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Suppression of microRNA-29 Expression by TGF-β1 Promotes Collagen Expression and Renal Fibrosis

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ABSTRACT

Synthesis and deposition of extracellular matrix (ECM) within the glomerulus and interstitium characterizes renal fibrosis, but the mechanisms underlying this process are incompletely understood. The profibrotic cytokine TGF-β1 modulates the expression of certain microRNAs (miRNAs), suggesting that miRNAs may have a role in the pathogenesis of renal fibrosis. Here, we exposed proximal tubular cells, primary mesangial cells, and podocytes to TGF-β1 to examine its effect on miRNAs and subsequent collagen synthesis. TGF-β1 reduced expression of the miR-29a/b/c/family, which targets collagen gene expression, and increased expression of ECM proteins. In both resting and TGF-β1–treated cells, ectopic expression of miR-29 repressed the expression of collagens I and IV at both the mRNA and protein levels by targeting the 3′untranslated region of these genes. Furthermore, we observed low levels of miR-29 in three models of renal fibrosis representing early and advanced stages of disease. Administration of the Rho-associated kinase inhibitor fasudil prevented renal fibrosis and restored expression of miR-29. Taken together, these data suggest that TGF-β1 inhibits expression of the miR-29 family, thereby promoting expression of ECM components. Pharmacologic modulation of these miRNAs may have therapeutic potential for progressive renal fibrosis.

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Diabetic nephropathy (DN) is a major microvascular complication of diabetes and a leading cause of death among diabetic patients. It is characterized by excessive extracellular matrix (ECM) synthesis and accumulation leading to glomerular and tubular basement membrane thickening as well as and mesangial expansion. The resulting glomerulosclerosis and tubulo-interstitial fibrosis that ultimately develop represent the final common pathway for many CKDs. Recent studies have also demonstrated that microRNAs (miRNAs) contribute to disease processes and are likely to play a specific role in the development of fibrosis, by regulating the expression of target genes at the level of protein translation and mRNA stability.

The principal effector cells in this disease process are activated mesangial cells and fibroblasts, as well as myofibroblasts, approximately one third of which arise from tubular epithelium via the process of epithelium-like to mesenchymal transition (EMT),[1] as well as from other sources.[2–4] Activation occurs as a consequence of the elevated levels of growth factors, cytokines, and reactive oxidative species in

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the context of hyperglycemia, all of which contribute to diabetes-related renal damage. Among these factors, the major driver of renal injury is TGF-β1, which acts through the downstream Smad signaling pathway to drive many of the gene expression changes and pathology observed in renal fibrosis.

A novel recent finding is that TGF-β1 regulates the expression of certain miRNAs. Gregory *et al.* demonstrated that the miR-200 family are downregulated by TGF-β1 in Madin-Darby Canine Kidney cells, in which a feedback loop exists between this family and the downstream targets of miR-200, ZEB1, and ZEB2.[5] This action of TGF-β1 has been replicated in other cell types.[6–9] Kato *et al.* demonstrated an increase in miR-192 and a decrease in miR-215 in mesangial cells in response to TGF-β1, which was associated with increased collagen I expression.[10] We previously demonstrated that TGF-β1 and connective tissue growth factor (CTGF), which caused increased ECM protein expression in proximal tubular epithelium-like cells, could modulate expression of miR-192/215.[11] In that work, we reported that although TGF-β1 and CTGF differentially regulated miR-192/215 expression and thus E-cadherin expression via targeting ZEB2, these miRNAs did alter not ECM protein expression.[12] More recently, we also reported a role for miR-200a in the regulation of TGF-β2 expression and fibrogenesis in renal cells.[13]

Another miRNA, miR-29, has been increasingly noted to be associated to fibrosis. For example, Sengupta *et al.* reported that miR-29c was downregulated in nasopharyngeal carcinomas and was associated with increased expression of ECM proteins.[14] In this study, we demonstrate the role of the miR-29 family as a regulator of collagen synthesis and fibrosis *in vitro* and *in vivo,* using three animal models of renal fibrosis and therapeutic intervention in one of those models. Furthermore, we assess the reversibility of these changes in miRNA-29 in response to a renoprotective therapy.

RESULTS

The miR-29 Family of microRNAs Is Regulated by TGF-β1 in Proximal Tubular Epithelium-Like Cells

Proximal tubular cells (NRK52E) were treated with TGF-β1 (10 ng/ml) under high glucose (25 mM) conditions for 3 days, after which morphology, protein, gene, and miR-29 expression levels were assessed. As we previously reported, TGF-β1 resulted in the classic EMT-like morphologic changes, including significantly increased expression of collagens I and IV (Figure 1A) and protein (Figure 1, B and C).[11,12] Typically, E-cadherin expression was significantly decreased. TGF-β1 also reduced the expression of the miR-29 family (miR-29a, -29b, -29c) in these cells with significant decreases observed after both a short (3 days) and longer (>10 days) exposure (Figure 1, D and E, respectively). The relative expression of the miR-29 family members in NRK52E cells is shown in Figure 1F, demonstrating a 10-fold higher abundance of miR-29a relative to miR-29b and a five-fold higher abundance relative to miR-29c.

The miR-29 Family Regulates the Expression of Collagen in Proximal Tubular Epithelium-Like Cells

The 3′untranslated region (UTR) of most collagens contains target sites for miR-29–mediated translational repression (TargetScan). Ectopic expression of pre–miR-29a/b/c or pre–miR-Control (miR-C) in NRK52E cells demonstrated significantly decreased collagens I, III, and IV mRNA levels compared with miR-C transfected cells (Figure 2A). Western blot analysis revealed that miR-29a/b/c transfected cells also had significantly reduced collagen I and IV protein levels compared with miR-C (Figure 2, B and C), confirming the mRNA expression data in Figure 2A. Transfected NRK52E cells expressed 1000-fold higher levels of miRNA compared with nontransfected cells (Supplemental Figure 1).

miR-29 Regulates Collagen I and IV Expression in Human Podocytes

Experiments were conducted in conditionally immortalized human podocytes.[12,15] Cells were seeded and then differentiated for 12 days at 37°C.[12] TGF-β1 treatment tended to decrease the expression of miR-29 family members, but only the miR-29b and miR-29c decreases were significant (Figure 2D). Treatment with TGF-β1 significantly increased collagen I, III, and IV mRNA levels, as well as α-smooth muscle actin (αSMA) and vimentin (Figure 2E). Ectopic expression of miR-29a/b/c in podocytes also resulted in significantly decreased collagen IV mRNA as well as significantly attenuated the induction of all collagens in TGF-β1–treated podocytes (Figure 2E). miR-29a/b/c also attenuated the increase in αSMA and vimentin induced by TGF-β1 (Figure 2E). miR-C alone did not prevent the increase in αSMA, vimentin, or collagen expression induced by TGF-β1 (Figure 2E). Transfection of podocytes with miR-29a/b/c significantly attenuated the TGF-β1 induction of collagen I protein expression (Figure 2F). This result is consistent with our observations in proximal tubular cells demonstrating that the miR-29 family is involved with the repression of collagen protein expression.

miR-29 Regulates Collagen Expression in Primary Mouse Mesangial Cells

Experiments were also conducted in primary mouse mesangial cells collected from C57Bl6/J mice as previously described.[16] Treatment with TGF-β1 resulted in significantly increased collagen I, III, and IV mRNA expression (Figure 3A). The increased collagen expression was associated with a significant decrease in miR-29a, miR-29b, and miR-29c levels (28%, 46%, and 37%, respectively; $P<0.05$ compared with controls) (Figure 3B). Transfection of mesangial cells with miR-29a/b/c in the absence or presence of TGF-β1 resulted in significantly reduced collagen I levels (Figure 3, C and D, respectively). This decrease in collagen 1 protein in miR-29 transfected cells was also reflected at the mRNA level (Figure 3E), in which miR-29 reduced basal level collagen I mRNA but also prevented the induction of collagen I mRNA in TGF-β1–treated cells.

