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Full Review

RNA expression signatures and posttranscriptional regulation in diabetic nephropathy

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ABSTRACT

In the last decade, the integration of molecular approaches including transcriptome and miRNome analyses uncovered pathological mechanisms involved in the progression of diabetic nephropathy (DN). Using these techniques, molecular marker candidates [both messenger RNA (mRNA) and miRNA] have also been identified which may enable the characterization of patients at high risk for progression to end-stage renal disease. The results of such studies are urgently needed for a molecular definition of DN and for targeted treatment to improve patient care. The heterogeneity of kidney tissue and the minute amounts of RNA isolated from renal biopsies remain a challenge for omics-studies. Nevertheless, several studies have succeeded in the identification of RNA expression signatures in patients with diabetes and kidney disease. These studies show a reduced expression of growth factors such as VEGF and EGF, and an increased expression of matrix components and matrix-modulating enzymes, an activation of specific NF-κB modules, inflammatory pathways and the complement system. microRNAs are involved in the fine-tuning of mRNA abundance by binding to the 3′ untranslated region of a target mRNA, which leads in most cases to translational repression or mRNA cleavage and a decrease in protein output. Here, we review the platforms used for miRNA expression profiling and ways to predict miRNA targets and functions. Several miRNAs have been shown to be involved in the pathogenesis of DN (e.g. miR-21, miR-192, miR-215, miR-216a, miR-29, let-7, miR-25, miR-93, etc.). Functional studies provide evidence that miRNAs are not only diagnostic tools but also represent potential therapeutic targets in DN.

Keywords: chronic renal insufficiency, diabetic kidney disease, diabetes mellitus, diabetic nephropathy, gene expression

GENE EXPRESSION PROFILING IN RENAL TISSUE

On the histological level diabetic nephropathy (DN) is characterized by glomerular basement membrane thickening, mesangial expansion, nodular (Kimmelstiel-Wilsoon) or global glomerulosclerosis. These glomerular changes are often accompanied by tubular atrophy, interstitial fibrosis and vascular lesions (arteriolar hyalinosis and arterioslcerosis) [1, 2]. In the light of this heterogeneity of lesions, kidney biopsy remains an important tool to estimate the risk of progression to end-stage renal disease (ESRD) and to discriminate between diabetic and non-diabetic kidney disease. However, kidney biopsy is usually performed only in a minority of patients who present with nephrotic range proteinuria and where non-diabetic kidney disease is somewhat likely. In the vast majority of patients with DN, kidney biopsy is not performed, and the magnitude of albuminuria and decline in renal function are used for diagnosis and estimation of prognosis of DN. Novel biomarkers estimating the risk to develop progressive DN have been extensively studied, but these results have to be validated in sufficiently powered and well-designed studies [3]. Transcriptomic and miRNomic techniques together with systems biology tools provide an opportunity to identify even more accurate marker molecules. These markers have the potential to elucidate the pathogenesis of DN, serve as diagnostic and prognostic tools and hopefully will improve patient care in future.

The pathological changes described above are preceded and promoted by events such as infiltration of inflammatory cells, fibroblast activation and proliferation, excessive production and deposition of extracellular matrix (ECM) components and rarefaction of peritubular capillaries [4, 5]. At the molecular level, these processes are triggered by and lead to *de novo* intrarenal expression of transcripts representing damaging as well as protective/regenerative biological pathways [6]. Profiling RNA expression using omics-techniques describes the simultaneous alterations of thousands of genes. Using systems biology tools such as pathway analysis, differential gene expression can be further translated into activation/inhibition of specific biological pathways and networks. The results of such studies have substantially contributed to our understanding of renal disease [7].

