

Loss of MicroRNA-192 Promotes Fibrogenesis in Diabetic Nephropathy

Aleksandra Krupa, Robert Jenkins, Dong Dong Luo, Aled Lewis, Aled Phillips, and Donald Fraser

Institute of Nephrology, School of Medicine, Cardiff University, Cardiff, United Kingdom

ABSTRACT

The role of microRNAs (miRs), which are endogenous RNA oligonucleotides that regulate gene expression, in diabetic nephropathy is unknown. Here, we performed expression profiling of cultured proximal tubular cells (PTCs) under high-glucose and control conditions. We identified expression of 103 of 328 microRNAs but did not observe glucose-induced changes in expression. Next, we performed miR expression profiling in pooled RNA from formalin-fixed, paraffin-embedded tissue from renal biopsies. We studied three groups of patients with established diabetic nephropathy and detected 103 of 365 miRs. Two miRs differed by more than two-fold between progressors and nonprogressors, and 12 miRs differed between late presenters and other biopsies. We noted the greatest change in miR-192 expression, which was significantly lower in late presenters. Furthermore, in individual biopsies, low expression of miR-192 correlated with tubulointerstitial fibrosis and low estimated GFR. *In vitro*, treatment of PTCs with TGF- β 1 decreased miR-192 expression. Overexpression of miR-192 suppressed expression of the E-Box repressors ZEB1 and ZEB2, thereby opposing TGF- β -mediated downregulation of E-cadherin. In summary, loss of miR-192 expression associates with increased fibrosis and decreased estimated GFR in diabetic nephropathy *in vivo*, perhaps by enhancing TGF- β -mediated downregulation of E-cadherin in PTCs.

J Am Soc Nephrol 21: 438–447, 2010. doi: 10.1681/ASN.2009050530

Tubulointerstitial fibrosis is recognized as a key determinant of progressive chronic kidney disease (CKD). Epithelial-to-mesenchymal transition (EMT) of proximal tubular epithelial cells (PTCs), driven largely by the cytokine TGF- β , is integral to this process. MicroRNAs (miRs) are small, endogenous posttranscriptional regulators of gene expression. Recent data link decreased expression of miR-200 family members to EMT in cancer^{1–3}; however, the role of miRs in EMT associated with nonmalignant disease such as renal fibrosis is less clear. Furthermore, although alterations in miR expression are a subject of intense study in cancer (reviewed by Garzon *et al.*⁴), variations in miR expression in nonmalignant disease are not well explored, in part because standard diagnostic techniques in nonmalignant disease yield RNA quantities that are challenging to use for hybridization array-based miR profiling.

The purpose of this study was to investigate the role of miRs in the pathogenesis of diabetic nephropathy. Initial array experiments suggested that elevated glucose did not induce changes in miR expression in PTCs *in vitro*. Subsequent experiments used a PCR-based array system (TaqMan Low Density Array; Applied Biosystems) to profile miR expression in pooled samples from formalin-fixed, paraffin-embedded (FFPE) renal biopsy tissue of patients with established diabetic nephropathy. The

Received May 21, 2009. Accepted November 10, 2009.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Donald Fraser, Institute of Nephrology, Cardiff University, Heath Park Campus, Cardiff, UK, CF14 4XN. Phone: +44-0-2920748449; Fax: +44-0-2920748470; E-mail: fraserdj@cf.ac.uk

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data showed widespread changes in miR expression in patients with fibrosis at the time of biopsy, most particularly decreased miR-192 expression in those presenting with advanced nephropathy. Subsequent experiments confirmed correlation of miR-192 expression with histopathologic and functional indicators of adverse renal prognosis. Additional *in vitro* data demonstrated downregulation of miR-192 expression in PTCs incubated with TGF- β , and that enforced expression of miR-192–opposed TGF- β –dependent EMT events, linking loss of miR-192 expression mechanistically to EMT in renal fibrosis.

RESULTS

Elevated glucose causes significant alterations in PTC gene expression⁵ and is implicated as an important causal stimulus in diabetic nephropathy. We performed initial experiments to examine whether glucose altered miR expression in PTCs. We cultured HK-2 cells in control or high-glucose medium for 48 hours before quantifying miRs by mirVana Array. We detected 103 of 328 human miRs examined (Figure 1A). For a complete listing of expression data, see supplemental data). No significant differences in miR expression were observed in cells cultured in high glucose compared with normal glucose (Figure 1). These data suggested that the PTC miR expression profile was not influenced by glucose in the short term. To determine whether changes in miR expression were associated with diabetic nephropathy in the longer term, we examined miR expression in renal biopsies from a cohort of patients with established diabetic nephropathy. These patient biopsies were previously characterized in terms of histopathologic indices of scarring.⁶ Three predetermined groups of patient samples were studied, separated on the basis of rate of loss of estimated GFR (eGFR) in follow-up. These were progressors (loss of eGFR >5 ml/min per yr), nonprogressors (<5 ml/min per yr), and late presenters (eGFR <15 ml/min at time of biopsy). Patient characteristics are presented in Table 1.

Pooled RNA from each group was subjected to TaqMan Low Density Array analysis (Applied Biosystems). A total of 103 of 365 miRs examined were present. miR expression pattern varied across all three groups, with the greatest differences evident in late presenters. Comparative miR expression in progressors *versus* nonprogressors is plotted in Figure 2A and in late presenters *versus* combined data for progressors and nonprogressors in Figure 2B. For full listings of expression data, see supplemental data.

