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MicroRNAs: Potential Mediators and Biomarkers of Diabetic Complications

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Abstract

The incidence of diabetes is escalating worldwide and consequently, this has become a major healthcare problem. Moreover, both type 1 and type 2 diabetes are associated with significantly accelerated rates of microvascular complications including retinopathy, nephropathy and neuropathy, as well as macrovascular complications such as atherosclerotic cardiovascular and hypertensive diseases. Key factors have been implicated in leading to these complications including hyperglycemia, insulin resistance, dyslipidemia, advanced glycation end products, growth factors, inflammatory cytokines/chemokines and related increases in cellular oxidant stress (including mitochondrial) and endoplasmic reticulum stress. However, the molecular mechanisms underlying the high incidence of diabetic complications, which often progress despite glycemic control, are still not fully understood. MicroRNAs (miRNAs) are short non-coding RNAs that have elicited immense interest in recent years. They repress target gene expression *via* post-transcriptional mechanisms and have diverse cellular and biological functions. Herein, we have discussed the role of miRNAs in the pathobiology of various diabetic complications, their involvement in oxidant stress, and also the potential use of differentially expressed miRNAs as novel diagnostic biomarkers and therapeutic targets.

Complications Associated with Diabetes

The incidence of diabetes worldwide, and most notably in the United States, is increasing at an alarming rate and is expected to get worse since, besides those diagnosed with diabetes, there are millions of people showing signs of insulin resistance, glucose intolerance and pre-diabetes. Diabetes is a metabolic disorder that results in elevated fasting blood sugar levels due to the inability to produce sufficient insulin or due to insulin resistance. Type 1 diabetes (T1D) is an autoimmune disorder characterized by the destruction of pancreatic beta cells and loss of insulin production. Type 2 diabetes (T2D) involves progressive insulin resistance and beta cell dysfunction. Some of the adverse effects of T1D and T2D include microvascular complications such as retinopathy (which can lead to blindness), nephropathy (which can result in end stage renal disease and ultimately kidney failure) and painful neuropathy (which can lead to amputations). In addition, macrovascular complications of diabetes include coronary artery disease, atherosclerosis, hypertension and stroke [1, 2].

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The micro- and macro-vascular complications observed in the diabetic population result from the actions of various pathological factors and pathways functioning independently or in combination. Several signaling pathways have been found to be activated by hyperglycemia including increased flux into the hexosamine and polyol pathways, increased levels of oxidant stress, increased formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC) and mitogen activated protein kinases (MAPKs), as well as actions of cytokines/chemokines and several growth factors including angiotensin II and transforming growth factor-beta1 (TGF- β 1) [3–9]. Hyperglycemia induced activation of these pathways has been linked to increased mitochondrial superoxide anion formation, NADPH oxidase activation and associated oxidant stress [10, 11]. Therefore, while reactive oxygen species such as superoxide anions may be short-lived, their effects can be long lasting due to subsequent activation of multiple downstream signaling pathways. They also co-operate with reactive nitrogen species like nitrotyrosine and peroxynitrite to further enhance diabetes induced cellular dysfunction [12]. Hyperglycemia and consequent increases in oxidant stress, AGEs, PKC and MAPKs lead to increased activation of NF- κ B proinflammatory transcription factor which promotes the expression of key inflammatory genes [13–15]. Inflammation plays a major role in several diabetic complications and also contributes to beta cell failure in T1D as well as insulin resistance in T2D. In addition, hyperglycemia-induced activation of TGF- β 1 leads to increased expression of profibrotic genes associated with diabetic nephropathy. Diabetes-related oxidant stress including mitochondrial oxidant stress and other cellular stresses like endoplasmic reticulum (ER) stress are important pathogenic factors in diabetic complications and interestingly, they have been shown to involve NOX4 and Nrf2 which are downstream of high glucose or TGF- β signaling [16–21]. Overall, the consequences of hyperglycemia result in deleterious changes in the expression patterns of various inflammatory and other pathologic genes and proteins which contribute to the development of vascular complications in diabetes [22].

While these biochemical mediators have provided important information, most current therapies for common diabetic complications are not fully effective. It is therefore critical to evaluate additional molecular mechanisms and how they might be reversed, because the aforementioned diabetic complications can be life threatening and extremely debilitating if left untreated. Furthermore, some patients with diabetes may continue to experience complications despite subsequent glycemic control, a phenomenon termed metabolic memory which can be a major challenge in clinical management [23, 24]. Herein, we identify microRNAs as key emerging players during the development of numerous diabetic complications and how they may provide a window of opportunity for the development of new therapeutic modalities to ultimately reduce the mortality associated with diabetes and its complications.