Renal gene expression profiling can be performed from various sources of kidney tissue. In clinical routine, renal biopsies are formalin-fixed and paraffin-embedded (FFPE) before further staining. Obtaining sufficient amounts of RNA with adequate quality for microarray or RNA-sequencing analysis from FFPE sections to study long RNA molecules (>200 nucleotides) remains a challenge. Usually, RNA from such samples is chemically cross-linked and degraded, and has been primarily used for reverse transcription-quantitative PCR (RT-qPCR), ideally using short amplicons [8]. However, Hodgin *et al.* demonstrated the principal feasibility of transcriptomics analysis from archived routine FFPE-sections [9]. Using T7 promoter-based RNA amplification, the authors identified transcripts in microdissected glomeruli, which differentiated between classic focal-segmental glomerulosclerosis (FSGS)/collapsing FSGS and controls/minimal change disease (MCD), respectively. Currently, most researchers use either frozen (cryo-cut) biopsy sections [10] or RNA later preserved biopsies at low temperatures [8]. For microRNA gene expression analysis, the fragmentation status of the RNA appears to be less important as various methods (RT-qPCR, microarray hybridization, RNA sequencing) have been successfully used on challenging samples such as FFPE or body fluids like serum or plasma (see further).

Kidney tissue is a complex 3D structure consisting of several compartments and numerous cell-types. Cell- or compartment-specific transcriptomic responses can be identified by isolation of tubuli [11] or glomeruli [12] by laser-capture microdissection, sieving of the glomeruli [13] or by dissection of the whole tubulointerstitial compartment [14]. The origin of the RNA has to be taken into account when conclusions are drawn, particularly, when datasets are compared between cohorts, between human subjects and animal models or between humans and *in vitro* cell-culture experiments.

TRANSCRIPTOMICS IN HUMAN DIABETIC NEPHROPATHY

The results of selected transcriptomic studies in human DN are summarized in Table 1. After isolation of human glomeruli by sieving, Baelde *et al.* identified differential gene expression between two patients with DN and two control subjects. Ninety-six genes were upregulated and 519 genes were downregulated in glomeruli from subjects with diabetes mellitus [13]. Gene expression and pathway analysis of those genes, which were decreased in DN suggested a disturbed cytoskeleton formation and an accumulation/reduced degradation of extra-cellular matrix components. Also, the diminished expression of certain tissue repair-related genes, such as bone morphogenetic protein-2 (BMP-2), fibroblast growth factor-1 (FGF-1), insulin-like growth factor binding protein-2 and connective tissue growth factor (CTGF) suggested a reduced tissue repair capacity. Finally, the reduced glomerular messenger RNA (mRNA) levels of vascular endothelial growth factor (VEGF) may be responsible for impaired endothelial repair potential and capillary rarefaction.

Lindenmeyer *et al.* performed gene expression profiling in the tubulointerstitial compartment from renal biopsies of patients with diabetes and nephropathy, and from pretransplantation biopsies of living donors, which served as control subjects [15]. The growth factors VEGF and epidermal growth factor (EGF) showed reduced expression, while matrix components such as collagens I and IV, fibronectin 1 and vimentin as well as matrix-modulating enzymes (matrix metalloproteinase-2, -7 and -14 and tissue inhibitor of metalloproteinase 1 and 3) showed increased expression in subjects with diabetes. A functional network analysis showed a central role for VEGF and EGF in DN, and the reduced expression of VEGF and EGF particularly in the proximal tubular cells was confirmed by immunohistochemical staining. The decrease of VEGF was associated with a reduced peritubular capillary density and an increase in proteinuria in patients with DN. These results are in contrast to increased VEGF expression in rodent models of DN [19–21] and in human diabetic retinopathy [22], suggesting different pathophysiological mechanisms of these microvascular changes.

Proteinuria and hyperglycaemia have been suggested as factors contributing to endoplasmatic reticulum (ER) stress response in the kidney of patients with DN. In a transcriptomic analysis of genes involved in 'unfolded protein response' (UPR; i.e. ER stress) it has been shown that subjects with progressed DN show a significantly different expression of UPR genes than patients with stable DN [16]. The same upregulation of selected UPR genes HSPA5, HYOU1 and XBP1 was detected in patients with MCD, although the extent of gene and protein expression was less than in patients with DN. Nevertheless, this transcriptomic study points to proteinuria as a significant cause of UPR in kidney tissue particularly in human DN. Interestingly, the lack of proapoptotic signals in both DN and in MCD cases suggests an adaptive, protective UPR, at least in early stages of DN.

