Anti-Inflammatory Role of MicroRNA-146a in the Pathogenesis of Diabetic Nephropathy

Kirti Bhatt,* Linda L. Lanting,* Ye Jia,* Sailee Yadav,* Marpadga A. Reddy,* Nathaniel Magilnick,†‡ Mark Boldin,† and Rama Natarajan*

*Department of Diabetes Complications, †Department of Molecular and Cellular Biology, and ‡Irell and Manella Graduate School of Biological Sciences, Beckman Research Institute of City of Hope National Medical Center, Duarte, California

ABSTRACT
Inflammation has a critical role in the pathogenesis of diabetic complications, including diabetic nephropathy (DN). MicroRNAs have recently emerged as important regulators of DN. However, the role of microRNAs in the regulation of inflammation during DN is poorly understood. Here, we examined the in vivo role of microRNA-146a (miR-146a), a known anti-inflammatory microRNA, in the pathogenesis of DN. In a model of streptozotocin-induced diabetes, miR-146a−/− mice showed significantly exacerbated proteinuria, renal macrophage infiltration, glomerular hypertrophy, and fibrosis relative to the respective levels in control wild-type mice. Diabetes-induced upregulation of proinflammatory and profibrotic genes was significantly greater in the kidneys of miR-146a−/− than in the kidneys of wild-type mice. Notably, miR-146a expression increased in both peritoneal and intrarenal macrophages in diabetic wild-type mice. Mechanistically, miR-146a deficiency during diabetes led to increased expression of M1 activation markers and suppression of M2 markers in macrophages. Concomitant with increased expression of proinflammatory cytokines, such as IL-1β and IL-18, markers of inflammasome activation also increased in the macrophages of diabetic miR-146a−/− mice. These studies suggest that in early DN, miR-146a upregulation exerts a protective effect by downregulating target inflammation-related genes, resulting in suppression of proinflammatory and inflammasome gene activation. Loss of this protective mechanism in miR-146a−/− mice leads to accelerated DN. Taken together, these results identify miR-146a as a novel anti-inflammatory noncoding RNA modulator of DN.


Diabetic nephropathy (DN) is the leading cause of CKD and ESRD.1–6 Development and progression of DN involve a complex interplay among metabolic, hemodynamic, growth, and inflammatory factors.1,3,7–13 The progressive decline in renal function during DN is a result of a multitude of pathologic changes in the kidneys, including glomerular and tubular hypertrophy, macrophage infiltration, extracellular matrix (ECM) accumulation in multiple renal cells, mesangial expansion, endothelial dysfunction, and podocyte injury.1,3,7–12,14–16 These pathologic changes clinically manifest as proteinuria and a steady deterioration in GFR.3,4,16 Identification of molecular pathways that contribute to the pathophysiology of DN is imperative for the development of new therapeutic strategies. Conversely, identification of factors that exert adaptive and protective roles in the early stages of DN can be exploited to prevent progression to ESRD.

Augmented inflammation is a hallmark of diabetes,17,18 and this proinflammatory state plays a critical role in the development and progression of DN.10,19,20 Elements of the diabetic
milieu, such as hyperglycemia and advanced glycation end-products are thought to play an important role in creating a proinflammatory environment in both peripheral circulation and renal tissues. Moreover, the initial renal glomerular dysfunction is thought to result in macrophage infiltration due to increased expression of inflammatory adhesion and chemoattractant proteins. Infiltration of macrophages, the major inflammatory cells that mediate renal inflammation during DN and local chemokine production, lead to additional immune cell migration into the kidneys, as well as interaction with resident renal cells, which further exacerbates renal inflammation, injury, and fibrosis. Along with renal cells, macrophages are also a key source of profibrotic and proinflammatory factors, such as TGF-β1, IL-1β, PDGF, TNF-α, IL-6, and plasminogen activator inhibitor-1 (PAI-1), which have been associated with DN pathogenesis. However, the key factors mediating early gene expression changes and signaling pathways that modulate renal macrophage activation and infiltration in DN are not yet completely understood.