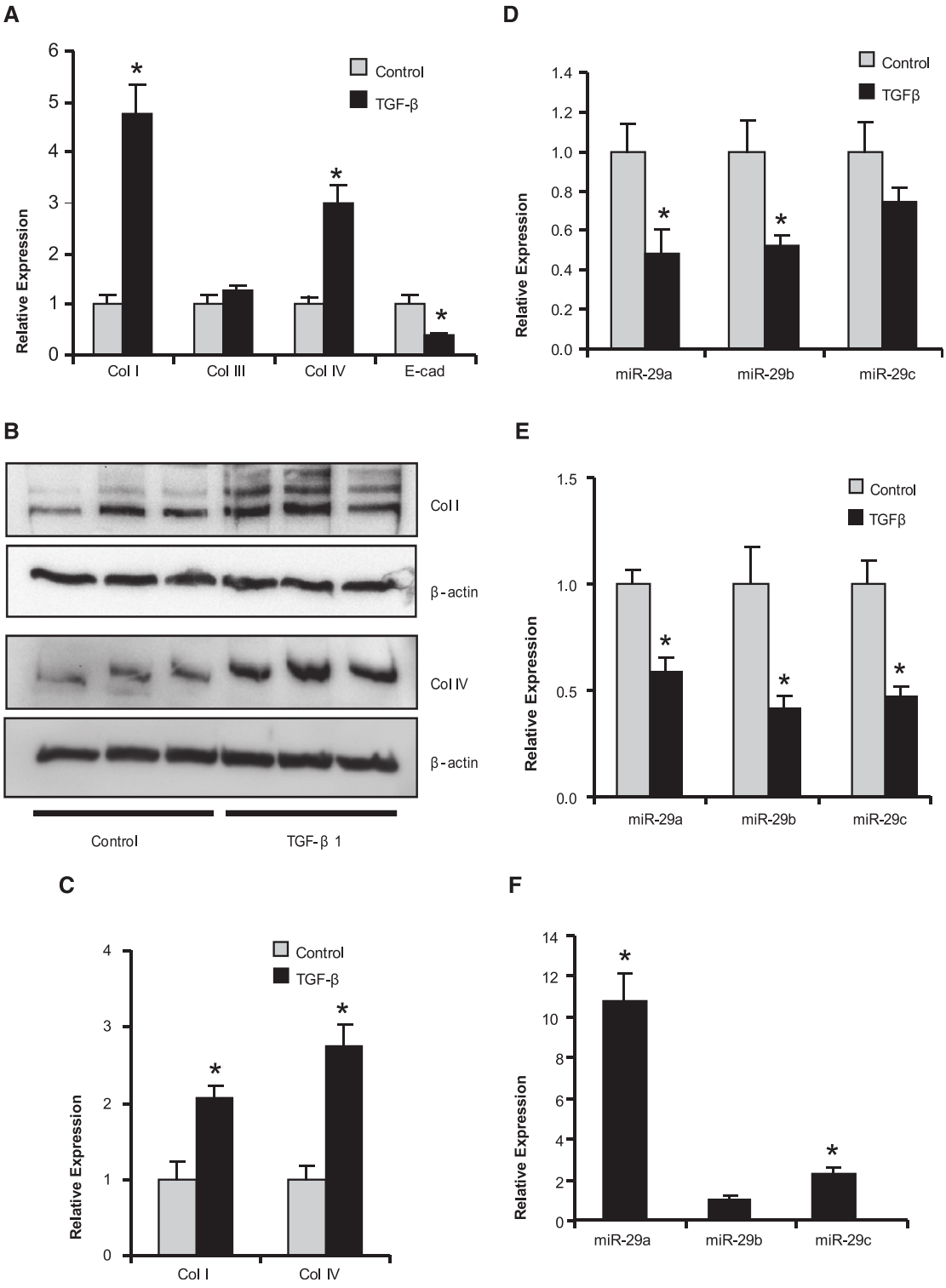

Figure 1. TGF-β1 induces EMT-like changes in proximal tubular epithelium-like cells. (A) NRK52E cells were cultured in the presence of TGF-β1 (10 ng/ml, 3 days). After treatment with TGF-β1, collagen I and IV gene expression were significantly increased by real-time quantitative PCR, whereas E-cadherin expression was significantly decreased (*$P<0.05$ compared with control). (B) Western blot analysis demonstrated that collagen I and collagen IV were both elevated at the protein level after TGF-β1 treatment and (C) these increases were significant (*$P<0.05$ compared with control). (D) Expression of miR-29a and miR-29b were both significantly decreased in cells treated with TGF-β1 for 3 days (*$P<0.05$ compared with control). The decrease in miR-29-c was not significant. (E) In longer-term TGF-β1–treated cells (10 days), all three members of miR-29 were significantly reduced (*$P<0.05$ compared with control). (F) Relative expression levels of the miR-29 family members in control NRK52E cells compared with miR-29b. (*$P<0.05$ compared with miR-29b).

