Association of Elevated Urinary miR-126, miR-155 and miR-29b with Diabetic Kidney Disease

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ABSTRACT

Effective diabetic kidney disease (DKD) biomarkers remain elusive, and urinary microRNAs (miRNAs) represent a potential source of novel non-invasive disease sentinels. We profiled 754 miRNAs in pooled urine samples from DKD patients (n = 20), detecting significantly increased miR-126, miR-155 and miR-29b compared to controls (n = 20). These results were confirmed in an independent cohort of 89 DKD patients, 62 diabetic patients without DKD and 41 controls: miR-126 (2.8-fold increase; $p<0.0001$), miR-155 (1.8-fold; $p<0.001$) and miR-29b (4.6-fold; $p = 0.024$). Combined receiver operating characteristic curve analysis resulted in an area under the curve of 0.8. A relative quantification threshold equivalent to 80% sensitivity for each miRNA gave a positive signal for 48% of DKD patients compared to 3.6% of diabetic patients without DKD. Laser capture microdissection of renal biopsies followed by RT-qPCR detected miR-155 in glomeruli, proximal and distal tubules, while miR-126 and miR-29b were most abundant in glomerular extracts. Subsequent experiments showed miR-126 and miR-29b enrichment in glomerular endothelial cells (GEnCs) compared to podocytes, proximal tubular epithelial cells and fibroblasts. Significantly increased miR-126 and miR-29b were detected in GEnC conditioned medium in response to tumour necrosis factor-alpha and transforming growth factor-beta 1, respectively. Our data reveal an altered urinary miRNA profile associated with DKD and link these variations to miRNA release from GEnCs.

Keywords: microRNAs, urine, biomarker, diabetic kidney disease, chronic kidney disease

Abbreviations:

ACR	albumin:creatinine ratio
CKD	chronic kidney disease
DKD	diabetic kidney disease
eGFR	estimated glomerular filtration rate
GEnC	glomerular endothelial cell
KDIGO	kidney disease: improving global outcomes
NKF KDOQI	National Kidney Foundation kidney disease outcomes quality initiative
LCM	laser capture microdissection
MDRD	modification of diet in renal disease
miRNA	microRNA
PTC	proximal tubular epithelial cell
RIISC	renal impairment in secondary care study

Introduction

Recent estimates suggest that 1 in 12 of the global population suffers from diabetes mellitus, approximately 40 % of those affected will go on to develop diabetic kidney disease (DKD).[1] DKD is the leading cause of end-stage renal disease and predisposing factors include genetic causes, ethnicity, hyperglycaemia, insulin resistance, intraglomerular hypertension and hyperfiltration.[2,3]

Hyperglycaemia results in numerous deleterious consequences including upregulated cytokine synthesis, renin-angiotensin system activation, generation of advanced glycation end products and reactive oxygen species, and increased protein kinase C activity.[4,5] Nitric oxide and NF-κB pathway-driven loss of endothelial and vascular modulation have been implicated in insulin resistance, and early DKD may be associated with insulin signaling defects specific to the podocyte.[6] These insults result in loss of glomerular filtration rate and ultimately to renal failure from mesangial hyperexpansion, nodular glomerulosclerosis and tubulointerstitial fibrosis.[7]

Detection of urinary microalbuminuria currently forms the basis of DKD progression monitoring, varying from normal mean albuminuria values around 10 mg/day to a diagnosis of microalbuminuria at 30–300 mg/day and macroalbuminuria above 300 mg/day.[8] Prognosis is complicated, since not all microalbuminuric patients progress to overt nephropathy. A number of novel biomarkers have been assessed for utility in DKD but none are being used as routine clinical markers, and they may lack specificity and sensitivity to predict individual DKD patient outcomes. In light of the above, novel markers that can discriminate aetiology, progression and/or response to treatment remain highly desirable.

MicroRNAs (miRNAs) are ubiquitously-expressed short noncoding RNAs that regulate the expression of most protein coding genes in the human genome, and detection of miR-192, miR-194, miR-215, miR-216, miR-146a, miR-204 and miR-886 is elevated in the kidney.[9] Urinary miRNAs represent a highly promising novel source of non-invasive biomarkers that are stabilised via argonaute 2 protein/exosome association and are rapidly and precisely detected by RT-qPCR.[10]

Reports have suggested a role for miRNAs in the pathology of DKD,[11,12] including previous work from this laboratory showing decreased miR-192 in biopsies from late-stage DKD patients with diminished renal function.[13] However, comparatively little is known about the abundance of urinary miRNAs in DKD patients.

We hypothesised that alterations in urinary miRNA profiles would be associated with DKD. We identified candidate DKD biomarkers by comparing miRNA profiles in urine samples from a patient discovery cohort with those from unaffected controls. Selected candidates were then measured in a larger, independent cohort. Subsequently, laser capture microdissection of renal biopsies and *in vitro* cell culture were used to investigate the sources of our candidate urinary miRNA DKD biomarkers with respect to nephron domain and cell type.

Materials and Methods

Study Participants

DKD was defined in accordance with the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease (CKD).[14] Accordingly, CKD should be attributable to diabetes in the presence of macroalbuminuria (in the absence of urinary infection), or in the presence of microalbuminuria with concomitant diabetic retinopathy, or in type 1 diabetes of at least 10 years duration.[14] The initial profiling study cohort of 20 DKD patients and 20 healthy controls was obtained from the Wales Kidney Research Tissue Bank, University Hospital of Wales, Cardiff. The DKD group was predominantly male (85%), mean age 72 years (SD +/- 8.7). DKD patients were CKD stage 3-5 (pre-dialysis), with mean eGFR of 29 ml/min/1.73m^2 (SD +/- 8.5) and a mean urinary Albumin:Creatinine ratio (ACR) of 13.5 mg/mmol (SD +/- 14.5). The control group (n = 20) in the profiling cohort were 50% male, mean age 47 years (SD +/- 11.0) with no microalbuminuria (ACR<3 mg/mmol). For further details on ACR categories see Table 1.

