SHORT COMMUNICATION

miR-802-5p is a key regulator in diabetic kidney disease

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Background: Diabetic kidney disease has substantial burden and limited therapeutic options. An inadequate understanding of the complex gene regulatory circuits underlying this disorder contributes to the insufficiency of current treatment strategies. MicroRNAs (miRNAs) play a crucial role as regulators of functionally related gene networks. Previously, mmu-mir-802-5p was identified as the sole dysregulated miRNA in both the kidney cortex and medulla of diabetic mice. This study aims to investigate the role of miR-802-5p in diabetic kidney disease. **Materials and Methods:** The validated and predicted targets of miR-802-5p were identified using miRTarBase and TargetScan databases, respectively. The functional role of this miRNA was inferred using gene ontology enrichment analysis. The expression of miR-802-5p and its selected targets were assessed by qPCR. The expression of the angiotensin receptor (Agtr1a) was measured by ELISA. **Results:** miR-802-5p exhibited dysregulation in both the kidney cortex and medulla of diabetic mice, with two- and four-fold over-expressions, respectively. Functional enrichment analysis of the validated and predicted targets of miR-802-5p revealed its involvement in the renin-angiotensin pathway, inflammation, and kidney development. Differential expression was observed in the Pten transcript and Agtr1a protein among the examined gene targets. **Conclusion:** These findings suggest that miR-802-5p is a critical regulator of diabetic nephropathy in the cortex and medulla compartments, contributing to disease pathogenesis through the renin-angiotensin axis and inflammatory pathways.

Key words: Diabetic nephropathy, microRNAs, systems biology

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INTRODUCTION

Chronic kidney diseases (CKDs), affecting about 9% of the human population, are considered the 12th leading cause of mortality all around the world.[1] Diabetic kidney disease (DKD), the most common type of CKDs, accounts for about half of the cases of end-stage kidney disease.[2,3] Considering this fact, it is an urgent need for more investigations focusing on the discovery of more efficient therapies. However, the poor understanding of the disease's underlying molecular mechanisms is still the main hurdle. Noncoding RNAs play pivotal roles in the maintenance of physiological functions with their dysregulation being a core component of the pathogenic mechanisms.[4] Specifically, micro-RNAs (miRNAs) regulate a bundle of functionally related genes[5] and are key role players in the pathogenesis of a variety of complex disorders including DKD.[6] In a previous study,[7] we developed and validated a murine model of DKD and performed a microarray profiling of miRNAs in the kidney cortex and medulla. Notably, the effect of the anatomical compartment (cortex vs. medulla) on the expression of miRNAs was higher than that of the disease state (DKD vs. normal), indicating the divergence of the molecular processes in these two subsections. The only miRNA that was ubiquitously differentially expressed in both compartments was mmu-miR-802-5p. The current study aimed to decipher this miRNA's role in DKD.

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MATERIALS AND METHODS

Animal model preparation

The kidney cortex and medulla tissues harvested from a validated murine model of DKD in our previous study[7] were exploited. In order to develop this model, ten DBA/2j male mice were equally divided into control and DKD groups. Diabetes was induced by intraperitoneal administration of 40 mg/kg (total amount: 200 mg/kg) of streptozotocin for 5 consecutive days. After 3 months of the last dose, kidney injury was validated based on different biochemical and histopathological parameters and kidney tissues were harvested. All protocols for using animal modeling were approved by the Isfahan University of Medical Sciences Ethics Committee (IR.MUI.MED.REC.1399.933).

Bioinformatics analysis

miRNA annotation of mmu-miR-802-5p was extracted from the mirBase database.[8] Predicted and validated targets of miR-802-5p were retrieved using TargetScan v. 7.1[9] and miRTarbase[10] databases, respectively. Network construction and gene ontology (GO) term enrichment analysis were performed using the CluePedia[11] and the ClueGo plugins[12] of Cytoscape software. Adjusted $P \leq 0.05$ was considered a statistically significant threshold for GO enrichment analysis.

Quantitative polymerase chain reaction

RNA extraction and cDNA synthesis were carried out as described elsewhere.[6,7] For real-time polymerase chain reaction (PCR), 1 µl cDNA, 5 µl high ROX™ SYBR Green master mix (Ampliqon, Herlev, Denmark), 0.5 µl forward and 0.5 µl reverse primers, and 3 µl double distilled water were mixed, and then the reaction was carried out using Corbett Real-Time machine (Carlsbad, USA). The temperature profile consisted of an initial step at 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. REST software[13] was used to analyze the results. The sequences of primers are provided in Supplementary Table 1.

