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Citation for final published version:

Wonnacott, Alexa , Denby, Laura, Coward, Richard J. M., Fraser, Donald J. and Bowen, Timothy 2022. MicroRNAs and their delivery in diabetic fibrosis. *Advanced Drug Delivery Reviews* 182 , 114045. 10.1016/j.addr.2021.114045

Publishers page: <http://dx.doi.org/10.1016/j.addr.2021.114045>

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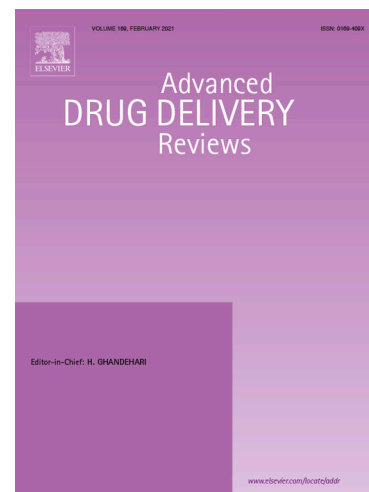
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PII: S0169-409X(21)00438-5
DOI: <https://doi.org/10.1016/j.addr.2021.114045>
Reference: ADR 114045

To appear in: *Advanced Drug Delivery Reviews*

Received Date: 19 March 2021
Revised Date: 21 September 2021
Accepted Date: 4 November 2021



Please cite this article as: A. Wonnacott, L. Denby, R. JM Coward, D.J. Fraser, T. Bowen, MicroRNAs and Their Delivery in Diabetic Fibrosis, *Advanced Drug Delivery Reviews* (2021), doi: <https://doi.org/10.1016/j.addr.2021.114045>

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MicroRNAs and Their Delivery in Diabetic Fibrosis

Alexa Wonnacott^a, Laura Denby^b, Richard JM Coward^c, Donald J Fraser^a, Timothy Bowen^{a,*}

^a Wales Kidney Research Unit, Division of Infection & Immunity, School of Medicine,
College of Biomedical and Life Sciences, Cardiff University, Heath Park, Cardiff CF14 4XN, UK

^b Centre for Cardiovascular Science, Queen's Medical Research Institute,
University of Edinburgh, Little France Crescent, Edinburgh EH16 4TJ, UK

^c Bristol Renal, Dorothy Hodgkin Building, Bristol Medical School,
University of Bristol, Bristol BS1 3NY, UK

* Corresponding author at: Wales Kidney Research Unit, Division of Infection & Immunity, School of Medicine, College of Biomedical and Life Sciences, Cardiff University, Heath Park, Cardiff CF14 4XN, UK. Tel.: +44 29 2184 8467.
E-mail address: bowent@cardiff.ac.uk (T. Bowen).

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Abstract

The global prevalence of diabetes mellitus was estimated to be 463 million people in 2019 and is predicted to rise to 700 million by 2045. The associated financial and societal costs of this burgeoning epidemic demand an understanding of the pathology of this disease, and its complications, that will inform treatment to enable improved patient outcomes. Nearly two decades after the sequencing of the human genome, the significance of noncoding RNA expression is still being assessed. The family of functional noncoding RNAs known as microRNAs regulates the expression of most genes encoded by the human genome. Altered microRNA expression profiles have been observed both in diabetes and in diabetic complications. These transcripts therefore have significant potential and novelty as targets for therapy, therapeutic agents and biomarkers.

Keywords: microRNA; diabetes; fibrosis; chronic kidney disease, noncoding RNA; nanotechnology, microRNA-based therapeutics

Abbreviations: AAV, adeno-associated virus; ACE, angiotensin-converting enzyme; Ad, adenovirus; ADPKD, autosomal dominant polycystic kidney disease; AGE, advanced glycation end-product; AKI, acute kidney injury; AKT2, RAC-beta serine/threonine-protein kinase; APC, activated protein C; ARB, angiotensin II receptor blocker; ASO, antisense oligonucleotide; CDP, cyclodextrin-containing polycations; CKD, chronic kidney disease; DKD, diabetic kidney disease; DPP-4, dipeptidyl peptidase-4; ECM, extracellular matrix; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERBB4, ERB-B2 receptor tyrosine kinase 4; EV, extracellular vesicle; FOXO, forkhead box transcription factor; GFR, glomerular filtration rate; GLP-1 RA, glucagon-like peptide-1 receptor agonist; HCV, hepatitis C virus; IL, interleukin; JAK/STAT, janus kinase/signal transducer and activator of transcription; LNA, locked nucleic acids; LV, lentivirus; mRNA, messenger RNA; miRNA, microRNA; MMP, matrix metalloproteinase; MRA, mineralocorticoid

receptor antagonist; mTOR, mammalian target of rapamycin; NF- κ B, nuclear transcription factor- κ B; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; NP, nanoparticle; NRF2, nuclear factor erythroid 2-related factor 2; PAR, protease-activated receptor; PEG, polyethylene glycol; PI3K, phosphoinositide 3-kinase; PKD, polycystic kidney disease; PLGA, polylactide-co-glycoside; PTEC, proximal tubular epithelial cells; PS, phosphorothioate; RAAS, renin-angiotensin-aldosterone system; RISC, RNA-induced silencing complex; ROS, reactive oxygen species; RT-qPCR, real time - quantitative polymerase chain reaction; siRNA, short interfering RNAs; SGLT2i, sodium-glucose co-transporter-2 inhibitor; T2DM, type 2 diabetes mellitus; TGF- β 1, transforming growth factor-beta 1; TLR, toll-like receptor; TNF, tumour necrosis factor; UTR, untranslated region, UUO, unilateral ureteric obstruction; VEGF, vascular endothelial growth factor.

1. Introduction

1.1. Fibrosis

A common complication of diabetes mellitus, the pathological process of fibrosis leads to loss of homeostasis and subsequent dysfunction of tissues and organs. Fibrosis is frequently likened to aberrant wound healing, where excessive deposition of extracellular matrix (ECM) components and subsequent tissue remodeling lead to scarring and perturbation of tissue and organ function. The fundamental and complex mechanisms driving fibrosis [1], are becoming clearer via the application of techniques including single-cell RNA and assay for transposase-accessible chromatin sequencing [2, 3]. Fibrosis may affect the eyes, heart, liver, lungs and kidney, as described in more detail below and elsewhere in this volume.

1.2. Regulation of gene expression in pathology and treatment of diabetic fibrosis

1.2.1. The human genome

The diploid human genome is composed of approximately 6 giga base-pairs of DNA [4]. The median and mean sizes of a human protein coding gene have been calculated as 22.6 and 66.7 kilo base-pairs, respectively [5]. Prior to the completion of the Human Genome Project [6], predictions of the number of protein coding genes encoded by the human genome often reached 100,000 or more [4, 7]. The subsequent identification of around 20,000 such functional messenger (m)RNA sequences [5, 7] has emphasized the importance of the regulation of gene expression in both human susceptibility to disease (e.g. [8, 9]) and definition of closely related species that share a high percentage of primary DNA sequence identity, such as humans and higher primates [10].

The sequencing of the human genome and development of technologies for high-throughput sequencing of its RNA outputs provide critical genetic components for systems biology [11]. Systems biology uses computational analysis of data from multiple omics approaches and clinical

analyses to deliver the goal of personalized medicine: focused, individual-specific treatment to optimize patient outcomes [11-14].

1.2.2. Noncoding RNAs

It is now clear that while less than 2% of the human genome sequence encodes protein coding mRNAs, more than 95% is processed into RNA transcripts, the majority being noncoding RNAs [7, 8]. Many laboratories are now investigating the biological and clinical significance of this pangenomic noncoding RNA transcription [8]. Conventionally divided into “long” and “short” noncoding RNAs, some functional transcripts have been grouped into families, and these include the microRNAs [15].

1.2.3. MicroRNAs

In eukaryotes, the regulation of gene expression by small RNAs is mediated by argonaute proteins [16]. MicroRNAs (miRNAs) are endogenous RNA transcripts that regulate the expression of most genes encoded by the human genome. MiRNAs are transcribed by RNA polymerase II as primary (pri)-miRNAs that are then processed to pre-miRNAs, exported from the nucleus and elicit their effects as short, single-stranded mature miRNAs between 18-24 nucleotides in length (Fig. 1). MiRNAs interact with argonaute proteins and other key factors in the RNA-induced silencing complex (RISC) to destabilise and/or repress translation of target mRNA sequences [17-19].

Fig. 1 – Key stages of miRNA biogenesis.

1.2.4. MiRNA repression of mRNA translation

MiRNAs bind imperfectly with miRNA-specific recognition sequence motifs in the 3'-untranslated regions (3'-UTRs) of target mRNAs [20]. The part of the miRNA sequence most important for

target recognition, the seed sequence, spans nucleotides 2-8 at the 5' end of the miRNA. Based on seed sequence similarity, miRNAs can be subdivided into families. In mammals, the 90 most evolutionarily conserved miRNA families each have hundreds of conserved mRNA targets, and most of these miRNAs are required for normal development or physiology [19, 20].

The process of target recognition by miRNAs is complex, and combined experimental molecular biological and modelling approaches are revealing further details. In one recent study, McGeary and colleagues adapted RNA Bind-n-Seq [21] (a computational method to characterize sequence and structural specificity of RNA binding proteins) and a convolutional neural network to the study of miRNA-target interactions [20]. Analyses of mammalian miRNAs miR-1, let-7a, miR-7, miR-124 and miR-155, and the nematode transcript lys-6, identified specific noncanonical target sites and differences in canonical target-site affinities for each transcript [20]. Since 3'-UTR length is typically orders of magnitude greater than that of miRNA seed sequences, these UTRs frequently bear multiple putative binding motifs for one or more miRNAs, which can result in cooperative action of multiple miRNAs [22]. MiRNAs also function in transcriptional autoregulation systems, modulating expression of transcription factor and other mRNAs [23].

1.3. MiRNAs in diabetic fibrosis

MiRNAs play key roles in major organ fibrosis [24-26]. More specifically, miRNAs have been implicated in the fibrosis of organ systems covered in more detail elsewhere in this volume including diabetic retinopathy [27-29], diabetic cardiomyopathy [30, 31], diabetic liver fibrosis [32, 33] and diabetic pulmonary fibrosis [34]. Key miRNA functions also modulate kidney development and maintenance of homeostasis, as well as driving the pathology of numerous renal diseases including diabetic kidney disease (DKD) [35-42].

While identification of general patterns of miRNA disease associations is not straightforward, numerous studies have now presented evidence for association of miR-21 with profibrotic pathologies including DKD [37, 43-45]. By contrast, numerous studies have posited anti-fibrotic functions and/or protection against rapid disease progression for members of the miR-29 family [37, 46, 47]. Specific instances of the effects of these miRNAs in the pathology and treatment of DKD appear in later sections of this review.