The confirmation cohort was drawn from two secondary care facilities: the Wales Kidney Research Tissue Bank (as above) and the Renal Impairment In Secondary Care (RIISC) study, University Hospital of Birmingham, UK.[15] 89 patients with DKD, including 3 patients with type 1 diabetes, and 41 healthy controls were recruited across the two sites. An additional control group of 62 diabetics without DKD were recruited from Cardiff, including 17 patients with type 1 diabetes. Ethical approval

106 was granted by the Wales Kidney Research Tissue Bank Governance Committee and
107 the South Birmingham Local Research Ethics Committee, respectively.
108
109 Patient demographics and clinical parameters are shown in Table 1. All patients
110 were recruited from specialist nephrology and diabetes care services at the two sites
111 during the period spanning autumn 2010 to autumn 2013. DKD patients from the
112 RIISC study cohort were predominantly advanced nephropaths as per RIISC protocol
113 inclusion criteria: briefly, patients with CKD stages 4-5 (pre-dialysis), or CKD stage 3
114 and accelerated progression and/or proteinuria as defined by the UK National
115 Institute for Health and Care Excellence 2008 CKD guideline for secondary care
116 review. The diabetic patient control group all had a diagnosis of diabetes by standard
117 American Diabetes Association criteria,[16] but without evidence of DKD (i.e. not
118 fulfilling the KDOQI criteria).
119
120 At initial clinic visit, renal function was recorded using estimated glomerular
121 filtration rate (eGFR), calculated using the modification of diet in renal disease
122 (MDRD) equation[17]. Urine samples were aliquoted for albumin:creatinine ratio (ACR)
123 assessment and for RNA extraction (see below). ACR cut-offs for disease severity
124 were defined as per Kidney Disease: Improving Global Outcomes (KDIGO) 2012
125 guidelines.[18]
126
127 **Urine Collection, RNA Isolation and RT-qPCR analysis**
128 Urine samples were collected and RNA extraction from 350 µl of urine, generation of
129 cDNA from equal volumes of RNA extracts and RT-qPCR were then carried out as

described in detail elsewhere.[10] TaqMan assays (Thermo Fisher Scientific, Paisley, UK) used in this study were: hsa-miR-29b-3p (ID 000413); hsa-miR-126-3p (ID 002228); hsa-miR-155-5p (ID 002623); hsa-miR-191-5p (ID 002299). Relative quantities were calculated using the $2^{-\Delta\Delta Ct}$ method, and miRNA expression was normalized to hsa-miR-191-5p.[10]

MiRNA profiling by TaqManArray Human MicroRNA Cards

Urinary miRNAs were reverse transcribed using the Megaplex Primer Pools (Human Pools A v2.1 and B v3.0, Thermo Fisher Scientific) with a predefined pool of 381 reverse transcription (RT) primers for each Megaplex Primer Pool. A fixed volume of 3 µl of RNA solution was used as input in each RT reaction, and RT reactions were performed according to the manufacturer's recommendations. RT reaction products were amplified using Megaplex PreAmp Primers (Primers A v2.1 and B v3.0, Thermo Fisher Scientific), the samples were then diluted to a final volume of 100 µl and control subject and DKD patient products were pooled as follows.

To exclude the possibility that gender, age and eGFR status had extreme effects on miRNA expression profiles, the following pooling strategy was followed. Control pool (CP)1: urine samples from 5 females of average age 44.8 years; CP2: 5 females, 57.6; CP3: 5 males, 35.2; CP4, 5 males, 53.2. Patient Pool (PP)1: urine samples from 5 CKD3 patients with an eGFR between 43.3 and 36 mL/min per 1.73m^2; PP2: 5 stage 3 patients, 35 - 31; PP3: 5 stage 4/5 patients, 27.3 - 23; PP4: 5 stage 4/5 patients, 22 - 12.9.

TaqManArray Human MicroRNA Cards A v2.1 and B v3.0 (Thermo Fisher Scientific) were used to quantify 754 human miRNAs. Each array included 377 test miRNAs, 3 endogenous controls and a negative control. Quantitative (q)PCR was carried out on an Applied Biosystems 7900HT thermo cycler (Thermo Fisher Scientific) using the manufacturer's recommended program.

Laser Capture Microdissection (LCM) from Renal Biopsy Samples

Glomeruli, proximal tubular and distal tubular profiles were microdissected from 6-µm sections obtained from five FFPE archived renal biopsies from unaffected people using the Arcturus Pixcell IIe infrared laser enabled LCM system (Thermo Fisher Scientific).

Cell Culture

Human conditionally immortalised glomerular endothelial cell (GEnC) and human podocyte (ATC) cell lines were propagated at 33°C as described previously.[19,20] After 5 (GEnC) and 14 (ATC) days, cells were transferred to 37°C incubation to inactivate the SV40 T antigen and permit differentiation, prior to experimental use. Where stated, GEnCs were growth arrested for 24 h and then treated with TNF-α (10 ng/mL) or TGF-β1 (1 ng/mL) at either normoglycaemic (5 mM) or hyperglycaemic (25 mM) D-glucose concentrations for 24 h. Proximal tubular epithelial cell (PTC) line HK-2[21] and fibroblast[22] cultures were maintained as described elsewhere. Cells and culture medium obtained from each well were used for RNA extraction as described above.

178 **Statistical analysis**

179 MiRNA profiling data were analysed using Thermo Fisher Scientific's DataAssist
180 Software (version 3.01), NormFinder Software (http://moma.dk/normfinder-
181 software; last access 21/02/18) and GraphPad Prism 6 (version 6.0d). Pearson
182 Correlation Coefficients was used to detect clusters of similarity in miRNA threshold
183 cycle values between each pool group in patients, and between each pool group in
184 controls. To identify a suitable reference gene for the normalization of miRNA
185 expression in this study, the NormFinder algorithm was applied to the expression
186 data obtained from the Human TaqMan miRNA Arrays. Analysis comparing miRNA
187 levels between subjects with DKD and controls was carried out using GraphPad Prism
188 version 6 version 6.0d. Values for p below 0.05 were considered statistically
189 significant. MiRNA profiling data sets can be found in Gene Expression Omnibus
190 (https://www.ncbi.nlm.nih.gov/geo; accession number GSE114477).
191

Results

Altered urinary miRNA detection in DKD patients

To select candidate miRNAs that may act as DKD biomarkers, we first compared data from unbiased expression profiling of 754 miRNAs in urine samples from 20 DKD patients and 20 unaffected controls. Analyses were performed on 4 patient and 4 control pools, each composed of urine samples from 5 individuals as recommended by Zhang and colleagues.[23] Samples were pooled prior to profiling to minimize the contribution of subject to subject variation and to make substantive features easier to find, and thereby identify biomarkers common across individuals.[24] Previous analysis suggested that 40 individuals might optimally be pooled across 8 arrays,[23] which was our chosen pooling approach.