Using a combined approach of unbiased RNA expression profiling with a hypothesis-driven pathway analysis, Schmid *et al.* identified a set of mainly inflammatory genes that were differentially regulated in the tubulointerstitial compartment of renal biopsies from subjects with progressive DN as compared with early and stable DN [14]. A group from these differentially regulated stress response genes directly linked to the NF-κB pathway, indicating an induction of this pathway in progressive DN. Further analysis of the expression values of downstream NF-κB target mRNAs identified the involvement of

Table 1. Selected transcriptomic studies in human DN

Authors	Subjects	Tissue	Study approach	Primary outcomes	Major results
Baelde *et al.* [13]	DN ($n = 2$) CTRL ($n = 2$)	Glomeruli	Unbiased: DN versus CTRL	DEGs 96 ↑ and 519 ↓	BMP-2 ↓, FGF-1 ↓, IGFBP-2 ↓, CTGF ↓, VEGF ↓ Validated by qPCR of VEGF
Lindenmeyer *et al.* [15]	DN ($n = 11$) CTRL ($n = 3$) MCD ($n = 4$)	Tubulointerstitium	Targeted common hypothesis (202 genes) and pathway analysis: DN versus CTRL	DEGs 38 ↑and 11 ↓ Functional network in DN	EGF ↓ VEGF-A ↓ Validated by IHC and qPCR Reduced capillary density
Schmid *et al.* [14]	DN ($n = 13$) CTRL ($n = 7$) MCD ($n = 4$)	Tubulointerstitium	Unbiased and targeted (232 NF-κB targets): DN versus (CTRL + MCD)	1349 DEGs between DN, CTRL and MCD	NF-kB targets ↑ in progressive DN
Lindenmeyer *et al.* [16]	DN ($n = 11$) CTRL ($n = 3$)	Tubulointerstitium	Targeted hypothesis (28 ER stress genes): DN versus CTRL	No DEGs in mild DN compared with CTRL 6 ↑ in established DN compared with CTRL	ER stress ↑ Unfolded protein response ↑
Woroniecka *et al.* [17]	DN ($n = 9$) CTRL ($n = 13$)	Glomeruli	Unbiased: DN versus CTRL, pathway analysis	1700 DEGs Altered pathways	Podocyte-specific RNAs ↓ Fibrosis/inflammation ↑ Complement pathways ↑
Woroniecka *et al.* [17]	DN ($n = 10$) CTRL ($n = 12$)	Tubulointerstitium	Unbiased: DN versus CTRL, pathway analysis	1831 DEGs Altered pathways	Fibrosis/inflammation ↑ Complement pathways ↑
Hodgin *et al.* [18]	DN ($n = 22$) MN ($n = 21$) ND ($n = 5$) DBA STZ mice BKS db/db mice BKS eNOS$^{-/-}$ db/db mice	Glomeruli	Genome-wide cross-species comparison (overlap) of transcriptional networks Human: Albuminuria versus no albuminuria ($n = 1$) Mouse: Each model versus control ($n = 3$)	Model-specific, species-specific and cross-species networks and gene nodes	Common genes and pathways in human and all three mouse models: JAK/STAT, VEGFR, IL-7, FGF, HIF-1α

DN, diabetic nephropathy; CTRL, controls (zero-hour biopsies of kidney donors); MCD, minimal change disease; DEGs, differentially expressed genes; IHC, immunohistochemistry; qPCR, quantitative real-time PCR; ER, endoplasmatic reticulum; MN, membranous nephropathy; ND, non-diabetic.

a specific NF-κB transcription factor module (NFKB_IRFF_01) rather than the activation of the whole NF-κB pathway in progressive DN.