1.4. MiRNAs as biomarkers and therapeutic targets

Biomarkers are characteristics that are measured objectively and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention [48]. Biomarkers play important roles in drug development through their use as surrogates in early phase clinical trials, and potentially also via their use in enrichment of trial populations for those who stand to benefit from the therapy. Lack of effective biomarkers has hampered DKD therapy development and here recent studies highlight a potential role for miRNAs.

Urinary miRNAs are suitably robust for routine biomarker analysis, since these transcripts are stabilized by association with argonaute 2 protein and /or extracellular vesicles (EVs) including exosomes [49]. Indeed, miRNAs can be quantified precisely using RT-qPCR-based methods in liquid biopsies including urine, peritoneal dialysis effluent and renal transplantation perfusate [49-52]. These techniques have been used to demonstrate elevated urinary miR-29b, miR-126 and miR-155 in DKD [53] as well as increased abundance of miR-223 in bacterial peritonitis [50], miR-21 in peritoneal fibrosis [54] and six miRNAs in delayed graft function following kidney transplantation [55]. A miRNA signature of protective kidney ischemic preconditioning from the renal proximal tubule has also been described recently [56].

Identification of aberrant miRNA expression profiles in disease not only identifies potential biomarkers but, in combination with data on the physiological distribution of miRNAs (e.g. in EVs) and their mechanism of action in the repression of gene expression, provides clues to disease mechanisms, potential therapeutic targets and means of drug delivery [40, 57-61].

The subject of this review is the therapeutic delivery of miRNAs in the context of DKD. This is a young field, and much remains to be elucidated in transitioning from understanding the roles of miRNAs in DKD pathology to their use as therapy. We have already discussed the significance of noncoding RNAs and the place of miRNAs within this wider family of noncoding genomic transcripts. We will now delineate the particularities of DKD within the context of diabetes and its fibrotic complications, consider the rationale and practicalities for miRNAs as a DKD therapeutic, and survey the challenges that remain to be overcome.

2. The clinical need for miRNA delivery as an antifibrotic therapy

2.1. The clinical impact of diabetic fibrosis

The complications of diabetes may be described as macrovascular or microvascular in origin. Manifestations of macrovascular disease include coronary artery disease, stroke and peripheral vascular disease, which generally occur as a result of underlying obstructive atherosclerotic diseases affecting larger arteries. These frequently catastrophic events represent the commonest causes of death in patients with type 2 diabetes mellitus (T2DM) [62]. Microvascular complications encompass retinal, renal and neuropathic disease and are widely considered to be consequences of hyperglycemia, with several landmark studies demonstrating improved outcomes of microvascular complications with adherence to strict glycemic control [63-65]. Estimated healthcare costs associated with diabetes amount to over 400 billion dollars per year in the USA [66], while the UK national health service (NHS) spends 10% of its entire budget on complications of diabetes, over 14 billion pounds per year [67].

In addition to these established diabetic target organs, increasing evidence of diabetes-related pathology in other major organs including liver and lung means that it can be considered as a true multi-system disease characterized by chronic inflammation [68, 69]. Target organs demonstrate structural alterations in ECM deposition and thickening of basement membranes that reflect aberrant wound healing which ultimately leads to fibrosis [70, 71]. Detailed mechanisms of organ fibrosis are described elsewhere in this volume. The clinical manifestations and impact of this central pathophysiology are discussed briefly below.

2.1.1. Diabetic retinopathy

Hyperglycemia, inflammation and hypoxia are established drivers of diabetic retinopathy, the clinical progression of which correlates with accumulation of ECM components such as collagens, laminin and fibronectin [72, 73]. The combination of fibrotic tissue scarring and neovascularisation of the retina create irregular tractional forces that may result in retinal detachment and visual loss [74]. This fibrotic end-stage complication of diabetes is the leading cause of preventable blindness worldwide [75].

2.1.2. Diabetic cardiomyopathy

Hyperglycemia-induced diabetic myocardial fibrosis is characterized by increased type III collagen accumulation that predisposes to myocardial stiffness and loss of contractile function [76, 77]. Histopathology studies have demonstrated the presence of diabetic cardiac fibrosis independent of coronary atherosclerosis and hypertension, which frequently co-exist in patients with diabetes [78]. Heart failure carries significant prognostic implications, and these patients are at two-fold increased risk of death and 33% more likely to be hospitalized [79, 80].

2.1.3. Non-alcoholic fatty liver disease

T2DM-driven progression of liver cirrhosis independent of other metabolic risk factors such as hypertriglyceridemia, obesity and insulin resistance remains controversial [81]. Nevertheless, meta-analyses show that over 50% of prevalent patients with T2DM have a diagnosis of non-alcoholic fatty liver disease [82], and that these patients are twice as likely to develop the inflammatory histological phenotype non-alcoholic steatohepatitis, which is characterized by stellate cell activation and transformation into ECM-producing myofibroblasts [83, 84]. This pro-fibrotic state confers risk of progression to advanced liver cirrhosis and hepatocellular carcinoma [85], and is the most rapidly increasing indication for liver transplantation in the USA [86].

2.1.4. Diabetic pulmonary fibrosis

Not traditionally considered a diabetic target organ, mounting evidence from epidemiological studies suggests that diabetes is an independent risk factor for idiopathic pulmonary fibrosis, manifesting as reduced lung capacity, compliance and diffusing capacity [87, 88]. It is postulated that the hyperglycemic milieu initiates inflammatory and fibrotic lung changes mediated by, for example, advanced glycation end-product (AGE) accumulation, oxidative stress and transforming growth factor-beta (TGF- β)-activated epithelial-to-mesenchymal transition [89, 90]. Diabetes-related pulmonary fibrosis may therefore represent a considerable underappreciated burden on the quality of life of patients with diabetes.

2.2. The clinical impact of DKD

DKD is a complication of both T1DM and T2DM, and is characterized by an increase in urinary albumin excretion and progressive decline in renal function. DKD develops in 15-40% of patients with T1DM with peak incidence at approximately 15-20 years disease duration [91]. The prevalence and progression of DKD in T2DM is more variable, with 5-20% of patients developing

this complication. Non-modifiable risk factors for DKD development include aging and genetic/ethnic susceptibility [92]. Well-established modifiable risk factors include the often-interrelated existence of obesity, hyperglycemia, dyslipidemia and hypertension. More recently, there has been a focus on insulin resistance as an independent risk factor for the development of DKD [93]. Individuals with DKD are also at greater risk of acute kidney injury (AKI) episodes, and architectural changes to the renal glomerulus associated with recovery and repair, particularly to the podocytes, may accelerate DKD progression [94, 95].

The natural history of DKD usually begins with glomerular hyperfiltration, followed by progressive leakage of albumin into the urine that manifests initially as microalbuminuria (30-300 mg/24 hours) before progressing to macroalbuminuria (>300 mg/24 hours). These changes are associated with progressive reduction in kidney function and glomerular filtration rate (GFR), suggesting that diabetes initially affects the glomerular compartment of the kidney. Histologically, this hypothesis is supported by the classic glomerular observations of thickening of the glomerular basement membrane, mesangial hypercellularity, podocyte loss and hyaline Kimmelstiel-Wilson nodules depicted in Fig. 2. If unchecked, this pathology will progress to interstitial fibrosis throughout the kidney, supporting the hypothesis that earlier interventions are more effective in treating and/or slowing the progression of DKD.

Nevertheless, considerable heterogeneity in DKD pathophysiology means that some patients exhibit a more variable disease course. For example in non-albuminuric chronic kidney disease (CKD), 0.6-28.4% of patients with diabetes develop GFR decline before the occurrence of, or in the absence of, albuminuria [96]. This occurs most often in patients with T2DM, in whom varying contributions of normal aging, hypertension and arteriosclerosis may modulate disease progression and pathophysiology [96]. This heterogeneity may complicate prediction of the rate

of disease progression, necessitating frequent blood and urine monitoring in primary and secondary care facilities, and is a key consideration in drug development for DKD.

Kidney failure (formerly end-stage kidney disease) is defined by a GFR of <15 ml/min/1.73m², or treatment with renal replacement therapy [97]. Global glomerulosclerosis, interstitial fibrosis and tubular atrophy are key correlative histological findings. While not specific to DKD, these observations represent a final common CKD endpoint, irrespective of etiology [98]. DKD is, however, the leading cause of kidney failure and indication for commencement of renal replacement therapies (i.e. kidney dialysis and transplantation) in the developed world [99, 100]. Kidney failure, and hemodialysis therapy in particular, accelerate cardiovascular risk, which accounts for a significant proportion of excess dialysis-associated mortality [101].

Fig. 2 – The effect of the diabetic environment on the kidney and key features of DKD.

2.3. Management strategies for DKD

DKD management has traditionally centred on a non-targeted, non-individualized approach where the only significant deviation from blanket proteinuric CKD management has been the optimization of glycemic control. Earlier referral for transplantation work-up in eligible patients is sometimes preferred, in appreciation of the increased likelihood of requiring pre-operative cardiac/vascular optimization [102]. Consequently, considerable research efforts have focused on understanding the underlying mechanisms driving DKD progression, to direct novel therapeutic targeting. Modulating maladaptive immune responses using anti-inflammatory and anti-fibrotic mediators has been one approach, while there is increasing awareness that the anti-fibrotic actions of established diabetic medications contribute to their renoprotective effects, as summarized below.

2.3.1. Traditional management

The cornerstone of DKD management is risk factor modification to prevent disease progression. Many measures are common to general diabetes management, including lifestyle modifications to avoid obesity, cessation of smoking and regular exercise. Pharmacological intervention is often required to maintain strict blood pressure and glycemic control. Other treatments target the kidney more directly, including the use of angiotensin-converting enzyme inhibitors (ACEis) and angiotensin II receptor blockers (ARBs) to retard albuminuric progression. For the last two decades, this regimen has represented the gold standard treatment in minimizing loss of renal function and progression to kidney failure [103, 104]. In addition to the beneficial reduction of intraglomerular hypertension achieved from blockade of the renin-angiotensin-aldosterone system (RAAS), ACEis and ARBs reduce circulating TGF- β 1 levels [105] and confer anti-fibrotic effects. This has predominantly been attributed to the blockade of angiotensin II, a central effector in promoting mononuclear cell infiltration, ECM protein deposition and fibroblast differentiation [106].

However, long-term use of ACEis/ARBs in CKD may lead to angiotensin-II and aldosterone-escape, which can exacerbate salt and water retention and result in adverse cardiac effects such as myofibrosis, arrhythmias and congestive cardiac failure [107]. To overcome these effects, dual blockade of the RAAS with combinations of ACEis, ARBs, direct renin inhibitors and mineralocorticoid receptor antagonists has been investigated. However, clinical trials have concluded that additional risks including hyperkalemia and AKI outweigh any benefits [108-110]. Even when effective RAAS blockade is achieved, the risk of kidney failure can remain high and correlates with residual levels of albuminuria [111].