In Figure 1A the 12 data points in the upper right quadrant of the plot represent those miRNAs for which statistically significant fold-change increases were detected in patient urine compared to control samples, the 35 points in the upper left quadrant the corresponding downregulated miRNAs. The fold-change data for these 47 miRNAs are summarised in Figure 1B, and the 8 miRNAs exhibiting >5-fold change were subsequently selected as potential candidate biomarkers for further analysis.

Specific RT-qPCR assays were then used to analyse these miRNAs in each component urine sample pooled for profiling analysis. Statistically significant differences in miRNA detection between DKD patient and control urine samples were replicated for miR-126 (4.3-fold increase; $p = 0.0087$), miR-155 (22.9-fold; $p = 0.0024$) and miR-29b (4.9-fold; $p = 0.0002$) (Figure 1, C-E).

Elevated urinary miR-126, miR-155 and miR-29b detection in an independent DKD patient cohort

To test the above findings, miR-126, miR-155 and miR-29b were quantified in samples from an independent cohort of patients with established DKD from the Renal Impairment in Secondary Care Study (RIISC).[15] Samples from 89 patients meeting the criteria established in the UK National Institute for Health and Care Excellence 2008 criteria were available. An additional cohort of 62 patients with diabetes mellitus but without proteinuria or other evidence of DKD were included, as were samples from 41 people without evidence of diabetes or DKD (Table 1). We included diabetes patients without DKD as a third group in this analysis to identify DKD-specific miRNA detection changes and not purely hyperglycaemia-driven effects from our profiling comparison of DKD patients with control individuals.

Significant differences were again seen between DKD patients and controls for miR-126 (2.8-fold increase; $p<0.0001$; Figure 2A), miR-155 (1.8-fold; $p<0.001$; Figure 2B) and miR-29b (4.6-fold; $p = 0.024$; Figure 2C). Comparison of DKD patients with diabetic patients without DKD was statistically significant for miR- 126 (3.1-fold increase; $p<0.0001$) and miR-155 (1.6-fold; $p = 0.024$) with a trend to increased miR-29b (4.1-fold; $p = 0.121$) (Figure 2A-C).

RT-qPCR data for all 3 miRNAs were used to compare DKD patients and diabetic patients without DKD in the combined receiver operating characteristic (ROC) curve analysis shown in Figure 2D, giving an area under the curve (AUC) of 0.80. To analyse the contributions of each miRNA to the above ROC curve, individual specificity and likelihood ratios were calculated for relative expression (RQ) values

equivalent to a sensitivity of 80%.[25,26] Data displayed in Table 2 illustrate the magnitude of corresponding specificity values was miR-126 > miR-155 > miR-29b, and that combined miRNA data resulted in a ≥6.5% increase in specificity and likelihood ratio compared with individual miRNAs. These RQ data were then used as consecutive threshold values to discriminate between DKD and diabetic patients without DKD (D in Table 3) from the independent cohort. The discriminatory order was miR-29b (DKD/D = 5.62) > miR-126 (3.48) > miR-155 (2.23), and RQ values exceeding all 3 thresholds were obtained for 48.0% of DKD patients compared with 3.6% of diabetic patients without DKD (Table 3).

Laser capture microdissection shows increased glomerular abundance of miR-126 and miR-29b that is replicated in GEnC culture

Previous reports have linked changes in miRNA expression to DKD pathology, but have focused on whole tissue studies. For example, we showed association of decreased miR-192 expression with disease progression in DKD biopsies by *in situ* hybridisation.[13]

In the present study we used laser capture microdissection (LCM) to isolate glomeruli, proximal and distal tubules (Figure 3A) from histologically normal formalin-fixed, paraffin-embedded (FFPE) renal biopsy samples, and analysed miR-126, miR-155 and miR-29b expression by RT-qPCR. In Figure 3B, a typical CD10-stained FFPE biopsy section is seen before and after LCM to isolate glomeruli, proximal and distal renal tubules. MiR-126, miR-155 and miR-29b were detected in extracts from all three nephron regions (Figure 3, C-E). Increased glomerular

abundances were observed for miR-126 (Figure 3C) and miR-29b (Figure 3E), while miR-155 was most abundant in the distal tubule (Figure 3D).

Conclusions regarding nephron region-specific miRNA expression from the above analyses are inherently limited, however, since tissue extracts are subject to trace contamination by cells from other nephron domains. Therefore, cellular miRNA localisation within each nephron region was subsequently investigated by RT-qPCR analysis of podocyte and endothelial cell (GEnC) cultures from the glomerulus, renal proximal tubular epithelial cells (PTC) and fibroblasts. Detection of miR-126 was significantly higher in GEnCs compared with other cell types (Figure 3F). Most miR-155 was detected in PTCs and least in GEnCs (Figure 3G), while miR-29b was most abundant in GEnCs (Figure 3H).

GEnC release of miR-126 and miR-29b in an *in vitro* model of hyperglycaemia is driven by TNF-α and TGF-β1, respectively

The above data localized the majority of miR-126 and miR-29b expression to the GEnC. We next sought stimuli by which miRNAs are released into the glomerular ultrafiltrate, and hence the urine. Data from animal models of diabetes show increased glomerular and PTC TNF-α expression, and renoprotective effects of TGF-β inhibitors have also been reported.[27,28] GEnC expression of our candidate miRNAs was thus analysed *in vitro* in response to TNF-α and TGF-β1 in normoglycaemia and hyperglycaemia (Figure 4, A-F).

The presence of TNF-α led to significantly increased miR-126 detection in GEnC conditioned medium at 5 mM and 25 mM D-glucose (Figure 4B), a pattern also seen for miR-29b following TGF-β1 addition (Figure 4D). These cytokines did not

increase GEnC expression of miR-126 (Figure 4A), or miR-29b (Figure 4C), a pattern consistent with increased release, but not expression, of miRNAs.

No significant changes in miR-155 were detected in response to elevated D-glucose with either cytokine, and data for TNF-α are shown (Figure 4, E and F). Similarly, changes in miR-126 following TGF-β1 addition, and for miR-29b in the presence of TNF-α, were not observed (data not shown). Elevated D-glucose alone did not change miRNA expression in GEnCs or conditioned medium (Figure 4, A-F).