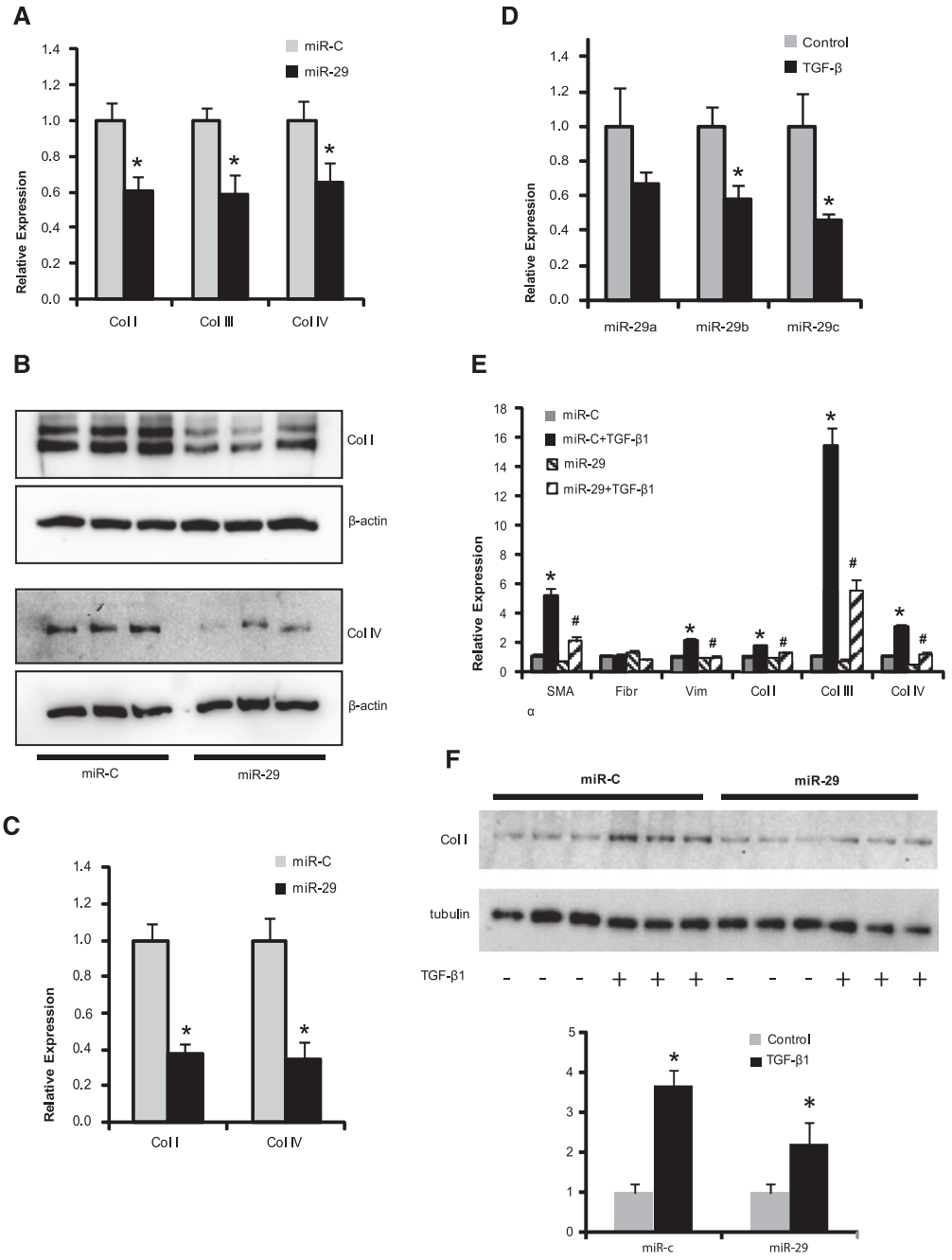

Figure 2. miR-29 represses the expression of collagen genes. (A) NRK52E cells were transfected with miR-29a/b/c (50 nM each) and RNA was harvested after 3 days for real-time quantitative PCR analysis. miR-29 significantly decreased expression of collagens I, III, and IV (*$P<0.05$ compared with control transfected cells). (B) Western blot analysis demonstrated a significant decrease in collagen I and collagen IV protein expression (*$P<0.05$ compared with control transfected cells), which was consistent with the RNA expression analysis. (C) The quantified Western blot data shown in graph format (*$P<0.05$ compared with control). (D) Conditionally immortalized human podocytes differentiated for 10 days at 33°C before treatment with TGF-β1 (3 days, 5 ng/ml). Treatment with TGF-β1 reduced expression of miR-29a/b/c (*$P<0.05$ compared with control). (E) Human podocytes were transfected with miR-29a/b/c or miR-C, with or without treatment with TGF-β (5 ng/ml, 3 days) and RNA isolated for expression analysis. TGF-β1 treatment increased expression of αSMA, vimentin, and collagens I, III, and IV (*$P<0.05$ compared with control). Transfection with miR-29a/b/c significantly decreased basal level expression of collagen IV relative to miR-C, and significantly attenuated TGF-β1–induced expression of αSMA, vimentin (Vim), and collagens I, III, and IV ($^{\#}P<0.05$ compared with control). (F) Collagen I protein expression as assessed by Western blot analysis in podocytes transfected with miR-29a/b/c or miR-C, with or without TGF-β1 treatment. Protein expression was significantly attenuated by miR-29a/b/c in TGF-β1–treated cells as shown in the graph below (*$P<0.05$ compared with control).

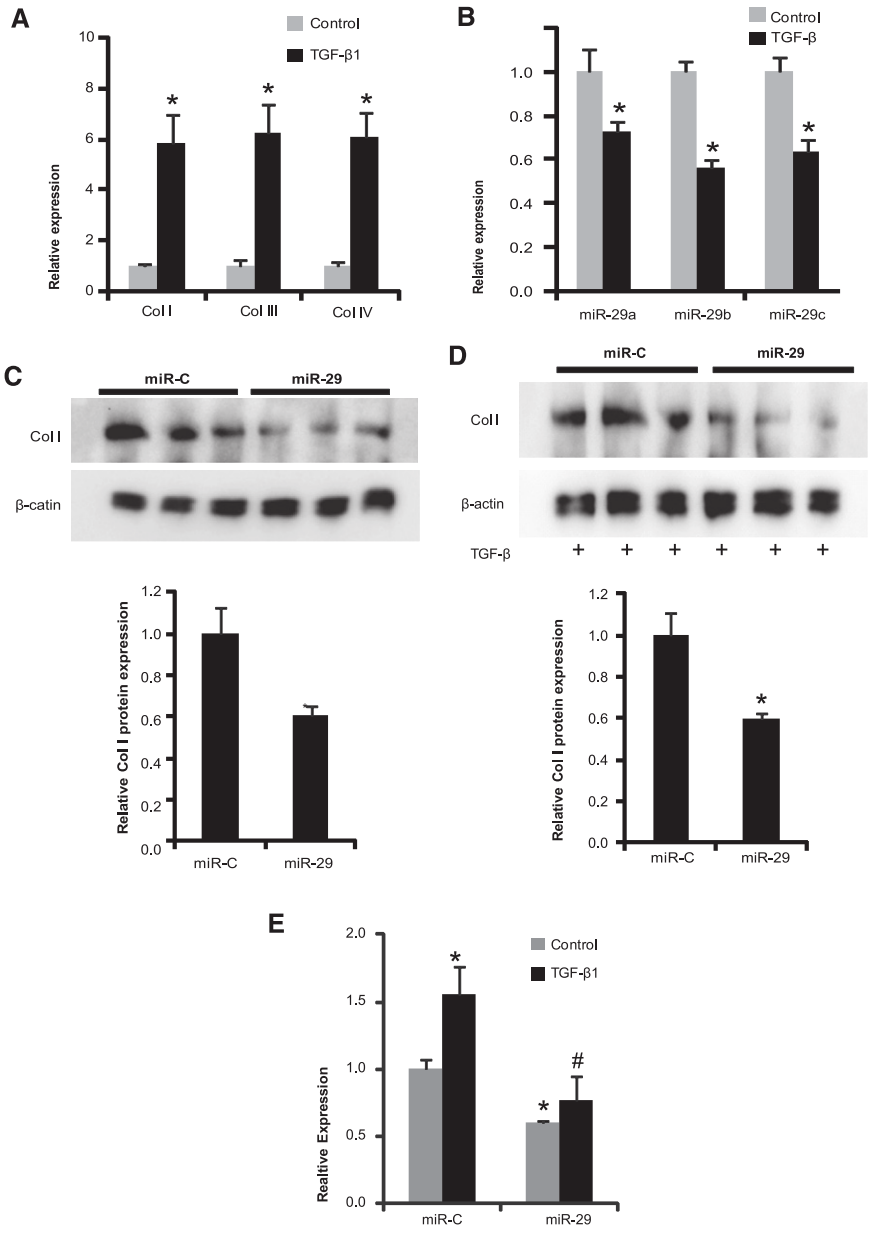

Figure 3. miR-29 repression of collagen 1 expression in primary mouse mesangial cells. (A) Primary mouse mesangial cells were treated with TGF-β1 for 3 days demonstrated significantly increased collagen I, III, and IV expression ($P<0.01$ compared with control). (B) TGF-β1 also resulted in significantly decreased miR-29a, miR-29b, and miR-29c expression ($P<0.05$ compared with control). (C) Mesangial cells were transfected with miR-29a/b/c (50 nM each) and harvested after 3 days. Western blot analysis demonstrated significantly decreased collagen I expression in transfected cells (graphed below, $P<0.05$ compared with control). (D) Similar experiments with TGF-β1 treatment demonstrated that miR-29 transfection could prevent the induction of collagen I by TGF-β1 treatment (graphed below, $P<0.05$ compared with control). (E) Real-time qPCR analysis of miR-29 transfected cells shows significantly decreased collagen I mRNA in control but also in TGF-β1–treated cells ($^{*}P<0.05$ and $^{\#}P<0.05$ compared with control).

Collagen I and Collagen IV 3′UTRs Are Targeted by miR-29

The miR-29 family shares the same seed sequence that is complementary to the conserved binding sites found in the 3′UTR of many collagen genes (Targetscan). The 3′UTRs of collagens Ia1, IVa1, and IVa3 contain several sites for miR-29. To confirm whether these collagens are real targets of the miR-29 family, three luciferase reporter constructs incorporating the 3′UTR of these collagens were made and tested in transfection experiments. Proximal tubular cells were co-transfected with the Renilla luciferase-collagen-3′UTR constructs, a β-galactosidase construct, and either pre-miR-29a/b/c or the pre-miR control. TGF-β1 was added 4 hours post-transfection and analyses for β-galactosidase and luciferase activities were performed 3 days post-transfection. As shown in Figure 4, A–C, TGF-β1 significantly increased the luciferase activity from each construct compared with control cells. The miR-29 family was able to significantly reduce basal luciferase activity in the absence of TGF-β, but more importantly was able to totally abolish the TGF-β–mediated increase in luciferase activity in each case (Figure 4, A–C). Conversely, where the miR-29 binding sites were mutated in the collagen I and IV 3′UTRs so that miR-29a/b/c could no longer bind, luciferase activity was unaltered by miR-29a/b/c (Figure 4D). These data confirm that the miR-29 family is a specific and potent translational repressor of collagen I, IVa1, and IVa3 by targeting the 3′UTR of these genes and thus can override the profibrotic effect of TGF-β1 on these genes.