Notably, expression of miR-192, identified as highly expressed in human kidney⁷ and as enriched in murine renal cortex,⁸ was higher in progressors and nonprogressors compared with late presenters (Figure 2B), suggesting loss of miR-192 expression in advanced diabetic nephropathy. miR-192 expression was quantified in 22 individual biopsy samples by quantitative reverse transcriptase–PCR (qRT-PCR), and lower expression in late presenters was confirmed (Figure 3A). Subsequently, we tested association between miR-192 expression and structural and functional clinical parameters. Signif-

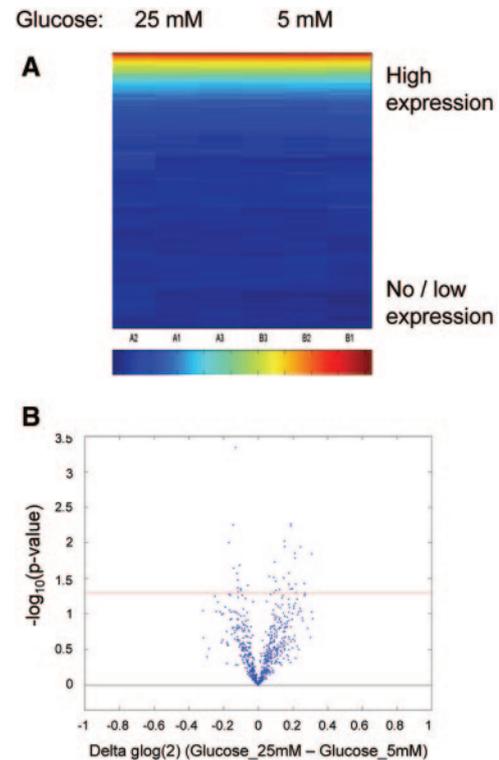


Figure 1. miR profiling in PTCs is shown. HK-2 cells were cultured in 25 mM glucose or control medium for 48 hours before RNA extraction and miR profiling using Ambion mirVana miRNA Bioarray. (A) Expression of 328 human miRs in HK-2 cells is represented on a heat map scale, with dark blue being low/no expression and red being maximum expression. Samples and miRs were grouped according to unsupervised Bicluster Analysis. (B) Volcano plot shows the relationship between change in miR expression between the two groups (25 mM glucose and control) and significance ($-\log_{10}$ of uncorrected P values). Horizontal red line represents $P = 0.05$ threshold.

icant correlations were observed between eGFR and miR-192 expression (Pearson correlation 0.768; $P < 0.001$; Figure 3C) and between fibrosis score and miR-192 expression (Pearson correlation -0.704 ; $P < 0.001$; Figure 3B).

The miR-200 family is extensively linked to EMT in cancer.^{1–3} To check the specificity of the changes observed in miR-192, we quantified miR-200b in the 22 individual biopsy samples by qRT-PCR. No significant variation was detected among progressors, nonprogressors, and late presenters (Figure 4A), and no correlation was seen between miR-200b expression and either fibrosis score (Figure 4B) or eGFR (Figure 4C).

To localize miR-192 expression within the kidney, we performed *in situ* hybridization for miR-192 using a double-digoxigenin–labeled locked nucleic acid probe. Hybridization signal was largely found overlying tubules and glomeruli, in keeping with expression of miR-192 in both glomerular and tubular cells. Consistent with the qRT-PCR data, very little signal was detected in areas of fibrosis (Figure 5). No signal was detected in control experiments performed without probe (data not shown).

Table 1. Patient characteristics

Patient Group	n	Gender	Age (Years)	eGFR	Change in eGFR (ml/min per yr)
Nonprogressors	9	1 F, 8 M	49.7 ± 3.8	46.2 ± 4.7	2.0 ± 0.5
Progressors	9	2 F, 7 M	54.8 ± 4.4	45.1 ± 10.1	9.4 ± 0.8
Late presenters	4	0 F, 4 M	56.3 ± 7.1	8.3 ± 0.9	NA

