaminyltransferase 2
C1GALT1  1 β 1,3 galactosyltransferase 1
SLE      Systemic lupus erythematosus
NF-Kβ    Nuclear Factor Kappa Beta
ANCA     Anti-Neutrophilic Cytoplasmic Antibodies associated Vasculitis
PR3      Proteinase 3
MPO      Myeloperoxidase
ADPKD    Autosomal Dominant Polycystic Kidney Disease
Col4a3-/- Homozygous for Collagen Type 4 alpha 3 Chain Absence
siRNA    Small Interfering Micro Ribonucleic Acid
PLGA     Poly lactic-co-glycolic Acid
DOPC     1,2-dioleoyl-sn-glycero-3-phosphocholine

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