Woroniecka and colleagues provided an unbiased summary of gene expression identified separately in microdissected glomeruli and tubuli from subjects with diabetic kidney disease [17]. In diabetic glomeruli, a decreased expression of the majority of probesets was observed, with a downregulation of almost all podocyte-specific transcripts (e.g. PLCE1, PTGDS, NPHS1, NPHS2, SYNPO, PLA2R1, WT1, CLIC5, PODXL), while transcripts associated with fibrosis and inflammation were increased (e.g. IGH, C3, COL1A2, CXCL6, COL6A3). Pathway analysis highlighted the regulation of complement pathway, focal adhesion and integrin and ECM receptor pathways in diabetic glomeruli. In diabetic tubular tissue an increased expression of genes particularly associated with fibrosis and inflammation was identified (IGH, IGL and multiple collagen transcripts), which was also confirmed using pathway analysis tools. Notably, of approximately 1700–1800 genes that were differentially regulated between controls and either the glomerular or the tubulointerstitial compartment, only 330 genes (≈19%) showed differential expression in both compartments, thus indicating a compartment-specific regulation of gene-expression in diabetic kidney disease. However, the complement system was identified as one of the significantly regulated pathways in both glomerular and also in the tubulointerstitial compartment with an increased expression of several members of the complement system (e.g. C3, CD55, C1QA, CD46, C1QB, CFB, C4A/C4B, C7, CFH, C3AR1, CR1 and C2). The glomerular expression of C3 was heterogeneous on both the mRNA and the protein level as shown by semi-quantitative immunohistochemistry (it was present in 50% of the samples), and it was associated with increased glomerulosclerosis.

Various murine models that mimick human diabetic kidney disease have been used to identify factors that cause or predict DN [23]. However, these models show rather early manifestations of DN such as moderately elevated albuminuria, mesangial expansion, mild glomerulosclerosis and reduction in glomerular podocyte number. In the majority of cases, however, human DN is a late and progressive manifestation of the disease. These differences have to be taken into account, when transcriptomic data are compared between murine models and human subjects. Hodgin *et al.* performed such a comparison of glomerular transcriptional networks between humans with early type 2 DN (microalbuminuria and normoalbuminuria, normal/elevated glomerular filtration rate), and three mouse models (streptozotocin DBA/2, C57BLKS db/db and eNOS-deficient C57BLKS db/db mice) using a systems approach [18]. Analysis of differentially regulated genes showed only little overlap between any two of the models, and level less commonality among all three. However, on the level of biological pathways cross-species overlaps between all mouse models and human subjects were found in the JAK/STAT signalling, VEGF and VEGF-R signalling, HIF-1α transcription factor network, IL-7 signalling and FGF signalling pathways, respectively. Interestingly, some pathway changes were seen only between the human subjects and a specific mouse model, e.g. enrichment of genes involved in

epidermal growth factor receptor-1 (EGFR1) signalling was only seen in a network shared between humans and streptozotocin DBA/2 mice. In addition, direction of gene expression changes was significantly discordant between late human DN and the mouse models, and much more similar when very early human DN (i.e. no albuminuria) was compared with the murine models. These findings suggest that transcriptional responses in mouse models of DN are similar to those in human subjects that occur very early in human DN, even before albuminuria develops. However, this limits their utility for analysis of late stages of DN.

LONG NON-CODING RNAS

Genome-wide studies have shown that the human genome is pervasively transcribed, producing thousands of non-coding RNA (ncRNA) transcripts, including small RNAs (e.g. miRNAs, piRNAs, snRNAs) and long non-coding RNAs (lncRNAs). MiRNAs have been most thoroughly investigated, function as negative regulators of gene expression and are predicted to target as much as 60% of all human protein coding genes (see below). LncRNAs are by definition longer than 200 nucleotides and display no evidence for protein coding potential. They are (alternatively) spliced, but have typically fewer exons and are expressed at lower levels compared with protein coding genes.

Many of these lncRNAs are associated with disease-linked SNPs or show pronounced tissue-specific expression profiles [24, 25], hinting at a possible role in human disease and development. The potential importance of lncRNAs (and ncRNAs as a whole) in development is further supported by the intriguing observation that organism complexity is strongly correlated to the proportion of the genome that is non-coding [26, 27]. To investigate the functional relevance of lncRNAs in various physiological conditions, the laboratory of John Rinn generated 18 mouse lncRNA knockout strains [28]. Strain characterization revealed peri- and postnatal lethality and developmental defects for various knock-outs demonstrating their importance in development. Unlike miRNAs, lncRNAs primarily regulate gene expression at the transcriptional level by binding and (re-)positioning transcription factors or proteins involved in the regulation of chromatin architecture [29]. Often, lncRNAs repress gene repression by actively recruiting the polycomb repressive complex PRC2 towards specific loci in the genome. This can occur both in *cis* and in *trans* and results in tri-methylation of H3K27, marking transcriptionally silent chromatin. One example is HOTAIR, a lncRNA transcribed from the HOXC locus that interacts with PRC2 [30]. Alternatively, lncRNAs can activate gene expression in *cis* through recruitment of the WDR5/MLL complex, resulting in tri-methylation of H3K4 [31] or recruitment of the Mediator complex, resulting in phosphorylation of H3S10 [32]. In both cases, chromosomal looping brings the lncRNA in close proximity to its target genes. Considering all available literature on lncRNA functions, four archetypes of molecular functions emerge that lncRNAs execute, i.e. signals, decoys, guides and scaffolds [29]. Several lncRNAs display characteristics from multiple archetypes that, alone or in combination, are critical for its function. HOTAIR is such an example, acting as a signal for positional identity in the body, serving as a scaffold by binding to protein complexes and acting as a guide by targeting ribonucleoprotein complexes to proper locations in the genome.