MicroRNAs: Biogenesis and target recognition

MicroRNAs (miRNAs) are endogenously produced short non-coding RNAs of about 20–22 nucleotides in length that have been shown to play a key role in mammalian post-transcriptional gene expression by repressing translation or inducing target degradation, ultimately resulting in gene silencing [25–27]. miRNAs are ubiquitously expressed throughout the mammalian system and therefore capable of regulating several key biological pathways and cellular functions [25–30]. miRNAs were first characterized in the early 1990s in a nematode, *Caenorhabditis elegans*. The first identified miRNA, lin-4, was found to play a critical role in controlling developmental stage transitions by down-regulating its target, lin-14, *via* antisense complementarity to the 3' UTR region of lin-14 [27, 31–34]. There are over 1000 miRNAs within the human genome and it is estimated that about 60% of the human protein-coding genes can be regulated by miRNAs [27], resulting in profound effects on the expression of numerous proteins. Although it has been roughly 20 years since their

discovery, our knowledge of miRNA functions and mechanisms of action is still relatively limited. Nevertheless, enormous efforts from several groups have now documented the role of miRNAs and their targets in various biological pathways, and cellular functions such as differentiation, growth, proliferation and apoptosis related to numerous disease states.

miRNA processing begins from their transcription by RNA polymerase II into primary transcripts (pri-miRNAs) in the nucleus (Figure 1). These pri-miRNAs are relatively long (up to several kilo bases) and may contain multiple hairpin-like structures. A microprocessor complex consisting of the ribonuclease (RNase III) endonuclease, Drosha, and its binding partner, DGCR8, binds to the hairpin structures in pri-miRNAs and processes them to precursor miRNAs (pre-miRNAs), which are roughly ~ 70 nucleotides long and have a stem loop structure [31, 35]. The pre-miRNAs are then exported out of the nucleus and into the cytoplasm by Exportin 5 where they undergo further processing. They are recognized and cleaved by another RNase III enzyme, Dicer to generate the mature miRNA duplex comprising ~22 nucleotides [31, 35]. One strand of the duplex is selected to be loaded onto the RNA-induced silencing complex (RISC), while the other strand gets signaled for degradation (Figure 1). RISC, a multi-protein complex containing Argonaute (Ago) proteins, along with the mature miRNA, is able to recognize and bind the 3'UTR region of the target mRNA *via* sequence complementarity. If there is perfect complementarity between the mRNA and the miRNA, the target will be cleaved. However, if there is imperfect complementarity to the 3'UTR region of the target mRNA, then the target's translation is inhibited or destined for degradation in processing bodies (P-bodies), ultimately leading to gene silencing [30, 31, 35].

However, recent findings show that miRNA biogenesis and target recognition are more complex. Drosha-mediated processing was found to be enhanced by Smads in response to TGF- β or bone morphogenetic proteins (BMPs) [36, 37], or by p53 [38] (Figure 1). Another example is with the let-7 family of miRNAs which have been identified as tumor suppressors that inhibit the expression of key oncogenes such as MYC, RAS and HMGA2 [39–41]. The RNA-binding protein Lin28 represses the let-7 miRNAs and their expression levels are thus inversely correlated [42, 43]. Piskounova et al. have identified Lin28A to be differentially localized mainly in the cytoplasm, whereas Lin28B localization is predominantly in the nucleus most likely due to the presence of nuclear localization signals. Lin28A and Lin28B have distinct mechanisms for binding and inhibiting let-7 miRNAs [44]. For instance, Lin28A recruits the TUTase for uridylation to inhibit pre-let-7 processing by Dicer, and this ultimately leads to the degradation of oligouridylated pre-let-7 RNAs. Lin28B, however, functions distinctly in that it binds to pri-let-7 miRNAs in the nucleus, which then blocks processing *via* the Microprocessor containing the ribonuclease Drosha and DGCR8. This posttranscriptional regulation of let-7 by Lin28 is essential for normal development in *C. elegans* [45]. These alternative mechanisms for Lin28A and Lin28B functions on let-7 biogenesis have potential implications for their use in drug therapy [44]. Interestingly, some miRNAs are processed without Dicer. miR-451, an important miRNA for erythropoiesis, is processed by Ago2 slicer activity in a Dicer-independent manner [46]. Ota et al. reported that RNA editing enzyme, adenosine deaminases which convert adenosine residues to inosine on double stranded RNAs, also enhance silencing of the target RNA by miRNAs [47]. Adenosine deaminase acting on RNA1 (ADAR1) was shown to form a complex with Dicer to enhance DICER mediated processing of siRNA and miRNA. This study demonstrates a novel mechanistic interaction between the RNA interference machinery (Dicer) and the RNA editing enzyme (ADAR1) which promotes miRNA processing[47].