Discussion

Diabetic kidney disease (DKD) is the leading cause of kidney failure requiring renal replacement therapy worldwide, but effective methods to identify and halt progression of disease-specific pathophysiological changes remain elusive. Current effective interventions such as control of blood glucose and blood pressure are challenging to achieve, costly and time intensive. Existing tests track DKD from diabetic diagnosis to kidney failure, but do not allow accurate prognosis for the individual patient. In addition, the absence of treatment response biomarkers hinders development of emerging DKD therapies. There is thus an unmet need for additional DKD biomarkers to target intervention and follow response to therapy.

In this study we set out to identify urinary miRNA DKD biomarkers. Increased detection of miR-126, miR-155 and miR-29b was observed in the urine of DKD patients in comparison with both unaffected individuals and diabetic patients without DKD. MiRNA localization and release studies further suggested specific release of miR-126 and miR-29b from GEnCs. This raised the possibility that urinary miRNA quantification might provide data on ongoing pathological processes, and so aid patient stratification and measurement of response to therapy.

Urinary miRNA biomarkers have several potential significant advantages over circulating miRNAs for adoption into existing treatment pathways alongside current biomarkers, including speed and cost of non-invasive sample access.[29] However, few urinary miRNA DKD biomarker data have so far been reported. Previous studies have focused on circulating miRNAs, and have generated conflicting data with respect to association of miR-126 with diabetes mellitus and/or DKD. A recent cross-sectional analysis of type 2 diabetes mellitus patients found a negative association with

317 plasma miR-126,[30] and similar findings have been reported for type 1 diabetes
318 mellitus and all complications.[31] By contrast, miR-126 detection did not change in
319 whole blood from type 2 diabetes mellitus patients and control subjects, but
320 decreased in DKD patient samples.[32] Furthermore, no change in plasma miR-126 was
321 observed in a study of paediatric type 1 diabetic patients.[33] These analyses provide
322 inconsistent data for the biomarker utility of circulating miR-126, in contrast to the
323 significant and reproducible increases we detected in miR-126, miR-155 and miR-29b
324 in DKD patient urine in the present study.

325 The DKD-specific alterations in urinary miRNA profiles detected in this study
326 may have functional significance. Our *in vitro* analyses localized miR-126 and miR-
327 29b principally to the GEnC, with miR-155 expression distributed evenly across the
328 nephron. Glomerular endothelial localization of miR-126 may reflect the role of this
329 transcript in vascular regulation. Targeted mouse miR-126 deletion resulted in
330 vascular abnormalities by removing inhibition of sprouty-related EVH1 domain-
331 containing protein 1 expression, thereby enhancing vascular endothelial growth
332 factor (VEGF) function.[34] A role in DKD pathology for VEGFA signalling between
333 GEnCs and podocytes has been proposed.[35] In addition, miR-126 repression of
334 vascular cell adhesion molecule 1 expression in human umbilical vein endothelial
335 cells regulates their response to pro-inflammatory adhesion molecules.[36] MiR-126
336 has also been implicated in the heterogenic inflammatory response of renal
337 microvascular endothelial cells.[37]

338 Increased expression of miR-155 has been observed in DKD patient renal
339 biopsies, in close correlation with increased serum creatinine.[38] Furthermore, miR-
340 155 deficiency attenuated renal damage and IL-17 expression was downregulated in

341 streptozotocin-induced DKD mice.[39] Together with miR-126, miR-155 has been
342 implicated in multiple forms of vascular remodelling and associated with
343 cardiovascular disease.[40]

344 Decreased miR-29b has been reported in early and advanced animal models
345 of diabetic renal fibrosis.[41] Chen and colleagues found that loss of renal miR-29b in
346 db/db mice led to increased albuminuria, TGF-β-mediated fibrosis and immune
347 injury, while restored miR-29b expression inhibited renal injury.[42] Indeed, while we
348 have focused on upregulated miRNAs in this study, we acknowledge the potential
349 importance of miRNA downregulations that we detected.

350 In the present study we localized miR-29b to the glomerular endothelium.
351 Reduction of collagen and laminin synthesis has been reported following forced miR-
352 29b expression in human corneal endothelial cells.[43] In apoE knockout mice, miR-29b
353 induced aortic endothelial permeability in response to a high fat diet, and brought
354 about aortic apoptosis by direct targeting of melatonin receptor mt1.[44] In addition,
355 upregulated miR-29b expression has been observed in human umbilical vein
356 endothelial cells exposed to hyperglycaemia.[45]

357 The cytokine-driven release from GEnCs observed for miR-126 (TNF-α) and
358 miR-29b (TGF-β1) reported here suggests that these cells may be the principal
359 source of elevated urinary miR-126 and miR-29b detected in DKD. We speculate that
360 this constitutes evidence for disease-related signalling down the nephron that will be
361 interesting to test in future studies. Indeed, we have demonstrated association of
362 urinary miRNAs with exosomes[10] and exosomal transport, which might facilitate
363 passage of miRNAs through the nephron, has been reported for all three candidate
364 biomarker miRNAs.

Exosome-mediated release of miR-126 from $CD34^+$ peripheral blood mononuclear cells is proangiogenic, and decreased miR-126 was detected in elevated glucose cell culture and diabetic patients.[46] MiR-155 is depleted in urinary exosomes from microalbuminuric type 1 diabetes mellitus patients.[47] Endogenous miR-29b, spontaneously released from beta-cells within exosomes, stimulates TNF-α secretion from spleen cells isolated from diabetes-prone NOD mice *in vitro*.[48]

In summary, we have used unbiased profiling approaches to identify a urinary miRNA signature associated with DKD, and have subsequently confirmed increased miR-126, miR-155 and miR-29b in an independent patient cohort. MiR-126 and miR-29b were identified as enriched in GEnCs, and released from these cells in response to DKD-related cytokines. Urinary miR-126, miR-155 and miR-29b are therefore promising DKD biomarkers, and the potential pathological significance of miR-126 and miR-29b release from GEnCs merits further evaluation.

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C.B. and K.S. performed the experimental work, generated and analyzed data, and helped draft the manuscript. A.W., C.C., L.N., R.J. and T.A. performed the experimental work, generated and analyzed data. M.J., P.H., C.D., S.S., P.Cor and P.C. discussed elements of experimental design and/or cohort composition. D.F. and T.B. designed the research. T.B. wrote the manuscript, which was edited by D.F. then amended and approved by each author.