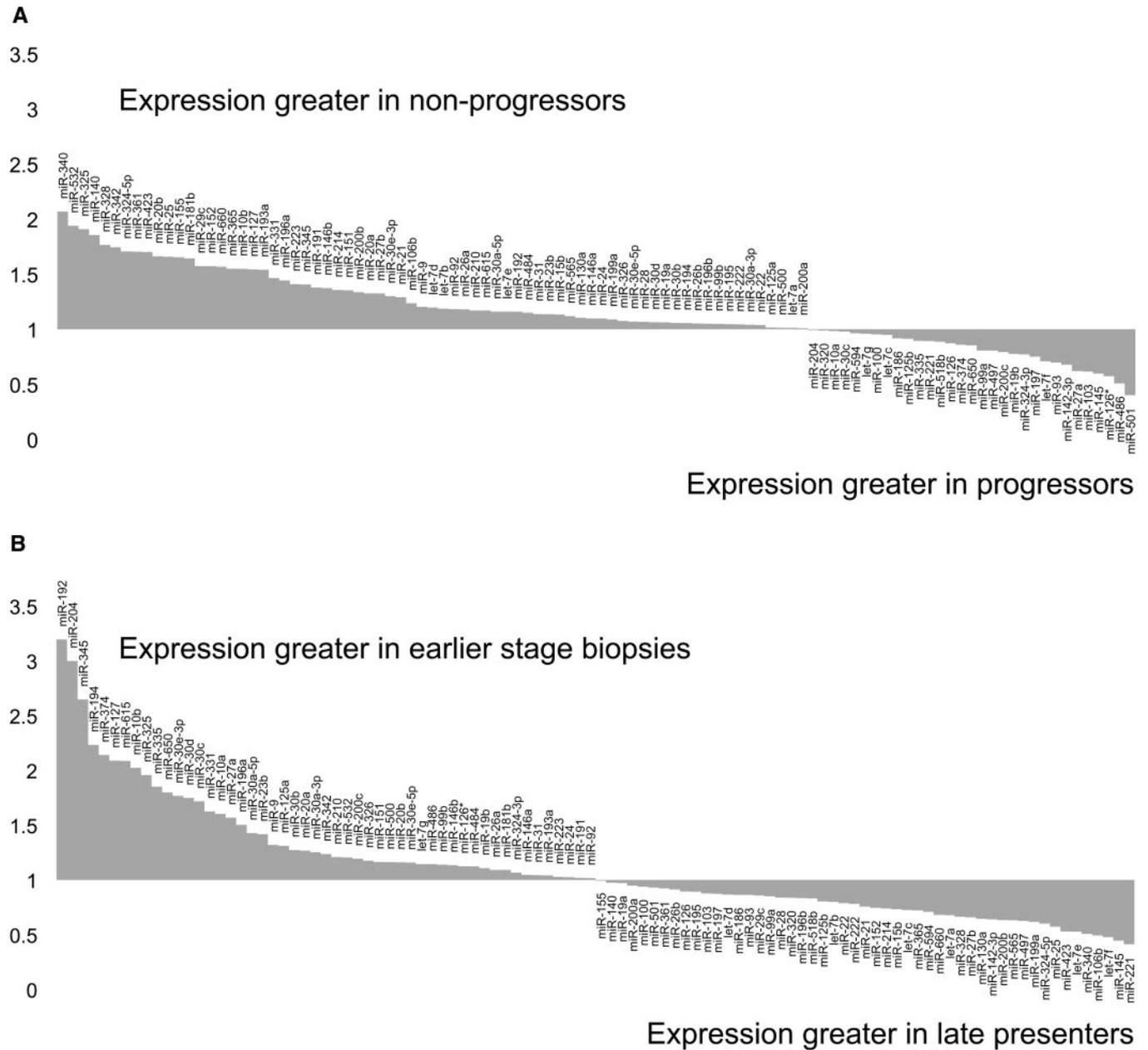


Figure 2. Taqman Low Density Array determination of miR expression in renal biopsy tissue from patients with diabetic nephropathy. Renal biopsies from patients with diabetic nephropathy were separated into three groups on the basis of progression after biopsy: Progressors (change in eGFR >5 ml/min per yr; n = 9), nonprogressors (change in eGFR <5 ml/min per yr; n = 9), and late presenters (eGFR ≤15 ml/min at biopsy; n = 4). After RNA extraction, expression of 365 miRNAs was quantified in pooled RNA for each of the groups using Taqman Low Density Array V1. Fold expression values are displayed for miRNAs expressed (threshold cycle <33). (A) Comparison of progressors versus nonprogressors. (B) Comparison of combined earlier stage biopsies (progressors+nonprogressors) versus late presenters.

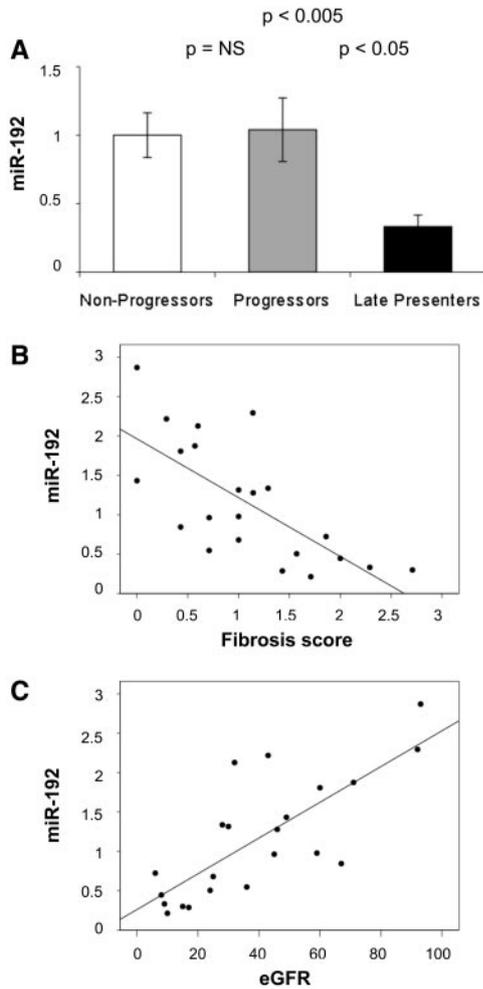


Figure 3. miR-192 expression in individual kidney biopsies and its correlation with severity of kidney disease. (A) miR-192 was examined in individual samples pooled for use in miR profiling (nine nonprogressors, nine progressors, and four late presenters) by qRT-PCR, using miR-16 as an endogenous control. Data are means \pm SEM. (B) Plot of miR-192 expression versus fibrosis score (Pearson coefficient -0.704 ; $P < 0.001$). (C) Plot of miR-192 expression versus four-variable MDRD eGFR (Pearson coefficient 0.768 ; $P < 0.001$).

These data indicate that miR-192 expression correlates inversely with loss of renal excretory function and with the presence of tubulointerstitial fibrosis in renal biopsy tissue from patients with established diabetic nephropathy. A key step in fibrogenesis in the kidney and, hence, in progression of CKD is EMT of tubular epithelial cells in response to TGF- β .⁹ ZEB1 (also known as δ EF1) and ZEB2 (also known as SIP1) are E-Box-binding proteins that repress E-cadherin transcription and hence abrogate E-cadherin-mediated intercellular adhesiveness. These actions of ZEB1 and ZEB2 are an important early stage of EMT,¹⁰ although it is important to acknowledge that EMT is not the only source of the renal myofibroblast in renal fibrosis, which may also originate from bone marrow, endothelial, and resident fibroblast precursor populations.