Recently, MCP1 (monocyte chemoattractant protein-1-induced protein 1) was identified as a broad suppressor of the miRNA biogenesis pathway by cleaving the terminal loops of

pre-miRNAs, thereby counteracting the effects of Dicer endonuclease [48] and resulting in the inhibition of miRNA synthesis (Figure 1). An inverse correlation was observed between MCPIP1 and Dicer function in human lung cancer patients, suggesting that low expression of Dicer is associated with poor prognosis [48]. Another recent paper reported that BCDIN3D is an RNA methyltransferase of 5' monophosphate ends of miRNA precursors which inhibits miRNA processing. Inhibition of BCDIN3D could enhance miR-145 maturation and suppress breast cancer tumorigenesis [49]. Another report showed that in endoplasmic reticulum (ER) stress-induced apoptosis, IRE1 α RNase, an ER transmembrane kinase-endoribonuclease (RNase) activation caused rapid decay of certain miRNAs (miRs -17, -34a, -96, and -125b) that repress translation of Caspase-2 [50]. Selective degradation of Dicer and Ago2 by autophagy was also suggested to regulate miRNA activity [51]. Thus, emerging evidence has revealed additional mechanisms of miRNA biogenesis, and it is becoming increasingly clear that modulators of miRNA processing also have physiological and pathological functions.

Numerous bioinformatics and computational approaches have been developed to best predict mRNAs targeted by miRNAs in vertebrate species. The most commonly used method, TargetScan, uses the seed sequence of the miRNA to predict miRNA target sites conserved among orthologous 3'UTRs of vertebrates [52]. miRNA targets are identified by finding complementary Watson-Crick (and non-Watson-Crick GU Wobble base pair) seed matches that are conserved in the 3'UTR regions of target mRNAs [52]. However, one of the caveats to this approach is the high frequency of false-positive and false-negative predictions. Several biological studies have shown that perfect complementarity (7-nt match) to the seed region of the miRNA is no longer essential for a miRNA-mRNA interaction [53–57]. For example, *lin-4* [54], *let-7* [55] and *lscy-6* [57] targets have all been identified to contain imperfect seed binding sites in *C. elegans*.

A genome-wide map of miRNA binding sites within the mouse brain was decoded by utilizing a novel method known as Ago-HITS-CLIP (High Throughput Sequencing Cross-linked Immunoprecipitation). This technique involves direct recovery of CLIP containing the Ago protein, followed by HITS of the RNA interaction sites that are recovered and then analysis of the sequences on the basis of seed matches [58, 59]. Recently, this approach led to the interesting discovery that not all Ago binding sites followed the classical “seed pairing” rule, and 27% of the Ago-mRNA clusters identified in the mouse brain were considered orphans due to the fact that they had no predicted seed matches in the Ago-bound miRNAs [58].

The ability of miRNAs to alter or fine tune the expression of key regulators in various physiological processes and pathophysiological disease states makes them novel targets for diseases such as diabetic complications. This is exemplified by reports showing that deletion of key enzymes for miRNA processing, such as Dicer or Drosha, in mice can result in severe defects in hearts and kidneys [60–67]. Identifying dysregulated miRNAs, and an in-depth understanding of their mechanisms of actions and functional roles would provide better insights for the development of new biomarkers and therapeutic targets. This review is focused on the emerging role of miRNAs in diabetic complications including their involvement in redox regulated cellular stresses known to be present in diabetes such as oxidant stress, ER stress and ischemia.

MicroRNAs associated with Diabetic Complications

Diabetic Nephropathy (DN)

DN is the first complication of diabetes in which miRNAs were implicated and to date, several miRNAs have been identified in cell and animal models of DN. DN is a progressive

kidney disease and a severe microvascular complication that can lead to end-stage renal disease and painful dialysis. Major hallmarks of DN include expansion of the glomerular mesangium (hypertrophy), tubulointerstitial fibrosis and glomerular basement membrane thickening due to the accumulation of extracellular matrix (ECM) proteins such as collagen, and podocyte dysfunction along with proteinuria [68, 69]. TGF- β levels and signaling are augmented in renal cells during the progression of DN and this leads to potent induction of these fibrotic events and renal dysfunction [69–72]. Our group reported an upregulation of several miRNAs (miR-192, miR-200b/c, miR-216a and miR-217) in TGF- β -treated mouse renal mesangial cells (MC) and in renal glomeruli of mouse models of diabetes [Streptozotocin (STZ)-injected T1D and db/db T2D mice] relative to the corresponding non-diabetic control mice [70, 73–75]. Our studies revealed that miR-192 actions lead to the up-regulation of key fibrotic genes, namely Collagen type I alpha2 (Col1a2) and Col4a1 in MC. In addition, miR-192 up-regulates other miRNAs miR-216a/217 and miR-200b/c in MC by targeting the E-box repressors, Zeb1/2, to relieve repression at their promoters, and this amplifying circuit augmented collagen expression. Interestingly, we observed that the miR-216a/miR-217 cluster activates Akt kinase by targeting Pten in TGF- β treated mouse MC thereby uncovering a miRNA mediated mechanism for TGF- β induced Akt kinase activation and cellular hypertrophy [75]. miR-200b/c can also regulate collagen expression and promote the auto-regulation of TGF- β in mouse MC by inhibiting Zeb1 [74]. Recent reports showed that expression levels of TGF- β 1, p53 and miR-192 were all increased in the renal cortex of diabetic mice, and this was associated with enhanced glomerular expansion and fibrosis relative to non-diabetic mice [76]. Furthermore, mice with genetic deletion of *miR-192 in vivo* were found to be protected from key features of DN. *In vitro* studies revealed that TGF- β induces reciprocal activation of miR-192 and *p53*, via the miR-192 target Zeb2 in a feedback amplification loop, leading to increased MC ECM gene expression and hypertrophy related to DN [76, 77]. An inverse correlation between miR-192 and *Zeb2* was also observed in glomeruli of human subjects with early DN [76]. Other studies show that the miR-192 promoter is also regulated by Smads, Ets-1 and chromatin remodeling *via* histone acetylation in response to TGF- β in renal cells [78, 79]. Altogether, these studies have identified miR-192 as a master miRNA regulator under TGF- β -treated cells or diabetic conditions, with increased levels of miR-192 contributing to enhanced expression of ECM genes associated with DN [70, 73–77, 79].

