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Post-transcriptional gene-expression regulation by micro RNA (miRNA) network in renal disease ☆

Tamás Kaucsár [1], Zsuzsanna Rácz [1], Péter Hamar *

Semmelweis University, Institute of Pathophysiology, H-1089, Budapest Nagyvárad t. 4, Hungary

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ABSTRACT

Micro RNAs (miRNAs) are a recently discovered class of small, non-coding RNAs with the function of post-transcriptional gene expression regulation. MiRNAs may function in networks, forming a complex relationship with diseases. Alterations of specific miRNA levels have significant correlation with diseases of divergent origin, such as diabetic or ischemic organ injury including nephropathy, and malignant diseases including renal tumors. After identification of disease-associated miRNAs, there are two options of influencing their tissue expression. The function of miRNAs can be inhibited by antisense oligonucleotides (ASOs), which have been shown to silence specific miRNAs *in vivo*. Moreover, miRNA activity can be also mimicked or enhanced by delivering chemically synthesized miRNAs. Thus, modifying the expression of miRNAs is a potential future gene-therapeutic tool to influence posttranscriptional regulation of multiple genes in a single therapy. In this review we focus on key renal miRNAs with the aim of revealing the pathomechanisms of renal diseases.

Nucleic acid therapy with oligonucleotides and short interfering RNA (siRNA) are under clinical evaluation presently. Similar therapeutic strategies, to influence miRNA function is also already under clinical investigation in RNA interference trials. We summarize here studies specifically aimed at the modification of miRNA expression.

Research on the post-transcriptional regulation of gene expression by miRNA may reshape our understanding of renal pathophysiology and consequently may bring new diagnostic markers and therapeutic agents.

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* Corresponding author.
E-mail address: hampet@net.sote.hu (P. Hamar).

[1] The 2 authors: TK and Zs R contributed equally.

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1. Introduction

In the postgenomic era, investigation of the human transcriptome has revealed that the genome encodes many thousands of functional RNAs not transcribed into proteins (non-coding RNAs) [1]. Micro RNAs (miRNAs) compose a large family: about 1% of the genes encoded in the genome belong to the miRNA family [2]. First described in 1993 [3] miRNAs are short, being composed of only 18–25 nucleotides (nt). Presently, it is hypothesized, that miRNA sequences play an important role in gene-expression regulation, through RNA interference (RNAi), controlling protein synthesis from most human genes at the posttranscriptional level (post-transcriptional regulation of gene-expression) [4]. Emerging knowledge surrounding the role of miRNAs in the regulation of post-transcriptional protein expression has dramatically altered the view of how target genes are regulated.

The regulatory functions of our body comprise networks. The hypothesis, that miRNAs exert their regulatory function in networks is supported by the high number of non-coding RNAs which are functionally active, e.g. miRNAs that that have been shown to target signaling molecules [5]. Furthermore, some genes encoding miRNAs are closely located (clustered) in the genome (see the "Kidney specific miRNAome" in this review) and in some cases different miRNAs control a single mRNA target or vice versa a single miRNA may influence expression of multiple different target proteins. MiRNA expression profiles during different disease states can be determined by microarray studies. Such systemic approaches together with individual analysis of different miRNAs provide insight into an exciting new regulatory network of the miRNAome.

1.1. Micro RNAs (miRNAs): generation and mechanism of action (Fig. 1)

MiRNAs are generated from endogenous hairpin structured transcripts throughout the genome [2]]. MiRNA encoding genes are transcribed by RNA polymerase II (pol II) providing long precursor transcripts, known as primary miRNAs (pri-miRNAs) [6]. After transcription, still inside the nucleus, Drosha RNase: a type III nuclear ribonuclease cleaves nucleotides from the pri-miRNA, processing it into shorter pre-miRNAs and defining their 3' end. Efficient cleavage requires a double-stranded RNA-binding domain (dsRBD) containing cofactor: DiGeorge syndrome critical region (DGCR)-8. The stem-loop (hairpin) structured pre-miRNA has a characteristic 5' phosphate and 3' hydroxy termini with a two nucleotide 3' single-stranded overhanging end [7]. This end structure is recognized by the nuclear export factor Exportin-5 (Exp5/Xpo5), which uses Ran-GTP as a co-factor [8] and transports the pre-miRNA to the cytoplasm [9]. Further cytoplasmic processing by Dicer (another type III ribonuclease in the cytoplasm) performs a second cleavage at the hairpin structure, and defines the 5' end of the mature miRNA. The Dicer also uses a dsRNA-binding domain (dsRBD) containing cofactor: TAR (HIV-1) RNA binding protein 2 (TRBP2). As a result of the cleavage by Dicer a double-stranded 18- to 25-nucleotide-long miRNA is generated [10]. The mature miRNA is one of the strands of the double stranded (ds)RNA (miRNA/miRNA* duplex). One of the two strands is loaded on an Argonaut family protein (AGO1): the catalytic site of the RNA induced silencing complex (RISC), thus assembling the RISC-ribonucleoprotein complex. Unlike siRNAs which bind to AGO2, miRNAs bind to AGO1.

The guide strand of the miRNA is incorporated into the RNA-induced silencing complex (RISC) [11], and remains stably associated with RISC, becoming the mature miRNA. The opposite (passenger) strand is disposed. The miRNA guides RISC to the target messenger (m)RNA with complementary sequence.

Translation of the target messenger RNA (mRNA) is silenced in case of incompletely complementary sequence, and the mRNA is spliced up (cleaved) by the RISC in case of fully complementary sequence. As endogenous miRNAs often contain mismatches, the more common (primary) mechanism is translational repression: AGO1 does not cleave the mRNA, but binds to it and allosterically inhibits translation. Unlike RNAi induced by siRNA, cleavage (degradation) of the mRNA occurs more seldom, only by complete match between the miRNA and the mRNA.

It is interesting, that many distinct ways exist to obtain post transcriptional gene silencing by miRNA interference. These mechanisms are reviewed and detailed by Eulalio in 12. Briefly protein translation can be inhibited at translation initiation (1) by inhibiting different eukaryotic translation initiation factors (eIF)s or (2) at translation elongation. Furthermore, instead of translation inhibition, (3) co-translational degradation of the nascent polypeptide chain or (4) without interfering with the translation machinery by sequestering and processing mRNAs in discrete cytoplasmic foci: P bodies are possible ways of post-transcriptional gene silencing by miRNA [12].