MicroRNAs (miRNAs) are short, non-coding RNAs that have recently emerged as essential regulators of gene expression in normal physiology and disease states. The role of miRNAs in kidney diseases has been an area of intense investigation in the past several years. Work from our laboratory and other groups has elucidated the role of numerous miRNAs in the pathophysiology of DN. However, the in vivo functional roles of miRNAs in renal inflammation during DN are not clear. In this study, we found that the expression of microRNA-146a (miR-146a), a previously reported modulator of inflammation, is elevated in macrophages and kidneys during DN. Functional and mechanistic studies in the miR-146a-deficient mice showed that miR-146a plays a crucial protective and anti-inflammatory role during the pathogenesis of DN. Overall, this study has identified a novel miRNA-regulated inflammatory component in DN.

RESULTS
Expression and Functional Analysis of the Role of miR-146a in Early DN
To determine whether miR-146a expression is altered during DN, we used a well established model of diabetes induction in

---

Figure 1. Increased severity of early diabetic nephropathy phenotypes in miR-146a deficient mice. (A) WT mice were injected with vehicle (control) or STZ as described in the Concise Methods. Seven weeks after diabetes induction, renal cortical tissues were isolated and analyzed for miR-146a expression by qPCR analysis (n=6). (B) Incidence of diabetes in WT and miR-146a−/− mice, represented as the proportion of STZ-injected mice displaying diabetic phenotype as confirmed by blood glucose levels (n=15). (C) Glomerular area and mesangial matrix expansion in WT and miR-146a−/− mice kidneys (7-week STZ) (n=6). (D) Representative images of PAS-stained renal cortical sections after 7-week STZ. Original magnification, ×400. (E) Inflammatory and fibrotic gene expression in WT and miR-146a−/− mice kidneys (7-week STZ) (n=6). In all panels, data are presented as mean±SEM. *P<0.05 and @P<0.01 (statistically significant difference) compared with respective control group; $P<0.01 (statistically significant difference) compared with WT STZ group.
mice by streptozotocin (STZ) injection. The expression of miR-146a was significantly increased in the kidney cortex 7 weeks after diabetes induction (Figure 1A). To determine the functional role of miR-146a in the pathogenesis of DN, we used miR-146a−/− mice.35 We initially confirmed that loss of miR-146a did not affect the percentage incidence of STZ-mediated diabetes induction in the miR-146a−/− mice, as determined by blood glucose measurements (Figure 1B). Next, short-term experiments (7 weeks of diabetes) showed that the diabetic miR-146a−/− mice had significantly greater increase in glomerular area compared with the wild-type (WT)-STZ counterparts (Figure 1, C and D). Moreover, whereas the expression levels

**Figure 2.** miR-146a−/− deficient mice develop severe DN. WT and miR-146a−/− mice were injected with vehicle (control) or STZ, and diabetic mice were followed for several weeks thereafter. Urine was collected between 11 and 15 weeks, and mice were euthanized at 16 weeks after diabetes induction. (A) Renal cortical miR-146a expression in WT mice at 16 weeks (n=6). (B) Urinary volume levels from vehicle and STZ-injected WT and miR-146a−/− mice at 11, 13, and 15 weeks after diabetes induction (n=6). (C) Urinary protein levels at the same time periods (n=6). (D) Urinary albumin levels at 15 weeks (n=6). (E) Representative images of PAS staining of the renal cortical sections of mice at 16 weeks. Original magnification, ×400. (F) Quantitation of glomerular area and mesangial matrix expansion from PAS-stained sections (n=6). (G) Masson trichrome staining in the four groups. Original magnification, ×400. (H) Representative Western blot analysis of renal nephrin expression in WT and miR-146a−/− mice at 16 weeks. (I) Densitometric analysis of nephrin levels was performed using ImageJ software, and values were normalized to loading control for each lane. The relative protein levels were calculated compared with control group (n=4). In all panels, data are presented as mean±SEM. *P<0.05 and **P<0.01 (statistically significant difference) compared with respective control group; †P<0.05 and ‡P<0.01 (statistically significant difference) compared with WT STZ group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
of proinflammatory and profibrotic genes, such as IL-1β, MCP-1 (Ccl2), CD11b, and PAI-1, showed significant increases in kidney cortex of both WT and miR-146a−/− diabetic mice kidneys; however, relative to WT-STZ mice, their expression in miR-146a−/− STZ kidneys was even greater (Figure 1E). Tnf-α showed a trend toward higher expression in the miR-146−/− STZ mice versus WT-STZ mice but did not reach statistical significance (Figure 1E).