2.3.2. Next generation glucose-lowering medication

Sodium-glucose co-transporter-2 inhibitors

Following a long period of stagnation in DKD management, the advent of new glucose-lowering therapies with additional renoprotective effects has generated considerable excitement in the field. Sodium-glucose co-transporter-2 inhibitors (SGLT2is) promote renal glucose excretion (glycosuria) from the proximal tubule, leading to reduced plasma glucose, weight loss and reduced blood pressure. Initial cardiovascular outcome trials of SGLT2is demonstrated apparent renoprotective effects, which prompted dedicated renal phase 3 outcome studies. In 2019 and 2020, two large multicentre randomized trials, CREDENCE and DAPA-CKD, were stopped early, having both demonstrated overwhelming clinical efficacy in pre-specified endpoints including reduction in doubling of serum creatinine, kidney failure, renal death or cardiovascular death with use of Canagliflozin and Dapagliflozin, respectively [112, 113].

Mechanisms of SGLT2i-induced renoprotection are hypothesized to go beyond reducing obesity and improving glycemic control, and include favourable systemic and glomerular hemodynamics, altered tubuloglomerular feedback mechanisms and natriuretic peptide effects [114, 115]. SGLT2 inhibition may also ameliorate oxidative stress [116] and confer anti-inflammatory/anti-fibrotic effects [117, 118]. These effects may be mediated via angiotensin-II suppression (as above), attenuation of the hypoxia inducible factor pathway and reduction in inflammatory and fibrotic mediators such as tumour necrosis factor (TNF) receptor-1, interleukin (IL)-6, and fibronectin-1 [119-121].

SGLT2is are increasingly used in mainstream DKD therapy in patients with estimated (e)GFR > 30 ml/min/1.73m², and ongoing clinical trials may lower this eGFR initiation threshold. The unique mechanism of SGLT2i action confers adverse effects including increased incidence of genital infections and euglycemic diabetic ketoacidosis. Increased amputation and fracture risk have

also been reported, albeit infrequently [122], thus longer and larger analyses will be needed to confidently assign causality and to determine if such effects will preclude SGLT2i use in a minority of patients.

Incretin-based therapy

Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) improve glycemic control by stimulating glucose-dependent insulin release from pancreatic β -cells and suppressing glucagon release. Clinical trials of GLP-1 RAs have demonstrated reduction in major cardiovascular events and a variable, though consistent, reduction in albuminuria [123]. The mechanism of this effect is unclear, but reduced albuminuria and improved tubulointerstitial architecture in GLP-1 RA-treated rats was associated with reduction in markers of inflammation and fibrosis such as TNF- α and fibronectin [124]. GLP-1 RAs also normalise oxidative stress markers in the renal tissues of diabetic rats, independently of glucose-lowering action [125]. It is therefore likely that renoprotective effects are mediated via a combination of extra-renal hemodynamic and glucose-lowering effects in conjunction with the GLP-1R-independent effects on renal inflammation [126-129].

Blocking the enzymatic degradation of GLP-1 using dipeptidyl peptidase-4 (DPP-4) inhibitors has demonstrated similar anti-fibrotic renoprotective effects in diabetic mouse models. Inhibition of endothelial-to-mesenchymal transition and restoration of anti-fibrotic miR-29 family members ameliorated kidney fibrosis, implicating these miRNAs as potential regulators of DPP-4 in the kidney [130]. Clinical trials have demonstrated cardiovascular safety and reduced albuminuria in patients with T2DM and renal dysfunction, but offer no protection against decline in eGFR and/or kidney failure [131, 132].

2.3.3. Endothelin receptor antagonists

Endothelin-A receptor activation has been implicated in glomerular inflammation, oxidative stress, podocyte damage and vasoconstriction of the afferent vessels, resulting in increased albumin permeability [133]. Endothelin also promotes collagen deposition and ECM remodelling in experimental models, independently of hemodynamic modularity effects [134, 135]. Clinical trials of endothelin receptor antagonists have been dogged by serious adverse effects and low event rates resulting in premature termination, obscuring reports of delayed onset of kidney failure, renal replacement therapy and death in pre-selected albuminuric-responders using Atrasentan [136, 137].

2.3.4. Mineralocorticoid receptor antagonists

Mineralocorticoid receptor antagonists (MRAs) have demonstrated pleiotropic renoprotective effects in pre-clinical studies, including hemodynamic alterations and reduction in oxidative stress, inflammation and fibrosis [138]. Animal models of DKD show improvements in histological and biochemical markers of fibrosis in response to MRAs [139, 140]. Clinical use of steroidal-MRA agents has, to date, been limited by consequential hyperkalemia. It was anticipated that this would be overcome using the novel non-steroidal MRA agent, Finerenone. However, FIDELIO-DKD reported modest renoprotective effects that remained offset by significant hyperkalemia in the treatment group [141].

2.3.5. Anti-inflammatory and anti-fibrotic mediating treatments trialed in DKD

Numerous other non-glucose-lowering pharmacological agents targeting inflammatory and fibrotic mediators in DKD have been trialed with limited success, and notable examples are described briefly below.

AGE inhibitors

AGEs are established drivers of oxidative stress and renal inflammation, and maybe generated in response to hyperglycemia [142]. Blocking AGE synthesis using vitamin B6 derivative Pyridoxamine retarded DKD progression in animal studies [143], and showed marginal improvements in serum creatinine in limited subgroups of patients with diabetes [144, 145].

Anti-inflammatory agents

Anti-inflammatory DKD targets trialed in preclinical studies range from specific inhibition of inflammatory mediators including cell adhesion molecules, chemokines and cytokines, to broader targeting of intracellular signaling pathways like the Janus kinase/signal transducers and activators of transcription (JAK/STAT), the nuclear transcription factor-kappa B (NF- κ B) and the nuclear factor erythroid 2-related factor 2 (NRF2) pathways [146, 147], but few have completed clinical trials and none are currently in routine use.

Targeting of overactive JAK/STAT pathway signaling with Baricitinib reduced albuminuria and biomarkers of inflammation including intercellular adhesion molecule 1, TNFR 1 and 2, serum amyloid A, and (urinary) monocyte chemoattractant protein-1 in a phase 2 clinical trial, but it remains unknown if this will reduce long-term DKD progression: no change in renal function was observed during the 24-week study period [148].

Bardoxolone Methyl, an NRF2 activator and hence potent anti-oxidant was initially reported to increase eGFR in DKD [149], but the larger BEACON trial was terminated due to increased heart failure [150]. However, advocates believe it may prove clinically useful in selected patient groups with low pre-existing susceptibility, and its role is being reassessed in further clinical trials [151].

Extra-renal effects of anti-inflammatory therapies may also have a significant impact on mortality in patients with CKD and diabetes, as demonstrated in the CANTOS trial of IL-1 β inhibitor Canakinumab, which resulted in significant reduction in major cardiovascular events [152].

Anti-fibrotic agents

Targeting of the TGF- β superfamily retards DKD progression and protects against renal fibrosis in some animal studies [153, 154], but contradictory findings showing increased renal inflammation have also been reported [155]. In clinical trials, Pirfenidone, an inhibitor of TGF- β 1 production and activity, restored eGFR in individuals with DKD without significant attenuation of albuminuria [156]), suggesting that Pirfenidone ameliorates tubulointerstitial fibrosis without affecting glomerular injury. A phase 2 trial of TGF- β 1-specific humanized neutralizing monoclonal antibody demonstrated neither benefit in eGFR nor albuminuria in DKD [157].

The PREDAIN trial showed a reduction in albuminuric DKD progression with the use of RAAS blockers in addition to Pentoxifylline, a phosphodiesterase inhibitor that decreases TGF- β 1 and TNF- α activity, and two further phase 4 trials of this drug are currently in recruitment [158].

2.4. Common themes in clinical translation of DKD therapeutics

The current mechanistic understanding and implementation of DKD therapeutic agents can be summarized thus:

1. Well-established treatments of proven clinical efficacy in DKD are increasingly recognized as having anti-inflammatory and anti-fibrotic effects, especially those targeting the RAAS.

2. The mechanistic continuum from the maladaptive, chronic inflammatory state through to the development of established fibrosis presents multiple target options in DKD. However, success in experimental models infrequently translates, with many high-profile clinical trials abandoned due to adverse side effects or lack of clinical efficacy.
3. Many molecular pathways exert complex pleiotropic effects that can be simultaneously deleterious and protective in the progression of DKD. For example, systemic TGF- β suppression may ameliorate fibrotic effects, but may also block beneficial anti-inflammatory functions. Such contradictory effects may contribute to the failure of these agents in clinical trials.

3. Mechanisms of DKD and target identification

3.1. Pathogenetic mechanisms of DKD

The underlying mechanisms that drive DKD pathology remain unknown, but experimental data suggest the diabetic environment results in multiple signaling pathway dysregulation and contribute to fibrosis and decline in renal function. Since miRNAs regulate at least 60% of mammalian protein coding genes [159], it is highly probable that aberrant miRNA expression plays a significant role in determining the diabetic phenotype, and an increasing body of evidence points toward the critical action of miRNA regulators in numerous established diabetic processes. We describe below pathogenetic pathways experimentally implicated in DKD progression and prominent examples of associated regulatory miRNAs.

3.2. Altered insulin signaling

Cellular insulin resistance appears to be a key factor in the development of DKD. With time, cellular insulin resistance develops in patients with T1DM [160, 161] or T2DM [162]. Furthermore, those who develop kidney disease are more likely to have a family history of insulin

resistance [163, 164], require higher doses of insulin to control their disease [165] and have elevated homeostatic model assessment of insulin resistance scores [166]. Additionally, recent evidence from a large Scandinavian diabetic population study of over 30,000 patients showed that at diagnosis, severely insulin resistant patients were significantly more likely to develop nephropathy [93]. These findings have since been replicated in a German cohort [167].

MiRNAs are long-established mediators of insulin resistance in classical peripheral target organs. In the livers of patients with diabetes, and in mouse models, miR-103 and miR-107 upregulation and subsequent repression of target caveolin-1 prevents insulin receptor stabilization at the cell membrane [168]. Members of the miR-29 family are recognized to be master modulators of glucose utilization in skeletal muscle via multiple targeting effects on insulin receptor substrate 1, hexokinase 2, glucose transporter 1, phosphoinositide-3-kinase regulatory subunit 3 and RAC-beta serine/threonine-protein kinase (AKT2) [169, 170].

In models of obese mice, adipose tissue miR-181b levels are reduced, leading to increased expression of pleckstrin homology domain leucine-rich repeat protein phosphatase-2, inactivation of AKT and impaired insulin signaling. MiR-181b rescue improved glucose tolerance through enhanced insulin-mediated AKT phosphorylation at Ser473 and induction of endothelial nitric oxide synthase, nitric oxide activity and forkhead box O1 (FOXO1) phosphorylation. These effects were seen specifically in white adipose tissue, and not in liver or in skeletal muscle, highlighting the cell specificity of miRNA action in the regulation of insulin responses [171].