1.2. MiRNA nomenclature

The continuous discovery of new miRNAs necessitates a consistent gene naming scheme. Therefore, every mature miRNA has a "miR" prefix (precursor miRNAs are denoted with "mir") and a unique identifying number, which are assigned sequentially, in order of discovery. Identical miRNAs have the same identifying number, even between different organisms. The host organism can be designated by an abbreviated 3 or 4 letter prefix (e.g., hsa-miR for *Homo sapiens*, mmu-miR for *Mus musculus*, etc.). Furthermore, identical miRNAs encoded in different chromosomal locations (in case of multiple copies) have numbered suffixes (ascending in order of discovery, (not chromosome number), e.g.: hsa-miR-194-1 and hsa-miR-194-2 are located on chromosome 1 and 11, respectively). Paralogous miRNA sequences, which differ only by one or two nucleotides have lettered suffixes (e.g., hsa-miR-200a, hsa-miR-200b, hsa-miR-200c). Where two different mature miRNAs are processed from the same hairpin precursor, the ending (3' or 5') of the arm of provenance has to be specified (e.g., miR-17-5p, miR-17-3p) or an asterisk can be applied to the less predominantly expressed transcript (strand) (e.g., miR-199*). The miRNA encoding genes are named using the same three-letter prefix, which can be modified according to the conventions of the host organism (capitalization, hyphenation or italics). Nevertheless, online databases also exist (e.g., http://rfam.janelia.org/) to prevent accidental overlap when naming newly discovered miRNAs. The new identifying number will be assigned just after the paper describing the miRNA has been accepted for publication [13,14].

1.3. MiRNA function

MiRNAs are involved in gene regulation in different processes such as embryonic [15] or hematopoetic [16] development, apoptosis [17], or tumor initiation and progression (*miR-17–92* cluster, *miR-21*, *miR-372*) [18].

MiRNAs are involved in many physiological [2] and pathophysiological processes [19]. For example, miRNAs have a crucial role in endocrine functions. *MiR-375*, for instance, is thought to act by inhibiting the expression of myotropin, which induces the exocytosis of insulin granules [20]. It has also been described that specific miRNAs play an important role in the heart during development in mice or in human cardiac conductance [21], cardiomyopathies [22] or hypertrophic growth response.

The most investigated role of miRNAs in nephrology is in oncogenesis. However, the involvement of miRNAs in many other renal diseases is under intense investigation, including diabetic nephropathy, immunologic renal diseases such as allograft rejection and autoimmune renal diseases and genetically determined renal diseases such as polycystic kidney disease.

1.4. Influencing miRNA expression in vivo

Members of the miRNAome are explored in different disease states by genome-wide search tools such as microarrays, and substantial data has been already accumulated in several disease states and organ systems, including the kidney. Data obtained with microarray analysis has to be validated by quantitative qPCR [23]. A new tool: next generation sequencing can also be applied to detect multiple miRNAs from an experimental sample [24]. In many pathological processes, miRNA levels have been found to be up- or down-regulated. A functional investigation of selected miRNAs is ongoing. Presently, experimental strategies aimed at interfering with miRNAome are based on transfection of small, pre-determined nucleic acid sequences into target cells.

Generally, miRNA expression can be influenced similarly to previously established oligonucleotide and/or siRNA technologies: miRNAs are short RNAs such as siRNAs, thus intracellular delivery of a double stranded, short RNA (siRNA) with identical nucleotide sequence will enhance or imitate miRNA function, whereas an siRNA designed to silence a miRNA will knock down the given miRNA effect. Thus, previously acquired experience with so far investigated techniques for siRNA/oligo delivery can be utilized in functional miRNA experiments.

1.5. Nucleic acid therapy — problems and solutions of delivery

The major problem of *in vivo* therapies with nucleic acids (siRNA, miR, ASO, pDNA) (nucleic acid therapy or nucleic acid-based next generation biopharmaceuticals [25] is delivery itself into target organs and target cells [26]. Instability of nucleotides in the extra cellular surrounding and high sensitivity to degradation by nucleases, can lead to inactivation of the applied nucleic acid [27]. Half-life of siRNA in body fluids is ca. 2 min [28]. Furthermore, small molecules are rapidly cleared from the bloodstream by the kidney [29]. Thus, injected small nucleic acids shortly disappear by enzymatic digestion and renal clearance. Finally, nucleic acids are negatively charged, thus they do not likely penetrate cell membranes and enter cells [30]. Strategies to overcome these problems are described in detail by other reviews on short interfering RNA (siRNA) delivery [26,30], for more details, see the RNAi review of this issue by Stokman et al,). Here we shortly list presently known strategies developed to enhance *in vivo* half-life of therapeutic nucleic acids and to promote their delivery to target organs and cellular uptake. Most of these chemical modification strategies have been developed and tested in non-mammalian species such as the zebra fish. They have a theoretical potential in renal research and therapy. If available, we mention renal specific experience and give some more details later in this review in the chapter on functional investigations of miRNAs in the kidney.

1. Physical forces: Naked, unmodified as well as chemically modified nucleic acid delivery can be amplified by enhanced pressure injections (local or systemic: hydrodynamic tail vein injection) — first applied for RNA interference in the kidney by us [31]: the solvent bolus protects from nuclease degradation, and the hydrodynamic pressure forces the nucleic acid into the interstitium of parenchymal organs, and induces pore openings on parenchymal cell membranes. Such pore openings can be enhanced by local application of ultrasound (sonoporation [32] or electric field, similarly to *in vitro* electroporation [33,34].
2. Chemical modifications of the therapeutically applied nucleic acids include modifications of the ribose-phosphate backbone [35], or terminal modifications: addition of functional groups such as methyl, alkyl [36] or cholesteryl groups [29]. Furthermore, more fundamental chemical modifications have been investigated such as morpholinos (nonionic/uncharged DNA analogs, commercially available Vivo-Morpholinos) [37,38] or locked nucleic acid analogues (LNA) [29,39]. LNA modifications have been applied in siRNA [40] as well as in miRNA [41] studies. Although it would be a feasible technique, no information is available yet in the context of influencing miRNA expression of the kidney. Chemical modifications aim to enhance resistance to nuclease enzymatic breakdown, but preserve function. In some cases chemical modifications may lead to loss or reduction of nucleic acid function (unpublished observations).
3. Delivery can be enhanced by packaging the therapeutic nucleic acids into vectors or instead of delivery of the therapeutic nucleic acids themselves, plasmid DNA (pDNA) encoding the therapeutic nucleic acid is applied widely. Nucleic acids or encoding plasmids can be delivered by viral (adenovirus, adeno associated virus, lentivirus) or non-viral: chemical complex delivery systems. Chemical complexes are formed between positively charged polyion complexes (PIC) and negatively charged nucleic acids (for eg.: cyclodextrin [42], poly-ethylene-glycol (PEG)) [43]. Carriers (transfection reagents) include cationic *liposomes* (for eg.: Lipofectamine™ RNAiMax® /Invitrogen/) [44] in which nucleic acids are encapsulated in lipid vesicles, *lipoplexes* (self assembling multi-lamellar lipid complexes) [45,46], or cationic polymers: *polyplexes* [47] (for eg.: poly-ethilene-imine (PEI) [29], polyamines: such as poly-L-lysine or siPORT® /Ambion/). More recently, "nanocarriers" such as carbon *nanotubes* [48], *iron nanoparticles* combined with a magnetic field [49] *or gold nanorods* [50] have also been developed. These vectors or nanocarriers protect the nucleic acids from renal filtration, enzymatic degradation and enhance cellular uptake. Electrostatic surface coating of delivery particles can enhance or target their delivery [51]. However, recently, renal toxicity [52] as well as renal clearance of nanoparticles have been reported [53], thus, before clinical application of these strategies, toxicological investigations will be necessary.
4. Conjugation of the nucleic acid or the nucleic acid-delivery particle with cell surface receptor ligands can enhance cell specificity (targeting ligands) and cellular uptake [27].
5. Therapeutic nucleic acid delivery can be enhanced by depo-products (carriers) with prolonged deliberation of the therapeutic nucleic acid or complex particle such as gelatine [54], hydrogels [55], athelocollagen [56] or chitosan [29]. Such depo products could be deposited under the kidney capsule, for local delivery of miRNA targeting nucleic acid therapy, however, to our best knowledge, this approach has not been tested yet in the context of miRNA.