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554

555 **Figure legends**

556 **Figure 1** Urinary miRNA detection in urine samples from DKD patients and control
557 subjects. **A:** Volcano plot showing the detection profile of the 377 urinary miRNAs in
558 TLDA Card A in DKD patients (n = 20; four pools of five patients) and controls (n = 20;
559 four pools of five controls). The dotted horizontal line represents a p value boundary
560 of 0.05. **B:** Fold change of miRNA detection between DKD patients and controls.
561 DataAssist Software (Thermo Fisher Scientific) was used to perform relative
562 quantification for sample comparison, to perform t-test sample group comparisons,
563 and to produce the graphic output shown. (**C**-**E**) RT-qPCR analysis shows significant
564 differences in detection of (**C**) miR-126, (**D**) miR-155 and (**E**) miR-29b between
565 patients and control urine in the component urine samples pooled for profiling
566 analyses (**A** and **B**). DKD patients versus controls for **C**: miR-126, **D**: miR-155, **E**: miR-
567 29b (n = 20 for each group). Analysis was carried out by unpaired 2-tailed t-test with
568 Welch's correction. Profiling data analysis using the NormFinder algorithm identified
569 miR-191 as optimal for normalisation of our RT-qPCR data. Data were normalized to
570 endogenous control miR-191 and are presented as mean +/- SEM. $^{**}P < 0.01$, $^{***}P <$
571 0.005 and $^{*****}P < 0.0005$.

572

573 **Figure 2** RT-qPCR detection of selected miRNAs in patients and control subjects.
574 Relative expression was significantly different in 89 DKD patients compared with 62
575 diabetic patients without DKD and 41 controls for (**A**) miR-126 and (**B**) miR-155, and
576 significantly different in DKD patients compared with controls for (**C**) miR-29b. **A**:
577 DKD patients versus diabetic patients without DKD and controls; **B**: DKD patients and
578 diabetic patients without DKD; DKD patients versus controls; **C**: DKD patients versus

579 controls (n = 89 DKD patients, 62 diabetic patients without DKD, 41 controls).
580 Analysis was carried out by unpaired 1-tailed t-test with Welch's correction. Data
581 were normalized to endogenous control miR-191 and are presented as mean +/-
582 SEM. (**D**) Combined receiver operating characteristic curve analysis for miR-126, miR-
583 155 and miR-29b, area under the curve (AUC) = 0.80. Data were generated using the
584 pROC package in R-3.2.3. *P < 0.05, ****P < 0.001 and ******P < 0.0001.
585
586 **Figure 3** Localization of miRNA expression by laser capture microdissection (LCM)
587 and cell culture. **A:** Key functional nephron domains include the glomerulus (G), the
588 proximal tubule (PT) and the distal tubule (DT). **B:** A CD10-stained FFPE renal biopsy
589 sample before and after excision of glomeruli by LCM. Bars = 100 μm. (**C**-**E**) Relative
590 expression of miR-126, miR-155 and miR-29b, respectively, in LCM-isolated Gs, PTs
591 and DTs from 5 renal biopsies of healthy individuals. **C:** G versus PT; G versus DT (n =
592 5 biopsies). (**F**-**H**) Relative expression of miR-126, miR-155 and miR-29b, respectively,
593 in *in vitro* cultured HK-2 renal proximal tubular epithelial cells (PTCs), fibroblasts,
594 podocytes and conditionally immortalized glomerular endothelial cells (GEnCs). **F:**
595 GEnCs versus fibroblasts; GEnCs versus PTCs and podocytes; **G:** PTC versus GEnCs; **H:**
596 GEnCs versus podocytes (n = 4). Analysis was carried out by one-way ANOVA analysis
597 with Tukey's multiple comparison test. Data were normalized to endogenous control
598 miR-191 and are presented as mean +/- SEM. *P < 0.05, **P < 0.01 and ****P <
599 0.001.
600
601

Figure 4 MiRNA expression in GenCs and GenC conditioned medium in response to hyperglycaemia and DKD-related cytokines. Following 24 h culture in 5 mM or 25 mM D-glucose, relative expression in GEnCs and GEnC conditioned medium, respectively, of (**A** and **B**) miR-126 in response to 10 ng/ml TNF-α, (**C** and **D**) miR-29b in response to 1 ng/ml TGF-β1 and (**E** and **F**) miR-155 in response to 10 ng/ml TNF-α, and in untreated cells. **B:** 5 mM D-glucose versus 5 mM D-glucose plus TNF-α, and 25 mM D-glucose versus 25 mM D-glucose plus TNF- α; **C:** 5 mM D-glucose versus 5 mM D-glucose plus TGF-β1, and 25 mM D-glucose versus 25 mM D-glucose plus TGF-β1; **D:** 5 mM D-glucose versus 5 mM D-glucose plus TGF-β1; 25 mM D-glucose versus 25 mM D-glucose plus TGF-β1 (n = 4). Analysis was carried out by one-way ANOVA analysis with Tukey's multiple comparison test. Data were normalized to endogenous control miR-191 and are presented as mean +/- SEM. **P* < 0.05 and ***P* < 0.01.

626 **Table 1** Demographic and clinical parameters of patients recruited from 2 centres:
627 Wales Kidney Research Tissue Bank, Cardiff (University Hospital Wales) and
628 Birmingham (University Hospital Birmingham, Renal Impairment in Secondary care
629 (RIISC) study cohort) (n = 151)

630

Feature	Patients (n=151)		Controls (n=41)
	Diabetic (n=62)	DKD (n=89)	
Male n (%)	37 (58)	55 (62)	18 (44)
Non-Caucasian n (%)	13 (21)	33 (37)	
Mean Age (years, SD)	52 +/- 16.1	62 +/- 13.6	55 +/- 15.4
eGFR ml/min/1.73m^2			
Mean (SD)	78 +/-16.3	30 +/- 20.9	
Median (IQR)	84 (72-90)	22 (17-38)	
CKD stage n(%)			
No CKD/CKD G1 (eGFR ≥ 90)	23 (37)	2 (2)	
CKD G2 (eGFR = 60-89)	32 (52)	10 (11)	
CKD G3 (eGFR = 30-59)	5 (8)	17 (19)	
CKD G4 (eGFR = 15-29)	2 (3)	45 (51)	
CKD G5 (eGFR <15)	0	15 (17)	
ACR* mg/mmol n(%)			
A1-Normal-high normal (ACR<3)	54 (87.1)	15 (16.9)	
A2-Moderately increased (ACR 3-30)	8 (12.9)	25 (28.1)	
A3-Severely increased (ACR>30)	0 (0)	49 (55.0)	

631 *Albumin:Creatinine ratio (ACR) group cut-offs and nomenclature derived from
632 KDIGO 2012 recommendations.