MICRORNAS IN HEALTH AND DISEASE

Given their spatio-temporal expression patterns, miRNAs are involved in fine-tuning of mRNA abundance in a tissue-specific manner. They act by regulating gene expression through sequence-specific binding to the 3′ untranslated region (3′ UTR) of a target mRNA, but several lines of evidence indicate that miRNAs can also bind to other regions of a target mRNA [33, 34]. The miRNA–mRNA interaction usually causes translational repression and/or mRNA cleavage and thus reduces the final protein output; as such broadly contributing to tissue specificity of mRNA expression in many human tissues [34]. A significant proportion of the protein coding fraction of the genome is under the influence of one or more miRNAs [35]. Functionally, miRNAs can target mRNA molecules involved in many biological processes, including cell growth and development, cell fate and apoptosis. Given that miRNA transcripts affect nearly every aspect of cellular function, it is not surprising that they play a critical role in the aetiology of a wide variety of disease manifestations [36].

miRNAs have been implicated in many types of cancers, as well as specific cardiac and neurologic diseases [34–40]. Furthermore, studies have identified tissue-specific miRNA signatures that have the potential to act as diagnostic markers in human disease [41–45]. Patterns of miRNA expression alone classified tumour samples according to their tissue of origin [46]. Remarkably, the panel employed in this analysis was composed of only a few hundred miRNAs, yet was more effective at identifying tumours of unknown origin than approaches using thousands of mRNAs.

Due to the tissue-specific deregulation of miRNA expression in cancer, multiple studies have explored the potential usefulness of miRNA expression profiles as biomarkers of diagnosis, prognosis and response to treatment, in cancer and other diseases [38]. In this setting, miRNAs hold a major advantage over mRNA biomarkers as they are characterized by superior stability in degraded RNA samples [45]. This enables miRNAs to be accurately quantified in challenging samples such as archived FFPE tissue blocks [46], for which mRNA measurements are no longer possible or very challenging, or in clinically relevant body fluids (Figure 1) [47].

MICRORNA EXPRESSION PROFILING

Several measurement platforms have been developed to determine relative miRNA abundance in biological samples using different technologies such as small RNA sequencing, RT-qPCR and (microarray) hybridization. Each of the available platforms is characterized by specific features such as required amount of input martial and number of miRNAs detected.