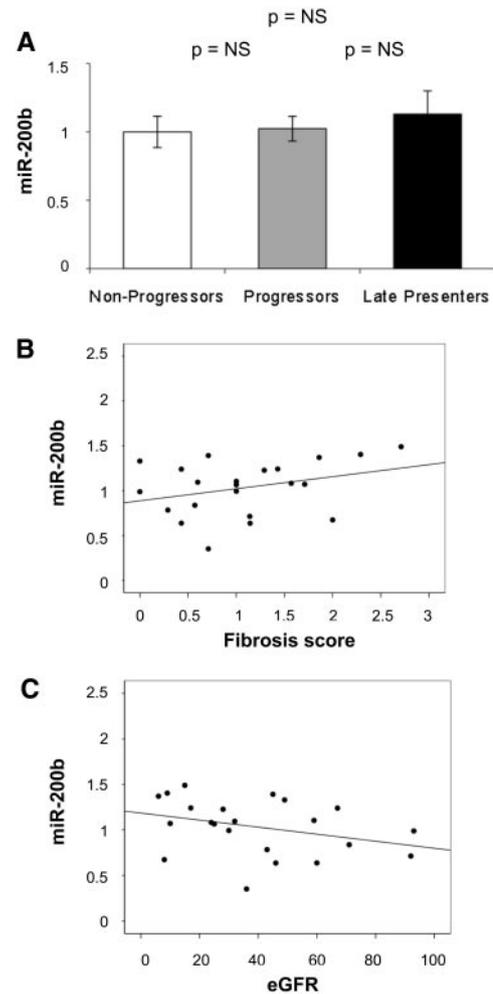


Figure 4. miR-200b expression in individual kidney biopsies and its correlation with severity of kidney disease are shown. (A) miR-200b was examined in individual samples pooled for use in miR profiling (nine nonprogressors, nine progressors, and 4 late presenters) by qRT-PCR, using miR-16 as an endogenous control. Data are means \pm SEM. (B) Plot of miR-200b expression versus fibrosis score (Pearson coefficient 0.321 ; $P = 0.146$). (C) Plot of miR-200b expression versus four-variable MDRD eGFR (Pearson coefficient -0.331 ; $P = 0.133$).

ZEB1¹¹ and ZEB2¹² are also validated targets for miR-192. To examine the role of TGF- β in decreased miR-192 expression in the fibrotic kidney and to examine whether decreased miR-192 expression was functionally related to development of renal fibrosis, we performed *in vitro* experiments using the PTC line HK-2. Incubation with 10 ng/ml TGF- β for time points to 96 hours led to induction of vimentin, plasminogen activator inhibitor 1 (PAI-1), and ZEB2 and to repression of E-cadherin expression (Figure 6, A through E and G through K). Subsequently, we examined the effect of TGF- β on PTC miR-192 expression. Ninety-six hours of incubation with 10 ng/ml TGF- β led to a 51% reduction in miR-192 (Figure 6, F and L).

Next, we studied the effect of enforced expression of miR-192 in PTCs. Transient transfection with miR-192 expression

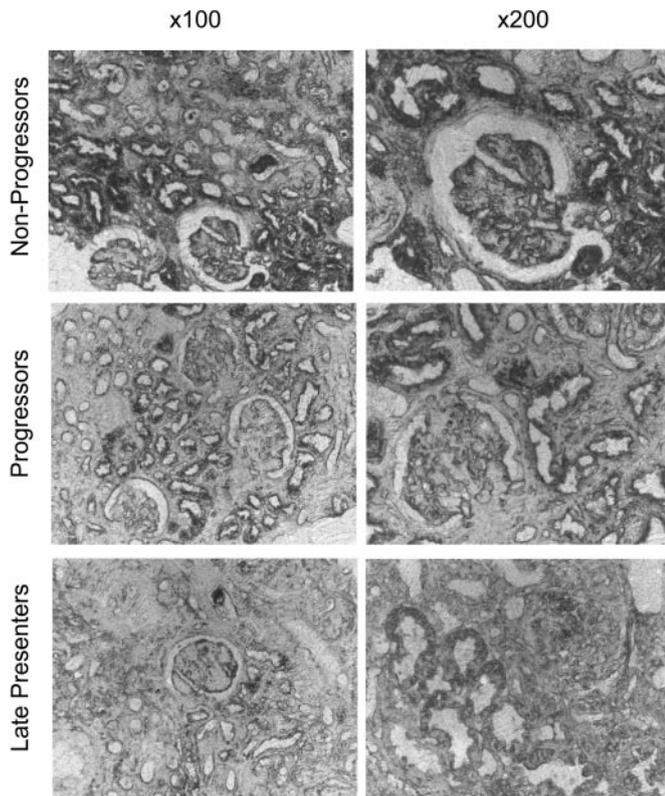


Figure 5. *In situ* hybridization for miR-192 is shown. Sections from two biopsies in each group (nonprogressors, progressors, and late presenters) were stained for miR-192 using a double-digoxigenin-labeled locked nucleic acid probe. No staining was seen in control experiments performed using secondary antibody in the absence of probe (data not shown). Sections were examined and photographed under phase contrast microscopy. Representative $\times 100$ and $\times 200$ images are shown.

construct did not induce detectable changes in PTC phenotype or TGF- β -dependent gene expression patterns (data not shown); therefore, HK-2-derived clonal cell lines exhibiting stable miR-192 overexpression were developed (Figure 7A). Enforced expression of miR-192 reduced basal ZEB1 and ZEB2 expression and opposed the induction of ZEB2 seen in cells incubated with 10 ng/ml TGF- β for 96 hours (Figure 7, B and C). Enforced miR-192 expression led to a 17-fold increase in E-cadherin expression (Figure 7D).

E-cadherin expression was decreased after incubation of miR-192-overexpressing cells with 10 ng/ml TGF- β for 96 hours but remained seven-fold higher than control values (Figure 7D). E-cadherin expression after enforced expression of miR-192 was also quantified by immunoblotting and by fluorescence microscopy. Increased E-cadherin expression and retention of E-cadherin expression after 96 hours of incubation with 10 ng/ml TGF- β was seen in clones overexpressing miR-192 (Figure 8). These data are consistent with targeting of the E-Box repressors ZEB1 and ZEB2 by miR-192 and hence a major role for miR-192 in defining E-cadherin expression in PTCs.