Other reports have also shown the role of additional key miRNAs in the progression of DN. miR-377 enhanced fibronectin expression in MCs *via* down-regulation of manganese superoxide dismutase and p21-activated kinase [80]. The same study also reported that miR-192 expression levels were increased in high glucose (HG)-treated MCs. miR-192 was also reported to be up-regulated in STZ-injected T1D mice fed with a high fat diet and the effects were enhanced in farnesoid X receptor knockout mice [81]. Long et al. reported decreased miR-93 expression levels in glomeruli of diabetic db/db mice, and in HG-treated podocytes and renal microvascular endothelial cells [82]. The decreased levels of miR-93 correlated with increased expression of VEGF-A, suggesting VEGF-A could be a direct target of miR-93 and implicating an anti-angiogenic role for miR-93. Conversely, they reported that miRs -29c, -192, and -200b were up-regulated under similar conditions [83]. The increase in miR-29c was shown to activate Rho kinase *via* targeting of Spry1, leading to an accumulation of ECM proteins and podocyte apoptosis; these effects of miR-29c were verified both *in vitro* and *in vivo*. On the contrary, Wang et al. reported miR-29 family members were down-regulated, resulting in increased levels of their targets, collagens I, III, IV in studies performed with diabetic ApoE $^{-/-}$ mouse kidneys, and in proximal tubule epithelial cells, MC and podocytes treated with TGF- β [84]. The discrepancies in these two studies with miR-29 were attributed to the different animal models of diabetes used and types of cell stimulations. Studies in other non-diabetic animal models of renal fibrosis have also demonstrated a profibrotic role for miR-192 and an anti-fibrotic role for miR-29 family

members [78, 85, 86]. miR-192 and miR-215 were recently reported to be up-regulated in MC treated with TGF- β and glomeruli from diabetic db/db mice and to induce phenotype transition of MC by targeting CTNNBIP1, a β -catenin interacting protein, through Wnt/3-catenin signaling related to DN[87].

On the other hand, a few studies have suggested that miR-192 is decreased under diabetic conditions and this leads to increased fibrosis. In one report decreased miR-192 levels was associated with the severity of DN and fibrosis in diabetic patients, although normal levels of miR-192 in healthy kidneys were not defined [88]. Wang et al. reported decreased levels of miR-192 in diabetic ApoE $^{-/-}$ mice, and in TGF- β treated tubular and other renal cells [89]. While these discrepancies might be due to the differences in models studied and the varying responses of miRNAs in multiple renal cell types in heterogenous tissues being studied, they also underscore the complexities of examining disease-related effects of miRNAs due to their cell-specific responses within the kidney. In the case of miR-192, the cell-type specific response to TGF- β might be explained by the p53 or Ets-1 status since TGF- β -induced miR-192 expression was abolished in MC from p53 $^{-/-}$ or Ets-1 $^{-/-}$ mice [76, 79]. In other studies, miR-200a was shown to target TGF- β 2 in cultured proximal-tubular epithelial cells, creating another circuit since TGF- β 1 increased TGF- β 2 expression *via* decreases in miR-200a in these cells [90]. miR-21 has recently been studied by several groups. Dey et al. reported that miR-21 was up-regulated in the renal cortex of the OVE26 T1D mice leading to mTOR activation and the targeting of Pten, all of which are related to DN pathogenesis [91]. miR-21 has also been evaluated as a therapeutic target for DN in mouse models [92]. In contrast, miR-21 was also reported in one study to be down-regulated in db/db mice and its over-expression blocked MC proliferation [93]. miR-25 was shown to be down-regulated in diabetic rat kidneys and HG-induced MCs [94]. Interestingly, Nox4 was identified as a direct target of miR-25, suggesting that decreased miR-25 can upregulate Nox4 to promote oxidant stress which ultimately can result in renal dysfunction in rats since Nox4 has been implicated in the pathogenesis of DN [20, 21, 94] (Figure 2).