Functional investigations of miRNAs include miRNA expression blockade with AntiSense Oligonucleotides (ASOs) or enhancement with different nucleic acid structures designed to target any miRNA of interest [164].

Specifically, in order to enhance delivery of nucleic acids aimed to modify miRNA function, the following chemical modifications are applied primarily. *Two'-O-methylation* of the sugar moiety or *phosphorothioate backbone* can provide miRNA analogues with a prolonged half-life without interfering with efficiency and specificity and are thus, the most commonly used chemical modifications for miR delivery.

1.6. Inhibition of miRNA function

MiRNAs can be blocked at multiple levels (Fig. 1). A non-sequence specific, direct method to reduce miRNA activity is to interrupt its synthesis by targeting components of the miRNA biogenesis machinery. However, this method might lead to global reduction of all miRNAs and related side-effects.

More specifically, targeted degradation of the pri-miRNA transcript in the nucleus can be achieved with antisense OligoDeoxyriboNucleotides (**ODN**). RNaseH recognizes RNA–DNA duplexes,

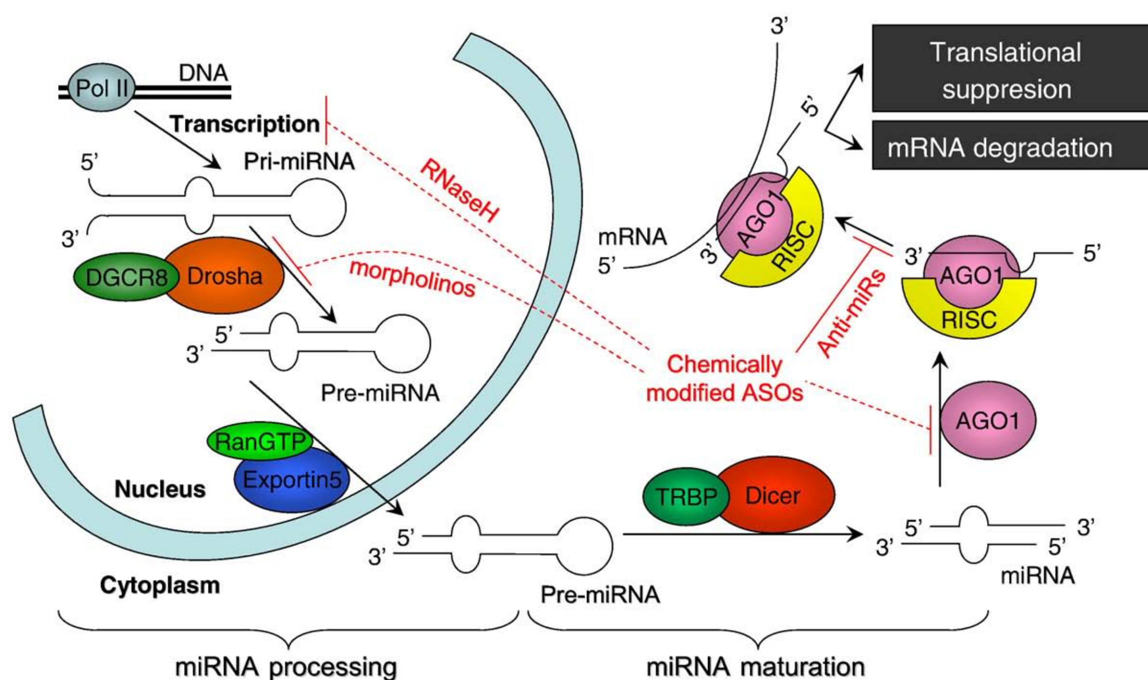

Fig. 1. The miRNA machinery and sites of intervention- Micro RNA biogenesis and function (based on [160–162], reviewed in [163,164]. Pol II: RNA polymerase II. DGCR-8: DiGeorge syndrome critical region (cofactor of Drosha RNase III). RanGTP: cofactor of Exportin-5. TRBP2: TAR (HIV-1) RNA binding protein 2 (cofactor of Dicer (a cytoplasmic RNase III, which cuts the hairpin of the pre-miRNA. RISC: RNA-Induced Silencing Complex. AGO1: Argonaut protein (the catalytic site of the RISC). mRNA cleavage or translational suppression depends on the level of complementarity. Possible interventions (red): chemically modified AntiSense Oligonucleotides (ASOs) can block the RISC active site or inhibit mature miRNA binding to the RISC, but can interfere with miRNA processing early steps as well.

cleaving the RNA strand: the pri-miRNA with ODN complementary sequence [57,165]. However, whether this is an effective approach to target miRNAs requires further study [58]. Targeting the hairpin structure by short interfering (si)RNA, or RNaseH-ODN in the pri-miRNA/pre-miRNA state is not likely to be effective due to difficulties in accessing the loop structure with a short nt sequence and which may be protected by pre-miRNA binding factors [52].

Morpholino modified antisense oligonucleotides (morpholinos, Fig. 2) were also used to target miRNA precursors in zebra fish embryos to inhibit miRNA maturation at Drosha or Dicer processing [59].

The most effective miRNA inhibitors act on the mature miRNA (Fig.1) [58]. Anti-miRNA Oligonucleotides (AMOs) are actually AntiSense Oligonucleotides, a class of ASOs that are chemically engineered short RNAs, which effectively and specifically silence miRNAs. Unlike RNaseH-ODNs, AMOs target mature miRNA in the cytosol, more specifically in the RISC.