The importance of kidney-specific insulin sensitivity has come to light only recently. Insulin signaling in the glomerular podocyte is of particular interest (see Fig. 3). This cell type is critical in preventing albuminuria, and more than 50 genetic mutations affecting the podocyte result in nephrotic syndrome [172]. The human podocyte is insulin-responsive and expresses many of the

key components of classically insulin-responsive cell types such as adipocytes and muscle cells, including glucose transporter type 4 [173]. A podocyte-specific insulin receptor knockout transgenic mouse has been engineered to elucidate the importance of insulin signaling in these cells [174]. These animals developed albuminuric kidney disease with a number of features replicating DKD including thickened glomerular basement membrane, podocyte loss and glomerulosclerosis. Significantly, kidney disease developed in a normoglycemic environment, supporting the hypothesis that cellular insulin resistance is a major driver of kidney disease.

Further podocyte studies modulating other downstream elements of the insulin signaling pathway have shown severely detrimental effects on kidney function. These include loss of AKT2 in the phosphoinositide 3-kinase (PI3K) pathway, which is a critical determinant of podocyte survival after nephron loss [175], and activation of the mammalian target of rapamycin (mTOR) complex 1 resulting in aberrant localisation of slit diaphragm proteins and enhanced podocyte endoplasmic reticulum (ER) stress that manifests in the recapitulation of DKD changes in non-diabetic mice [176]. Loss of glycogen synthase kinase 3 results in podocyte apoptosis due to loss of the differentiated phenotype [177]. Finally, recent work suggests that insulin resistant environments cause dysregulation of other important signaling pathways in the podocyte and throughout the kidney that could be beneficially modulated in DKD, including the neuropeptide Y axis [178].

Differential expression of podocyte miRNAs in DKD (Fig. 3) has been well documented in a variety of experimental models [179]. The miRNA-specific mechanisms in the regulation of podocyte-specific insulin signaling remain incompletely defined, but gathering evidence suggests that the pleiotropic nature of miRNA function might be exploited to therapeutic effect via a “multi-target hit” approach. For example, miR-217 is upregulated in sera from patients with T2DM, and miR-217 levels correlate positively with albuminuria [180]. In podocytes, miR-217 directly targets the

phosphatase and tensin homologue, a negative regulator of PI3K/AKT/mTOR signaling, and inhibition of miR-217 restores defective autophagy pathways, inhibits reactive oxygen species (ROS) and apoptosis, and increases nephrin expression and glucose uptake [181].

Fig. 3 – Altered signaling pathways implicated in DKD, and differentially expressed podocyte miRNAs.

3.3. Glycocalyx regulation

Diabetes results in loss of the glycocalyx layer lining the luminal aspect of the systemic [182] and glomerular endothelium [183]. This loss takes place at an early stage in the development of DKD, and is evident in mouse models and human biopsy studies. In diabetic conditions endothelial cell damage occurs as the glycocalyx is lost via shedding of the heparan sulfate proteoglycan syndecan-4, which is mediated by matrix metalloproteinase (MMP) 2 and MMP9 [184]. Regulation of MMP expression by miRNAs thus represents a potential therapeutic avenue. MiR-21 promotes MMP2 and MMP9 expression while simultaneously repressing endogenous metalloproteinase inhibitor 3 [185]. This mechanism has been posited to explain the reduction in pro-inflammatory IL-1 β , TNF- α and alleviated kidney damage seen in diabetic rats treated with anti-miR-21 [186]. Other miRNAs that regulate MMP9 expression include miR-129 and miR-335, which act indirectly by inhibiting specificity protein 1-driven MMP9 expression [187].

External growth factors also modulate the glycocalyx and thus represent potential miRNA targets, including vascular endothelial growth factor (VEGF)-C [188] and VEGF-A165b, the inhibitory isoform of VEGF-A [183], as described further below.

3.4. TGF- β 1 signaling

The pro-inflammatory multi-functional cytokine TGF- β 1 is widely acknowledged as a key modulator of DKD, and is involved in the development of glomerulosclerosis and the evolution of interstitial fibrosis. TGF- β 1 controls an array of biological processes, these include upregulating both the synthesis and cross-linking of key ECM components that is a major driver of DKD pathology. Inhibition of TGF- β 1 activity in the kidney is therefore a potentially attractive therapeutic approach for DKD, and some favorable renal outcomes have been observed using neutralizing TGF- β antibodies in mouse models of T1DM [189] and T2DM [190]. Manipulation of miRNAs that mediate TGF- β 1 signaling, such as miR-21 and miR-29b (see section 1.3) represent one of the most clinically advanced applications of miRNA-based therapeutics; the mechanics and delivery of these are detailed later in this review.

However, global targeting of TGF- β 1 in DKD is not straightforward since inhibition of this cytokine has detrimental effects elsewhere in the body including primary aldosteronism and impaired natriuresis, processes which themselves would be detrimental in DKD [191]. MiRNA regulation of TGF- β 1 signaling introduces a further dimension of complexity: even at the glomerular-specific level there is evidence that the same miRNA family, despite possessing identical seed sequences, may exert anti- and pro-fibrotic effects on different cells under diabetic conditions. In mesangial cells, and in db/db mouse renal cortex, miR-29b is downregulated, leading to upregulation of collagen matrix deposition via a TGF- β /Smad3-dependent mechanism [192]. Expression of miR-29c is upregulated in the diabetic podocyte, leading to repression of target sprouty homolog-1 and resultant podocyte apoptosis, with anti-miR-29c treatment resulting in attenuated proteinuria and reduced ECM deposition in db/db mice [193]. These nuances of miRNA regulation represent one of many challenges to be overcome in the development of miRNA-based therapeutics (see section 5).

3.5. Inflammatory pathways

There is increasing recognition of the importance of the role of chronic inflammation, and by extension of those miRNAs that regulate these inflammatory pathways, in the pathogenesis of DKD. Systems biology approaches have identified JAK/STAT signaling as a potentially important pathway in the progression of DKD [194]. In both early and late DKD, there is increased expression of numerous JAK/STAT genes in several renal cell types. Furthermore, enhancing JAK2 selectively in glomerular podocytes of diabetic mice has detrimental effects [195], and the aforementioned trial of JAK1/JAK2 inhibitor Baricitinib in patients with DKD has shown beneficial effects in albuminuria reduction [148]. MiR-150b is recognized as a modulator of this pathway via direct targeting action of suppressor of cytokine signaling 1, although evidence of the beneficial effects of anti-miR-150b in reducing interstitial fibrosis *in vivo* is predominantly from non-diabetic models [196].

MiR-34b targets both the IL-6 receptor and downstream JAK2/STAT3 signaling, with miR-34b overexpression shown to inhibit apoptosis and the expression of TNF- α , IL-1 β , IL-6 and caspase-3 in tubular epithelial cells [197].

NF- κ B pathway activation also represents an important stimulus for DKD propagation. MiR-451 regulates this pathway via repression of large multifunctional protease 7, an activator of NF- κ B signaling. Intraperitoneal injection of miR-451 mimic resulted in attenuation of microalbuminuria, glomerular damage and blood glucose levels in db/db mice [198], supporting a role for this miRNA in modulating the inflammatory response in DKD.

MiR-146a is frequently dysregulated in the context of chronic inflammatory states, where it acts as an anti-inflammatory mediator by down-regulating components of the NF- κ B pathway such as toll-like receptor (TLR)4, IL-1 receptor-associated kinase 1 (IRAK1) and TNF-receptor-associated factor [199]. MiR-146a knockout in streptozotocin-induced diabetic mice results in

accelerated histological and biochemical progression, and upregulation of proinflammatory and profibrotic genes, supporting a protective role for miR-146a in DKD [200]. The role of miR-146a has been further delineated at a podocyte-specific level, where loss of this miRNA de-represses notch homolog 1 and ERB-B2 receptor tyrosine kinase 4 (ERBB4) targets, inducing a podocyte injury which was counteracted by the use of ERBB4/ epidermal growth factor (EGF) receptor inhibitors *in vitro* [201]. Approaches to the delivery of miR-146a to protect the podocyte against noxious inflammatory stimuli are discussed later in this review.

3.6. Oxidative and ER stress

Several studies have reported dysregulated expression of cytoprotective genes that provide defence against oxidative stress in DKD. NRF2 activation protects against hyperglycemia-induced glomerular changes and attenuates mesangial hypertrophy, due in part to inhibitory effects on TGF- β 1 and subsequent reduction in ECM production [202]. NRF2 is a direct target of miR-27a [203] and the adipokine omentin-1 has been used to down-regulate miR-27a expression, relieving NRF2 repression thus decreasing oxidative stress and improving renal function in db/db mice.

Other miRNA targets in oxidative stress pathways include miR-125b, which contributes to hyperglycemia-induced ROS generation and apoptosis in renal tubular epithelial cells by targeting ACE2 [204], and miR-25 has been proposed to modulate mitochondrion-derived oxidative stress in experimental DKD models via validated targets cell division control protein 42 homolog and nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) [205, 206].

Recent experimental data suggest that miRNA-based therapeutics may be used to manipulate podocyte responses to both oxidative and ER stress. Xu *et al.* reported that miR-423 expression is reduced in biopsy tissue of patients with DKD, and in podocytes treated with high glucose *in*

vitro. Enforced expression of miR-423 inhibits ROS generation by targeting NOX4 and is thereby protective against hyperglycemia-induced podocyte damage [207]. MiR-27a upregulation induces ER stress in podocytes via FOXO1 repression [208] and downregulation of miR-27a inhibits ER stress and ER-associated apoptosis in the renal cortex of db/db mice [209].

Mitochondrial dysfunction and oxidative stress are involved in a variety of human disorders, and may be central to the development of DKD. Brownlee's original observation that the microvascular diabetic target organs share a common reliance on mitochondrial energy production [210], coined the "unifying hypothesis", laid the foundation for investigating mechanisms of mitochondrial dysfunction in DKD. While the precise mechanisms remain hotly debated [211], increased mitochondrial ROS production, changes in mitochondrial morphology and decreased mitochondrial biogenesis/ATP depletion have all been implicated in DKD pathogenesis [212, 213]. Mitochondrial miRNAs, whether imported from the cytosol or transcribed from the mitochondrial genome itself [214], have been implicated in the dynamic regulation of these mitochondrial responses to the diabetic *milieu*. Kato *et al.* recently described the role of miR-379 in DKD by disabling adaptive mitophagy via direct target mitochondrial fission-1 protein, and showed that miR-379 knockout mice protected against DKD development [215]. The widely and highly expressed miRNA, miR-21, has also been implicated in dysregulation of mitochondrial fatty acid oxidation by targeting peroxisome proliferator activated receptor- α , and this mechanism contributes to miR-21-induced fibrosis [216].