Thus, to-date several miRNAs have been reported to be involved in promoting or attenuating the progression of DN [77, 95]. Given the known mediatory role of oxidant stress in the pathogenesis of DN, further studies are needed to determine how oxidant stress may regulate the expression of miRNAs and vice versa in the diabetic kidney and whether redox signaling and transcription factors are involved. Increased understanding of such underlying mechanisms will aid in the development of better therapies for DN.

Diabetic Retinopathy (DR)

DR is one of the most common microvascular complications of diabetes which, if left untreated, can lead to blindness [96, 97]. The role of miRNAs in the progression of DR has recently been evaluated. An in-depth miRNA-expression profiling analysis of miRNAs in the retina and retinal endothelial cells (RECs) from Streptozotocin (STZ)-induced T1D rats [98] revealed up-regulation of several NF- κ B responsive miRNAs, including miR-146, miR-155, miR-132 and miR-21. Notably, vascular endothelial growth factor (VEGF) responsive miRNAs and the p53-responsive miR-34 family were up-regulated in both retinas and RECs of the diabetic rats. Recently, Feng et al. observed a decrease in miR-146a in HG treated endothelial cells from large vessels and retinal microvessels and in retinas from T1D rats [99]. They identified fibronectin to be a direct target of miR-146a, suggesting that miR-146a down-regulation could be a key mechanism for increased extracellular matrix protein production in diabetes. Furthermore, intravitreal injection of miR-146a mimics restored retinal miR-146a and decreased fibronectin levels in diabetes suggesting that such translational approaches could be developed for DR. Another group reported decreased levels of miR-200b in HG-induced endothelial cells and in retinas of STZ-injected diabetic rats and showed that VEGF was a direct target of miR-200b [100]. miR-200b mimic

treatments *in vitro* and *in vivo* were able to attenuate VEGF mRNA and protein expression levels. As expected, miR-200b antagomirs showed reciprocal effects. Since anti-VEGF therapies are being evaluated for DR, it is worth evaluating miRNAs that are known to target VEGF. Another report found that miR-29b and its potential target RAX (an activator of the pro-apoptotic PKR signaling pathway), were both localized in the retinal ganglion cells as well as the cells of the inner nuclear layer of the retinas from STZ-induced diabetic rats and control rats [101]. The authors suggested that increased levels of miR-29b during the early stages of diabetes may be protective against apoptosis of retinal neurons *via* the PKR pathway. Given the success of intravitreal delivery approaches for retinal disorders, treatments with mimics of “protective” miRNAs such as miR-146a, miR-29b and miR-200b could be developed as treatment modalities for DR.

Diabetic Cardiovascular Complications, Cardiomyopathy and Vascular Inflammation

Diabetes is associated with significantly increased rates of cardiovascular complications including atherosclerosis, hypertension and restenosis which involve the dysfunction of key vascular and inflammatory cells like endothelial, vascular smooth muscle and monocytes. Increased glucose levels results in abnormal cell signaling by enhancing oxidant stress and augmenting several key inflammatory and fibrotic players which leads to many of these cardiovascular disorders [4, 102–104]. In addition, diabetic cardiomyopathy is a complication that can lead to heart failure. Several studies have evaluated the role of miRNAs in cardiac complications.

miR-1 and miR-206 were found to be up-regulated in neonatal rat cardiomyocytes cultured with HG and this occurred *via* the serum response factor (SRF) and the MEK1/2 pathway [105]. In addition, these miRNAs negatively regulate heat shock protein60 (Hsp60) thereby contributing to HG-mediated apoptosis in cardiomyocytes. miR-1 was up-regulated in STZ-injected diabetic mice during the development of cardiomyopathy and could target Pim-1, which possesses anti-apoptotic properties [106]. Care et al. observed down-regulation of miR-133 expression levels in models of mouse and human myocardial hypertrophy [107]. They found that miR-133 targets RhoA (a GDP-GTP exchange protein) and Cdc42 (a signal transduction kinase) and that inhibition of miR-133 with antagomirs led to sustained cardiac hypertrophy, whereas its over-expression inhibited cardiac hypertrophy. Similarly, Feng et al. demonstrated down-regulation of miR-133a in a model of cardiomyocyte hypertrophy in STZ-injected diabetic mice *via* its effects on MEF2A/C, IGF-1R, SGFK1 [108]. miR-133 was found to regulate Glut-4, an insulin sensitive glucose transporter, by targeting KLF15 in cardiomyocytes [109], while Glut-4 itself is targeted by miR-223 [110]. In another study, miR-373 was down-regulated in cardiomyocytes of STZ-injected diabetic mice and in HG-treated rat cardiomyocytes and targeted MEF2C [111]. Recently, the expression levels of several miRNAs were examined in left ventricle biopsies from diabetic heart failure patients compared to non-diabetic heart failure patients. Results showed that key miRNAs were differentially expressed including miR-34b/c, miR-199b, miR-210, miR-650 and miR-223 [112]. Predicted targets of these miRNAs included genes with functions related to heart failure and cardiac dysfunction. Several groups have demonstrated that miRs-29, -30, and -21 are able to regulate ECM-related genes (such as collagens and connective tissue growth factor), and thus linked to cardiac fibrosis and heart failure. It is worthwhile to determine whether these miRNAs also play a role in diabetic heart disease [113–115].