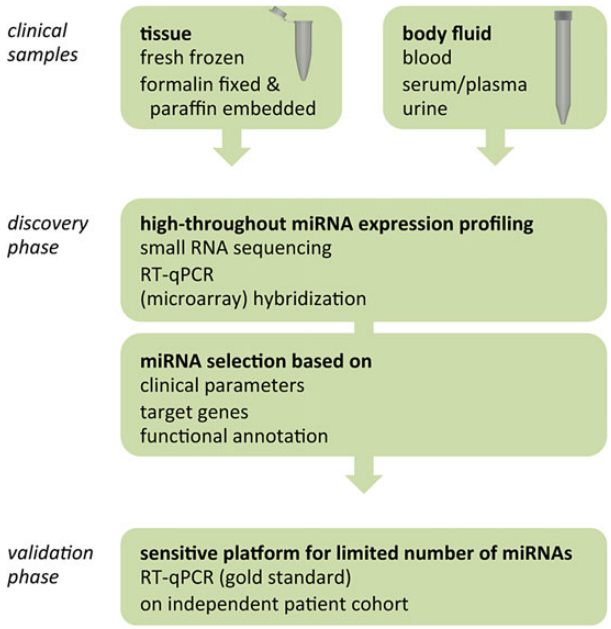

FIGURE 1: Workflow for the discovery of miRNA biomarkers of disease. Given their stability, miRNAs can be accurately quantified in challenging clinical samples such as formalin-fixed paraffin-embedded (FFPE) samples and different body fluids, making them ideal biomarkers from a technical point of view. First, a discovery study is performed in which a large series of miRNAs are quantified in a well-characterized sample set. This allows the identification of miRNAs that are able to discriminate between different disease subgroups, response to treatment, etc. In addition, knowledge regarding miRNA targets and functional annotation might aid in the prioritization of candidate miRNAs. Next, the discriminative power of the candidate miRNAs is validated in an independent patient cohort. RT-qPCR is the gold standard technique for cost-effective and accurate quantification of a limited panel of miRNAs in a large series of samples. Profound understanding of the strengths and limitations of different profiling platforms as well as sample requirements will contribute to a robust study design that increases the power of discovering miRNA biomarkers for disease.

While sequencing-based methods know no restraints regarding the latter, they generally require more input material, which might be limited for precious clinical samples. RT-qPCR and hybridization methods on the other hand, can only profile those miRNAs for which assays or probes are available.

Because of the small size of mature miRNAs, the high degree of homology between miRNA family members and the low abundance of miRNAs in body fluids, miRNA expression profiling is technically challenging; and the choice of profiling platform is highly dependent on the research question and samples to be profiled. To guide researchers in their choice of optimal miRNA profiling platform for their specific application, Mestdagh and colleagues set up a microRNA quality control (miRQC) study, in which they comprehensively assessed quantitative miRNA gene expression platforms [48]. To this end, a set of 20 standardized positive and negative control samples was profiled on 12 commercially available miRNA profiling platforms, and robust quality metrics were developed to objectively assess platform performance in terms of reproducibility, sensitivity, accuracy, specificity and concordance of differential expression. The results from this miRQC study indicate that each miRNA expression profiling method has its strengths and weaknesses, which help to guide informed selection of a quantitative microRNA gene expression platform for particular study goals.

In terms of miRNA expression profiling for discovery of biomarkers for clinical applications, a study design consisting of two phases, discovery and validation, is the gold standard (Figure 1). In the discovery phase, a first set of clinical samples, consisting of distinctive clinical subgroups according to the research question, is profiled on a platform that covers as many miRNAs as possible (e.g. massively parallel sequencing). Sequencing platforms are sensitive when RNA is not limiting; however, they lose sensitivity for low-input-amount RNA samples [48], ruling out the use of samples with limited RNA in the discovery phase (here, PCR-based methods are generally used). In the second phase, selected miRNA markers identified in the first phase should be validated on an independent patient cohort. Furthermore, with an average validation rate for differentially expressed miRNAs of only 54.6% between any two platform combinations, it is strongly advised that initial screening or discovery studies are followed by targeted validation using an alternative platform or technology, for example RT-qPCR, which is considered as the gold standard in validation studies [48].

PREDICTING MICRORNA TARGETS AND FUNCTIONS

Upon identification of miRNA biomarkers for a particular disease, gaining insights into the molecular processes that are regulated will contribute to the broader understanding of the pathophysiological role of the respective miRNAs. Functionally characterizing miRNAs relies on identifying the biologically relevant target mRNAs that they regulate. To this end, numerous bioinformatic and experimental approaches have been developed to identify miRNA target genes. Many computer programs, including Targetscan [49], Pictar [50], Mirò [51], miRanda [52], Mirmap [53], miRDB [54] and Diana Lab [55] predict target genes based on 3′-UTR complementarity sequences. Although miRNAs are about 22 nucleotides in length, the 5′ miRNA seed region, spanning 6–8 nucleotides, is the most crucial component for recognizing and binding to target sites in the 3′UTRs of genes [56]. Most miRNA target prediction programs exploit this complementarity as well as the fact that true sites tend to be conserved between related species, with different algorithms using slightly different criteria for each process [57]. In addition, (Gibbs) free energy, a measure of the stability of a biological system, and site accessibility, a measure of the ease with which a miRNA can locate and hybridize with an mRNA target, are often taken into account [57].