Ectopic Expression of miR-29 Causes Increased E-Cadherin Expression

TGF-β1 causes EMT in epithelium-like cells, leading to the loss of E-cadherin and disruption of cell junctions, eventually resulting in fibrosis.[12] Some studies have also demonstrated that E-cadherin expression is inversely regulated by collagen levels.[17–19] Given that miR-29 represses collagen expression at the level of translation, we tested the hypothesis that miR-29 may indirectly affect E-cadherin expression. Typically, E-cadherin mRNA levels were significantly decreased by TGF-β1 treatment (Figure 1A); however, ectopic expression of miR-29a/b/c significantly increased E-cadherin mRNA levels (Figure 4E). Immunofluorescence analysis confirmed the increased expression of E-cadherin in miR-29–transfected cells even in the presence of TGF-β1 (Figure 4F).

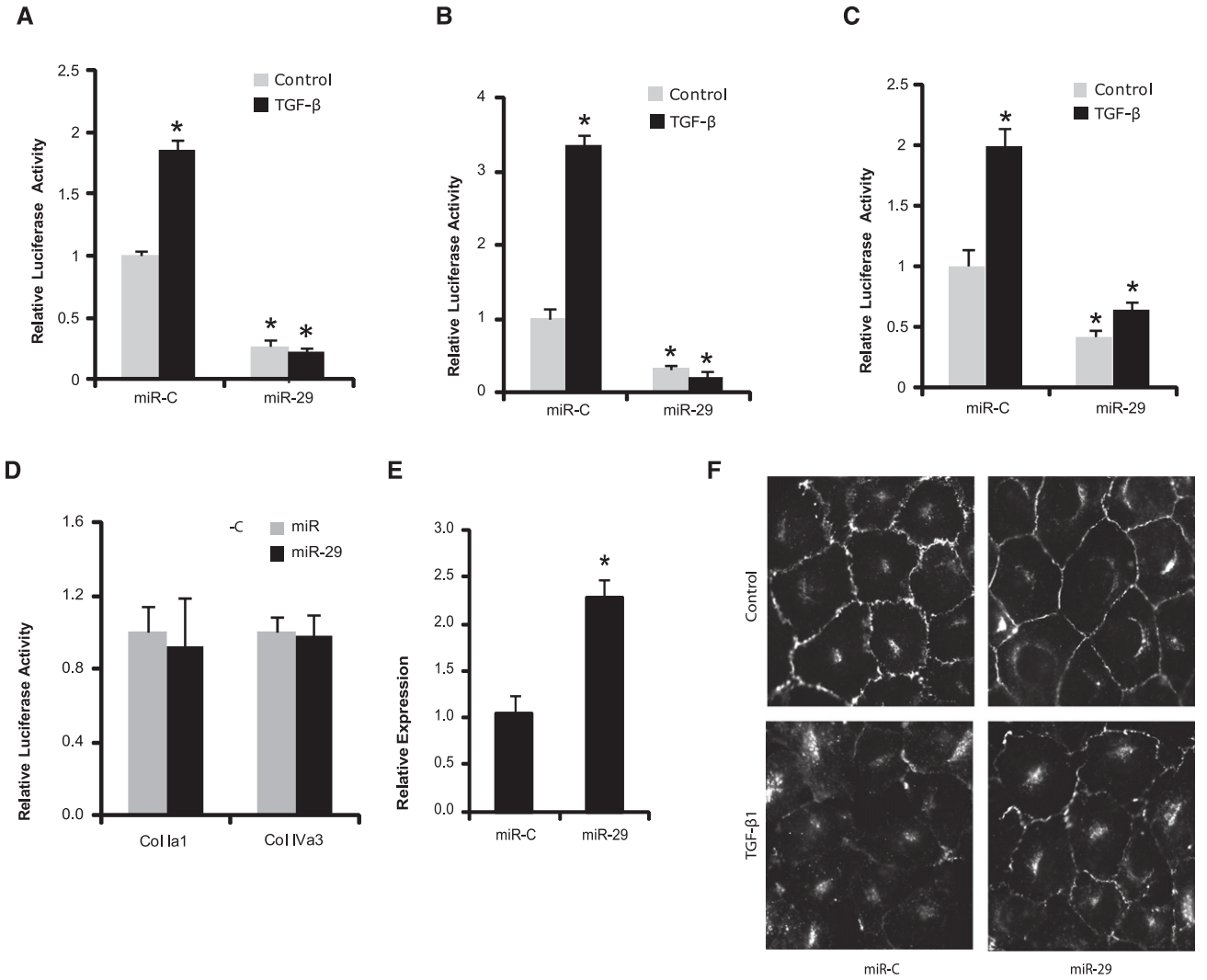

Figure 4. The 3′UTR of collagens I and IV is regulated by miR-29a/b/c. NRK52E cells were transfected with collagen 3′UTR luciferase reporter plasmids (1 μg), β-galactosidase plasmid (0.2 μg), and either miR-C (150 nM) or miR-29a/b/c (50 nM each), and cells were analyzed for β-galactosidase and luciferase activity after 3 days. TGF-β1 significantly increased luciferase activity in cells containing constructs with the 3′UTR of (A) collagen I, (B) collagen IVa1, and (C) collagen IVa3 (*$P<0.05$ compared with control transfected cells). In each case, miR-29a/b/c prevented this increase and even reduced luciferase activity below control transfected cells (*$P<0.05$ compared with control transfected cells). (D) Similar experiments were carried out using luciferase constructs with collagen Ia1 and IVa3 3′UTRs containing mutated miR-29 binding sites. miR-29a/b/c had no effect in those constructs in which the miR-29 binding sites were mutated. (E) Ectopic transfection of NRK52E cells with miR-29a/b/c also resulted in significant elevation of E-cadherin mRNA levels (*$P<0.05$ compared with control transfected cells). (F) Ectopic expression of miR-29a/b/c also prevented the decrease in E-cadherin expression induced by TGF-β1, as observed by immunofluorescence.

miR-29 Is Decreased *In Vivo* in a Model of Early Diabetic Renal Fibrosis

To further explore the relationship between the miR-29 family and renal fibrosis in diabetic kidney disease, we examined gene, protein, and miR-29 expression levels in the cortex of kidneys from STZ-diabetic apoE KO mice at 10 weeks of diabetes. This model of chronic hyperglycemia and dyslipidemia results in augmented renal fibrosis,[20] and represents a disease state with various pathologic similarities to the early diabetic renal disease seen in humans. All three miR-29 family members were decreased in diabetic animals compared with controls (Figure 5A); however, only the changes in miR-29a and miR-29c were significant. The altered expression of miR-29 family members was associated with increased expression of collagen IV and CTGF mRNA (Figure 5B) and collagen IV protein (Figure 5C). The relative expression of miR-29a/b/c in mouse kidney (Figure 5D) indicates a 14-fold abundance of miR-29a compared with miR-29b and a four-fold increase compared with miR-29c, similar to the trend observed in proximal tubular epithelium-like cells (Figure 1F). *In situ* hybridization with digoxigenin-labeled probes revealed that miR-29a and miR-29 are expressed in proximal tubular cells and