3.7. Coagulation pathway

Coagulation proteases are key elements in hemostasis and blood clotting. They are also intimately associated with inflammation, a prominent feature of DKD, and have important roles in disease development [217]. Coagulation proteases signal through protease-activated receptors (PARs). These receptors are expressed in multiple kidney cell types, and their

expression alters dynamically during diabetes. There is compelling evidence that activation of components of the clotting cascade can slow the development of DKD. This includes activated protein C (APC) signaling through the PAR-1 receptor. This signaling is modulated by endothelial thrombomodulin, which prevents diabetic-induced apoptosis and mitochondrial dysfunction in the podocytes and glomerular endothelial cells of the renal glomerulus [218]. Protective effects have been reported for maintenance of high APC levels during long-term diabetes, and recent data show that protein S, an essential cofactor for APC, is highly expressed in the glomeruli of diabetic rats early in disease development but is lost at later stages [219]. Indeed, maintaining protein S levels protects against longer term DKD progression. Expression of the protein S1 gene is regulated by miR-155 and miR-494 [220, 221], thus highlighting these miRNAs as potential therapeutic targets for DKD.

3.8. Other potential therapeutic targets

Several physiological processes involving crosstalk between the cells that maintain kidney function in homeostasis are dysregulated in DKD, and could potentially be therapeutically targeted. These include the VEGFs produced by the podocyte and which signal to glomerular endothelial cells. Experimental evidence suggests that under homeostasis VEGF-A expression is tightly regulated to maintain kidney function, since either too much [222, 223], or too little [224, 225], is detrimental. Both miR-16 and miR-93 [226] target VEGF-A in hyperglycemic conditions, and injection of miR-16-packaged exosomes attenuates podocyte injury in diabetic rats [227].

However, VEGF-A is challenging to target in a therapeutic context, and a more detailed understanding of the pathways regulating VEGF-A secretion and its actions in DKD will be required to determine if miRNA-based therapeutics might be used to “fine-tune” the VEGF-A response in DKD. A number of other “growth factors” that signal to kidney, such as growth hormone, insulin-like growth factors, and EGF have been identified as potential DKD initiators,

the full details of which are beyond the scope of this review, but are described in detail elsewhere [228-230]. Manipulation of the complex regulatory relationships between miRNAs and growth factors is a burgeoning field in cancer therapeutics, including the potential to modulate the efficacy of existing cancer treatments [231], and success here may inform future developments in DKD treatment.

4. The mechanics of miRNA delivery to the kidney

To exploit the power of miRNAs as master regulators of gene expression, several key factors must be considered. Current strategies of manipulating miRNA levels involve either i) decreasing the abundance of the selected miRNA(s), thus restoring the activity of suppressed target mRNAs, or ii) increasing the abundance of the selected miRNA(s) to suppress key mRNA targets. The ability of miRNAs to target multiple points in the same pathway may increase their potency.

However, for successful therapy, it is vital to ensure that miRNA targeting observes appropriate precautions. MiRNAs are expressed in multiple cell types and may be critical in the maintenance of homeostatic cellular phenotypes, thus alteration of miRNA expression may result in unexpected and unwanted off-target effects that may lead to harmful side-effects. Only around 30% of miRNAs in murine kidney cells maintain consistent cell-specific enrichment in normal and injured states [232], and certain miRNAs have the potential to modulate the expression of many mRNA targets. While this might be a positive feature in specific therapeutic contexts, it might also increase the likelihood of off-target effects, and miRNA delivery vectors must be designed with these caveats in mind.

4.1. MiRNA-based therapeutics

To increase the abundance of a selected miRNA, “miRNA mimics” can be used. These mimics are chemically-synthesized double-stranded RNA molecules which imitate the naturally occurring

mature miRNA duplexes produced via miRNA biogenesis [17]. By contrast, miRNA antagonists, which are also known as “anti-miRs”, “antagomiRs”, or miRNA-specific antisense oligonucleotides (ASOs), reduce the ability of miRNAs to bind to their mRNA targets [233, 234]. Anti-miRs have complementary sequences to their corresponding target miRNAs, with which they hybridize via Watson-Crick base-pairing within the miRNA seed sequence or beyond this region [235].

4.2. Optimization of miRNA-based therapeutics

Local delivery of miRNA therapeutics to the kidney in mouse models has been achieved by renal vein delivery [236, 237] and tissue selectivity improved using kidney-specific promoters [236]. However, given its invasive nature, direct application to the kidney is not the most realistic option for clinical application, and systemic administration is a more attractive method. One key issue is the exclusion limit of the glomerulus of approximately 50 kDa. Since the average mass of a nucleotide is 325 Da, DNA/RNA molecules greater than 150 base-pairs may be excluded by the glomerular filtration barrier on the basis of size alone. The passage of anti-miRs and miRNA mimics may also be inhibited by other factors including charge, the addition of bulky conjugates, and unfavourable nanoparticle (NP) technology. Further considerations include the sensitivity of exogenous RNAs to i) degradation by host ribonuclease enzymes and ii) escape from the endosomal compartment during cellular internalization, which also leads to degradation. Indeed, unmodified oligonucleotides have a half-life of only a few minutes [238], and are therefore not suitable for the treatment of renal disorders such as DKD via systemic administration.

Fig. 4 – Chemical modification of miRNAs and delivery modalities of miRNA-based therapeutics.

Various chemical modifications have been used to improve miRNA stability, including the addition of a 2'-O-methyl group, incorporation of phosphorothioate (PS) modifications where the non-bridging oxygen atom is replaced by a sulphur atom, or the replacement of RNA nucleotides with locked nucleic acids (LNAs) [239, 240] (Fig. 4). The addition of PS modifications makes miRNAs more resistant to degradation by plasma nucleases, increasing their circulating half-life to up to 30 minutes and the clearance half-life to hours [241]. Modifications also affect biodistribution. Studies in rat using gallium-radiolabelled oligonucleotides showed the highest uptake in bone marrow, kidney, liver, spleen and urinary bladder, with high kidney uptake observed specifically for those with PS or 2'-O-methyl conjugation [242].

Kidney fibrosis is a feature of DKD, and the natural propensity for the kidney to accumulate oligonucleotides is potentially advantageous in the context of miRNA-based therapy. Indeed, studies using an ASO to miR-214 with PS modification demonstrated anti-fibrotic effects in mice, and biodistribution data showed a kidney:liver uptake ratio of 5:1 [243]. LNA oligonucleotides have higher binding affinity for their target nucleotide sequences than unmodified sequences [244]. The effectiveness of anti-miR LNAs complementary to the target miRNA seed region alone has been demonstrated, and this approach can be used to target miRNA families [234].

4.3. Delivery systems for miRNA-based therapeutics to the kidney

Fig. 5 – The nephron, the functional unit of the kidney.

The nephron is the functional unit of the kidney (Fig. 5). One kidney possesses approximately 1.3 million nephrons, each consisting of a series of functional domains from the glomerulus, where blood filtration occurs, to the proximal renal tubule, loop of Henle and distal renal tubules, where

much of the secretion and reabsorption activity pivotal to renal function takes place, and then the collecting duct where final concentration of urine occurs. To date, regional targeting delivery of miRNA therapeutics to different functional regions of the nephron has been relatively poorly explored, but there is potential to combine different technologies to achieve this aim.

The ideal delivery vehicle to achieve specific targeting of the kidney with miRNA-based therapies would be administered systemically and have efficient cellular uptake in the desired region of the kidney, be stable under physiological conditions, have minimal immunomodulatory effects, and would effectively and specifically interact with its target(s).

For efficient uptake of miRNA-based therapies, numerous vector technologies have been employed to overcome poor cellular uptake, including viral and synthetic delivery systems. Viral delivery of synthetic miRNAs is very efficient using adenovirus (Ad), adeno-associated virus (AAV) and lentivirus (LV) vector systems [245]. However, none of these vectors has natural tropism for the kidney, and this limits their utility when optimizing a delivery system.

The goal of systemic delivery is complicated by the outcomes of numerous studies indicating that vector delivery to the kidney via this route is inefficient, in contrast to other organs where Ads and AAVs permeate into many tissues [246]. Furthermore, the kidney has evolved highly specific permeability mechanisms to control solute and protein entry into the parenchymal cells. Local delivery of AAVs has shown selective kidney uptake with AAV9 inducing expression in medullary and cortical regions whilst AAV6 and AAV8 only induce medullary expression [236], but local delivery is unlikely to be clinically viable.

Viruses can be genetically modified and engineered with targeting moieties expressed on capsid/envelope to increase specific kidney uptake [247, 248], and renal-specific expression can be conferred by putting the target transcripts under the control of kidney-specific promoters

[236]. Phage display technology [249] has been used to identify sequences that are enriched in the kidney and their display on the Ad capsid allowed improved kidney uptake. However, the presence of the targeting moiety did not overcome the natural tropism of Ad for the liver [248, 250]. The addition of cell-specific promoters would add a further layer of selectivity, restricting miRNA expression to the specified cell type, e.g. podocyte-specific expression under the nephrin promoter. Further hurdles facing viral-based delivery systems include Ad immunogenicity, the transient nature of AAV expression, and safety concerns regarding the genomic integration of LVs. Synthetic delivery systems which take advantage of the continuing developments in nanotechnology remain the most attractive for kidney-specific miRNA therapy.

Nanotechnology has provided several platforms with which it is possible to target the kidney selectively for the delivery of therapeutic agents. NPs, multifunctional nanocarriers which may be inorganic or organic, can be engineered to deliver drugs and oligonucleotides to the kidney [251] (Fig. 4). NPs are composed of a core that contains the therapeutic agent, encapsulated in a protective layer which can be modified to protect the NP from degradation in the circulation. In addition to the NP surface, targeting ligands including antibodies, proteins, peptides and aptamers may be added. For example, a liposome-polycation-hyaluronic acid NP formulation was modified using a tumour-targeting single-chain antibody fragment, which allowed specific delivery of miR-34a to the lung *in vivo* [252]. NP size can also be adjusted to enhance accumulation in specific organs, and size-dependent NP retention by the kidney means that the mesangium can be targeted with gold-based polyethylene glycol (PEG) NPs of 75 nm diameter [253].

The combination of focused ultrasound combined with gas-filled microbubbles (e.g. the clinically-approved SonoVue) has proved effective in enhancing non-invasive renal-specific administration of miRNA-based therapeutics. In the presence of gas microbubbles, ultrasound

causes plasma membrane pore formation in a process called sonoporation, increasing cellular permeability and thus miRNA uptake [254].

Lan and colleagues have successfully used this method to target the kidney. Initially a miR-21 knockdown plasmid was administered in a mouse model of unilateral ureteric obstruction (UUO), which attenuated miR-21 abundance by 20% and resulted in reduced renal collagen expression [255]. This group also significantly reduced fibrosis post-UUO by delivering a miR-29 plasmid under tetracycline-controlled transcription using ultrasound and microbubbles [256]. Depletion of miR-29b following AGE treatment of rat mesangial cells *in vitro* has also been shown by these researchers, and the fibrotic effects resulting from AGE treatment were attenuated by miR-29b replacement in this model [192]. Similarly, loss of miR-29b was associated with inhibited DKD progression in db/db mice, and miR-29b therapy inhibited renal injury and microalbuminuria [192].