1.6.1. Chemical modification

Of AMOs is usually applied to stabilize the AMOs against nuclease degradation, improve affinity for target miRNA and to promote tissue uptake for *in vivo* delivery. Prolongation of *in vivo* half — life of small RNAs is a crucial problem. Furthermore, improving hybridization affinity for the target RNA is necessary, as RISC-bound miRNA has a strong binding capacity for the target mRNA. Possible chemical modifications include *2′sugar modifications, locked nucleic acid (LNA)* as well as *phosphorothioate backbone* modifications of the AMO (reviewed in [185]). All of the 2′ modifications improve affinity to target RNA. The phosphorothioate backbone, reduces target affinity, however provides resistance to nuclease degradation, and is thus, usually applied for *in vivo* delivery of AMOs. Krüztfeldt and colleagues used *2'-O-methylated* (*2'-OM*) sugar, phosphorothioate backbone and a cholesterol moiety containing a *single — stranded* RNA (also called antagomir) [60]. Three low volume (end volume = 0.2 ml) tail vein injections significantly reduced *miR-16, miR-122, miR-192* and *miR-194* expression *in vivo* in many target organs: lung, liver, heart, intestine, bone marrow, ovaries and adrenals including the kidney [61]. Furthermore, they also characterized the properties and function of AMOs in mice. They demonstrated that AMOs require a length >19-nt for highest efficiency to discriminate between a single nucleotide mismatch of the target miR [61].

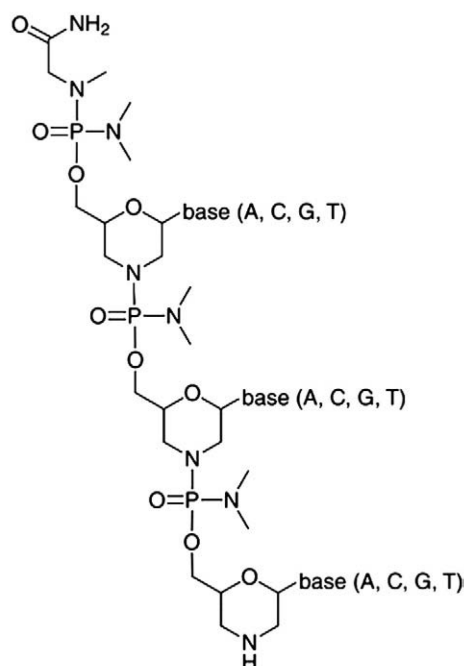

Fig. 2. (A) The structure of a Morpholino 3-mer [165].

1.6.2. Locked nucleic acids (LNAs)

are a class of nucleic acid analogues, with high binding affinity to complementary mRNA targets leading to mRNA inhibition. Strong RNA binding ability [61] of LNA enable their utilization to inhibit miRNAs [62]. Similar to the 2'-OM AMO approach, LNA AMOs prevent miR–RISC interaction [63]. LNA AMOs enable specific miRNA detection by northern blot analysis [64] and in situ hybridization [65]. LNA AMOs have already been successfully used for inhibition of miRNA function *in vitro* [66] and might be utilized in cancer diagnostics and therapeutics [67]. LNA AMOs, injected intravenously, effectively antagonized *miR-122* in mouse liver [68] and non-human primates [40]. Depletion of *miR-122* by tail vein injection of unconjugated and phosphorothioated AMOs into mice reduced plasma cholesterol without toxicity. Furthermore, intraperitoneal injection of phosphorothioate backbone LNA AMO was also efficient [64]. Inhibition of *miR-21* was similarly effective with 2'-OM, LNA AMO or cationic liposomes [69]. The 2'sugar, phosphorothioate backbone and LNA AMOs are commercially available.

1.6.3. AMOs function

According to one hypothesis, AMOs bind to the single stranded sense miRNA loaded into the RISC, hence preventing miRNA-RISC binding to the complementary mRNA [70]. Another hypothesis is that they interfere with miRNAs (complementary pairing) before loading into the RISC [58]. Recently, miRNA-AMO duplexes were demonstrated to degrade in a distinct cytosolic compartment from processing (P) — bodies, thus antagomir induced miRNA degradation is probably independent of previously described RNA interference (RNAi) pathways [61]. However, further research is necessary to elucidate the acting mechanisms of these molecules and to discover further methods of miRNA regulation [162]. The formation of stable heteroduplexes between LNA AMO and miRNA can be detected by northern analysis [71].

1.7. Enhancement of miRNA function

Besides inhibition, enhancement of miRNA function is also possible by enhancing endogenous miRNA function or by inserting short, double stranded RNA sequences (mimics) into cells with an identical nt sequence to the target miRNA.

Restoring miRNA function is important if pathologic processes are coupled with miRNA loss of function or reduced expression. Based on structural–functional homologies, exogenous short interfering RNAs (siRNAs) introduced into target cells may function as regulatory miRNAs.

1.7.1. Delivery of shRNA coding vectors

To experimentally induce a miRNA function, cells or organs are transfected with miRNA encoding short hairpin RNAs (shRNAs: pre-miRNA hairpin sequences) that mimic natural miRNA molecules. Following intracellular delivery, pre-miRNA hairpin sequences are processed into mature miRNAs by Dicer. Short hairpin RNA coding vectors provide a powerful method for miRNA expression [72]. Thus, transfection with pre-miRNA hairpin sequences mimic or increase the desired miRNA effects.

Besides delivery, another road-block to nucleic acid therapy is the incompletely mapped side-effect spectrum. Possible side-effects can be off-target effects including the induction of the antiviral interferon response, or sequence mismatched silencing of other miRNAs or mRNA-protein expression. Furthermore, it has been reported, that overloading the endogenous miRNA machinery may be harmful, even lethal [73]. However, optimal dosing may circumvent this problem [74]. Regarding clinical applications, presently, lethal diseases such as cancer or diseases of compartmentalized organs such as the eye or lung are the primary targets of nucleic acid therapy. These compartmentalized organs have the advantage, that they can be accessed directly (i.e. nose, eye) and not only through the systemic circulation, thus systemic side effects such as the interferon response or off-target silencing in not targeted organs can be avoided. Direct access may also enable more efficient delivery, and protection from RNase degradation in the blood. Furthermore, miRNA regulation in endocrine systems such as pancreatic insulin production has been investigated experimentally in rodents.