633

634

635

636 **Table 2** Specificity values, likelihood ratios and RQ thresholds for miR-126, miR-155,

637 miR-29b and all three miRNAs above an 80% ROC curve sensitivity threshold

638

	Sensitivity (%)	Specificity (%)	Likelihood Ratio	RQ Threshold
3 miRNAs	80.21	63.64	2.206	> 1.148
miR-126	80.41	57.14	1.876	> 0.6762
miR-155	80.61	52.00	1.679	> 0.9110
miR-29b	80.61	40.00	1.344	> 0.8058

639

640 **Table 3** DKD and diabetic patients without DKD (D) patient numbers and percentages

641 above an 80% ROC curve sensitivity threshold for miR-126, miR-155, miR-29b and all

642 three miRNAs

643

	Patients above 80% sensitivity threshold				
	miR-126	miR-155	miR-29b	3 miRNAs	Total
D	23	30	13	2	55
DKD	80	67	73	47	98
	Percentage of patients above 80% sensitivity threshold				
	miR-126	miR-155	miR-29b	3 miRNAs	
D	41.8	54.6	23.6	3.6	
DKD	81.6	68.4	74.5	48.0	

644

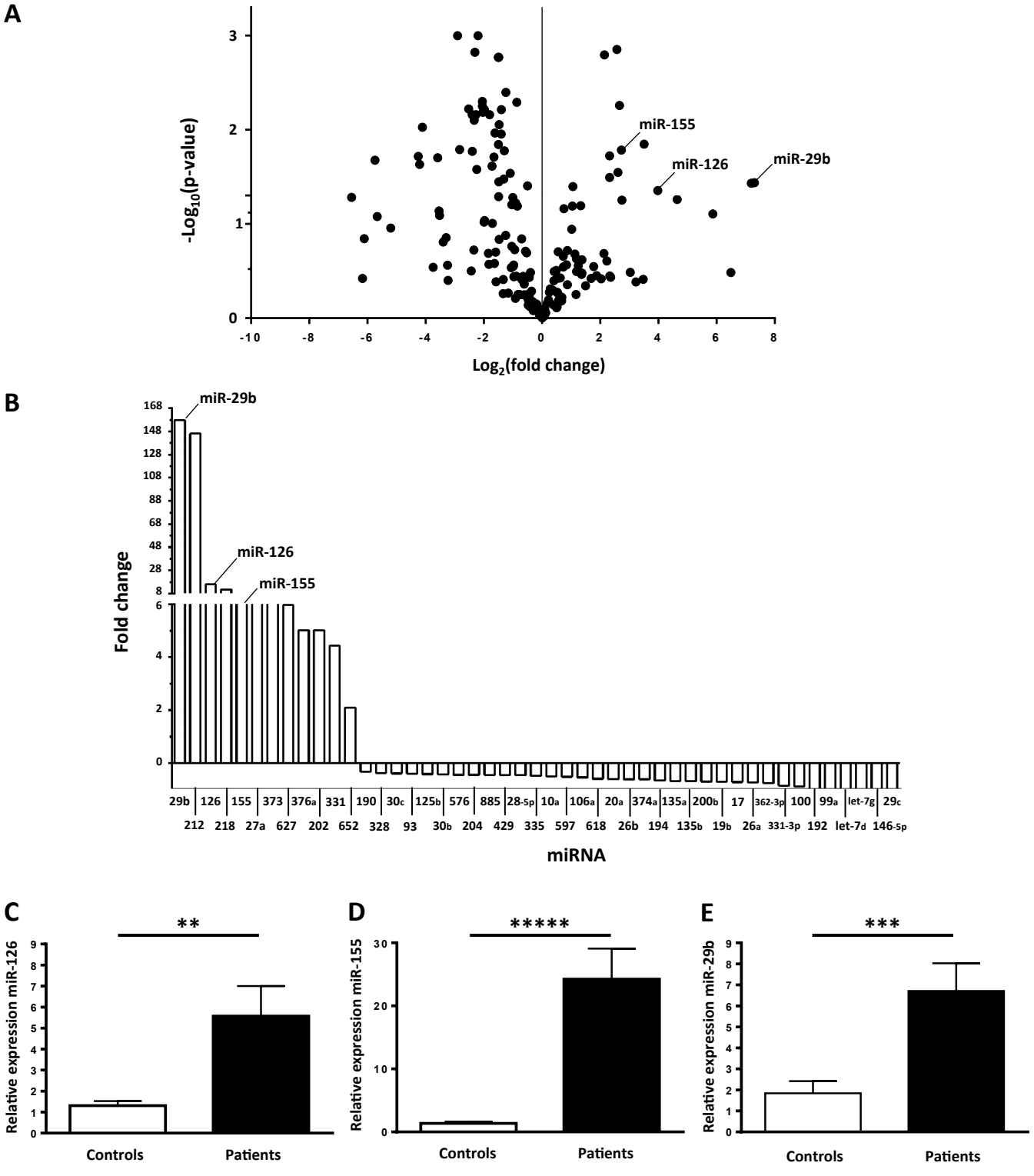

A
-Log10(p-value)
Log2(fold change)
miR-155
miR-126
miR-29b
B
Fold change
miR-29b
miR-126
miR-155
29b 126 155 373 376a 331 190 30c 125b 576 885 28-5p 10a 106a 20a 374a 135a 200b 17 362-3p 100 99a let-7g 29c
212 218 27a 627 202 652 328 93 30b 204 429 335 597 618 26b 194 135b 19b 26a 331-3p 192 let-7d 146-5p
miRNA
C
**
Relative expression miR-126
Controls
Patients
D
*****
Relative expression miR-155
Controls
Patients
E
***
Relative expression miR-29b
Controls
Patients