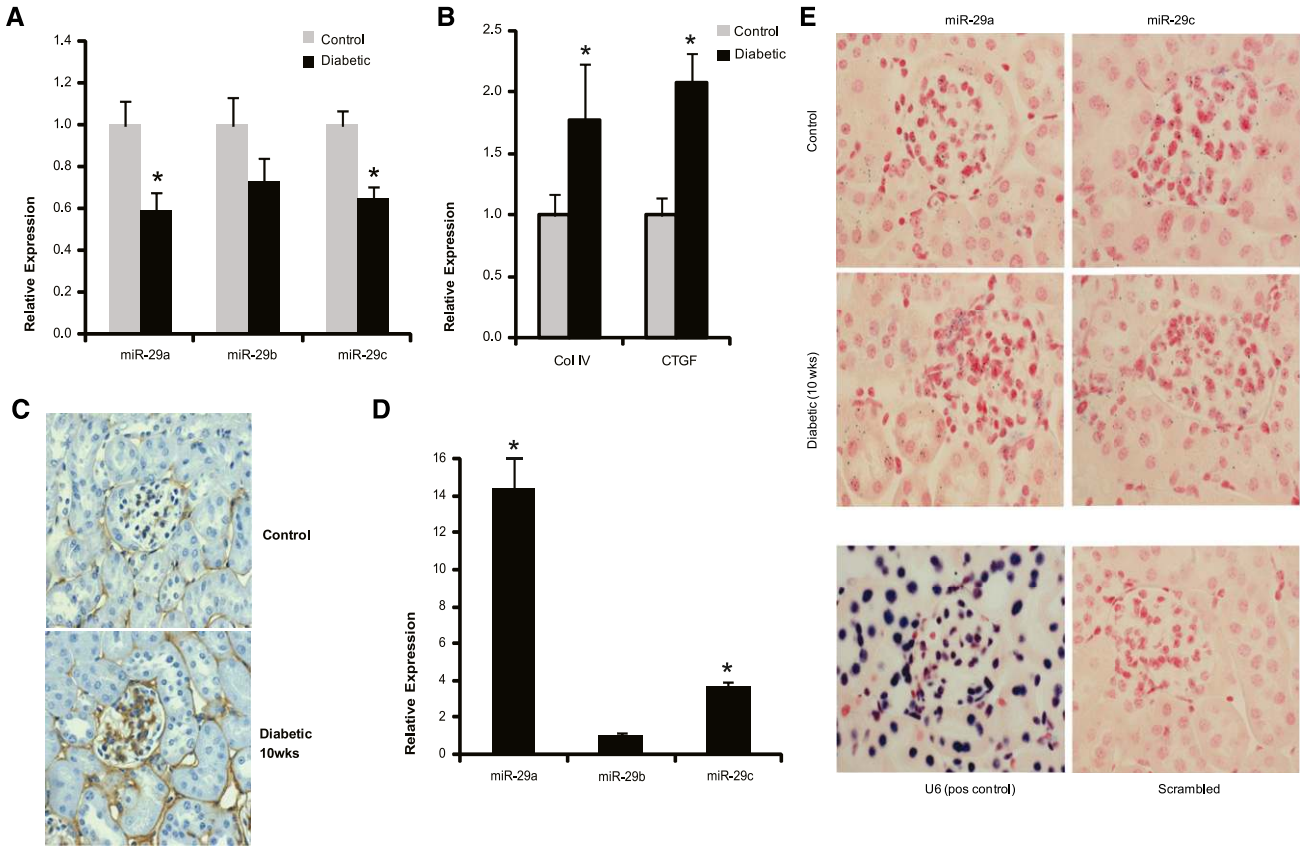

Figure 5. Changes in miR-29a/b/c expression in diabetic mouse kidney. (A) Total RNA was extracted from kidney cortex of control and 10-week diabetic apoE mice (n=7 per group). The expression of miR-29 family members was assessed by real-time quantitative PCR, revealing significantly decreased expression of miR-29a and miR-29c in renal cortex of diabetic animals (*P<0.05 compared with control). The decrease in miR-29b was not significant. (B) Gene expression analysis revealed significantly increased levels of collagen IV and CTGF in diabetic animals (*P<0.05 compared with control). (C) Immunohistochemical analysis confirmed increased collagen IV expression in the kidneys of diabetic animals. (D) The relative expression of the miR-29 family members in control animals is shown relative to miR-29b. (E) Endogenous miR-29a and miR-29c were detected in proximal tubular glomerular cells in control and 10-week diabetic apoE$^{-/-}$ mouse kidney using LNA probes (Exiqon).

glomerular cells in control and 10-week diabetic apoE$^{-/-}$ kidney (Figure 5E).

miR-29a/b/c Expression in Advanced Diabetic Renal Fibrosis: Effect of Nephroprotective Treatments

miR-29 expression at more advanced stages of DN was studied in UNx STZ-diabetic rats.[21] The rats were studied 18 weeks after induction of diabetes. Compared with nondiabetic animals, diabetic rats demonstrated reduced weight gain, increases in blood glucose (BG), glycosylated hemoglobin levels, 24-hour urinary protein excretion, and more advanced structural lesions as assessed by increases in glomerulosclerosis and tubulointerstitial fibrosis scores (Supplemental Table 1).

Expression of selected ECM proteins known to be increased in DN was determined by immunohistochemistry and immunoblotting (Figure 6). Compared with nondiabetic animals, D-VE rats demonstrated increased renal cortical immunoreactivity of collagen I, collagen IV, fibronectin, and laminin (Figure 6A), in particular in the tubulointerstitial compartment. The renal cortical protein abundance of fibronectin, collagen I, and laminin was further quantified by immunoblotting, which showed increases in all of these proteins in untreated diabetic (D-VE) rats as compared with control animals (Figure 6, B and C). These diabetes-induced changes were associated with decreased levels of miR-29a and miR-29c (Figure 6D), which is agreement with our observations in diabetic mice (Figure 5A). In contrast, miR-29b expression in the kidney was not altered by diabetes (Figure 6D).

Further measurements in this model of DN have evaluated effects of nephroprotective treatments on renal miR-29 expression in conjunction with their effects on markers of renal fibrosis. The rats were treated with the ρ-associated kinase (ROCK) inhibitor fasudil or with angiotensin receptor blocker (ARB) losartan. The treatments with fasudil as well as with losartan attenuated the development of proteinuria and structural lesions in diabetic rats (Supplemental Table 1), and reduced renal immunoreactivity and protein abundance of collagens, fibronectin, and laminin compared with D-VE rats (Figure 6, A–C). In