MiR-146a–Deficient Mice Develop Features of Accelerated DN

Proteinuria is a major characteristic of DN progression. In our first set of experiments (7 weeks), histologic and gene expression changes observed indicated early stages of DN but proteinuria was not evident. Therefore, we then performed experiments at 11–16 weeks after diabetes induction, a time period during which we have previously observed significant proteinuria.36 In this longer-term model, significant upregulation of miR-146a expression was observed in the kidneys of diabetic WT mice (Figure 2A). Of note, histologic and biochemical markers of DN increased significantly at 16 weeks, and urine volume and proteinuria increased substantially and progressively at 11, 13, and 15 weeks in the diabetic miR-146a−/− mice relative to WT group (Figure 2, B and C). Urinary albumin levels were also significantly elevated in the diabetic miR-146a−/− mice compared with the WT group at 15 weeks (Figure 2D). Histologic analysis using periodic acid-Schiff (PAS) and trichrome staining showed that diabetes-induced glomerular hypertrophy and fibrosis were significantly greater in the miR-146a−/− mice (Figure 2, E–G). Additionally, Western blot analysis in the kidney cortex showed greater decreases in nephrin levels in diabetic miR-146a−/− mice relative to diabetic WT mice, indicating greater podocyte injury in the former (Figure 2, H

Figure 3. Loss of miR-146a expression results in increased proinflammatory and fibrotic gene expression in kidneys. Kidney cortices from 16-week STZ or control WT and miR-146a−/− mice (n=6) were processed for gene expression analysis of proinflammatory genes (A–C) Tnf-α, MCP-1, and IL-1β; profibrotic genes (D–G) Col1a2, Col4a2, PAI-1, and TGF-β; and miR-146a target genes (H and I) Irak1 and Traf6. In all panels, data are presented as mean±SEM. *P<0.05 and **P<0.01 (statistically significant difference) compared with respective control group; #P<0.05 and $P<0.01 (statistically significant difference) compared with WT STZ group.
Importantly, phenotypic characterization of the untreated nondiabetic miR-146a−/− mice showed that renal histology, urinary albumin, and renal gene expression were similar between the WT and miR-146a−/− mice (Supplemental Figure 1). Overall, these findings suggest that loss of miR-146a does not affect normal renal function; during DN, however, miR-146a deficiency leads to a remarkable increase in the severity of DN.

miR-146a Deficiency Results in Increased Proinflammatory and Fibrotic Gene Expression under Diabetic Conditions

To examine the inflammatory signaling in the kidneys of these longer term (16-week) diabetic mice, mRNA levels of Tnf-α (Figure 3A), MCP-1 (Figure 3B), and IL-1β (Figure 3C) were analyzed by quantitative PCR (qPCR). These genes, implicated as key players in renal inflammation,10 were induced to a much greater extent in the diabetic miR-146a−/− mice kidneys than in the WT mice. Furthermore, key ECM and profibrotic genes, collagen type I α2 (Col1a2), Col4a2, PAI-1, and TGF-β1 were also more highly induced in the miR-146a−/− mice compared with WT-STZ mice (Figure 3, D–G). Importantly, Traf6 and Irak1, two inflammation-related and miR-146a target genes,34 were downregulated in the diabetic WT group but significantly elevated in the diabetic miR-146a−/− kidneys (Figure 3, H and I). Thus, loss of miR-146a results in a marked augmentation of proinflammatory and fibrotic gene expression during DN.

Increased Renal Macrophage Infiltration in Diabetic miR-146a−Deficient Mice

miR-146a is highly expressed in immune cells,34,37 especially macrophages, which led us to further focus on macrophages. Interestingly, F4/80 staining of kidney sections from 16-week diabetic mice showed that renal macrophage infiltration was significantly increased by miR-146a deficiency (Figure 4, A and B). These results were further validated by qPCR analysis of CD11b expression (Figure 4C). Next, we examined whether macrophages contribute to the increased miR-146a expression in diabetic kidneys. Combined in situ hybridization of miR-146a and immunostaining of F4/80 in the kidney cortex demonstrated that intraglomerular miR-146a staining is increased in diabetic kidneys, and this colocalizes with F4/80 staining (Figure 4D).