Figure 1

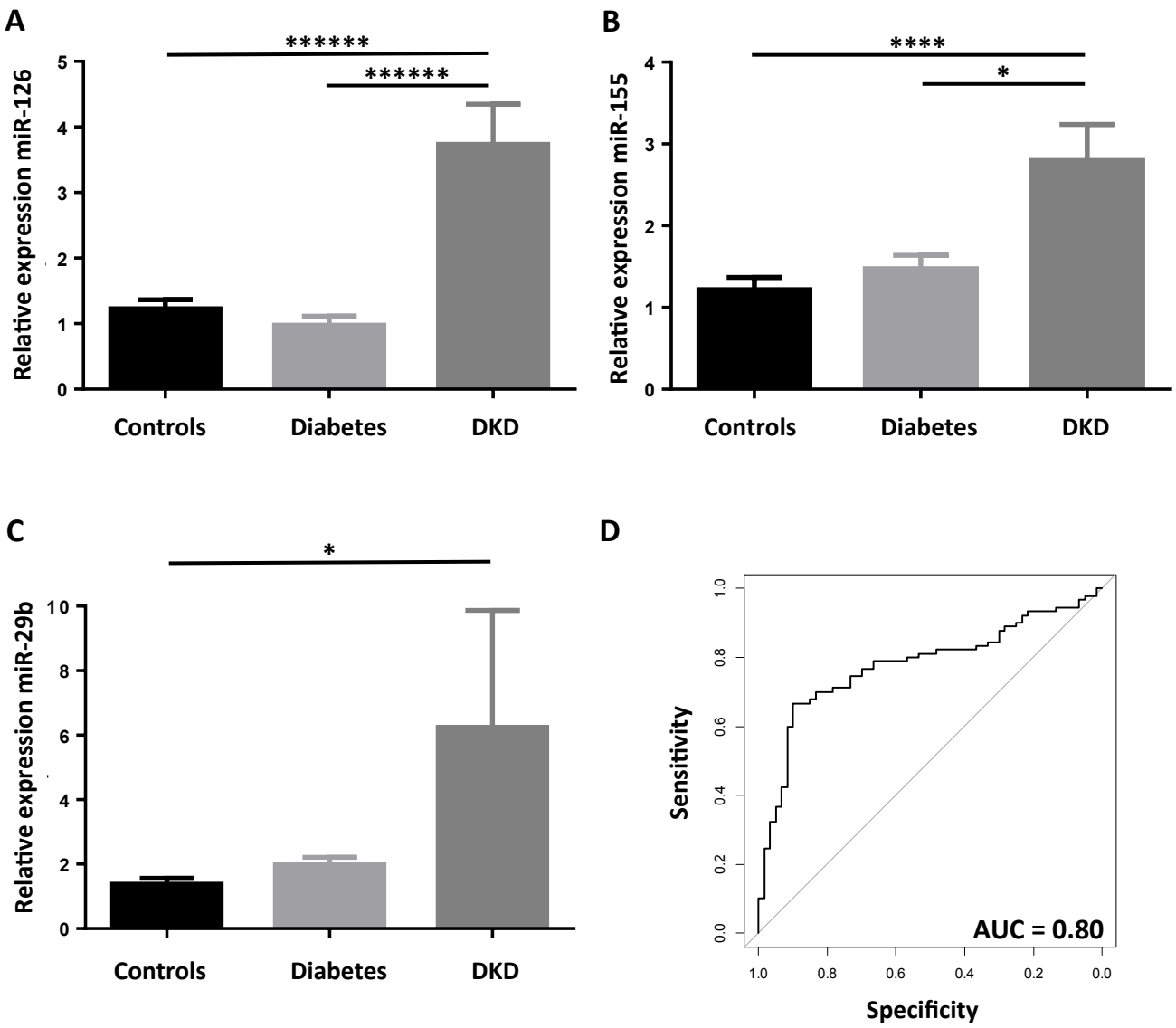
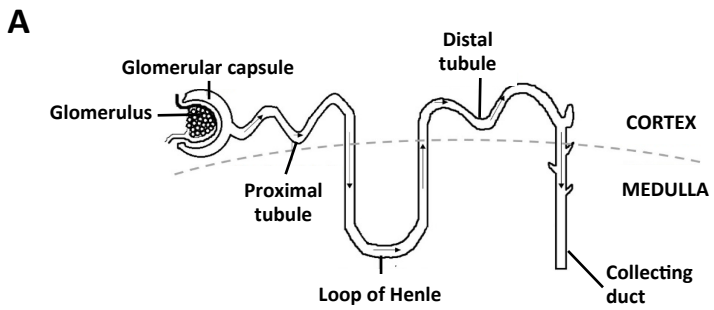
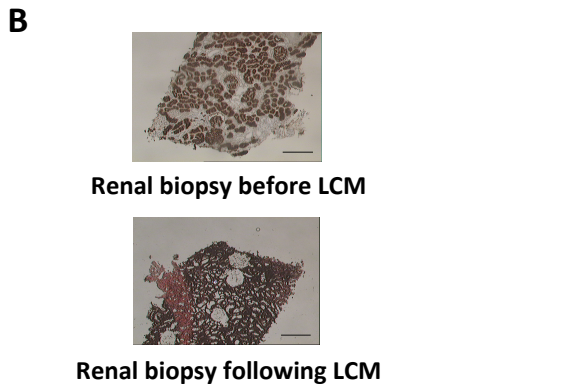
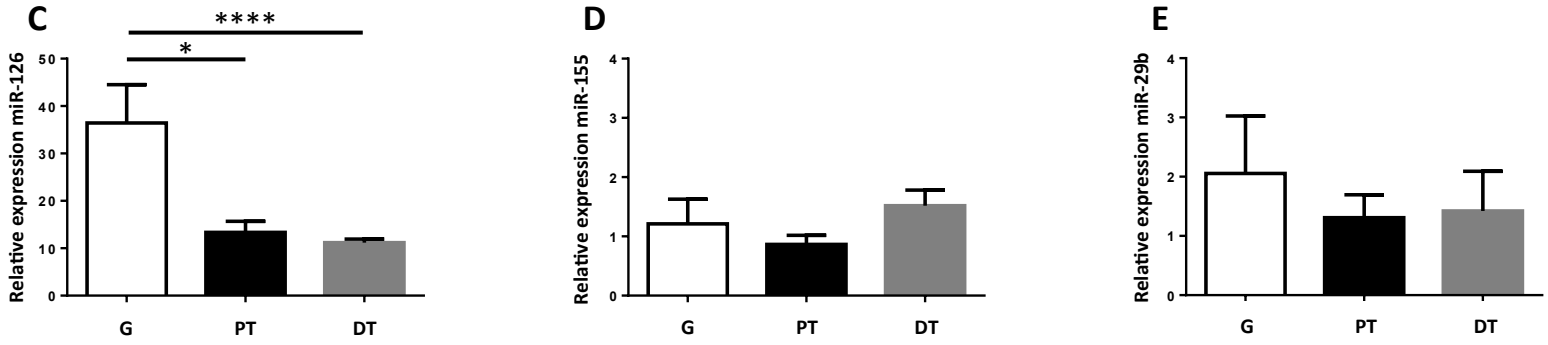