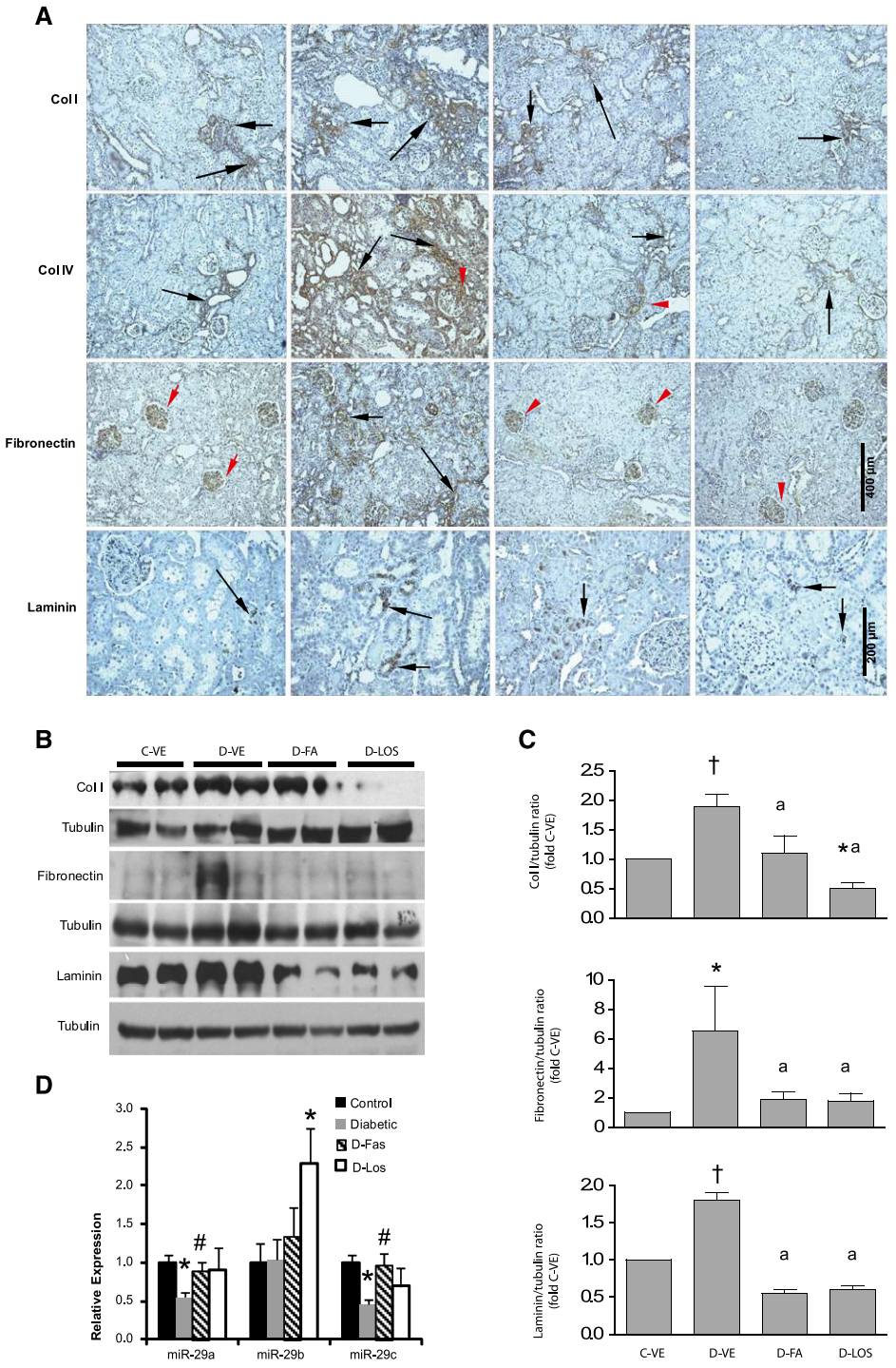

Figure 6. Renal expression of ECM proteins in diabetic rats. (A) Representative microphotographs (×200) show cortical sections from nondiabetic animals (C-VE), vehicle-treated diabetic rats (D-VE), fasudil-treated diabetic animals (D-FA), and diabetic animals treated with losartan (D-LOS) probed with primary antibodies raised against collagens I (Col) and IV (Col IV), fibronectin (×100), and laminin (×200). Arrows show immunoreactivity of proteins of interest in the tubulointerstitial compartment, and red arrowheads show examples of positively stained glomeruli. (B) Protein abundance of collagen 1, fibronectin, and laminin in renal cortical homogenates was further quantified by Western blotting. (C) Representative blots are shown and densitometric analysis is shown (*$P<0.05$, $^{\dagger}P<0.01$ versus C-VE; $^{a}P<0.05$ versus D-VE.) (D) The expression of miR-29a/b/c was assessed in this model by real-time quantitative PCR. miR-29a and miR-29c are significantly reduced in diabetic kidney cortex in this model. Fasudil treatment restores expression to that of control animals. Losartan treatment partially restores expression (*$P<0.05$ compared with control; $^{\#}P<0.05$ compared with diabetic animals).

fasudil-treated animals, these beneficial effects were associated with a reversal of the decreased expression of renal cortical miR-29a and miR-29c (Figure 6D). A similar trend in miR-29a and miR-29c expression was observed in D-LOS rats, albeit the reversal was not significant (Figure 6D). Unlike fasudil, treatment with losartan resulted in a marked increase in miR-29b expression compared with the control and other groups of diabetic rats (Figure 6D).

Decreased miR-29a/b/c in Advanced Nondiabetic Kidney Disease

We explored the expression of the miR-29 family in an advanced form of renal disease, adenine-induced renal fibrosis. In this model, there is marked tubulointerstitial fibrosis as revealed by trichrome staining (Figure 7A), with strong upregulation of collagen I (Figure 7B). This model was also associated with a significant decrease in expression of miR-29a/b/c (Figure 7C), which is consistent with our *in vitro* observations. RNU6B, a small nuclear RNA used as a housekeeping gene in these experiments, was not altered in this model.

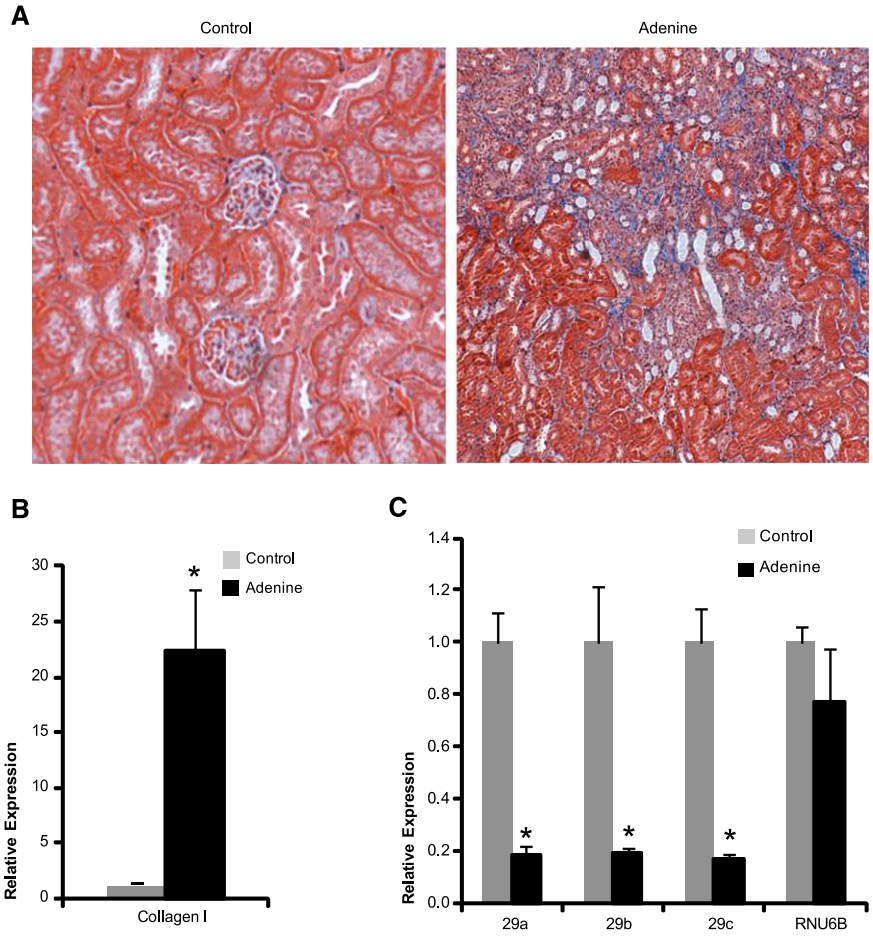

Figure 7. Changes in miR-29a/b/c expression in the adenine-induced renal fibrosis model. (A) Trichrome staining of tissue sections from renal cortex from control and adenine-fed C57bl6 mice after 4 weeks of treatment (n=3 per group). Blue staining indicates high levels of collagen in the adenine-fed mouse kidney compared with control. (B) mRNA was extracted from the renal cortex of control and adenine-fed C57BL6 mice. Gene expression was assessed by real-time quantitative PCR, revealing significantly increased expression of collagen I (*P<0.05 compared with control). (C) The expression of miR-29a/b/c, as assessed by real-time qPCR, showed significant reduction for all miR-29 family members (*P<0.05 compared with control). The expression of the housekeeping gene used in these studies, RN6UB, was not altered.

DISCUSSION

The important role of miRNAs as regulators of physiologic and pathologic processes in human health and disease has recently been recognized. In this study, we demonstrated that the miR-29 family plays an important regulatory role in TGF-β1–mediated fibrogenesis in renal tubular epithelium-like cells and podocytes. We demonstrated under high glucose conditions that TGF-β1 decreased the expression of miR-29a/b/c in tubular epithelium-like cells and podocytes, and that this was associated with increased expression of collagens I, III, and IV. The miR-29 family reduces collagen I, III, and IV expression by targeting the 3′UTR of these mRNAs to repress translation. Our observations are consistent with recent studies demonstrating that miRNAs also regulate the mRNA levels of target genes. We demonstrated that miR-29a/b/c was able to reduce collagen mRNA levels in both proximal tubular epithelium-like cells and podocytes. This occurred despite the absence of any region of complete homology between miR-29a/b/c and the target genes, and confirms that miRNAs regulate gene expression also by altering message stability as previously reported.[22–24] The miR-29 family is therefore an important downstream mediator of TGF-β1–mediated fibrogenesis.

To extend our studies to more physiologically and clinically relevant conditions, we further studied miR-29 in three different *in vivo* models of renal injury that represent different stages of early and advanced fibrotic disease demonstrating tubulointerstitial changes. We clearly demonstrated that miR-29a, miR-29b, and miR29c were decreased in the kidney of all three models of renal disease. Decreased miR-29 expression was associated with increased expression of collagens and other ECM proteins. Furthermore, these studies confirm that the diabetic milieu can induce changes in the expression of certain miRNAs in the kidney.