This ultrasound and microbubbles combination was used recently to co-deliver ASOs to miR-21 and miR-10b to deep pig tissues, including kidney, using poly lactic acid-co-glycolic acid (PLGA)-PEG NPs [257]. At 4 hours post treatment, histological investigation for hemorrhage, inflammation and edema attributable to the treatment showed no damage, suggesting that this treatment was safe [257].

Extracellular vesicles (EVs), including exosomes, have also been used to deliver miRNA cargoes, and encapsulation within exosomes or microparticles stabilises miRNAs. Recovery post-AKI was induced in kidney cells by the miRNA cargo of mesenchymal stromal cell-derived EVs [258].

As noted above, miR-29 loss has been observed in DKD progression and its replacement attenuated fibrosis [192]. Recently, Jahangard *et al.* generated a stable rat bone marrow mesenchymal stem cell line that overexpressed miR-29b, and this increased expression was also

seen in the exosomes produced by these cells [259]. Stem cell exosomes enriched in miR-29b were then transplanted into the hippocampal CA 1 region of rats in an Alzheimer's disease model, and showed positive effects on deficits in spatial learning and memory [259]. These findings present the possibility that a combination of ultrasound and microbubbles with miR-29b-enriched exosomes could be used for specific restoration of miR-29b in DKD.

The generation of "targomiRs", EGF receptor-targeted bacterial minicells loaded with miRNA mimic, represents another potential delivery system for miRNA-based therapeutics. Testing in clinical trial showed an acceptable safety profile as well as some early signs of activity in patients with malignant pleural mesothelioma [260]. The identification of a suitable targeting ligand in the kidney could extend the use of this delivery system for DKD.

4.4 Kidney targeted miRNA-based therapy

Podocytes are critical in the pathogenesis of DKD. Podocyte loss correlates with glomerulosclerosis and proteinuria [261-263], and a series of elegant studies in 2008 established that miRNA expression is crucial to podocyte homeostasis [264-266]. Subsequent transcriptional profiling of mRNAs and miRNAs, in combination with quantitative proteomic analyses, has provided further insights into the molecular interactions via which the healthy podocyte phenotype is maintained [267].

Numerous podocyte-linked miRNAs have been implicated in DKD pathogenesis. These include the highly expressed miR-146a, which is lost in preclinical models of DKD and in the glomeruli of patients with T2D, the latter loss is associated with increased albuminuria and glomerular damage [201]. Strategy designs to replace this lost miR-146a expression are informed by the knowledge that podocytes express the neonatal Fc receptor, which NPs can be modified to target [268], after which they are taken up via endocytosis and deliver their cargo directly to these cells.

Podocytes also express integrin $\alpha\beta3$, allowing podocyte-specific use of NPs modified with $\alpha\beta3$ -specific ligand cyclo-(Arg-Gly-Asp-D-Phe-Cys) [269]. Podocyte-specific targeting has been achieved previously for short interfering RNAs (siRNA), synthetic RNA duplexes that represent an alternative to miRNA-based therapeutics for manipulation of target gene expression in an anti-fibrotic context [270, 271], and therefore podocyte-specific delivery of miRNA mimics or anti-miRs is likely to be a feasible approach for miRNA-based therapy.

Under homeostasis mesangial cells play a role in glomerular hemodynamics and clearance of immune complexes, and produce a plethora of ECM proteins to support the glomerular capillaries. Mesangial expansion is a feature of early DKD, during which a combination of cell growth and increased ECM deposition leads to glomerulosclerosis and reduced renal capacity. Uniquely, NPs of specific size can target the mesangium [253]. In addition, efficient mesangial delivery of siRNAs encapsulated in cyclodextrin-containing polycations NPs (CDP/NPs) has been observed [272]. This mesangium-specific delivery can be further augmented by addition of a targeting ligand for the mannose receptor to the CDP/NP, since renal mannose receptor expression is restricted to the mesangial cells. Delivery of encapsulated siRNAs was successful as both mouse and human mesangial cells internalized siRNA/CDP-NPs, and *in vitro* delivered encapsulated siRNAs were shown to knockdown green fluorescent protein in a reporter mouse strain following intravenous administration [272]. These strategies offer further alternatives to target mesangial miRNAs.

Renal proximal tubular epithelial cells (PTECs) preferentially take up ASOs, providing clear opportunities to target the tubules with miRNA-based therapies. Several studies have shown renal accumulation of ASO-based anti-miRs following systemic administration [216, 243, 273, 274]. Tubular uptake of LNA-miR-214 post systemic delivery decreases renal tubular autophagy in DKD, reducing renal hypertrophy and albuminuria [275], and a chitosan derivative-based gene

delivery system has successfully been used to target tubular cells [276]. N, N-diethyl N-methyl and N-triethyl chitosan were synthesized from chitosan polymer, these derivatives were complexed with fluorescein isothiocyanate-dextran green/red fluorescent protein plasmid vectors and fluorescently-labelled miRNAs, and successfully transfected renal epithelial cells [276]. Protein uptake into PTECs is mediated by 2 receptors, megalin and cubilin. The megalin:cubilin complex is expressed on the apical PTEC membrane and Gao *et al.* found that chitosan polyplex NPs containing an siRNA against aquaporin 1 accumulated *in vitro* in PTECs and *in vivo* were exclusively found in cells expressing megalin, suggesting that a megalin-dependent endocytic pathway was responsible for uptake [277].

Delivery of an anti-miR-21 to mouse kidney *in vivo* has been achieved using small-sized cationic low molecular weight chitosan-modified PLGA NPs. This inhibitor showed antifibrotic effects in TGF- β 1-treated rat kidney epithelial cells from cell-line NRK-52E *in vitro*, and in kidneys from a UUO mouse model *in vivo* [278]. This delivery system showed high cell uptake and renal-targeting characteristics, as well as excellent biocompatibility [278].

4.5 Safety of miRNA-based therapeutics

Off-target effects are a key consideration in the development of effective miRNA-based therapies. Oligonucleotides and their means of delivery can trigger an inflammatory response through activation of cell surface TLRs [279, 280]. PS ASOs containing CpGs are potent stimulators of the innate immune system, but can also activate innate immunity in the absence of these sequence motifs, albeit at much higher doses [280]. This immunostimulatory effect is induced by binding to TLR9 for both CpG- and non-CpG-containing PS ASOs, in a myeloid differentiation protein-dependent manner [281]. However, commonly used chemical modifications such as LNAs, or the incorporation of 2'-O-methyl uridine or guanosine nucleosides [282] mitigate some of these effects by mimicking endogenous capped mRNAs, and have the

added benefit of decreased immunogenicity [283]. Anti-miRs with 2'-O-methyl residues also inhibit TLR7 sensing in a sequence-dependent manner, suggesting that potential for off-target effects on TLR7/8 function should be taken into account in the therapeutic development and *in vivo* application of these anti-miRNA oligonucleotides [284].

Since increased abundance of key miRNAs has been implicated in DKD and subsequent renal fibrosis, the use of anti-miRs to decrease miRNA abundance offers a potential avenue for therapy. Data on over 2,400 patients from 32 phase 2 and phase 3 clinical trials using anti-miRs with typical 2'-O-methoxyethyl conjugation revealed no evidence of clinically significant renal impairment, and no significant incidence of AKI, which held true in sub-populations of patients with diabetes and those with renal dysfunction at baseline [285]. Human pharmacokinetic studies have shown that the kidney accumulates the highest concentration of 2'-O-methoxyethyl conjugated ASOs following systemic administration [285]. This accumulation in the renal cortex is first order and saturates, therefore significantly increased ASOs or metabolite concentrations do not result from long-term treatment [285]. Comparative toxicological studies of high dose ASO use in healthy human volunteers and non-human primates have shown a number of toxicities in macaques with no class safety effects noted in healthy human volunteers, but nonhuman primates are thought to be more sensitive to these ASO-driven toxic effects than humans [286].

It is beneficial for the delivery of miRNA-mediated therapeutics that the kidney is a key site of ASO accumulation, but cellular distribution within the kidney is also vitally important. Distribution of 2'-O-methoxyethyl ASOs is altered in pre-clinical models of CKD, with a decrease to the renal cortex observed together with increased medullary accumulation [287]. Analyses in which 5'-Cy3-labelled-miR-21 ASOs, chemically modified ASOs to mature miR-21 containing PS linkages and modified sugar moieties including 2'-O-methoxyethyl were administered

subcutaneously to Col4a3^{-/-} mice that develop renal disease, anti-miR-21 was highly concentrated in the proximal tubule, but was also observed in distal tubules, endothelial cells, fibroblasts and F4/80+ macrophages [216, 274]. However, ASO-associated vasculitis and glomerulosclerosis have been observed in monkeys, apparently due to complement activation and endothelial injury [288], but may be a species effect [286].

5. Integration of miRNA therapy into renal patient treatment

5.1. Current state of miRNA clinical trials

Lifestyle modification, glycemic control, treatment of hyperlipidemia and other cardiovascular risk mitigation therapies are mainstays of treatment for patients at risk of DKD. The current epidemic of diabetes and DKD risk, and the limited success of the above treatment approaches in ameliorating its consequences, make the need for new therapies pressing. After many prior failures, the effects of the current generation of antidiabetic agents on renal and cardiovascular risks in this population are impressive. There remains, however, a significant shortfall of residual morbidity and mortality in those affected by DKD. MiRNAs have potential as contributors to redressing this gap in DKD treatment, as therapeutic targets, as therapeutic agents, and as biomarkers.

Moving RNA-based therapy into the clinic has proved challenging. In the past this has been due largely to the problem of safe and effective delivery of siRNAs to target cells. Various strategies are in development to address this, including a multiplicity of proposed chemical modifications to stabilize RNA molecules, systemic delivery systems, and local strategies including direct injection [289].

The challenge of delivery may be unequal in different tissues, with particularly high levels of uptake in liver and kidney following systemic administration [290]. This suggests that this therapeutic modality may be useful for kidney diseases, including DKD. Much of the initial clinical effort has focused on liver disease, and the field has received a major boost with the first RNAi-based therapy receiving US and European regulatory approval in 2018: Patisiran, an siRNA to transthyretin, reduces abnormal hepatic transthyretin production in people affected by hereditary transthyretin amyloidosis [291].

There are several candidate miRNA-based therapeutics in early phase clinical trials, including miRNA mimics and anti-miR sequences, some of which have progressed to phase 2 studies. Miravirsen, a miR-122 LNA anti-miR has shown potential for the treatment of hepatitis C virus (HCV) genotype 1. MiR-122 is essential for the completion of the life cycle of HCV expressed in the liver [292]. In phase 1 clinical trials, in some patients receiving high doses of Miravirsen monotherapy, HCV RNA was undetectable [293]. Due to its chemical modifications Miravirsen accumulates naturally in the liver, and therefore does not require a special delivery strategy. Multiple phase 2 clinical trials are currently underway.