Finally, we examined the specificity of the effect of miR-192

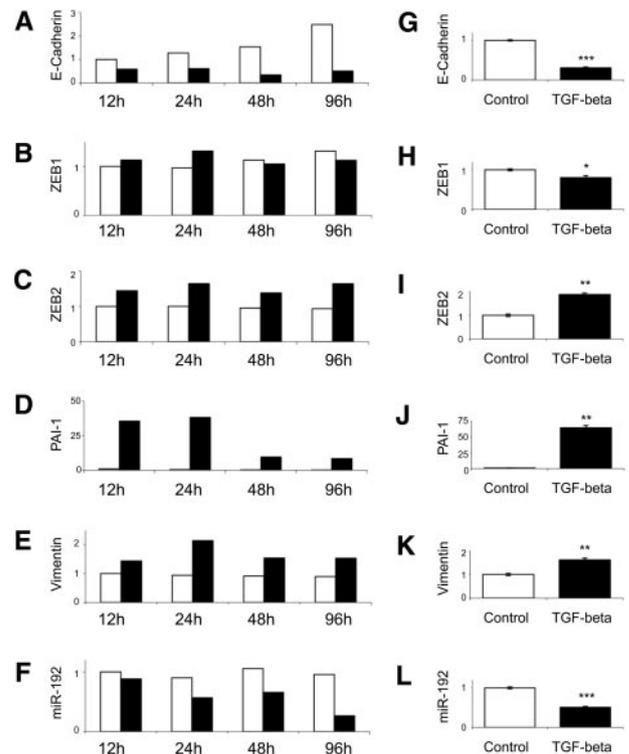


Figure 6. PTC responses to TGF- β were examined by qRT-PCR. (A through L) HK-2 cells were cultured without serum for 48 hours and then incubated with 10 ng/ml TGF- β (■) or control medium (□) for indicated time points (A through F) or 96 hours (G through L). Expression of E-cadherin (A and G), ZEB1 (B and H), ZEB2 (C and I), vimentin (D and J), PAI-1 (E and K), and miR-192 (F and L) was measured by qRT-PCR and normalized to GAPDH expression. miR-192 was normalized to miR-16. Error bars (G through L) represent SEM ($n = 3$). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

overexpression by studying other PTC responses to TGF- β . PAI-1 is induced by TGF- β *via* Smad-dependent transcriptional activation.¹³ Enforced expression of miR-192 did not alter basal or TGF- β -stimulated PAI-1 expression (Figure 9A). Vimentin expression is induced by TGF- β *via* Smad, AP1, and SP1 transcription factors.¹⁴ ZEB2 may also contribute to vimentin regulation in that siRNA-mediated ZEB2 knockdown represses basal vimentin expression *via* unknown mechanisms.¹⁵ Enforced expression of miR-192 led to decreased basal vimentin expression but did not inhibit induction of vimentin by TGF- β (Figure 9B). Other markers of EMT have been associated with the downstream effects of TGF- β , including HMGA2, Snail, Slug, and Twist. Notably, HMGA2 is induced by TGF- β and induces EMT *via* induction of transcriptional repressors of E-cadherin, including Snail, Slug, and Twist.¹⁶ Accordingly, expression of these genes was checked in cells exhibiting enforced expression of miR-192 or scrambled control sequence, after 96 hours of incubation with 10 ng/ml TGF- β or control medium. TGF- β induced expression of HMGA2, Snail, Slug, and Twist, which was not opposed by enforced expression of miR-192 (Figure 9, C through F). These

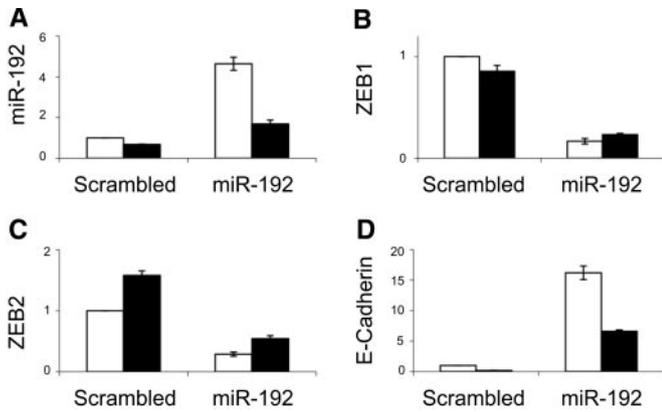


Figure 7. Altered E-cadherin and E-Box repressors expression in HK-2 cells overexpressing miR-192 by qRT-PCR is shown. Stable lines overexpressing miR-192 or a control short hairpin (Scrambled) were generated as described in the Concise Methods section. After 48 hours without serum, cells were incubated with TGF- β (■) or control medium (□) for 96 hours. (A through D) Subsequently, expression of miR-192 (A), ZEB1 (B), ZEB2 (C), and E-cadherin (D) was examined by qRT-PCR. miR-192 was normalized to miR-16, and the other genes were normalized to GAPDH. The experiment was performed for the control line and three independent cell lines overexpressing miR-192, all three giving similar results (results from one line are shown). Error bars represent SEM ($n = 3$).