1.8. Kidney specific miRNAome, renal disease specific alterations, and functional investigations of miRNAs in the kidney

Human and murine kidney — specific miRNA expression profiles have been already reported. The initial studies on miRNA expression in the kidney involved the isolation, detection, and validation of miRNAs from the whole kidney. Sun et al. compared miRNA expression in six different human organs, including the kidney, and found a highly kidney specific miRNA cluster which consist of *miR-192, 194, 204, 215 and 216*. They demonstrated, that *miR-194-1* and *miR-215* are both located on chromosome 1, at only 195 bp distance. Moreover, high-sequence homology was found between the precursor miR sequences of *miR-215* and *miR-192*. Interestingly *miR-192* is just 109 bp upstream of *miR-194-2*, on chromosome 11, and these two miRNAs could be regulated as a common transcriptional unit [75]. Another miRNA cluster related to the kidney was found by Sawera et al. They demonstrated that all the precursors and most of the mature miRNAs of the porcine *miR-17-92 cluster* were expressed in the kidney. Some of the precursors were also expressed in cerebellum, cortex, hippocampus and liver. The *mir-17-92* micro RNA cluster (represented by *miR-17, 18, 19a/b, 20, 25, 92, 93* and *106a/b*) is of particular interest, because of its evolutionary conservation [76]. In a study where a homology search was conducted using human miRNAs to query the pig genome, two of the miRNAs previously associated with kidney (*miR-92* and *miR-194*) and two other (*miR-31* and *miR-210*) were expressed in porcine kidney [77]. Another study, this time on mice, demonstrated that *miR-10b* and *miR-200b* were expressed exclusively in kidney, and *miR-192*, together with *miR-194*, was expressed both in kidney and liver [78]. Jin et al. reported that *miR-30* and *miR-200* were highly expressed in bovine kidney; meanwhile *miR-23b* and *miR-99a* were expressed in multiple tissues (muscle, kidney, liver, spleen, thymus, fat and brain). It is important to mention, that though several studies suggested that most miRNAs are conserved among related species, other studies provided evidence that many miRNAs are species specific [79].

Further research has been conducted in order to identify local miRNA expression profiles. This has shown that some miRNA are only present or are predominant in the cortex while others preferentially localize to the medulla, suggesting functional differences (Table 1.) [80].

Boggs et al. evaluated the expression of the *miR-17-92 cluster* in canine renal cortex and medulla. *MiR-17-3p* and *5p* had the highest expression in the renal medulla, though it was also present in the cortical region. Furthermore *miR-19a/b*, *miR-20* and *miR-92*, while present in both regions, were more prevalent in the cortex. *MiR-18* was expressed only in the renal cortex [81]. Another study used microarray and proteomic techniques to analyze the cortex and the medulla of rat kidneys and to obtain experimental evidence for predicted micro RNA targets. The most abundant miRNAs expressed in both regions were *let-7a/b/c* and *miR-26a*. In the renal medulla *miR-27a/b, miR-125 a/b* and *miR-200b/c* were most highly expressed, whereas *miR-192, miR-194* and *miR-203* expression was predominant in the renal cortex. Based on simultaneous proteomic expression profile changes cortical and medullar miRNA-target protein pairs were established by computational algorithms suggesting a role of cortical miRNAs in oxidative stress related processes [81].

Mapping the renal miRNAome with expression array studies was the first step. Next, miRNA expression patterns typical of diseases were explored, to provide information about the functional role of

Table 1
Localization of some renal miRNAs.

miR	Kidney expression pattern	References
Let-7a/b/c	Whole kidney	[75]
miR-10a/b	Whole kidney	[73]
miR-23	Whole kidney	[74]
miR-26a	Whole kidney	[75]
miR-30	Whole kidney	[74]
miR-31	Whole kidney	[72]
miR-99	Whole kidney	[74]
miR-204	Whole kidney	[70]
miR-210	Whole kidney	[72]
miR-215	Whole kidney	[70]
miR-216	Whole kidney	[70]
miR-18	Cortex	[74,76]
miR-19	Cortex>medulla	[71,76]
miR-20	Cortex>medulla	[71,76]
miR-92	Cortex>medulla	[71,72,76]
miR-192	Cortex>medulla	[70,75]
miR-194	Cortex>medulla	[70,72,75]
miR-203	Cortex	[75]
miR-27a/b	Medulla	[75]
miR-125a/b	Medulla	[75]
miR-17	Medulla>cortex	[71,76]
miR-200	Medulla>cortex	[73–75]

miRNA in disease. The microarray based studies generally identify large numbers of deregulated miRNAs in different pathologies. Therefore in this review we mention just those miRNAs which were further studied by the authors of the respective studies, or those which expression level had the greatest fold change value.

Probably the most investigated renal miRNA expression profile changes are those which occur during diabetic nephropathy (DN) [161]. Due to the pandemic increase in type 2 diabetes DN became the leading cause of renal failure. Thus, it is imperative to better understand the pathomechanisms of DN, which will lead to improved targeted therapies. *MiR-375* is an important regulator of insulin secretion: it has been identified as a pancreatic islet cell specific miRNA in mice, and it regulates insulin secretion by targeting myotropin [20]. Furthermore, *miR-21* was downregulated in early DN, thus chemically synthesized *miR-21*-containing plasmids were transfected into mesangial cells by viral vectors, leading to efficient elevation of *miR-21* expression. Upregulation of *miR-21* inhibited mesangial cell proliferation by targeting a phosphatase and tensin homolog (Pten), hence increasing levels of PIP3 and activation of Akt [82]. Akt activation was also achieved by *miR-216a* and *miR-217* upregulation through miRNA mimic oligonucleotides. These two miRNAs are co-expressed in the presence of high transforming growth factor (TGF)-β levels and function as Pten inhibitors [83]. Nevertheless, TGF-β produced by myofibroblasts in renal fibrosis is responsible for epithelial-mesenchymal transformation (EMT) and thus, is an important pro-fibrotic cytokine [84]. Consequently, many studies focused on the miRNA signaling network related to TGF-β secretion in DN. In mouse models (streptozotocin [85] induced as well as in genetically diabetic db/db [86]) of DN glomerular expression of *miR-192* was elevated and was accompanied by TGF-β - overproduction. TGF-β first inhibits zinc finger E-box binding home box 2 (ZEB2/SIP1) via *miR-192*, leading to E-box de-repression and collagen 1-α 1 and 2 (Col1a1 and 2) synthesis during diabetic nephropathy [87]. Subcutaneous injection of anti-*miR-192* oligo inhibited *miR-192* accumulation in diabetic mouse renal cortex and consequently RP23, *miR-216a, miR-217* and Col1a2 levels decreased, supporting the hypothesis that *miR-192* functions as a main regulator of other DN related miRNAs, and thus may have a therapeutic potential in DN [88]. The *miR-200* family is also upregulated by TGF-β and can also inhibit E-box suppressors, hence maintaining collagen synthesis [88]. In both human and mouse mesangial cells exposed to high glucose concentrations *miR-377* was highly up-regulated [89] suppressing its target, superoxide dismutase (SOD) 1/2, suggesting a role for *miR-377* in the regulation of oxidative stress. The altered antioxidant capacity could lead also to the observed increased fibronectin expression in diabetic nephropathy [90]. Furthermore, transgenic over expression of *miR-17* repressed fibronectin expression in mice, suggesting a possible therapeutic approach [90].