Kidney-directed miRNA-therapies include the targeting of miR-21, which is associated with kidney injury and renal fibrosis [216, 294], in people with Alport Syndrome. This X-linked disorder is characterized by kidney disease and caused by mutations in the gene sequences encoding type-IV collagen [295]. RG-012 is a miR-21-specific anti-miR with PS, 2'-O-methoxyethoxy and constrained ethyl modifications. When tested in a mouse model of Alport Syndrome, RG-012 demonstrated improved survival and a reduction of kidney disease progression [274], and it is currently under evaluation, as Lademirsen, in patients with Alport syndrome [296].

MiR-17 has also been targeted in the kidney for the treatment of autosomal dominant polycystic kidney disease (ADPKD). Extensive preclinical studies demonstrated that miR-17 is upregulated in human and murine forms of polycystic kidney disease (PKD), and deletion or inhibition of miR-17 attenuates cyst growth in mouse PKD models [273, 297, 298]. RGLS4326, a first-in-class oligonucleotide inhibitor of miR-17, is preferentially taken up in the kidney and collecting duct-derived cysts [299]. Furthermore, RGLS4326 displaces miR-17 from translationally active polysomes, and de-represses multiple miR-17 mRNA targets [299]. Despite a pause in a phase 1 multiple ascending dose study for RGLS432 in healthy volunteers, due in part to unexpected observations in a 27-week mouse chronic toxicity study, the study completed in July 2020. A first cohort of nine patients with ADPKD has been enrolled in a phase 1 clinical trial and received 1 mg/kg of RGLS4326 subcutaneously every other week for four doses (Clinical Trial Identifier NCT04536688). No serious adverse events have been reported, but a longer half-life in plasma for RGLS4326 was observed.

5.2. Challenges to miRNA-based therapeutics

Accumulation of miRNA-based therapies in the kidney suggests that these drugs will be useful for the treatment of kidney disease. However, selective cell uptake remains a key hurdle to ensure prevention of off-target effects. As discussed above, various strategies for targeting podocytes, mesangium and tubular cells have been demonstrated, but a crucial missing step is to combine these methodologies to target the most appropriate miRNA targets.

Ongoing work into the chemical modification of oligonucleotides will continue to improve these drugs, and to reduce their immune effects and toxicities. The Oligonucleotide Safety Working Group [300] has published extensive guidelines for assessing the various aspects of safety [301, 302]. Oligonucleotide-mediated toxicity falls under two main categories: (i) hybridization-dependent effects, including on- and off-target effects, and (ii) hybridization-independent effects

which are mostly caused by protein-binding properties [303]. MiRNA-based therapies represent a new class of drugs. Currently, there is an abundance of data from preclinical models, but very limited clinical data. We must therefore remain conscious that toxicity/off-target effects are potential problems that must be borne in mind when designing potential therapeutic agents.

5.3. Acceleration of therapy development

We still await the first miRNA-based drug to reach pharmaceutical breakthrough, but several are currently in clinical trials, and more are entering this stage of development [304]. There is now increased industrial interest, with numerous examples of large pharmaceutical companies acquiring small miRNA therapy companies.

MiR-132 has recently been targeted in studies on heart failure using an LNA anti-miR which was effective in preventing and reversing heart failure in preclinical models, including demonstration of efficacy in large animal models [305]. These findings have recently been translated to the clinic with the first-in-human phase 1b randomized, double-blind, placebo-controlled study of CDR132L, a first-in-class miR-132-specific ASO (Clinical Trial Identifier NCT04045405). Results from the study, in which 20 patients were randomized to CDR132L and 8 to placebo, found that CDR132L was safe and well tolerated, confirmed linear plasma pharmacokinetics with no signs of accumulation, and although underpowered, there was a suggestion of improved cardiac function. To address the previous safety concerns with first-generation ASOs, thrombocytopenia was pre-specified as a potential side effect, but platelet counts remained normal throughout [306]. Importantly, liver and kidney function showed no signs of toxicity at the doses used [306].

These important new studies targeting miR-17 and miR-132 suggest that robust preclinical studies, including testing with larger animal models combined with screening compounds for optimal pharmacological features, will increase the translation potential of miRNA-based

therapeutics. The field of miRNA biology remains young, and as we gain better insight into the mechanisms of action of specific miRNAs, more rational selection of the best candidates will facilitate advancement towards effective translation.

6. Conclusions and future perspectives

From the above it is apparent that miRNAs have highly significant potential both as biomarkers and therapeutic agents for fibrotic kidney disorders including DKD. With respect to their use as biomarkers, mindfulness of practical considerations will be essential if these transcripts are to fulfil their potential to improve patient outcomes. Interactions with industry on the detection of miRNA biomarkers from liquid biopsies consistently emphasizes the desirability of sensitivity, cost, speed and simplicity that avoids the need for end-user expertise. MiRNA biomarker detection methodologies that can be integrated as cost-effective, high-throughput protocols into existing care pathways include a recently described electrochemical method that is more sensitive than RT-qPCR. This technology is currently under development for use in the biochemistry laboratory and / or at the point of care [307, 308].

Analysis of global gene expression has recently undergone profound methodological transformations for laboratory and computational analyses that can inform approaches to identify and deliver miRNA-based therapeutics [309]. Single-cell RNA sequencing is a particularly powerful method that offers the opportunity to analyze organs at much higher resolution than previous bulk sequencing methods.

In the case of the kidney, each nephron comprises the glomerulus, proximal and distal convoluted tubules, loop of Henle and collecting duct (Fig. 5), discrete functional regions populated by resident cells with different morphological and functional phenotypes. Single-cell RNA sequencing has identified rare cell types and previously unknown fibrosis-specific cell states

[310]. This technology has been used to log infiltrating cell populations in progression and regression of fibrotic disease [311] and single-nucleus RNA sequencing has identified new classes of PTECs in renal fibrosis [312]. Non-invasive robust data have also been generated from urine samples [313]. Regrettably, single-cell RNA sequencing has not yet been adapted for analysis of miRNAs. Nevertheless, recent publication of an atlas of cell-enriched miRNA expression including proximal tubular cells, endothelial cells, macrophages and fibroblasts is the closest yet to single-cell resolution in the kidney [232].

Particularly, perhaps, for those of us who devoted many hours to manual Sanger nucleic acid sequencing, it is hugely encouraging that the resources now at our disposal generate detailed data allowing thorough investigation into human gene expression and its relationships with health and illness. In addition to single-cell sequencing approaches, the application of genomic sequencing to precision medicine promises to improve patient stratification and maximise healthcare resource allocation [314-316].

In closing, the emergence of miRNAs and other noncoding RNAs, and the interaction of these transcripts with protein-coding mRNAs and their products, has provided new dimensions to the way we think about gene products [317]. The human genome can no longer be interpreted as a static linear sequence. An appreciation of macro-level genome configuration should also inform our analyses [318, 319] including copy number variation and dosage effects [320-322]. Indeed, an awareness of the time axis that our findings inevitably reflect [323] remains pivotal to a full and meaningful appreciation of the data which we can now access and its influence on complex processes including the regulation of gene expression [324] and human health [325]. As Dobzhansky put it so succinctly “nothing makes sense in biology except in the light of evolution” [326].

Declaration of Competing Interests

TB and DF are inventors for patent WO/2017/129977 Chronic Kidney Disease Diagnostic. LD is the recipient of PhD studentship co-funding from Regulus Therapeutics and GSK.

Sources of Funding

LD is supported by a Kidney Research UK Senior Fellowship SF_001_20181122. RJMC has been supported by a Medical Research Council (MRC) Senior Clinical Research Fellowship [139] and currently MRC project grant MR/T002263/1. He also receives support from Kidney Research UK and from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 115974. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA with JDRF. The authors also acknowledge support from Kidney Research UK Project Grant Award RP44/2014 to TB. This is a publication of independent research funded by the National Institute for Health Research (NIHR) Invention for Innovation (i4i) Programme (Grant Reference no. II-LA-0712-20003). The Principal Investigator for the grant is TB. The views expressed in this paper are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health, UK. The Wales Kidney Research Unit is funded by Health and Care Research Wales.

Acknowledgements

AW, DJF and TB are part of the Wales Kidney Research Unit (WKRU). We thank Judit Sutak, Department of Cellular Pathology, North Bristol NHS Trust, Bristol, UK, for the histopathological images. We also express our gratitude to Servier Medical Art (<https://smart.servier.com>) for free access to high-quality medical images.

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FIGURE LEGENDS

Fig. 1 – Key stages of miRNA biogenesis. In the nucleus, each miRNA is transcribed from its specific genomic locus by RNA polymerase II as a primary miRNA or pri-miR transcript. Intracellular editing follows to produce the pre-miRNA (or pre-miR) that is then exported to the cytoplasm where it is edited further to the mature miRNA duplex. Following this, the selected mature miRNA strand of the RNA, an argonaute protein and other factors combine to form the RNA-induced silencing complex (RISC) via which the miRNA elicits its effects on mRNA translation. DGCR8 = DiGeorge syndrome critical region 8, TRBP = transactivation response element RNA-binding protein, AGO = argonaute (I-IV).

Fig. 2 – The effect of the diabetic environment on the kidney and key features of DKD. The diabetic environment (DE) can result in kidney damage with widespread sclerosis and fibrosis evident in periodic acid Schiff (PAS) stained kidney biopsy tissue from a patient with diabetic kidney disease shown bottom left. Comparison of the “normal” and “diabetic” glomerulus, when stained with Jones silver stain, shows nodular matrix accumulation, Kimmelstiel-Wilson lesions and sclerosis. In response to the diabetic environment podocytes are injured, and may become hypertrophic, foot process fusion occurs and cells may detach; mesangial cells become activated, proliferate and produce increasing amounts of extracellular matrix proteins, resulting in the development of glomerulosclerosis; and tubular cells become injured and undergo atrophy. This injury results in immune cell infiltration, fibroblast activation, and increased production and deposition of extracellular matrix by myofibroblasts, leading to tubulointerstitial fibrosis.

Fig. 3 – Altered signaling pathways implicated in DKD, and differentially expressed podocyte miRNAs. The diabetic environment (DE) results in aberrant kidney cell signaling. There is increased signaling via the AGEs, JAK/STAT and TGF- β pathways, and decreased insulin signaling. Podocyte to endothelial cell signaling of VEGF and nitric oxide is also affected. Podocyte injury by the DE results in altered miRNA expression profiles. Examples of mechanistic roles for the key miRNAs listed are given in the text. Further details on the podocyte-specific roles of each miRNA in DKD are summarized elsewhere [179].