Diabetic vascular complications have been linked with an up-regulation of inflammatory genes within vascular cells and inflammatory cells like monocytes, and a role for epigenetic mechanisms has also been implicated [23, 24, 116]. Some groups have reported inflammatory genes are potentially regulated *via* miRNAs during the progression of diabetic complications. We reported that miR-16 destabilizes the inflammatory cyclooxygenase-2 (COX-2) gene in monocytes under normal glucose conditions by binding to its 3'UTR

region. Meanwhile, S100b, a known pro-inflammatory ligand for advanced glycation end products (RAGE) receptor, could up-regulate COX-2 expression levels in these monocytes by down-regulating miR-16 levels [117].

In other studies, we examined the role of miR-125b in increased inflammatory gene expression and abnormal phenotype of vascular smooth muscle cells (VSMC) cultured from type-2 db/db diabetic mice compared to control db/+ mice [118, 119]. miR-125b levels were up-regulated in the diabetic db/db VSMC compared to control mice and was able to target and down-regulate Suv39h1, a histone methyltransferase that mediates histone H3-lysine-9 trimethylation (H3K9me3), a chromatin modification associated with repressed genes. In parallel, chromatin immunoprecipitation assays revealed that histone H3K9me3 levels were decreased at the promoters of key inflammatory genes, interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1). Furthermore, transfection of miR-125b mimics into normal db/+ VSMC conferred a diabetic phenotype of decreased SUV39h1 and H3K9me3 and increased expression of MCP-1 and IL-6, as well as enhanced monocyte binding. These studies implicate miR-125b as an important epigenetic regulator of inflammatory genes in type-2 diabetic db/db mice. They also suggest a novel interplay between miRNAs and the epigenetic machinery resulting in de-repression of key genes associated with the progression of diabetic inflammatory complications [119]. Another miRNA, miR-200b, was also up-regulated in VSMC from db/db mice. miR-200b targets and blocks the transcriptional repressor, Zeb1, which negatively regulates inflammatory genes by binding to E-boxes within their promoter region. Thus, the increase in miR-200b correlated with the increased levels of inflammatory genes which were also induced by miR-200b mimics [120].

Persistent HG causes endothelial cell dysfunction. Li et al. reported that exposure to HG induced miR-221 but inhibited c-kit expression in human umbilical vein endothelial cells (HUVECs). Antisense miR-221 oligonucleotide AMO-221 reduced the expression of miR-221 and restored c-kit protein expression in HUVECs treated with HG, suggesting that manipulation of the miR-221-c-kit pathway may be a new strategy for treating endothelial dysfunction in diabetic patients [121]. Wang et al. found increased levels of miR-320 in myocardial microvascular endothelial cells (MMVEC) from T2D GK rats which was associated with impaired angiogenesis [122]. Conversely, a miR-320 inhibitor enhanced insulin-like growth factor-1 (IGF-1) protein and angiogenic activity *in vitro* suggesting that miR-320 could be a potential therapeutic target for diabetic patients with impaired angiogenesis. Togliatto et al. reported that HG and AGE inhibit vascular endothelial cell proliferation by reducing miR-221/222 which target the cell cycle regulators, p27KIP1 and p57KIP2 [123]. Caporali et al. showed that HG induced miR-503 expression in EC [124]. miR-503 over-expression inhibited EC proliferation and migration while blocking miR-503 improved the EC functions cultured under HG. Since CCNE1 and cdc25A were identified and validated as direct miR-503 targets, down-regulated CCNE1 and cdc25A by HG was posited to lead to EC dysfunction. miR-503 expression was also enhanced in ischemic limb muscles of STZ-diabetic mice, in ECs enriched from these muscles, as well as in muscular specimens from the amputated ischemic legs of diabetic patients. Elevated levels of plasma miR-503 were detected in the diabetic individuals, suggesting *in vivo* relevance and miR-503 as a possible therapeutic target in diabetic patients with critical limb ischemia. Taken together, these reports provide further clues to the contribution of miRNAs in diabetes induced vascular dysfunction and cardiovascular complications.