Renal fibrosis is the final common pathway of end stage renal disease leading to renal failure in many different renal diseases such as diabetic, hypertensive or chronic allograft nephropathy. Renal fibrosis is usually initiated with glomerular damage, with podocyte detachment and focal sclerosis marked by urinary albumin loss. Albuminuria may induce subsequent tubular damage. In regulation of glomerular ultra filtration a number of podocyte associated miRNAs have been implicated. Podocytes are highly differentiated cells which are implicated in many progressive renal diseases and are responsible for maintaining the glomerular architecture and synthesis and composition of the slit diaphragm and the glomerular basement membrane, which compose the glomerular filtration barrier. Podocyte specific deletion of Dicer resulted in podocyte apoptosis, with consequent glomerular damage and proteinuria [91,92]. *MiR-23b, 24, 26a,* and *30* seem to be responsible for podocyte homeostasis [93,94]. Since its discovery, epithelial-mesenchymal transformation (EMT) is regarded as a key contributor to the progression of renal fibrosis [95]. *In vitro* studies have demonstrated a role for *miR-200* and *miR-205* in EMT. In accordance with previous studies [82], the target proteins of these two miRNAs were found to be ZEB1 and SIP1 (ZEB2) [96], which mediated EMT through the repression of the E-boxes in the E-caderin promoter [90].

One of the earliest associations between miRNAs and disease was made in the field of oncology. However, many aspects of this research can be applied to renal diseases such as fibrosis. Kort and colleagues found that *miR-17-92 cluster* (oncomiR-1) were upregulated in Wilm's tumor (WT) [97]. *MiR-34a*, a frequently identified miRNA in renal cancer was proved to be induced by the tumor suppressor gene product p53 [98]. CpG methylation of the *miR-34a* promoter was detected in several different cancer types leading to loss of *miR-34a* expression. Re-expression and inducible expression of *miR-34a* with a retroviral vector expressing pri-*miR34a* cDNA induced senescence and cell cycle arrest in carcinoma cell lines, demonstrating that, *miR-34a* is a tumor suppressor gene inactivated by CpG methylation and subsequent transcriptional silencing in a broad range of tumors including renal cancer [99]. *MiR-34* was over-expressed and associated with cancer cell proliferation in other renal cell carcinoma studies [100,101]. Some of the most commonly deregulated miRNAs (*miR-20a, 21* and *106a*) can modulate von Hippel-Lindau tumor suppressor (VHL) gene. Moreover, some RCC associated miRNAs (*miR-21, 26a, 27a, 106a* and *210*) can be induced also by hypoxia. Thus, hypoxia inducible factor 1, alpha subunit (HIF1α) is a potential target for the downregulated *miR-199**. Also, the expression of platelet-derived growth factor beta (PDGF-ß) polypeptide could be influenced by *miR-29*. These data highlight the importance of miRNA regulation in cancer angiogenesis [102] and renal fibrosis. In contrast to EMT modifications, in clear cell cancer *miR-141* and *miR-200c* were downregulated leading to E-cadherin over expression, while *miR-221* and *miR-22* were differentially expressed in chromophobe renal carcinoma [103]. Many other miRNAs were found to be upregulated in different RCC studies: *let-7f-2, miR-7-2, miR-28, miR-185* [104], *miR-32* [105], *miR-155* [94], *miR-17* and *miR-221* [106] suggesting that multiple miRNAs are involved in post-transcriptional miRNA regulation of gene expression in certain pathologic processes.

Differential miRNA expression has also been demonstrated in polycystic kidney disease (PKD) [107]. *MiR-31* and *miR-217* were downregulated, but *miR-21* was upregulated in PKD [101]. *MiR-15a* deficiency in PKD is responsible for upregulation of the cell cycle regulator: cyclin dependent kinase 25a (Cdc25a) leading to cystogenesis

[108]. Another regulator of cell proliferation: PKD2 is regulated by PKD-upregulated *miR-17* [109].

Lupus nephritis (LN) specific miRNAs are also of interest, since there is no curative therapy available [110]. Initial studies conducted by Dai et al. revealed that several miRNAs (*miR-184, miR-196a, miR-198* and *miR-21*) might be used in systemic lupus erythematosus (SLE) diagnosis as biomarkers. A further comprehensive study of (class II) human lupus nephritis identified 36 upregulated and 30 downregulated miRNAs in LN biopsies compared to healthy control subjects [111]. The same research group also studied miRNA profile in IgA nephritis and found 65 miRNAs with significant different expression levels (most downregulated: *miR-150, miR-615* and *miR-296*; most upregulated: *miR-124a, miR-662* and *miR-130b*) [112]. Also in IgA nephropathy Wang et al. found under expressed *miR-200c* and high expression levels of *miR-192, miR-205* and *miR-141* [113].

Another important process which is related to the immunological response and involves miRNAs is kidney transplantation. Microarray analysis in allograft biopsy specimens sustained the argument that miRNA expression patterns could be valuable biomarkers in clinical transplantation by reflecting the allograft status [109] Sui et al. identified 20 miRNAs differently expressed in acute rejection after renal transplantation [114]. These data may also help to better understand the pathophysiologic background of kidney graft rejection (Table 2).

Target validation: V: validated; pV: previously validated; P: predicted; AC: anti-correlated.

Target and Possible role: TGF-b: Transforming growth factor beta; IgA: Immunoglobulin A; Cdc25: cell division cycle 25 homologe; 3' UTR: 3' untranslated region; HIF: hypoxia inducible factor; mTOR: mechanistic target of rapamycin (serine/threonine kinase); VEGF: vascular endothelial growth factor; VHL: von Hippel-Lindau tumor suppressor; E2F1: Elongation 2 transcription factor 1; PTEN: phosphatase and tensin homolog; PI3K: phosphoinositide-3-kinase; Akt: serine/threonine-protein kinase (Ak (mouse strain) transforming protein: murine thymoma viral oncogene homolog 1); PDCD4: programmed cell death protein 4; TPM1: tropomyosin 1; SLC: solute carrier family (sodium/potassium/chloride transporters); TCF21: transcription factor 21; BAK1: B-cell CLL/lymphoma 2-antagonist/killer 1; Ezh2: enhancer of Zeste homolog 2; SPATA2: spermatogenesis associated protein 2; OGT: O-linked N-acetylglucosamine transferase; POLE4: polymerase (DNA-directed), epsilon 4; RSBN1: rosbin, round spermatid basic protein 1; KIAA1920: chondroitin sulfate proteoglycan 4 pseudogene 5 (CSPG4P5); Col4a2: collagen, type IV, alpha 2 chain; NKCC-2: Na-K-2Cl cotransporter; CycE: Cyclin E; CDK: cyclin-dependent kinase; SFRP1: secreted frizzled-related protein 1; ZEB2: zinc finger E-box binding homeobox 2 (ZFHX1B, other Designations: Smad-interacting protein 1, SIP1); SEMA6A: Semaphorin-6A (sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A); CD3: cluster of differentiation 3 (lymphocyte cell surface marker); LRRC2: leucine rich repeat containing 2; PARP8: poly (ADP-ribose) polymerase family, member 8; ZNFN1A4: IKAROS family zinc finger protein subfamily 1A, 4 (Eos); PCDHA: protocadherin alpha subfamily; PTPN13: protein tyrosine phosphatase, non-receptor type 13; RHOA: ras homolog gene family, member A; FN1: fibronectin 1; RAP1B: ras-associated protein 1b; GRIK2: glutamate receptor, ionotropic, kainite (Kainic acid) 2; KIT: cytokine receptor (CD117) tyrosine kinase with a a kinase insert (KI) (Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog); KLK: kallikrein-related peptidase; ERBB4: v-erb-a erythroblastic leukemia viral oncogene homolog 4; SOD: superoxide dismutase; PAK1: p21 protein (Cdc42/Rac)-activated kinase 1; EYA1: eyes absent homolog 1; HMGA2: high mobility group AT-hook 2; IMP1: insulin-like growth factor 2 mRNA binding protein 1; ARID3B: AT rich interactive domain 3B; HIC2: hypermethylated in cancer 2; GM632: zinc finger protein 512B (Znf512b); CDH1: cadherin 1; eGFR: estimated Glomerular Filtration Rate; Smad3: SMA (a Caenorhabditis elegans protein) – and MAD (mothers against decapentaplegic – a drosophila protein) related protein, (transforming growth factor beta receptor signaling molecules); α-SMA: alpha smooth muscle actin; ECM: extracellular matrix; DN: Diabetic nephropathy.