miR-146a Expression Is Increased in Nonrenal Macrophages from Diabetic Mice

We next examined whether miR-146a expression was also altered in nonrenal macrophages from diabetic mice. Indeed, miR-146a levels were significantly increased in peritoneal

Figure 4. Macrophage infiltration is increased in miR-146a−deficient diabetic kidneys. WT and miR-146a−/− mice kidneys were harvested from control or diabetic mice (16 weeks). (A and B) Representative sections from F4/80-stained cortices and quantitation of F4/80 staining from the renal cortex. Original magnification, ×400. (C) CD11b gene expression in WT saline and STZ (16-week) kidney cortex (n=6). (D) Combined in situ hybridization of miR-146a (green) and F4/80 (red) staining in the renal cortex of diabetic WT mice sections. Blue staining refers to 4′,6-diamidino-2-phenylindole–stained nuclei in the representative images. In all panels, data are presented as mean±SEM. *P<0.05 and **P<0.01 (statistically significant difference) compared with respective control group; #P<0.05 and $P<0.01 (statistically significant difference) compared with WT STZ group.
macrophages from 12-week-old diabetic db/db mice, a model of type 2 diabetes with renal dysfunction, relative to control db/+ mice (Figure 5A). miR-146a levels were also elevated in peritoneal macrophages isolated from control and STZ-injected mice (4-week diabetes) (n=5). Evidence shows that some gene expression changes observed in diabetic macrophages, such as Tnf-α, can be directly induced by hyperglycemia. Therefore, we cultured peritoneal macrophages in high glucose (HG, 25 mM) or osmotic control mannitol-treated peritoneal macrophages derived from normal C57BL/6 mice (n=4). We did not observe significant miR-146a induction under HG conditions (Figure 5C), even though, as expected, we did see increased inflammatory signaling in the macrophages (Tnf-α induction) treated with HG (Figure 5D). Next, we treated peritoneal macrophages with LPS or TNF-α and found a significant increase in miR-146a expression (Figure 5E), as reported elsewhere. These results demonstrate that diabetic miR-146a induction in macrophages may not be directly mediated by hyperglycemia but is likely induced in response to inflammatory mediators upregulated during diabetes.

miR-146a–Deficient Macrophages Have Increased Proinflammatory Phenotype under Diabetic Conditions

Mechanistically, peritoneal macrophages isolated from diabetic miR-146a−/− mice showed higher expression of inflammatory mediators and markers of M1 macrophage phenotype, including IL-1β, IL-18, iNOS, and Cox2, relative to diabetic WT mice (Figure 6, A–E). Increases in Tnf-α expression, however, was not different (Figure 6C). Notably, the markers of the anti-inflammatory M2 macrophages Arg-1, Ym1, CD206, and Retnla (Figure 6, F–I) were significantly reduced in diabetic miR-146a−/− mice compared with WT diabetic mice; in the latter, these markers were upregulated, suggesting loss of adaptive M2 responses in miR-146a−/− mice. Phenotypic characterization of macrophages from the WT and miR-146a−/− mice suggested that the baseline gene expression was similar between both groups, except for TNF-α (Supplemental Figure 2). In addition, Traf6 and Irak1, important effectors of NF-κB and known targets of miR-146a, were also significantly increased in macrophages of diabetic miR-146a−/− mice (Figure 6J). Transfection of miR-146a–mimic oligonucleotides into WT macrophages and subsequent treatment with TNF-α showed that miR-146a overexpression results in downregulation of Traf6 under normal and inflammatory conditions (Supplemental Figure 3). Moreover, TNF-α–induced induction of proinflammatory genes, such as IL-1β, Cox2, and iNOS, was also suppressed by miR-146a overexpression (Supplemental Figure 3). These results suggest that miR-146a induction during early DN may play a renoprotective role by tilting the balance toward anti-inflammatory phenotype in macrophages.