Each miRNA target prediction algorithm has a sizable rate of both false positive and false negative predictions [58], and thus more than one algorithm may be necessary to make predictions about any particular gene or microRNA; such

comparative analysis is possible via the decodeRNA web tool (http://www.decoderna.org) (formerly microRNA bodymap [59]), which allows to assess the overlap in miRNA target predictions from eight different databases (TargetScan, miRDB, MicroCosm, PITA, RNA22, DIANA, TarBase, and miRecords). In addition, *in vitro* or *in vivo* confirmation of *in silico* predictions has become a required component of the workflow that establishes a miRNA–mRNA interaction. The gold standard in miRNA target validation involves the exploration of direct miRNA gene targeting with a two-fold 3′UTR luciferase assay. In this assay, cotransfection of a miRNA mimic and a vector containing the 3′UTR of the gene of interest downstream of a luciferase reporter gene, will result in decreased luminescence signal when the transfected miRNA mimic targets the luciferase-3′UTR fusion transcript. An additional luciferase gene under control of a constitutive promoter, which is cotransfected into the cells, can function as a normalizing signal [60]. This assay is conclusive regarding a miRNA–mRNA interaction only when a parallel experiment with a mutated 3′UTR shows that lack of miRNA binding cannot affect luciferase activity.

Over the past years, the availability of genome-wide mRNA expression data has moved the functional annotation of miRNAs beyond the mere identification of their target genes. Different web tools have been developed to allow the multi-level integration of corresponding miRNA and mRNA gene expression levels and miRNA target prediction. Upon large-scale identification of miRNA targets via combination of both levels, biological functions can be assigned to each miRNA on the basis of enrichment of biological processes among the regulated mRNA targets. Bioinformatics tools, such as miRGator [61, 62], miRDB [54], miRò [51], MAGIA [63] and FAME [64], have been developed with target prediction as a built-in functional annotation. The more recently established decodeRNA web tool [59], allows to elucidate tissue-specific miRNA functions that goes beyond miRNA target prediction and expression correlation. The applied approach is based on a multi-level integration of miRNA and mRNA gene expression and miRNA target prediction in combination with transcription factor target prediction and mechanistic models of gene network regulation. In addition, decodeRNA enables prioritization of candidate miRNAs based on their expression pattern or functional annotation across tissue or disease subgroup, through a user-friendly user interface.

MICRORNA IN DIABETIC KIDNEY DISEASE

The involvement of miRNAs in the pathogenesis of chronic kidney disease has been reviewed in detail [65, 66]. Here we summarize the evidence on miRNAs that are particularly involved in diabetic kidney disease. TGFβ1, high glucose and oxidative stress are commonly accepted major players in DN. In 2004 Sun *et al.* [67] found kidney specific enrichment of various miRNAs, including miR-192, miR-215 and miR-216a. The first studies investigating miRNAs in animal models of DN have been performed at the end of the last decade. Up to now, a rapidly increasing number of miRNAs involved in pathogenesis of DN have been published. Especially, miR-192 has become a focus of research. *In vivo* inhibition of miR-192 significantly decreased renal fibrosis in STZ-induced diabetic mice [68]. It has been shown *in vitro* in mouse mesangial cells that miR-192 regulates a miRNA-circuit (including miR-200b/c) that amplifies TGFß1-signalling, promotes ECM-deposition by regulating the expression of collagens (e.g. Col1a2 and Col4a1) and accelerates tubulointerstitial fibrosis in DN [69]. In contrast, Krupa *et al.* showed that loss of miR-192 might promote fibrogenesis in progressed human DN [70]. Furthermore, Wang *et al.* proposed that miR-192 increases the expression of E-cadherin independently of TGFß1, but does not affect the expression of ECM proteins in proximal tubular cells [71]. These seemingly contradictory results point towards a compartment- and cell-specific characteristics of miR-192, at least in renal cells and in (diabetic) kidney disease. Target genes of miR-192 are Zeb1 and Zeb2, also called E-cadherin transcriptional repressors (TCF8/δEF1) and SMAD-interacting protein 1 (SIP1), respectively. TGFß1 induces a transcriptional cross-talk between miR-192 and p53 via Zeb2 leading to cellular hypertrophy of mesangial cells and ECM accumulation—two key features of DN [72].