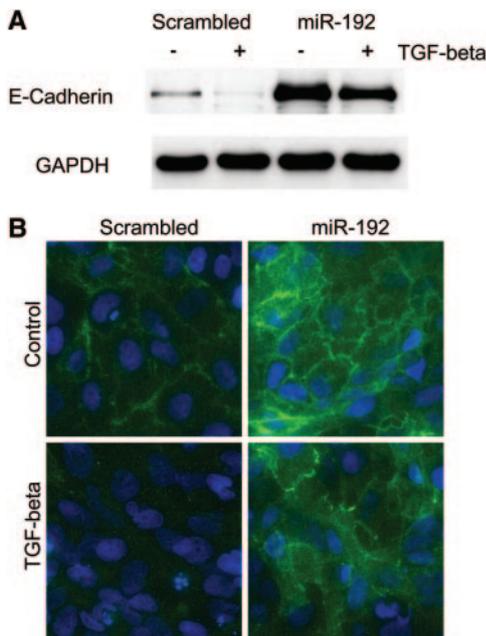


Figure 8. Altered E-cadherin expression in cells overexpressing miR-192 by immunoblot and fluorescence microscopy is shown. Stable lines overexpressing miR-192 or a control short hairpin (Scrambled) were growth-arrested in serum-free medium for 48 hours before incubation with 10 ng/ml TGF- β (■) or control medium (□) for 96 hours. (A and B) Then E-cadherin protein was examined by Western blot (A) or fluorescence microscopy (B). The representative results of at least three independent experiments are shown.

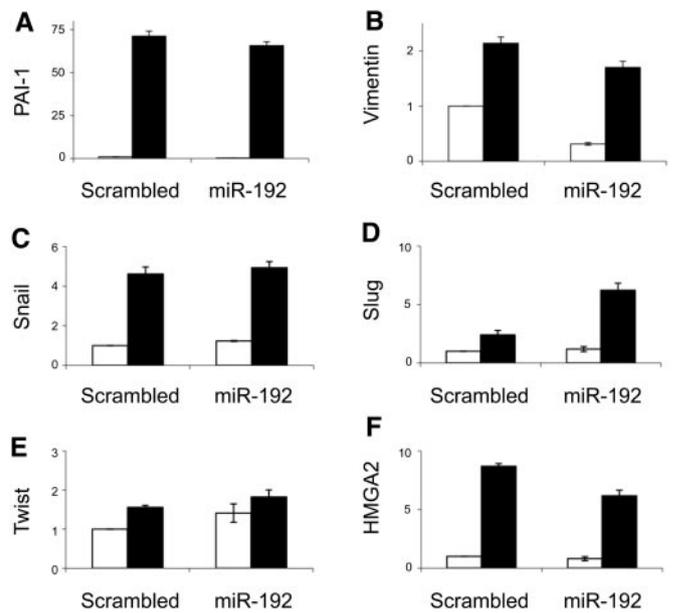


Figure 9. Expression of other EMT markers in HK-2 cells overexpressing miR-192 by qRT-PCR is shown. Stable lines overexpressing miR-192 or a control short hairpin (Scrambled) were growth-arrested in serum-free medium for 48 hours before incubation with 10 ng/ml TGF- β (■) or control medium (□) for 96 hours. (A through F) Expression of PAI-1 (A), vimentin (B), Snail (C), Slug (D), Twist (E), and HMGA2 (F) was examined by qRT-PCR. The expression was normalized to GAPDH. Error bars represent SEM ($n = 3$).

data support the specific effect of miR-192 in maintaining E-cadherin expression *via* repression of ZEB1 and ZEB2.

DISCUSSION

The major findings of this study are that PTC miR-192 expression is decreased in response to TGF- β and that loss of miR-192 correlates with tubulointerstitial fibrosis and reduction in eGFR in renal biopsies from patients with established diabetic nephropathy. Initial array studies suggested that, *in vitro*, PTC miR expression profile is highly stable and is not influenced by glucose alone in the short term. Subsequent PCR-based array examination of miR expression in pooled kidney biopsy samples categorized miR expression in patients with established nephropathy. Multiple differences in miR expression were detected between patients who progressively lost renal function after biopsy and those who did not and between those with advanced disease at the time of biopsy and those with earlier stage disease. These data identify miRs for further study and suggest that miR quantification may provide useful prognostic information for patients with diabetic nephropathy. In keeping with this, subsequent quantification of miR-192 in individual biopsy samples demonstrated correlation of loss of miR-192 expression with decreased eGFR and with histopathologic evidence of tubulointerstitial fibrosis on biopsy.

Subsequent *in vitro* work was undertaken to investigate causal links between loss of miR-192 expression and development of tubulointerstitial fibrosis. TGF- β 1 is an ubiquitous cytokine that functions in an autocrine or paracrine manner to elicit a multiplicity of effects, principally related to cell growth and extracellular matrix accumulation. It is widely implicated in the pathogenesis of renal fibrosis in human and experimental disease.^{17–20} TGF- β is postulated to be a major biologic signal that regulates the switch in tubular cell activities toward a profibrogenic phenotype.²¹ In this study, TGF- β was shown to decrease miR-192 expression in PTCs. Interestingly, all clonal cell lines studied retained miR-192 downregulation after incubation with TGF- β , despite replacement of the miR-192 promoter with a constitutively active promoter in the overexpression vector. This suggests that TGF- β acts posttranscriptionally to regulate miR-192 expression. TGF- β was recently shown to alter posttranscriptional processing of miR-21 *via* interaction of Smad proteins with Drosha, a core component of the nuclear miR processing complex.²² It is not known how widespread posttranscriptional regulation of specific miRNAs is by TGF- β .