Increased Inflammasome Activation in miR-146a–Deficient Macrophages

The nucleotide-binding domain and leucine-rich repeat pyrin domain (Nlrp3) inflammasome is a multiprotein complex activated by various stress signals, including LPS and oxidants, and functions to activate caspase-1, which is required for IL-1β and IL-18 processing. Inflammasome activation is seen in activated macrophages from diabetic db/db mice (Figure 5B). We did not observe significant inflammasome activation under HG conditions (Figure 5C), even though, as expected, we did see increased inflammasome signaling in the macrophages (Tnf-α induction) treated with HG (Figure 5D). Next, we treated peritoneal macrophages with LPS or TNF-α and found a significant increase in miR-146a expression (Figure 5E), as reported elsewhere. These results demonstrate that diabetic miR-146a induction in macrophages may not be directly mediated by hyperglycemia but is likely induced in response to inflammatory mediators upregulated during diabetes.

Figure 5. Macrophages from diabetic mice have increased miR-146a expression. (A) Real-time qPCR analysis of miR-146a expression in peritoneal macrophages from db/db and control db/+ mice (n=5). (B) Real-time qPCR analysis of miR-146a expression from peritoneal macrophages isolated from control and STZ-injected mice (4-week diabetes) (n=5). (C and D) qPCR quantification of miR-146a and Tnf-α from HG or osmotic control mannitol-treated peritoneal macrophages derived from normal C57BL/6 mice (n=4). (E) qPCR quantification of miR-146a from LPS– and TNF-α–treated peritoneal macrophages (n=6). In all panels, data are presented as mean±SEM. *P<0.05 and †P<0.01 (statistically significant difference) compared with respective control group. PM, peritoneal macrophages.
myeloid cells and has also been recently implicated in development of renal diseases, including DN. Because our data showed that IL-1β and IL-18 gene expression was significantly increased in the macrophages of diabetic miR-146a−/− mice, we also examined inflammasome activation. Gene expression analysis showed significantly increased expression of NLRP3 in miR-146a−/− diabetic macrophages (Figure 7A). Of note, by Western analysis we observed increased caspase-1 activation (marker of inflammasome activation) in the peritoneal macrophages (Figure 7, B and C) and kidney cortices (Figure 7, D and E) of diabetic miR-146a−/− mice relative to WT, as indicated by decreases in pro–caspase-1 and increases in cleaved caspase-1 levels. These results suggest that depletion of miR-146a leads to increased IL-1β and IL-18 gene expression as well as enhanced inflammasome activation.

**DISCUSSION**

Inflammation is a major contributor to the pathogenesis of DN. Macrophages are among the major inflammatory/immune cells that infiltrate into the kidneys under diabetic conditions and create a proinflammatory environment. This in turn affects almost all renal cells, contributing to ECM accumulation, fibrosis, cellular dysfunction, and eventually proteinuria. However, the cellular mechanisms that modulate the inflammatory signaling during DN are incompletely understood. Here we report the involvement of a potent anti-inflammatory miRNA, miR-146a, in the pathogenesis of DN. We observed that miR-146a was upregulated in the kidneys and macrophages of diabetic mice, and mechanistic studies showed that miR-146a plays a critical renoprotective role during DN by tilting the balance toward anti-inflammatory signaling.

Expression of immune-modulatory miRNAs, especially miR-146a, has been determined in different diabetic complications, including DN. While some studies have shown lower miR-146a expression during DN, others have shown higher expression. Our results (Figures 1, 2, and 7) show a significant up-regulation of miR-146a in the peritoneal macrophages as well as in the kidneys of STZ-induced diabetic mouse models. The reported alterations in the expression of miR-146a do not prove a causal role of miR-146a in the pathogenesis of DN. Thus, ascertaining the functional role of miR-146a in the development of DN in vivo was the major objective of this study, and for this we used miR-146a−/− mice. All in vivo experiments were performed in young mice (<6 months of age at the end of experiment), because after 6–8 months, some miR-146a−/− mice may develop autoimmune disorders, which could lead to confounding results. Importantly, STZ-mediated diabetes induction was not altered in miR-146a−/− mice (Figure 1), thereby providing a model with which to directly elucidate the role of miR-146a in DN.

The key features of DN, such as increases in glomerular area, proteinuria, a key clinical marker of DN, was also considerably increased in miR-146a−/− diabetic mice (Figure 2). Moreover, increases in proinflammatory and profibrotic gene expression in the diabetic WT kidneys at both 7 weeks and 16 weeks after diabetes induction were further augmented in the diabetic miR-146a−/− mice (Figures 1 and 2). These histologic, biochemical, and gene expression studies have provided the first direct in vivo evidence that miR-146a plays a protective role during the development of DN.
The development and progression of DN are highly complex, with diverse renal cells, including podocytes and endothelial, mesangial, and tubular cells, affected.  