Other miRNAs suggested to be involved in progressive DN are miR-29 and let-7. TGFß1 inhibits the expression of members of the miR-29a/b/c family in proximal tubule cells, mesangial cells and in podocytes, resulting in increased expression of ECM proteins such as collagens I and IV [73]. In mouse models of DN, a reduced let-7b expression was found, while the expression of the TGFß1-receptor (TGFß1R) was increased. In proximal tubule cells knockdown of let-7b resulted in increased expression of TGFß1R, while ectopic expression of let-7b repressed the TGFß1-receptor, reduced the expression of ECM proteins, decreased SMAD3 activity and attenuated the profibrotic effects mediated by TGFß1 [74].

Fiorentino *et al.* demonstrated renal upregulation of miR-21, miR-217 and miR-221 in diabetic mice and also in human DN [75]. These miRNAs target (either directly or indirectly via downregulation of SirT1) tissue inhibitor of metalloproteinase 3 (TIMP3), and it has been shown that loss of TIMP3 promotes diabetic renal injury [76]. In addition, miR-21 has been shown to target SMAD7/TGFß1- and NF-kB-pathways, aggravate epithelial-to-mesenchymal transition (EMT) and accelerate renal fibrosis in diabetic mice [77]. Inhibition of miR-21 by knockdown plasmids in a mouse model of DN ameliorated microalbuminuria, renal fibrosis and inflammation [78]. On the other hand, Lai *et al.* showed that loss of miR-21 in TGFß1-transgenic mice was associated with increased albuminuria, podocyte depletion and mesangial expansion. Inhibition of miR-21 was accompanied by increases in TGFß1/Smad3-signalling activity and expression of proapoptotic target genes. Furthermore, albuminuria in humans with DN correlated significantly to glomerular but not to tubulointerstitial miR-21 levels, which suggests miR-21 rather as an inhibitor of TGFß1-functions, at least in diabetic renal disease [79].

Increased availability of reactive oxygen species (oxidative stress) generated in large part from NADPH oxidase (Nox) family members represents a major cause of diabetic renal disease. In a rat model of DN and in glucose-treated mesangial cells expression levels of Nox4 were increased, which was

accompanied by decreased expression of miR-25. *In vitro* studies in mesangial cells identified Nox4 as a target of miR-25 [80]. Furthermore, high glucose downregulated the expression of miR-93 in podocytes in endothelial cells and also in the glomeruli of diabetic db/db mice, which correlated with increased expression levels of VEGF-A. On the other hand increased expression of miR-93 abrogated VEGF-A expression, and also prevented the effect of high glucose on VEGF downstream targets fibronectin and Col4a3, suggesting an anti-angiogenic and anti-fibrotic role of miR-93 [81].

MicroRNAs are differentially expressed in various stages of DN and are also found in the extracellular environment. Hence miRNAs can serve as biomarkers to predict the course of disease. Argyropoulos and colleagues analysed miRNA expression in the urine of patients with diabetes mellitus type 1, and they identified a set of 27 deregulated miRNAs across different clinical stages of diabetic renal disease [82]. These miRNAs and their targets map to pathways of known relevance in renal complications of diabetes, such as growth factor signalling, apoptosis, immunity, substrate metabolism and transmembrane transport, thus strongly suggesting the renal source and pathogenetic relevance of these miRNAs.

In summary, the amount of data on the involvement of miRNAs in the pathogenesis of DN increases continuously [65, 66]. Pharmacological modulation of some of these miRNAs may have therapeutic potential by inhibiting glomerular hypertrophy or deposition of ECM proteins in both the glomerular and the tubulointerstitial compartment. Furthermore, miRNAs may serve as biomarkers to predict progression of DN. Importantly, a multi-centre validation of the findings published to date is warranted. In addition, we will have to extend our knowledge of miRNA–mRNA interactions in general and in a cell-specific context (e.g. proximal tubule versus mesangial cells) in order to provide an appropriate basis for future studies in humans.

TRANSPARENCY DECLARATIONS

The authors declare no conflicts of interest.

CONFLICT OF INTEREST STATEMENT

None declared.

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