In tubular epithelial cells, repression of E-cadherin is an early event that precedes other alterations during EMT.²³ ZEB1 and ZEB2 are key transcriptional regulators of TGF- β -mediated suppression of E-cadherin.²⁴ Previous reports established a role for miR in maintenance of an epithelial phenotype *via* targeting of ZEB1 and ZEB2. Several reports established ZEB1 and ZEB2 as targets of miRNAs of the miR-200 family and showed that expression of miR-200 family miRNAs is associated with maintenance of an epithelial phenotype in cancer-derived cell lines.^{1–3,25} ZEB1¹¹ and ZEB2¹² are also targets of miR-192. Notably, miR-192 is enriched in the normal renal cortex⁸ and highly expressed in the kidney,⁷ whereas members of the miR-200 family are not. In this study, we show that miR-192 targets ZEB1 and ZEB2 in PTCs and that enforced expression of miR-192 leads to enhanced E-cadherin expression. These data suggest that miR-192 plays a major role in defining E-cadherin expression in PTCs and that TGF- β -dependent loss of miR-192 expression is an important contributor to alterations in PTC phenotype in renal tubulointerstitial fibrosis. It is important to consider, however, that ZEB1 and ZEB2 are not exclusively relevant for E-cadherin but regulate other genes, for example, collagen I in fibroblasts and mesangial cells. Intriguingly, ZEB2 has also been identified as a target of miR-192 in mesangial cells¹²; however, in the work of Kato *et al.*,¹² miR-192 expression was increased by serum or TGF- β in murine mesangial cells and in the glomeruli of diabetic mice. This suggests that mesangial cell and PTC miR expression profiles may exhibit divergent responses to profibrotic stimuli such as TGF- β and that the mechanisms underlying this may be a fruitful area for further study.

Extensive characterization of miR expression in malignancy shows that miR profiling has promise as a clinical tool for diagnostic and prognostic evaluation. To date, however, miR profiling with established array-based techniques has required

RNA quantities that preclude analysis of most nonsurgically obtained samples. In this article, we examined miR expression in renal biopsies from patients with diabetic nephropathy. We found that miR expression profiling was possible using PCR-based techniques in FFPE tissue from renal biopsy samples collected in a standard manner for routine evaluation. This approach could usefully be widely applied within nephrology, in which array studies may thus far have been limited in comparison with the extensive data available using cancer samples, more readily available after surgical resection of tumors. Beyond the advantages of studying tissue from patients rather than animal models, a key advantage of renal biopsy tissue over nephrectomy specimens is the relatively rapid fixation, given the substantial changes in miR expression that occur after a prolonged period of tissue hypoxia,²⁶ a potential confounding element in studies based on specimens obtained at surgical nephrectomy. Recent TaqMan Low Density Array data showing variations in miR expression profiles in biopsies from human renal allografts undergoing acute rejection²⁷ also support the potential widespread importance of miRNAs as biomarkers in renal disease.

CONCISE METHODS

Cell culture

Human PTC line HK-2 was obtained from ATCC (LGC Standards, Teddington, UK). The cells were maintained in DMEM (Life Technologies/Invitrogen, Paisley, UK)/Ham's F12 (Sigma, Poole, UK) supplemented with 10% FCS (Biosera, Ringmer, UK), hydrocortisone, insulin, transferrin, and sodium selenite (Sigma) as described previously.²⁸ Cells were grown to confluence and were serum deprived for 48 hours before stimulation with 25 mM glucose or 10 ng/ml recombinant TGF- β (R&D Systems, Abingdon, UK) under serum-free conditions.

mirVana Arrays

Expression of 328 human miRNAs was examined in HK-2 cells (control or exposed to 25 mM glucose for 48 hours) by Asuragen (Austin, TX), using Ambion mirVana miRNA Bioarray V2 as described previously.²⁹

Tissue Samples

FFPE kidney specimens of 22 patients with diabetic nephropathy, previously characterized by Lewis *et al.*,⁶ were examined. Renal function was defined as the eGFR calculated using the four-variable Modification of Diet in Renal Disease (MDRD) Study equation, and the stage of renal disease was derived from a serum creatinine value measured at the time of renal biopsy (National Kidney Foundation, 2002). Patients who had stage 5 CKD or required renal replacement therapy within 6 months of their renal biopsy were considered late presenters. Patients who had deterioration of ≥ 5 ml/min per yr in their eGFR (which also equated to a $>10\%$ change in the starting GFR) were termed progressors, and those who did not satisfy these criteria were termed nonprogress-

sors. Fibrosis scores were determined in the previous study as reported⁶ using the grading system of Shih *et al.*³⁰

miR Profiling Using TaqMan Low Density Arrays

RNA was extracted from FFPE samples using RecoverAll Total RNA Isolation Kit (Applied Biosystems, Warrington, UK), and RNA quality was assessed with RNA 6000 Pico Chip kit (Agilent Technologies, Wokingham, UK). For each group of patients (nonprogressors, progressors, and late presenters), 25 ng of pooled total RNA was used for multiplex reverse transcription reactions with miR-specific RT primers (Human Multiplex RT Set: Pools 1 to 8, 1.0; Applied Biosystems). Subsequently, expression of 365 miRs was tested by quantitative PCR using TaqMan Low Density Array Human MicroRNA Panel 1.0 (Applied Biosystems) run on an Applied Biosystems 7900HT instrument with detection limit set to Ct = 33, according to the manufacturer's instructions. The data presented are normalized to miR-16, identified as a suitable endogenous control using the geNorm VBA applet for Microsoft Excel.³¹

Generation of HK-2 miR-192 Stable Cell Lines

Plasmids for expression of miR-192 precursor or control short hairpin were prepared by insertion of annealed oligonucleotides into psiSTRIKE U6-Puromycin vector (Promega, Southampton, UK). HK-2 cells were transfected with those plasmids using Lipofectamine LTX Plus (Invitrogen), and stably transfected cells were selected using Puromycin (InvivoGen/Autogen Bioclear, Calne, UK). Three single lines with highest miR-192 expression were used in experiments. Oligonucleotides inserted to siSTRIKE Vector were as follows:

miR192 forward ACCGTGCACAGGGCTCTGACCTATGAA-TTGACAGCCAGTGCTCTCGTCTCCCCTCTGGCTGCCAAT-TCCATAGGTCACAGGTATGTTGCGCCTTTTTC and miR192 reverse TGCAGAAAAAGGCGAACATACCTGTGACCTATGG-AATTGGCAGCCAGAGGGGAGACGAGAGCACTGGCTGTCAA-TTCATAGGTCAGAGCCCTGTGCA and Scrambled forward ACCGCGTATCGTAAGCAGTACTAAGTTCTCTAGTACTGCTTACGATACGCTTTTTC and Scrambled reverse TGCAG-AAAAAGCGTATCGTAAGCAGTACTA-GAGAACTTAGTACTGCTTACGATACG.