Additionally, immune cells, especially macrophages, infiltrate the kidneys during DN. Interestingly, miR-146a is highly expressed in macrophages and can negatively regulate macrophage inflammation, which prompted us to determine its expression and function in diabetic macrophages. We found that renal macrophage infiltration during diabetes was further increased in the miR-146a−/− mice (Figure 4). At the same time, combined in situ hybridization of miR-146a and staining of macrophage marker F4/80 in diabetic kidneys revealed their colocalization (Figure 4). Mechanistically, the increase in miR-146a expression in macrophages was not directly mediated by hyperglycemia, at least in the time periods tested (Figure 5). Because miR-146a expression can be induced by low inflammatory conditions, the miR-146a expression seen in diabetic tissues is most likely induced by proinflammatory mediators present in the diabetic milieu.

It is noteworthy that the expression of miR-146a is very sensitive to levels of proinflammatory mediators and may follow a temporal or circadian pattern, and that, depending on the inflammation levels and tissues tested, miR-146a expression may show significant variations. Of note, the extent of miR-146a induction at 16 weeks after diabetes was much lower than at 7 weeks, suggesting a progressive decline of this “protective” miRNA during the development of DN. It is plausible that during long-term diabetes, miR-146a expression may gradually decrease, contributing to progression of DN. This temporal aspect could not be tested in our mouse models because they do not depict severe DN or renal failure.

The renal infiltration of macrophages during DN is preceded by the development of proinflammatory phenotype in peripheral monocytes/macrophages. The low but significantly elevated levels of circulating inflammatory mediators are thought to contribute to monocyte/macrophage activation. At the same time, macrophage polarization is also altered. To study the role of miR-146a in these early events, we isolated murine peritoneal macrophages after 4 weeks of diabetes induction from both WT and miR-146a−/− mice in order to examine the phenotype of macrophages using gene expression analysis. We found that in macrophages from diabetic WT mice, both the proinflammatory M1 macrophage markers (IL-1β, IL-18, iNos, and Cox2) and anti-inflammatory M2 markers (Arg-1, Ym1, CD206, and Retnla) were elevated (Figure 6). However, in macrophages from diabetic miR-146a−/− mice, the extent of induction in M1 gene expression was significantly greater than that observed in the WT group. Intriguingly, we found that the gene expression of M2 markers was significantly suppressed in the diabetic miR-146a−/− macrophages (Figure 6). These results demonstrate for the first time that miR-146a deficiency skews macrophage polarization toward a more inflammatory phenotype.

---

**Figure 7.** Macrophages from miR-146a−/−deficient mice have increased inflammasome activation under diabetic conditions. (A) Nlrp3 gene upregulation in peritoneal macrophages isolated from diabetic miR-146a−/− mice (n=5). (B) Western immunoblot of pro- and cleaved caspase-1 in peritoneal macrophages isolated from WT and miR-146a−/− mice 4 weeks after diabetes induction. (C) Densitometric analysis was done using ImageJ software, and values were normalized to loading control for each lane. The relative protein levels (peritoneal macrophages) were calculated compared with control group (n=4). (D) Western analysis of pro- and cleaved caspase-1 in kidney cortex from 16-week diabetes induction. (E) Densitometric analysis was done using ImageJ software, and values were normalized to loading control for each lane. The relative protein levels (kidney) were calculated compared with control group (n=4). In all panels, data are presented as mean±SEM. *P<0.05 and †P<0.01 (statistically significant difference) compared with respective control group; $P<0.05 and $P<0.01 (statistically significant difference) compared with WT STZ group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
miR-146a Protects against Diabetic Nephropathy

Figure 8. miR-146a plays an anti-inflammatory role during diabetic nephropathy. Proinflammatory signaling under diabetic conditions leads to miR-146a upregulation in macrophages; miR-146a is part of a negative feedback loop that limits macrophage activation, thus playing a protective role during diabetic nephropathy. TLR, toll like receptor.