A
******
******
Relative expression miR-126
5
4
3
2
1
0
Controls
Diabetes
DKD
B
****
*
Relative expression miR-155
4
3
2
1
0
Controls
Diabetes
DKD
C
*
Relative expression miR-29b
10
8
6
4
2
0
Controls
Diabetes
DKD
D
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
AUC = 0.80
1.0
0.8
0.6
0.4
0.2
0.0
Specificity

Figure 2





LCM-dissected nephron domains



Cell cultures

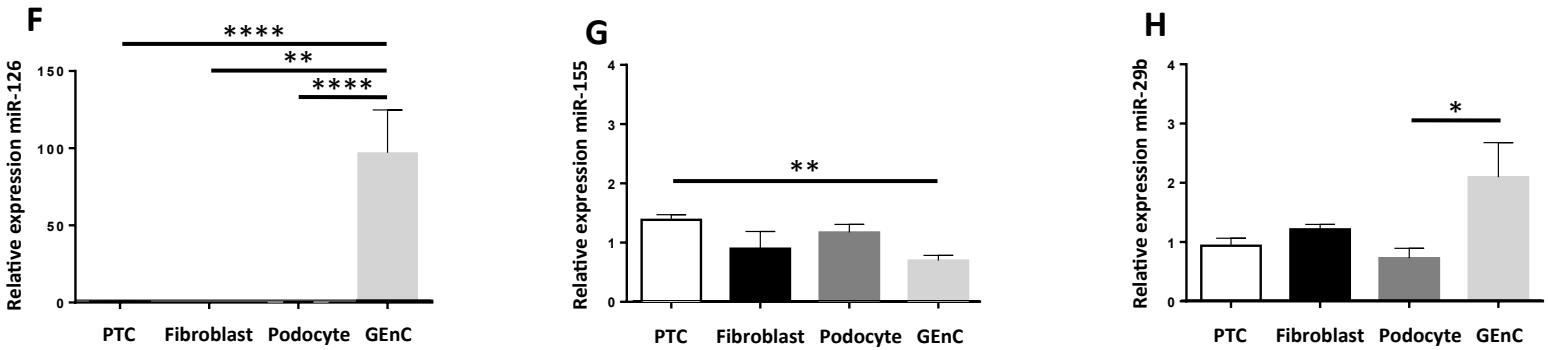


Figure 3

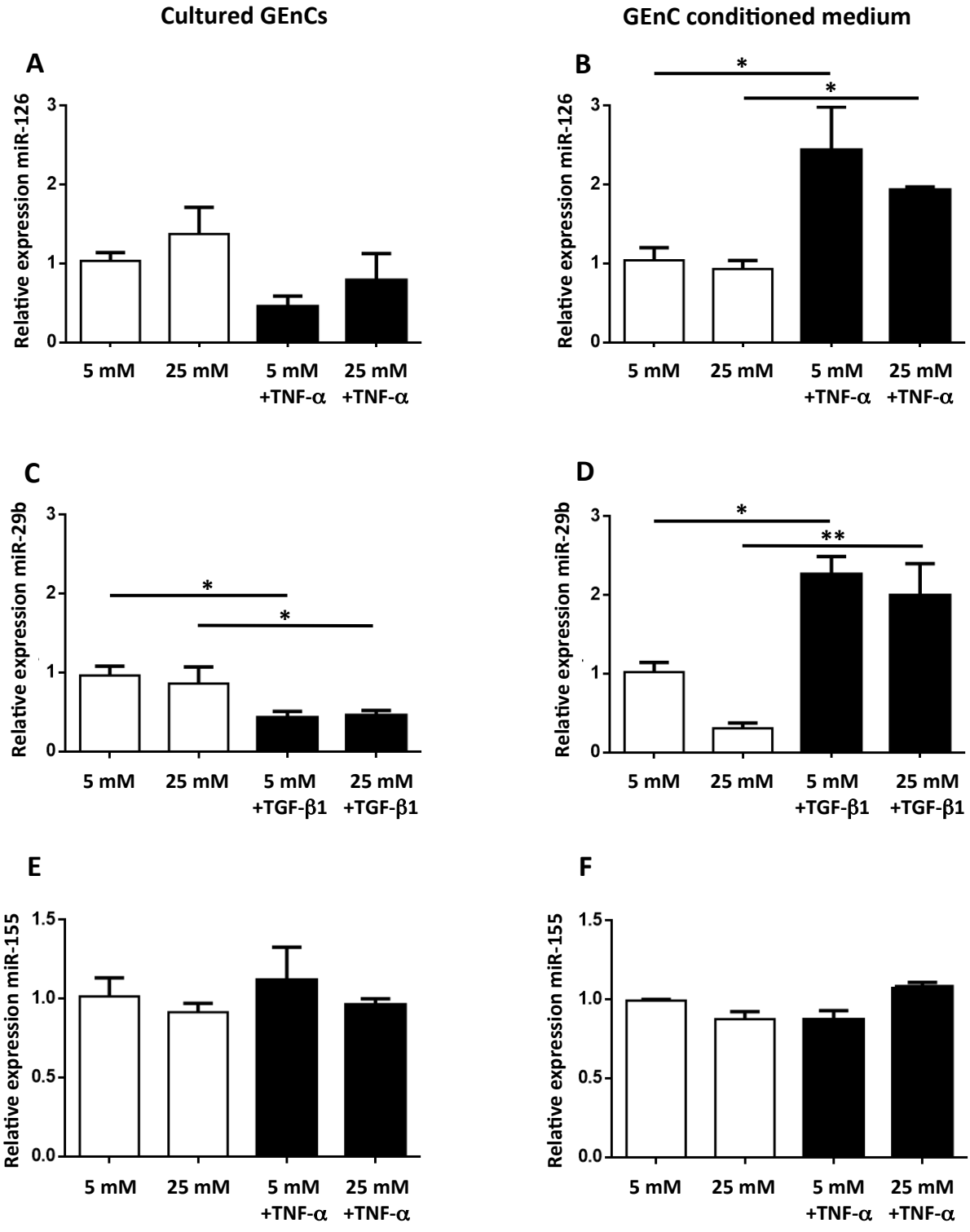

Cultured GEnCs
GEnC conditioned medium
A
B
C
D
E
F
Relative expression miR-126
Relative expression miR-29b
Relative expression miR-155
5 mM
25 mM
5 mM +TNF-α
25 mM +TNF-α
5 mM +TGF-β1
25 mM +TGF-β1
*
**

Figure 4