Several prosclerotic factors contribute to renal fibrosis, the most potent of which is TGF-β1,[25] which is increased in the

diabetic kidney.[26] Considering the well established roles of TGF-β1 in the pathophysiology of diabetes and renal disease, our observations are consistent with the effects of this growth factor in our *in vitro* experiments in two different renal cell lines. Our observations further confirm that miR-29 may be intricately involved in the development and progression of renal fibrosis independent of its underlying etiology, because our model of nondiabetic renal fibrosis also exhibited significantly reduced expression of the miR-29 family.

Similar associations have been observed in a number of pathologic settings in which fibrosis develops in different tissues, such as in the infarcted heart,[27] nasopharyngeal tumors,[14] liver fibrosis,[28] and systemic sclerosis,[29] suggesting that this mechanism of regulating collagen expression is likely to be generic across different tissues and diseases in which fibrosis is the final common outcome. Furthermore, recent studies suggest a similar relationship in the kidney in which miR-29b is reduced in a model of hypertensive renal disease in the kidneys of salt-sensitive rats.[30]

Although the evidence suggesting a role for miRNAs in renal pathophysiology has been rapidly increasing,[10,12,13,31–34] the effect of known nephroprotective treatments on the expression of miRNAs, or their possible contribution as protective agents against renal fibrosis, has yet to be established. In this context, we also evaluated miR-29 expression in diabetic UNx rats treated with the ROCK inhibitor fasudil or ARB losartan. Interestingly, both treatments influenced renal cortical miR-29 expression in association with their beneficial renal effects. Although treatment with fasudil reversed diabetes-induced decreases in expression of miR-29a and miR-29c and a similar trend was observed in diabetic uninephrectomized rats, losartan treatment resulted in a marked increase in miR-29b expression compared with other groups of rats. Because all three miR-29 types interfere with the profibrotic effects of TGF-β1, these observations suggest that modulation of protective miRNAs may contribute to nephroprotective actions of some pharmacological interventions in DN. This notion is further supported by an interesting parallel with renal involvement in Dahl salt-sensitive rats. As previously mentioned, this model of hypertensive renal injury is associated with lower miR-29 and, similarly to models of DN,[35,36] is responsive to treatment with ROCK inhibitors.[37] In addition, individual treatments seem to have alternative effects on the various miR-29 family members, although the net effect in the kidney is comparable. The mechanisms linking the ρ-ROCK pathway or AT1 receptor signaling cascades to miRNA expression remain unknown and require additional research.

We recently reported the association of several miRNAs in the development and progression of renal fibrosis as shown in Figure 8,[12,13] including miR-192,[12] miR-200a,[13] and now miR-29; however, the role of miR-192 remains controversial.[10,31] In contrast to our work, a recent study reported elevated expression of miR-29c under high glucose conditions in kidney endothelial cells and podocytes.[38] These investigators also reported increased expression of miR-29c in glomeruli from a model of type II diabetes, the db/db mouse, and that knockdown of miR-29c was able to prevent progression of DN. Furthermore, differences in the origin of cell lines examined and treatments, as well as differences in the animal models examined (insulin deficient versus type II diabetes), could explain some of the disparities among the various studies. Indeed, the differences between the various reports highlight the complex nature of miRNA research and methodology and the need for more comprehensive studies. It is likely that the role of miRNAs in renal fibrosis is very complex. Other miRNAs that have been implicated include miR-93,[39] miR-377,[34] miR-21,[40] and miR-216.[41]

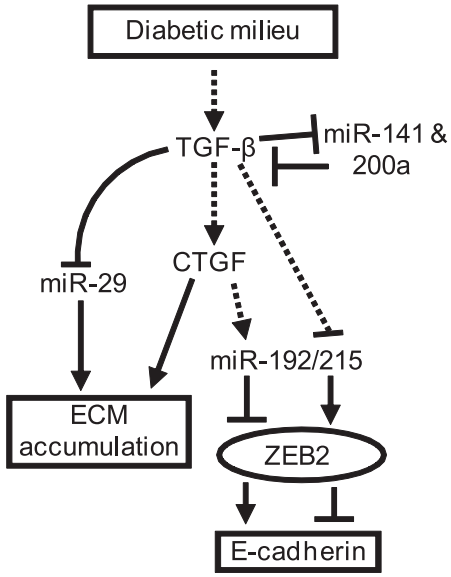

Figure 8. Schema of miRNAs and their potential role in renal fibrosis.

This work characterizes a novel axis in which the miR-29 family plays a pivotal role in regulating the effect of TGF-β1 on profibrotic genes in tubular epithelium-like cells and podocytes. The loss of expression of these miRNAs was demonstrated by treatment of cells with TGF-β1 and, importantly, in multiple models characterized by early or late-stage renal disease. Furthermore, we demonstrated that prevention of interstitial fibrosis restores the levels of these miRNAs, suggesting the important role that they play *in vivo* in regulating the pathogenesis that ensues ECM deposition, leading to loss of renal function. It remains to be explored as to how the balance of these novel molecules, which are clearly dysregulated in renal fibrosis, and the diabetic milieu further influence this complex situation.

CONCISE METHODS

Cell Culture

The rat kidney tubular epithelium-like cell line (NRK-52E) was use obtained from the American Type Culture Collection (Rockville, MD)

and maintained in DMEM as previously described.[11] For experimental treatments, serum was reduced to 2% and recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) was added. Human conditionally immortalized podocytes[15] were a gift (M. Saleem, Bristol Royal Hospital for Children, UK) and were cultured as previously described.[15] Podocytes were propagated and seeded at 33°C, and when cells were around 60% confluent they were transferred to 37.5°C for 10–14 days.[15] Under these conditions, the podocytes display the typical arborized pattern and express nephrin. For experimental treatments, serum was reduced to 2% and recombinant human TGF-β1 was added. High glucose conditions (25 mM) were used throughout.

RNA Extraction and Real-Time PCR

Gene expression was analyzed by real-time PCR (RT-PCR), performed as described previously [42] using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7500; Perkin-Elmer, Foster City, CA). Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7500 Sequence Detection System (Perkin-Elmer). To control for variation in the amount of DNA that was available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control 18S ribosomal RNA using an 18S ribosomal RNA TaqMan Control Reagent kit (ABI Prism 7500; Perkin-Elmer). Details of primers and TaqMan probes for most genes were previously reported.[11] Each experiment was conducted in six replicates. Results were expressed relative to control (untreated) cells, which were arbitrarily assigned a value of 1.

miRNA Assay

For miRNA analysis, cDNA synthesis real-time PCR was performed using TaqMan miRNA assays per the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Experimental groups were in replicates of six and normalized to RNU6B, Sno135, or U87, for rat, human, and mouse samples, respectively.

Transfection of miRNA Precursors and Inhibitors

NRK52E cells were seeded at 3×10^4 cells per well in 12-well plates. The next day, medium was replaced with OptiMEM (Invitrogen, Carlsbad, CA) and cells were transfected with pre-miRNAs (Applied Biosystems) using Oligofectamine (Invitrogen) at a final concentration of 100 nM. Pre-miRNA negative controls (miR-C) were used at the same concentration as the test reagent and cells were harvested 3 days post-transfection.