This study also addressed the mechanistic basis of diabetes-induced amplified proinflammatory signaling in the miR-146a−/− mice. The two well characterized targets of miR-146a are Irak1 and Traf6. In diabetic renal tissues (Figure 3) and isolated macrophages (Figure 6), miR-146a deficiency led to increased expression of Irak1 and Traf6. IRAK1 and TRAF6 promote NFκB-mediated upregulation of proinflammatory cytokines, such as IL-1β, IL-18, and TNF-α. miR-146a is part of a negative feedback loop that modulates this inflammatory response. Importantly, both IRAK1 and TRAF6 are downstream components of Toll-like receptor signaling, which contributes proinflammatory signaling during the development of diabetic nephropathy. Moreover, the reduction in the expression of Traf6 in the WT diabetic kidneys (Figure 3I) seen in our study has also been previously reported in human type 2 diabetic kidneys. On the basis of this, we propose a model (Figure 8) in which inflammation during the initial stages of diabetes is significantly suppressed by induction of miR-146a; therefore, deficiency of miR-146a results in enhanced expression of target genes, leading to increased IL-1β and IL-18 expression. This contributes to a proinflammatory shift (M1 phenotype) in the macrophages and NLRP3 inflammasome activation, which can accelerate DN progression.

Notably, our studies show that macrophages are one of the major cell types that express miR-146a and that its deficiency leads to major changes in the transcriptional and phenotypic characteristics of macrophages under diabetic conditions. However, because we have used a mice model with germline deficiency of miR-146a, we cannot rule out that the worsened DN is not due to the role of miR-146a in other immune and kidney cell types. For example, although we did not see miR-146a expression in podocytes, the diabetic miR-146a−/− mice showed significant podocyte damage, as determined by nephrin levels and proteinuria (Figure 2). It is unclear whether the podocyte damage is an indirect effect of elevated inflammation or due to a direct role of miR-146a in podocytes. We also did not assess whether miR-146a directly contributes to tubulointerstitial fibrosis. Future studies with tissue- or cell-specific knockout and transgenic mice models will likely address these critical questions. Collectively, this study has provided the first functional evidence for a protective, anti-inflammatory in vivo role of miR-146a in the pathogenesis of DN.

CONCISE METHODS

Animal Studies
miR-146a−/− mice on the C57BL/6 background were generated as described elsewhere. The db/db and control db/+ mice (12 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice studies were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the Beckman Research Institute of the City of Hope. The miR-146a−/− mice and the littermate WT controls were made diabetic with multiple low-dose STZ injections. Briefly, mice were fasted for 4 hours, followed by intraperitoneal STZ injections (50 mg/kg) for 5 consecutive days. Kidney cortex sections from 7-week and 16-week STZ-injected diabetic mice were obtained and processed as described previously.

Cell Culture and Materials
Thiglycollate-elicited peritoneal macrophages from all mice were isolated as described previously. Isolated peritoneal macrophages were treated with 25 mM glucose (HG) or mannitol (20 mM) plus glucose (5 mM) as an osmotic control group for 24 hours. LPS treatment was carried out at 10 ng/ml for 16 hours. miR-146a mimic and scrambled control oligonucleotides (Ambion) were transfected (40 pmol) into peritoneal macrophages using lipofectamine RNAiMax (Life Technologies, Carlsbad, CA) isolated from WT mice followed by TNF-α (10 ng/ml) treatment for 3 hours.

Quantitative Real-Time PCR
Total RNA extracted using the mirNeasy kit (Qiagen, Valencia, CA) was subjected to real-time qPCR analysis using the 2−ΔΔCt method as described elsewhere. U6 and actin were used as internal controls. cDNA was generated using the qScript miRNA cDNA synthesis kit (Quanta, Gaithersburg, MD) or GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). ABI-7500 (Applied Biosystems) was
used to perform real-time qPCR using SYBR Green chemistry (Applied Biosystems).