miRNAs related to cellular stress including oxidant stress and ER stress

Oxidant stress and ER stress have been shown to play major roles in diabetes and its complications. It is likely that miRNAs depicting differential expression in diabetes and in tissues/cells affected by diabetic complications are regulated by mechanisms and

transcription factors activated by these cellular stresses. It is therefore worth examining the role of redox mechanisms in the regulation of miRNAs affected under diabetic conditions. As mentioned earlier, miR-25 was down-regulated in diabetic rat kidneys and HG-induced MCs and the decrease of miR-25 could induce the expression of Nox4 to promote oxidant stress (Figure 2). Nox4 has been shown to be a key factor in the pathogenesis of DN [94]. miR-146a was also shown to target Nox4 in human endothelial cells with aging [125] and was down-regulated in HG-treated endothelial cells [99]. Another recent report showed that oxidant stress or ER stress enhances the production of reactive oxygen species (ROS) through reduction of miR-205 which targets prolyl hydroxylase 1 (PHD1) and concomitant decrease in heme oxygenase-1 (HO-1) and superoxide dismutase (SOD1/2) in human kidney tubular cell line, HK-2 [126]. We also showed that miRNA cascades (miR-192, miR-216a/217) activate Akt and inhibit FoxO3a/SOD2 (MnSOD) signaling in mouse kidney MC [75, 127]. As discussed earlier, miR-377 targets SOD1 and SOD2 in human MC [80], and also target HO-1 in combination with miR-217 in HUVEC [128]. Ochratoxin A (OTA), a mycotoxin with nephrotoxic and potential carcinogenic activity, could induce miR-200c and miR-132, and this promoted oxidant stress because Nrf2 is targeted by miR-200c, and HO-1 is targeted by miR-132 in renal proximal epithelial cells [129]. miR-211 was reported to regulate ER stress by attenuating the expression of Chop/gadd153 by directly targeting the Chop/gadd153 promoter *via* a novel nuclear post-transcriptional gene silencing mechanism [130]. miR-144 was shown to modulate oxidant stress associated with the severity of sickle cell anemia [131]. In breast cancer cells, miR-28 could regulate the redox sensitive transcription factor Nrf2 and hence affect cancer progression [132] while miR-200a could regulate Nrf2 activation by targeting Keap1 [133] (Figure 2). In ovarian cancer, miR-200 family also increased oxidant stress and sensitivity to chemotherapeutic agents (Paclitaxel) by targeting p38 [134].

Overall, these reports in the literature implicate HO-1, SOD1, SOD2 and NOX4 as major targets of key miRNAs regulated by diabetic conditions which therefore could fine-tune and contribute to enhanced ROS production (oxidant stress) and diabetic complications (Figure 2). In addition, several miRNAs (termed hypoxamirs) have now been shown to be induced in response to hypoxia [135]. A very recent paper also showed that hypoxia modulates miRNA maturation through Ago2 phosphorylation by EGFR (epidermal growth factor receptor) in breast cancer cells [136]. It would therefore be worthwhile in the future to further examine the roles of these and other miRNAs related to cellular stresses in the pathology of diabetic complications.

miRNAs as biomarkers and therapeutic targets for diabetic complications

There is heightened interest in evaluating miRNAs as potential biomarkers for various diseases especially due to improvements in the technologies for miRNA detection *in vitro* and *in vivo*. These include quantitative PCRs, microarrays and high throughput deep sequencing [137–141]. miRNAs are readily detectable in plasma and urine and their stability in these biofluids make them ideal candidate biomarkers for non-invasive and much needed early detection of diabetic complications. This is critical since such early detection can enhance clinical management, improve long term outcomes and greatly increase quality of life. Emerging evidence has emphasized the idea that circulating miRNAs in the plasma can be utilized as sensitive biomarkers for diseases like cancer, tissue injury and heart failure [137–141]. Recently, miRNA levels in the urine, urinary sediment and circulating miRNA levels have been examined in patients with kidney disease [142–147]. Very recently, a report showed that miR-192 and miR-205 levels were significantly higher in sera obtained from patients with primary focal segmental glomerulosclerosis and correlated with proteinuria and interstitial fibrosis [148]. These reports showing differential profiles of miRNAs in the biofluids of patients with DN and other diseases relative to healthy controls

support the use of miRNAs as candidate biomarkers of various diabetic complications. It is likely that renal, vascular and blood cell-associated miRNAs can be detected in urine and serum samples due to various transport mechanisms. However, the mechanisms by which miRNAs are trapped and transported in vesicles and *via* bound proteins are not fully understood. Furthermore, circulating miRNAs do not always correlate with tissue damage levels and may reflect other secondary processes. In spite of these caveats, there is still much enthusiasm for examining the profiles and levels of circulating miRNAs in biofluids as diagnostic biomarkers of diabetic complications.