Quantitative RT-PCR

miR-192 was quantified using a TaqMan MicroRNA Assay (Applied Biosystems). miR-16-specific stem-loop RT primer and qPCR primers were designed as described previously³² and used with MicroRNA Reverse Transcription Kit and Power SYBR Green Master Mix (Applied Biosystems). Before use in experiments, the primer set was analyzed for linear detection and efficiency approximately 100% within the range of 2 to 250 ng of HK-2 cell total RNA input, and single qPCR product was confirmed by melting curve analysis. For mRNA quantification, reverse transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was performed. The cDNA was then used in gene-specific qPCR with Power SYBR Green Master Mix and primers listed in Table 1. Relative quantities were calculated using the $2^{-\Delta\Delta Ct}$ method.³³ miR-192 expression was normalized to miR-16, and mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Initial analysis of multiple endogenous

controls established uniformity of expression and confirmed that the selected controls did not influence the results (U6 snRNA for small RNAs; TATA-box binding protein, β 2-microglobulin, human large ribosomal protein RPLP0, and HPRT1 for mRNA).

Primers for miR Detection

Primers for miR detection were as follows: miR-16RT ATCAAG-CGTCGCAGGAACATCACCCACATGTTACGATTGATCGCCA, miR-16 PCR forward TAGCAGCACGTAATATTGG, and miR-16 PCR reverse AGCGTCGCAGGAACATCA

Primers for mRNA Detection

Primers for mRNA detection were as follows: GAPDH forward CCTCTGACTTCAACAGCGACAC and GAPDH reverse TGTCAT-ACCAGGAAATGAGCTTGA, E-cadherin forward TCCCAATACATCTCCCTTCACA and E-cadherin reverse ACCCACCTCTAAG-GCCATCTTT, ZEB1 forward CAGGCAGATGAAGCAGGATGT and ZEB1 reverse TGACAGCAGTGTCTTGTGTGTAG, ZEB2 forward CCTTCTGCGACATAAATACGAACA and ZEB2 reverse AGTACGAGCCCGAGTGTGAGA, vimentin forward GGAGAAAT-TGCAGGAGGAGATG and vimentin reverse AAGGTCAAGACGT-GCCAGAGA, and PAI-1 forward TCTCTGCCCTACCAACATTC and PAI-1 reverse CGGTCATTCCCAGGTTCTCT.

miR-192 In Situ Hybridization

In situ hybridization for miR-192 was performed using double-digoxigenin-labeled LNA miRCURY probes (Exiqon, Vedbaek, Denmark) as described previously.³⁴ Briefly, 5- μ sections of FFPE kidney biopsy tissue were deparaffinized by sequential washes with xylene, 100 to 25% ethanol, and PBS. The sections were deproteinized with proteinase K and fixed with 4% paraformaldehyde. Prehybridization was performed with hybridization buffer (50% formamide, 5 \times SSC, 0.1% Tween 20, 50 μ g/ml heparin, and 500 μ g/ml yeast tRNA [pH 6]). Hybridization was performed with a probe concentration of 20 nM in hybridization buffer for 12 hours at 52°C. The sections were washed with 2 \times SSC, then multiple washes in 50% Formamide, 2 \times SSC, and finally PBS and 0.1% Tween 20. The sections were blocked with blocking buffer (2% sheep serum, 2 mg/ml BSA in PBS, and 0.1% Tween 20) and then incubated with anti-digoxigenin-alkaline phosphatase, Fab fragment (Roche, Mannheim, Germany) in blocking buffer for 12 hours. The sections were washed repeatedly in PBS and 0.1% Tween 20, and detection was performed using 1-Step NBT/BCIP plus Suppressor (Pierce) according to manufacturer's instructions. The sections were mounted in glycerol and viewed under phase contrast microscopy.

Immunoblotting

Cell extracts were prepared with RIPA Lysis Buffer (Santa Cruz Biotechnology, Heidelberg, Germany), and 15 μ g was fractionated on a 7.5% SDS polyacrylamide gel. After transfer to a nitrocellulose membrane, probing was carried out with anti-E-cadherin antibody (H-108, 1:1000; Santa Cruz Biotechnology). To ensure equal protein load in each lane, after visualization of E-cadherin by ECL method (GE Healthcare, Amersham, UK), membranes were stripped and reprobed with anti-GAPDH antibody (ab9485, 1:1000; Abcam, Cambridge, UK).

Immunofluorescence

After stimulation with TGF- β , cells on glass Lab-Tek Chamber Slides (Nunc/VWR Int., Leics, UK) were fixed with 3.7% PFA for 10 min. E-cadherin was probed with a murine mAb (HECD-1, 1:100; Abcam) and visualized by Alexa Fluor 555–conjugated anti-mouse secondary antibody (1:1000; Molecular Probes/Invitrogen, Paisley, UK). Hoechst dye (Sigma) was subsequently used to stain nuclei.

Statistical Analysis

Pearson correlation coefficients were calculated for fibrosis score or eGFR and miR-192 in kidney biopsies from patients with diabetic nephropathy using SPSS 14.0 software. Statistical evaluation was otherwise by *t* test, using Microsoft Excel, with *P* < 0.05 assumed to be significant.

ACKNOWLEDGMENTS

D.F. is funded by a clinician scientist award from the Welsh Office of Research Development. R.J. is funded by Kidney Research UK.

DISCLOSURES

None.

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