**Western Blot Analysis**

Protein was extracted from the cells and tissue and Western blot analysis was performed as previously described.61 The antibodies used were nephrin (Abcam, Inc., Cambridge, MA), caspase 1 (Abcam, Inc.), and glyceraldehyde-3-phosphate dehydrogenase (Sigma-Aldrich, St. Louis, MO). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

**Urine Protein and Albumin Assays**

Urine was collected over a period of 24 hours from mice in metabolic cages at 11, 13, and 15 weeks after diabetes. Urinary protein was measured as previously described36 using a protein estimation kit according to manufacturer’s instructions (Bio-Rad, Hercules, CA). Urinary creatinine was measured using a modified alkaline picrate method (Exocell, Inc., Philadelphia, PA), and albumin was measured using an ELISA kit (Albuwell M, Exocell, Inc.).

**In Situ Hybridization and Immunohistochemistry**

Combined *in situ* hybridization of miR-146a along with immunohistochemical staining for F4/80 was performed on frozen kidney sections from diabetic and control kidneys according to a previously established method.62 The FITC-labeled locked nucleic acid (LNA) oligonucleotide was used as a probe (Exiqon, Vedbæk, Denmark) to detect miR-146a, and scramble LNA was used as control. F4/80 antibody was purchased from eBioscience (San Diego, CA). Briefly, 7-μm kidney cortical cryosections were fixed and processed for prehybridization, followed by hybridization using LNA probes. The sections were then washed with 2× saline-sodium citrate buffer at a temperature 4–8°C above the hybridization temperature to remove unhybridized probe. To block endogenous peroxidases, the slides were incubated in H2O2 followed by washes with Tris-unhybridized probe. To block endogenous peroxidases, the slides were incubated in H2O2 followed by washes with Tris-saline-sodium citrate buffer at a 0.1 M Tris and 0.15 M NaCl, pH 7.5, and the signal was amplified using a Tyramide Signal Amplification kit (Perkin-Elmer, Waltham, MA). The sections were later processed for F4/80 immunostaining and mounted using Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Applied Biosystems) for visualization.

**Morphologic Examination**

Paraffin-embedded sections of kidney cortex from diabetic and control mice were used for immunohistochemistry and staining using F4/80 antibody. Analysis of histologic changes in the kidney by PAS and Masson trichrome staining was done according to previously reported studies.36,61 Image-Pro 6.1 software (Media Cybernetics, Inc., Bethesda, MD) was used to process the images for quantification of glomerular area and mesangial expansion index.

**Statistical Analyses**

Data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean±SEM as indicated. We used *t* tests to compare two groups of data and one-way ANOVA followed by the Tukey post *hoc* test analysis to compare multiple groups. *P*<0.05 was considered to indicate a statistically significant difference.

**ACKNOWLEDGMENTS**

We are grateful to members of the Natarajan laboratory for their helpful discussions.

This work was supported by grants R01 DK081705, R01 DK058191, and R01 DK065073 from the National Institutes of Health (NIH) to R.N. and a Juvenile Diabetes Research Foundation postdoctoral fellowship grant (3-PDF-2014-103-A-N) to K.B. Research reported in this publication included work performed in the Anatomic Pathology Core and the Animal Research Core, supported by the National Cancer Institute of the NIH under award number R01 CA353572.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

**DISCLOSURES**

None.

**REFERENCES**

15. Mathieson PW: The podocyte as a target for therapies—new and old.
Nat Rev Nephrol 8: 52–56, 2012

Contrib Nephrol 170: 36–47, 2011

17. Pickup JC: Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes.
Diabetes Care: 28: 833–835, 2004

Circulation 106: 2067–2072, 2002

19. Mora C, Navarro JF: In


Semin Nephrol 30: 290–301, 2010

Diabetes 52: 1256–1264, 2003


Physiol Rev 91: 827–887, 2011


27. Lorenzen JM, Haller H, Thum T: MicroRNAs as mediators and therapeutic targets in chronic kidney disease.


Am J Physiol Renal Physiol 300: F602–F610, 2011

Nat Rev Nephrol 11: 23–33, 2015

Int J Endocrinol 593956, 2014

32. McClellan A, Hagiwara S, Kantharidis P: Where are we in diabetic nephropathy?—

Diabetes Care: 34: 2810–2817, 2011

34. Boldin MP, Baltimore D: MicroRNAs, new effectors and regulators of NF-κB.


inflammatory phenotype in macrophages by a diabetes-induced long noncoding RNA. *Diabetes* 63: 4249–4261, 2014


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2015010111/-/DCSupplemental.