Collagen 3′UTR Luciferase Reporter Analyses

For transfection, NRK52E cells were seeded 1×10^5 cells per well in six-well plates the day before transfection. RL-reporter plasmids (0.5 μg/ml), the CMV-galactosidase construct, and miRNA were co-transfected using Lipofectamine 2000 (Invitrogen) in OptiMEM medium (Invitrogen). Cells were harvested 48 hours post-transfection using the luciferase reporter assay system (Promega, Madison, WI), with luciferase and galactosidase assays performed per the manufacturer's recommendations. All experiments were performed in triplicate with data pooled from at least two independent experiments. All 3′UTRs were isolated from rat genomic DNA by PCR with *Not*I and *Xba*I sites added before cloning into the pRL-reporter vector (Promega). The mutant 3′UTRs, synthesized (GenScript), were identical in sequence to the wild-type sequence except for the seed region in which the complementary sequence was used as previously described.[13]

Immunofluorescence

Cells were grown on coverslips, washed twice with PBS, fixed in 4% paraformaldehyde, permeabilized using 0.1% Triton X-100, and incubated in a blocking buffer (0.5% BSA in PBS, pH 7.4). Primary and secondary antibodies were diluted in blocking buffer and the cells with antibodies were incubated for 1–2 hours at room temperature. Coverslips were then mounted onto glass microscope slides using Prolong Gold antifade reagent (Invitrogen). Cells were viewed using epifluorescence on an Olympus BX61 microscope. The primary antibody was E-cadherin (1:200; Transduction Laboratories, Lexington, KY) and the secondary antibody was Alexa Fluor 488 goat rabbit-mouse (1:1000; Invitrogen).

In Vivo Studies

To explore the relationship between miR-29 and the development of fibrotic kidney disease, renal fibrogenesis was studied in three animal models of renal fibrosis, representing various stages of kidney disease. Early renal changes were examined in apoE knockout mice rendered diabetic by five daily intraperitoneal streptozotocin injections (STZ; Sigma Chemical Co, St. Louis, MO) injections as previously described[20] and compared with apoE KO mice that received vehicle (citrate buffer) alone (n=8 per group). We previously described that 10 weeks of diabetes in this particular mouse strain is associated with increased expression of matrix proteins in the kidney, particularly in the glomerulus.[12]

Male Sprague-Dawley rats subjected to right nephrectomy (UNx), followed after 2 weeks by intraperitoneal injection of STZ (55 mg/kg body wt) were utilized as the second model of progressive diabetic kidney disease. These rats display accelerated course of nephropathy with advanced glomerular and interstitial changes detectable 16–20 weeks after induction of diabetes.[21,36,43] Diabetic rats received daily evening injections of long-acting glargine insulin (Lantus; Eli Lilly, Indianapolis, IN) in doses individually adjusted to maintain BG approximately 300 mg/dl (17 mmol/L). BG levels were monitored at least weekly in all diabetic rats. In addition to measurements of miR profiles in advanced kidney disease, this model was also utilized for evaluation of possible effect of nephroprotective treatments on renal miR expression.

The findings in untreated UNx diabetic rats receiving vehicle (tap water) (n=7; D-VE) were compared with diabetic animals treated with 30 mg/kg per day of ROCK inhibitor fasudil (n=7; D-FA) (Calbiochem, San Diego, CA). This approach has demonstrated in the past decade an ability to exert strong protective effects in a variety of models of both nondiabetic and diabetic tubulointerstitial fibrosis.[36,37,44–47] Moreover, we have also studied a group of UNx diabetic rats treated with the ARB losartan (Merck, Whitehouse Station, NJ), 20 mg/kg per day[48] (n=7; D-LOS), as an established treatment of both clinical and experimental DN.[49,50]

Both drugs were administered in the drinking water. The rats were maintained on these treatments for 18 weeks. Age-matched,

uninephrectomized rats injected with a phosphate buffer and administered with vehicle were studied as nondiabetic controls (n=8; C-VE). At the end of the follow-up, the rats underwent measurements of systolic BP (tail plethysmography) and 24-hour urinary protein excretion in metabolic cages.[51] Within 2–3 days after the urinary collection the animals were anesthetized with inactin (100 mg/kg intraperitoneally) and blood samples were obtained for determination of BG and glycosylated hemoglobin. The left kidney was then harvested for further analyses, miRNA assay, Western blotting, and immunohistochemistry. Structural analysis of glomerulosclerosis and tubulointerstitial fibrosis scores was also performed (Supplemental Material). These studies were approved by the Portland Veterans Affairs Institutional Animal Care and Use Subcommittee.

In the final model, representing nondiabetic tubulointerstitial fibrosis, C57BL/6 mice were randomized to receive oral gavage with adenine (1 mg/kg per day) or vehicle (0.5% methylcellulose) for 4 weeks (n=4 per group). This results in marked tubulointerstitial fibrosis and nephron dropout, consistent histologically with changes seen in more advanced CKD.[13,52]

Western Blot Analysis

Whole-cell lysates were prepared from tissue-cultured cells and rat kidney tissue. Between 10–50 μg of protein was denatured, subjected to SDS-PAGE, and transferred onto polyvinylidene fluoride membrane by semidry transfer (Semi Dry Transfer Cell; BioRad). After transfer, all incubations were conducted on a rocking platform at room temperature. The membrane was blocked in 5% skim milk/TBST overnight, then incubated for 1 hour with collagen I antibodies and collagen IV antibodies (1:2000; Southern Biotech, Birmingham, AL). Collagen-1 antibody used for podocytes was from ABCAM (1:5000; Cambridge, MA). Membranes were washed with TBST and incubated with a peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Dako) for 1 hour in 5% skim milk/TBST. Immunoreactivity was detected using the Supersignal chemiluminescence kit (Sigma Aldrich) and the signal was captured and analyzed with the GelDoc XRS system (BioRad). To confirm equality of protein loading, all membranes were stripped and re-analyzed for tubulin or β-actin expression. Western blot analyses were performed at least in triplicate.

Rat kidney cortices were homogenized in lysis buffer containing protease and phosphatase inhibitors, and denatured proteins were electrophoresed and transferred to polyvinylidene fluoride as previously described.[43,53] After blocking, membranes were incubated overnight with collagen I (1:1500; Abcam, Cambridge, MA), fibronectin (1:2500; Sigma), and laminin (Santa Cruz) antibodies. Collagen I was analyzed under nonreducing conditions as recommended by the manufacturer. Immunodetection was accomplished using secondary antibodies conjugated with horseradish peroxidase (HRP) for 60 minutes (1:80,000; Pierce) in TBST containing 5% nonfat dry milk. Visualization was performed with an enhanced chemiluminescence Western blotting kit (Supersignal West Dura; Pierce, Rockford, IL). To confirm equality of loading, all membranes were stripped and re-analyzed for tubulin expression (Santa Cruz). Western blot analyses were performed at least in triplicate.

Immunohistochemistry

Four-micrometer paraffin kidney sections were used for immunohistochemical analyses as previously described.[54] Primary antibodies used were to collagen I and collagen IV (1:800, Southern Biotechnology, Birmingham, AL). Secondary antibodies were used as previously described.[54] Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Rat tissue samples were processed a little differently to that previously described.[53] In brief, the kidney sections were deparaffinized and were pretreated by steaming in 10% CITRA buffer (BioGenex, San Ramon, CA). After blocking, the slides were incubated overnight at 4°C with primary antibodies raised against collagens I and IV (1:100; Abcam, Cambridge, MA), fibronectin (1:100; Sigma), and laminin (1:50; Santa Cruz, Santa Cruz, CA), or with the same concentration of nonimmune mouse IgG as a control. Endogenous peroxidase activity was blocked with 3% H_2O_2 solution in methanol. The primary antibody was localized using the Vectastain ABC-Elite peroxidase detection system (Vector Laboratories, Burlingame, CA). This was followed by reaction with diaminobenzidine as chromogen and counterstaining with hematoxylin (Sigma). Sections of each diabetic kidney were processed in parallel with appropriate control tissue.

In Situ Hybridization

Six-micrometer paraffin sections were used to assess miR-29a and miR-29c expression in the kidneys of 10-week control and diabetic apoE KO mice. Double-labeled digoxigenin LNA-miR-29a and LNA-miR-29c probes were purchased (Exiqon), and the protocol used to detect endogenous miRNAs was the single-day *in situ* hybridization method (Exiqon)[55] per the manufacturer's recommendations. For negative control, a scrambled probe was used and U^ was used as a positive control probe (Exiqon).

Statistical Analyses

Values are shown as mean ± SEM unless otherwise specified. Statview software (Brainpower, Calabasas, CA) was used to analyze data by unpaired t test or by ANOVA and was compared using Fisher's protected least significant differences post hoc test. Nonparametric data were analyzed by the Mann–Whitney U test. $P<0.05$ was considered significant.

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DISCLOSURES

None.

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