As discussed in the sections above, several miRNAs have now been shown to be dysregulated under diabetic conditions and in tissues associated with complications and therefore present a new window of opportunity for therapeutic intervention. In order to determine whether they can serve as drug targets, the effects of down- or up-regulating them *in vivo* are being assessed in pre-clinical models. While still in its infancy, translational approaches with miRNAs as therapeutic targets for diabetic complications are also beginning to be considered [149]. The major focus of the clinical management of diabetes is not only glycemic control but also the prevention or reduction of debilitating complications. Combinatorial therapy with anti-miRNA and conventional drugs, or miRNA targeting alone could be a new approach. Recent advances in the synthesis of modified stable nuclease-resistant nucleic acids have provided us with better tools to develop more effective miRNA inhibitors and mimics for *in vitro* and *in vivo* delivery. For instance, locked nucleic acid (LNA) and modified anti-miRNAs (antimiRs or antagomirs) have been shown to be highly specific in inhibiting the miRNA of interest and are also being tested in clinical trials [75, 150, 151]. We recently demonstrated the high specificity and efficacy of LNA-modified antimiR-192 (LNA-antimiR-192) when injected into mice [75]. In addition to inhibiting miR-192 expression levels, downstream miRNAs (miR-216a, miR-217 and miR-200 family) and p53 were also reduced in renal cortex of normal as well as STZ-injected diabetic mice [74–76, 152]. Functional indices of renal fibrosis and hypertrophy, specifically collagens, TGF- β 1 and Akt activation were also attenuated in the anti-miR-192 injected mice [74–76, 152], suggesting that anti-miRNA therapies can be developed in the future for human diabetic renal disease. Interestingly, the mitotic inhibitor, paclitaxel (used in cancer chemotherapy) down-regulated miR-192 resulting in attenuated fibrotic damage in the remnant kidney model [86]. Reduced rates of DN progression were also noted in db/db mice injected with 2'-O-methyl antisense oligonucleotides targeting miR-29c [83]. Inhibition of miR-503 by miR-503 decoy or antisense inhibitor improved impaired angiogenesis in diabetic mice [124]. Other *in vivo* delivery modalities such as adeno-associated virus vectors and miRNA sponges are also being used [106, 153, 154]. Thus, utilizing antisense LNA oligos or antagomirs against key up-regulated miRNAs could be developed as novel therapeutic approaches for diabetic complications.

Several miRNAs are also reported to be down-regulated under diabetic conditions. If these miRNAs have protective effects, treatment with mimics of these miRNAs would be a therapeutic option for the associated complications. However, the instability of functional mature miRNAs makes this approach technically more difficult than with using antagomirs. Finally, since the differential regulation of miRNAs *in vivo* could be the result of defensive or other adaptive mechanisms, and one miRNA can theoretically have numerous targets, it is imperative that the *in vitro* and *in vivo* functional role of the miRNA be documented before any translational approaches *in vivo* are tested.

Summary

Our understanding of the biology and actions of miRNAs has greatly increased in recent years, but less is known about their *in vivo* role in disease states. Herein, we have reviewed

the role of miRNAs in several diabetic complications. We have discussed in depth the differential expression and complex signaling contributed by various miRNAs. The effects of these miRNAs depend upon cell-type specific patterns, the type of model systems utilized, time of sampling and the severity of the complications in the models studied. Furthermore, since one miRNA can potentially target multiple genes, it is challenging to develop miRNA-specific therapeutics due to potential off-target effects. Nonetheless, the miRNA field is advancing quickly and several miRNA biomarkers and anti-miRNA clinical trials are under current investigation. As our knowledge of the molecular mechanisms of how miRNAs are regulated and function *in vitro* and *in vivo* expands, so also will our capabilities to improve and optimize therapeutic approaches to more effectively target miRNAs to prevent the progression of diabetic complications. Furthermore, in the future, identification of additional miRNAs that regulate oxidant and/or ER stresses may further enhance these efforts aimed at harnessing the diagnostic and therapeutic potential of miRNAs for the complications associated with diabetes.

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Highlights

- Effects of diabetic conditions on microRNA expression (including biogenesis).
- Functions of microRNAs in progression of diabetic complications.
- Roles of microRNAs in oxidant stress induced by diabetic conditions.
- microRNAs as biomarkers of diabetic complications.
- microRNA-based therapeutic approach for diabetic complications.

Figure 1. Biogenesis of microRNAs and their actions

MicroRNAs are initially transcribed as pri-miRNAs which are processed into pre-miRNAs by the Drosha enzyme. Pre-miRNAs are further cleaved to result in double-strand RNA duplexes. Drosha processing can be regulated by p53 or Smads (activated by TGF-β or BMPs). Dicer processing can also be inhibited by MCPIP1 which is induced by MCP-1 (related to inflammation). The miRNA duplexes are then unwound by the action of a second enzyme, Dicer, and the mature miRNA guide strand is loaded into the RISC complex. Please refer to the main text for details. RISC, RNA-induced silencing complex; UTR, untranslated region; P-body, processing body.

Figure 2. miRNAs related to cellular stress (oxidant stress and ER stress)

Oxidant stress and ER stress play major roles in diabetes and its complications. Key miRNAs identified to regulate these processes *via* their relevant targets are depicted in the figure. Please see the main text for details.