Fig. 4 – Chemical modification of miRNAs and delivery modalities of miRNA-based therapeutics. A) Formulation of miRNA therapeutics can be altered by chemical modifications that result in improved pharmacokinetic properties such as nuclease resistance and improved target binding. B) MiRNA-based therapy delivery platforms include the use of nanoparticles (NPs) in which the miRNA mimic or anti-miR are encapsulated. NPs can be coated in polyethylene glycol (PEG), in a process referred to as PEGylation, to improve systemic circulation time and reduce immunogenicity. These drug delivery platforms can be further modified to include conjugation with specific ligands.

Fig. 5 – The nephron, the functional unit of the kidney. Blood arrives in the glomerulus from which the urinary filtrate is filtered into the lumen of the tubule. Ions are excreted and absorbed, and water is retrieved, through the different segments of the tubule, which are intimately linked to peritubular capillaries. This filtration process forms concentrated urine. The different functional regions of the nephron are shown together with the renal cortex, and inner and outer medulla.

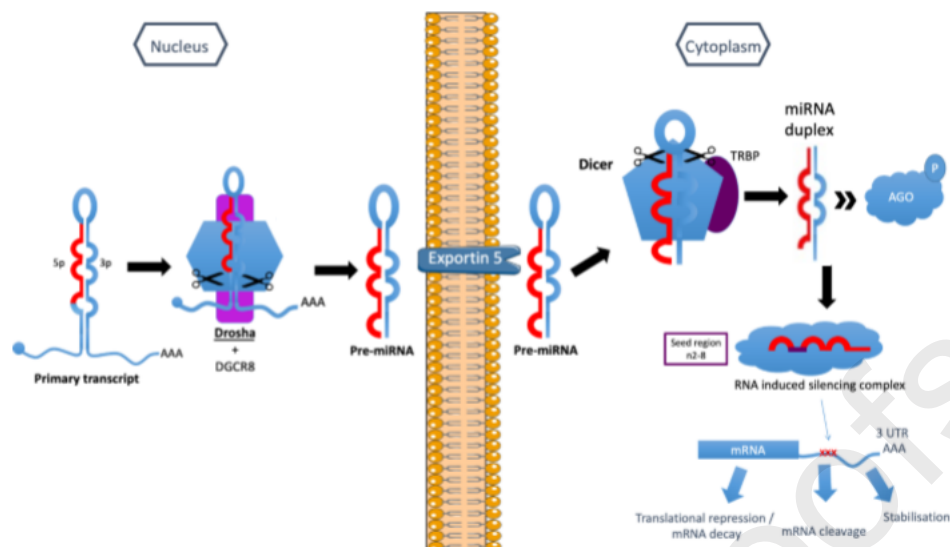


Figure 1

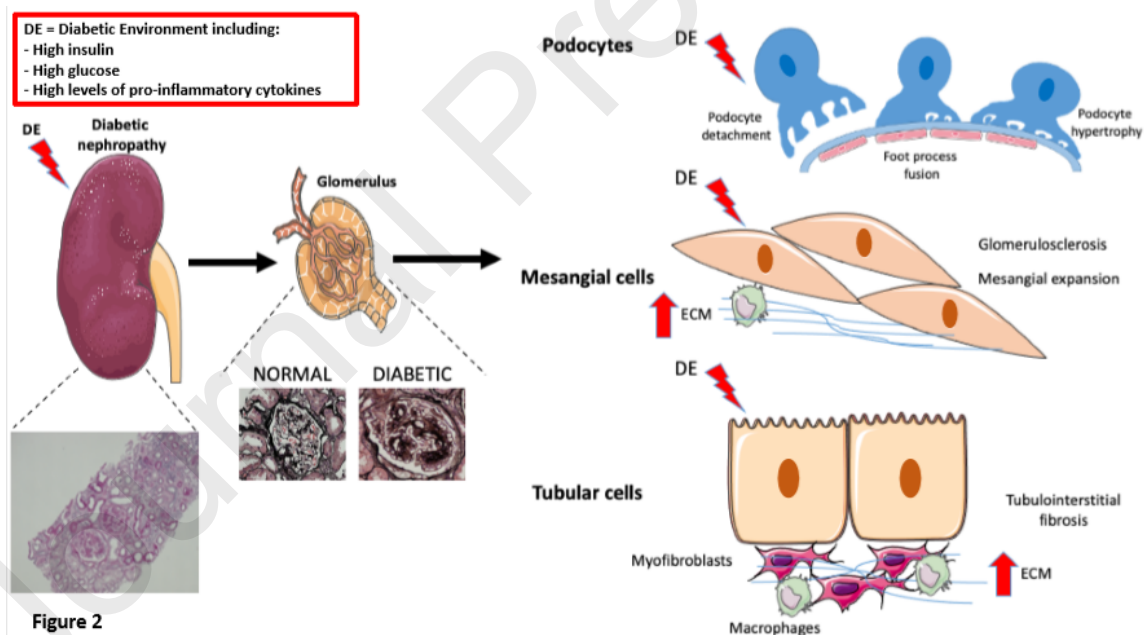


Figure 2

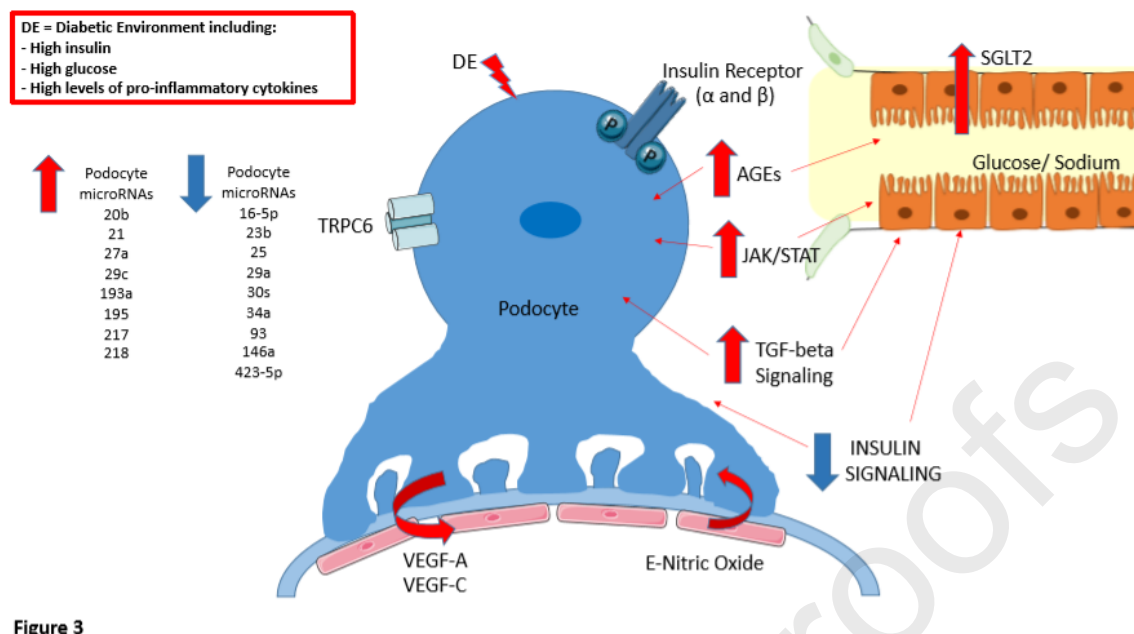


Figure 3

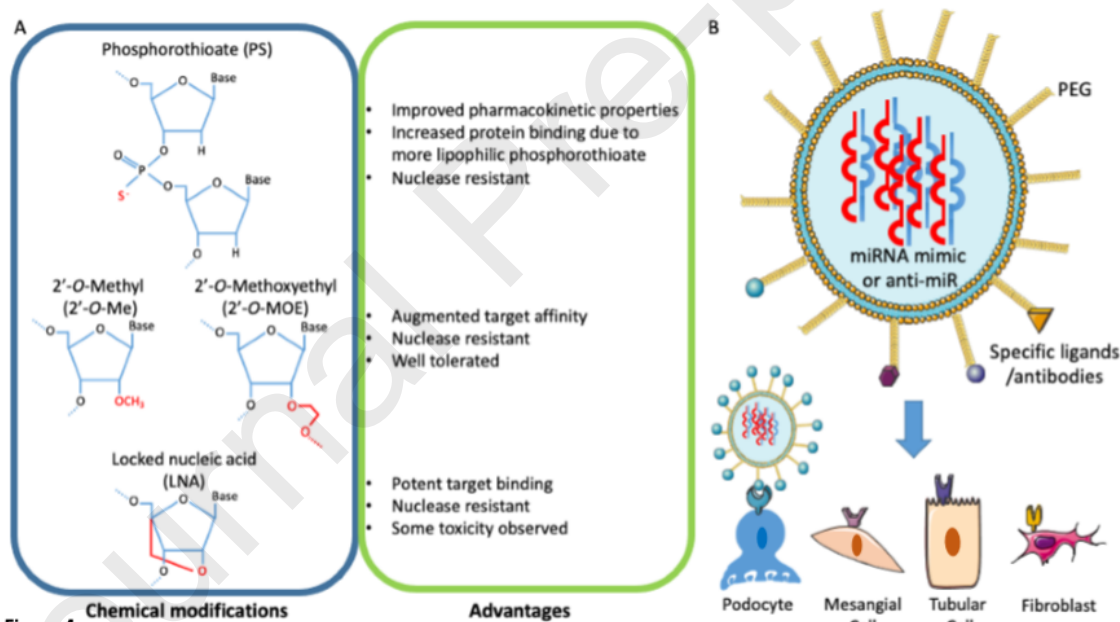


Figure 4

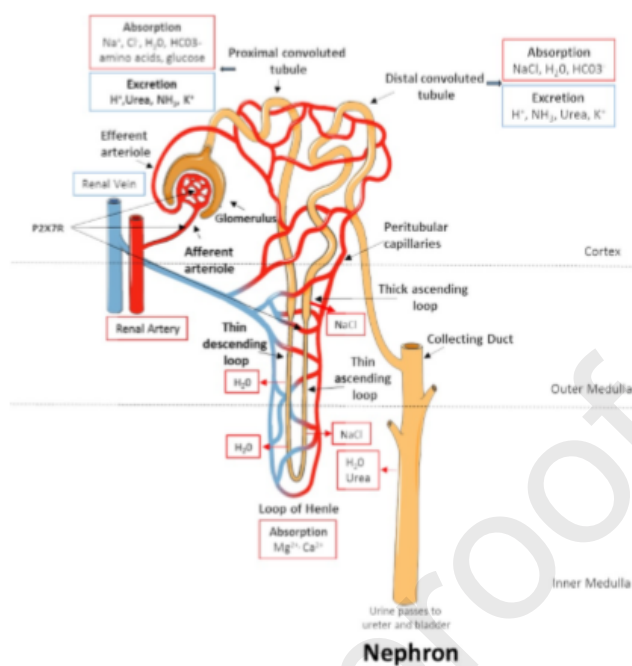


Figure 5