How this table was made: we searched the term “kidney microRNA” on the NCBI PubMed database and selected the articles about miRNA profile change in renal pathologies. From the studies publishing micro- or qPCR array data, we have taken into consideration only the first three most up- and down-regulated miRNAs. In this table only those miRNAs are included which are mentioned by two or more studies, or which have validated targets.

1.9. Diagnostic utilization of miRNAs:

Since numerous miRNAs appear to be disease specific, miRNA expression profiles or specific miRNA levels may be useful diagnostic or prognostic markers. Research of malignant diseases is in the spotlight. Biochips containing human cancer related miRNAs [145] are commercially available. Huang and colleagues described miRNA (*miR-29a, miR-92a*) plasma level with diagnostic relevance in advanced colorectal cancer [146]. Other miRNAs (*miR-146a, miR-223*) appear to be specific and sensitive biomarkers for sepsis but not systemic inflammatory response syndrome (SIRS) [147]. Furthermore, *miR-1* may be a novel biomarker for the diagnosis of acute myocardial infarction, without association with age, gender, blood pressure or diabetes mellitus [148]. For the detection of miRNAs as biomarkers *in vivo* real-time PCR (RT-PCR) can be used for quantitation of circulating miRNAs in the blood [149].

1.10. Non-renal applications: further functional investigations of miRNAs in vivo

In the previous chapter we summarized in-vivo functional investigations of miRNA function in the kidney. In this paragraph, we present a few papers dealing with functional investigations of miRNAs in vivo. Although, these studies are not related directly to the kidney, the described delivery methods could be applied to the kidney.

The primary focus of functional investigations of miRNAs is in the field of cancer research — oncomiRs. For more details on oncomiRs see the review by Cho [150].

Adeno-associated vector (AAV) infect both dividing, and non-dividing cells, can achieve high titers, and thus are a useful tool to deliver AMOs into parenchymal organs such as the kidney and to interfere with miRNA systems.

AAV efficiently delivered *miR-26* into hepatocytes, in mice, preventing the development of *liver cancer* through induction of tumor cell apoptosis [151,152]. Furthermore, AAV delivery of miRNA-based shRNA inhibition successfully prevented the development of an autosomal dominant *retinopathy* in mice by inhibition of peripherin-2 gain-of-function mutation [153].

Non-viral delivery has been also applied efficiently to target the miRNAome in a murine model of human prostate cancer. Synthetic *miR-16* was delivered successfully with atelocollagen. Atelocollagen is used for wound healing, vessel prosthesis or as a haemostatic agent [154]. Atelocollagen is obtained from collagen by pepsin treatment to lower its immunogenicity by freeing it from the highly antigenic telopeptides [155]. *MiR-16* was also administered as atelocollagen-*miR-16* complex *in vivo* via tail vein injection in a mouse model of prostate cancer. Treatment reduced cell proliferation and suppressed prostate tumor growth by regulating cell-cycle control associated cyclin-dependent kinase (CDK)1-2. This study demonstrated, that atelocollagen can efficiently deliver active miRNAs *in vivo* [156]. For further information on the role of miR-16 in oncogenesis see the recent review by Aqeilan RI et al [157].

Table 2
Reference table, summarizing deregulated miRNAs and their possible roles in different renal pathologies.

miR	Expression	Detection method	Kidney pathology	Target	Target validation	Possible role	Reference
15a	Down	MA, qPCR	Polycystic kidney disease	Cdc25A [3' UTR)	V	Cell proliferation, cyst growth	[115]
17-92 cluster/oncomiR-1	Up	MA, NB, qPCR	Renal carcinoma	HIFs, mTOR, VEGF and VHL	P		[116]
				E2F1	pV	Apoptosis, cell cycle regulation	[117]
						Cell proliferation	[118]
21	down	MA, qPCR	Diabetic nephropathy	PTEN (3' UTR)	V	Mesangial cells PI3K/Akt signal pathway	[119]
	up	MA	Diabetic nephropathy (TGF-b, glucose)				[120]
		MA, qPCR	Polycystic kidney disease				[121]
			Renal cell carcinoma	VHL, PDCD4, TPM1	P, pV	Induced by hypoxia	[122]
						Associated with carcinogenesis	[123]
				SLC12A1, TCF21	P, AC		[124]
26a	Down	MA	Renal cell carcinoma	BAK1, Ezh2, PTEN	pV	Induced by hypoxia, anti-apoptotic	[132]
27	Up	MA	Renal cell carcinoma	SPATA2, OGT, POLE4, RSBN1, KIAA1920	P	Induced by hypoxia	[125,132]
29a	Down	MA, NB, qPCR	Diabetic nephropathy (TGF-b, glucose)	Col4a2 (3'UTR)	V		[130]
29b	Up	MA, qPCR	Salt induced hypertension	Collagen genes (3' UTR)	V	Protection from renal medullary injury	[126]
30 family	Up	MA	Diabetic nephropathy (TGF-b, glucose)				[130]
30a-3p	Down	qPCR	Renal allograft acute rejection	NKCC-2	AC	Predicts renal graft function	[127]
34a	Up	NB, qPCR	Cisplatin induced acute kidney injury	CycE, CDK4, CDK6 and Cdc25C	pV	Cytoprotective	[128]
		rtPCR, qPCR	Renal cell carcinoma			Cell proliferation	[129]
						Oxidative stress	[133]
				SFRP1	P, AC		[134]
93	down	MA, NB, qPCR, iSH	Diabetic nephropathy	VEGF-A (3' UTR)	V		[130]
122	Up	MA, qPCR	Diabetic nephropathy				[129]
			Renal cell carcinoma				[132]
124a	Up	MA	IgA nephropathy				[131]
			Lupus nephritis			Potential diagnosis biomarker of lupus nephritis	[132]
141	Up	qPCR	Hypertensive Nephrosclerosis				[133]
			IgA nephropathy			Correlated with Vimentin	[134]
	Down	MA, qPCR	Renal cell carcinoma	ZEB2 (SIP1)	P	Transcriptional modulator for CDH1/E-cadherin	[135]
							[133]
						Tumor suppressor	[136]
				SEMA6A	P, AC		[134]
142-3p	Up	qPCR	Renal allograft acute rejection	CD3	AC	Predicts renal graft function	[137]
			Renal cell carcinoma				[133]
				LRRC2	P, AC		[134]
145	Down	MA, NB	Renal clear cell carcinoma	PARP-8	pV	Apoptosis	[127]
150	Down	MA	IgA nephropathy				[141]
			Lupus nephritis			Potential diagnosis biomarker of lupus nephritis	[142]
			Renal cell carcinoma				[132]
155	Up	qPCR	Renal allograft acute rejection	CD3	AC	Predicts renal graft function	[137]
			Renal cell carcinoma			associated with carcinogenesis	[133]
185	Up	MA, qPCR	Renal cell carcinoma	ZNFN1A4, SLC16A2, PCDHAC2, PCDHAC1, PCDHA8	P		[135]
				PTPN13	P, AC		[134]
192	Down	MA, qPCR	Diabetic nephropathy	ZEB1,2	AC	In "late presenter" DN patient; correlates with E-cadherin, tubulointerstitial fibrosis and reduction in eGFR	[137]
		qPCR	Renal fibrosis (TGF-b)	ZEB2 (3' UTR)	V	Regulate the transcription of E-cadherin	[138]

(continued on next page)

Table 2 (*continued*)

miR	Expression	Detection method	Kidney pathology	Target	Target validation	Possible role	Reference
	Up	MA, qPCR	Renal fibrosis (TGF-b)			Downstream mediator of TGF- /Smad3	[139]
			Diabetic nephropathy (glucose)				[140]
		qPCR	Diabetic nephropathy (TGF-b)	Z (3' UTR)	V	Collagen inducer	[141]
			Hypertensive Nephrosclerosis				[143]
			IgA nephropathy			Correlated with GFR decline rate and glomerular scar	[144]
199a*	Down	MA, qPCR	Renal cell carcinoma	HIFa	pV		[132]
200a	Down	MA	Renal cell carcinoma				[146]
	Up	qPCR	Hypertensive Nephrosclerosis			Correlated with ZEB2, α-SMA and Fibronectin	[143]
200b	Up	qPCR	Hypertensive Nephrosclerosis			Correlated with ZEB2, FSP-1 and Fibronectin	[143]
200c	Down	qPCR	IgA nephropathy			Correlated with E-cadherin and proteinuria	[144]
		MA, qPCR	Renal cell carcinoma	ZFHX1B (SIP1, ZEB2)	V	Transcriptional modulator for CDH1/E-cadherin	[145]
	132,133						
						Tumor suppressor	[146]
				VEGF	P, AC		[134]
205	Up	qPCR	Hypertensive Nephrosclerosis			Correlated with ZEB2, α-SMA	[143]
			IgA nephropathy			Correlated with GFR and tubulointerstitial scar	[144]
210	Up	MA, qPCR	Renal cell carcinoma				[145]
						Induced by hypoxia, anti-apoptotic	[132]
						Oxidative stress, hypoxia	[133]
215	Up	qPCR	diabetic nephropathy (TGF-b)				[151]
	Down	qPCR	Renal fibrosis (TGF-b)	ZEB2 (3' UTR)	V	Regulate the transcription of E-cadherin	[148]
216a	Up	qPCR	Diabetic nephropathy (TGF-b)	PTEN (3' UTR)	V	ECM gene expression, cell survival and hypertrophy	[142]
217	Up	qPCR	Diabetic nephropathy (TGF-b)	PTEN (3' UTR)	V	ECM gene expression, cell survival and hypertrophy	[152]
	Down	MA	Polycystic kidney disease	RHOA, FN1, RAP1B, GRIK2	AC		[131]
221	Specific	MA, NB	Renal clear cell carcinoma	KIT	pV	Tumor progression	[127]
223	Down	MA	Lupus nephritis			Potential diagnosis biomarker of lupus nephritis	[142]
	Up	qPCR	Renal allograft acute rejection	CD3	AC	Predicts renal graft function	[137]
224	Up		Renal cell carcinoma	KLK1	AC		[143]
	Up	qPCR	Renal clear cell carcinoma	ERBB4	P, AC		[134]
296	Down	MA	IgA nephropathy				[141]
	Down	MA	Lupus nephritis			Potential diagnosis biomarker of lupus nephritis	[142]
337	Up	MA, qPCR	Diabetic nephropathy (glucose)				[150]
			Renal fibrosis (TGF-b)				[149]
			Diabetic nephropathy (glucose)	SOD1, SOD2, and PAK1 (3' UTR)	V	Mesangial cell response to the diabetic milieu	[150]
562	Down	qPCR	Wilms' tumor	EYA1 (3' UTR)	V	Cell survival and proliferation	[144]
let-7f	Up	MA	Renal cell carcinoma	HMGA2, IMP1, ARID3B, HIC2, GM632	P		[135]
				KLK10	V		[153]

Abbreviations: detection methods: MA: Microarray; qPCR: real-time quantitative PCR; rtPCR: reverse-transcription PCR; NB: Northern blot; iSH: in situ hybridization.

Another transfection reagent: polyamine mixtures are widely used to deliver nucleic acid therapy. In a mouse model of human non-small-cell lung cancer *let-7b* AMO has been successfully delivered with siPORT® an amine transfection reagent (Ambion). Furthermore local delivery (intratumoral injection) of *let-7b* inhibited tumor growth and proliferation by both siPORT and lentiviral delivery [158]. *Let-7* seems to act by modulating apoptosis and cancer stem cell differentiation in both lung and breast cancer [159].

Similarly, renal diseases such as fibrosis may profit from the delivery of specific miRNA regulating cell-cycle or apoptosis in myofibroblasts, or controlling epithelial mesenchymal transformation. With better understanding of miRNA function in cell differentiation processes, stem cells

used for therapy of renal diseases could be directed to differentiate into lost, highly specialized renal cells (for e.g. podocytes).

2. Conclusion

The role of miRNAs is currently under intense investigation in many disease areas. After detecting expression profiles, research is now trying to influence expression of miRNA in different disease states. The kidney seems to have its own miRNA network, and disease-specific alterations may provide future diagnostic tools and therapeutic targets.

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