Sno-202 and Sno-234 were used as reference genes for miR-802 quantitative PCR (qPCR). For quantification of mRNAs, *Tfrc*, *Tbp*, *Hprt*, and *Gusb* were assessed in a pilot study, and finally, *Hprt* and *Gusb* were selected as reference genes based on their higher stability as revealed by BestKeeper, NormNinder, NormNinder, and geNorm algorithms, which were exploited through Cotton EST database.[14]

Enzyme-linked immunosorbent assay

For protein extraction, tissue samples were put in 1.5 ml microtubes each containing five glass balls, 50 µl RIPA buffer (Cytomatin Gene, Isfahan, Iran), and 1 µl protease inhibitor (P8340, Sigma-Aldrich, USA). The tissues were homogenized by a homogenizer machine (Precellys 24, Bertin instruments, France) in two rounds of 4500 rpm for 30 s. The suspension was then transferred to a new tube and centrifuged at 8000 rpm for 10 min. The supernatant was resuspended in 470 µl phosphate buffer solution (Merck, Darmstadt, Germany), and protein concentration measurement was performed using Bradford assay. The concentration of angiotensin receptor 1 alpha (Agtr1a) was measured using enzyme-linked immunosorbent assay kit (abx388581, Abbexa, United Kingdom) according to the manufacturer's instructions.

Statistical analysis

Expression values of miR-802 and its target genes were analyzed using the Mann–Whitney *U*-test with IBM SPSS version 25. We had no missing or out-layer data. $P < 0.05$ was considered the statistical significance threshold. In addition, Benjamini-Hochberg method was used for *P* value correction in Go term enrichment analysis.[15]

RESULTS

The expression of miR-802-5p was quantitatively assessed in the cortex and medulla samples previously harvested from a validated mouse model of DKD. In agreement with our earlier findings,[7] this miRNA showed statistically significant overexpression in both cortex and medulla of DKD mice compared to normal controls [Figure 1]. Notably, the expression value of this miRNA in the cortex and medulla is not statistically different. To provide clues on the functional roles of miR-802, its target genes were explored. Based on miRTarBase, and published literature,[16-20] five genes including *Pten*, *Agtr1a*, *Flot2*, *Map2k4*, and *Hnf1b* were recognized as validated targets of this miRNA. In

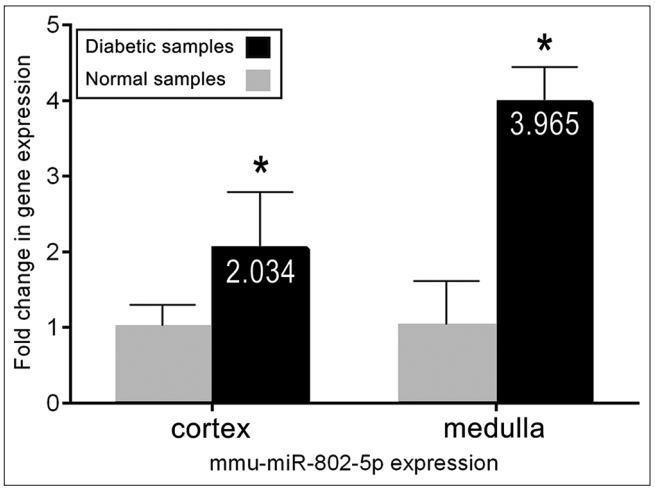

Figure 1: Mmu-miR-802-5p showed statistically significant overexpression in both the cortex and medulla of DKD mice compared to normal controls. The *sign indicates a $P < 0.05$. DKD = Diabetic kidney disease

addition, according to the TargetScan algorithm, 181 genes were predicted to be targeted by this miRNA [Figure 2a]. GO enrichment analysis of these validated and predicted targets suggests that miR-802 is involved in embryonic kidney development, inflammatory processes, and the renin–angiotensin–aldosterone system [Figure 2b].

It is previously shown that even the best available algorithms for miRNA target prediction suffer from low sensitivity and specificity.[21] Hence, we focused on those predicted miR-802 targets that are also targeted by at least one other DKD-associated miRNA detected in our previous survey.[7] Based on this filter, three targets, including *Kif4*, *Foxp1*, and *Ddit4*, were selected. The expression of these genes as well as the five validated genes [Figure 2c] was assessed by qPCR in the kidney cortex and medulla of DKD mice compared to controls. Considering the sensitivity of this technique to the selection of appropriate reference genes, four housekeeping genes were assessed and *Hprt* and *Gusb* were chosen as they were revealed to be more stable [Supplementary Figure 1]. *Pten* was the only target gene showing a statistically significant alteration [Figure 2d]. This gene was overexpressed both in the cortex and medulla. Although miRNAs are known to downregulate their targets, it should be noticed that the final expression level of a gene is the consequence of the balance of various regulators. Furthermore, miRNAs are expected to inhibit the translation of mRNAs and may not necessarily decline mRNA levels.

We assessed the protein expression of the angiotensin receptor, a target of miR-802 with well-recognized roles in renal homeostasis. Agtr1a was overexpressed in both compartments in diabetic conditions [Figure 2e] and

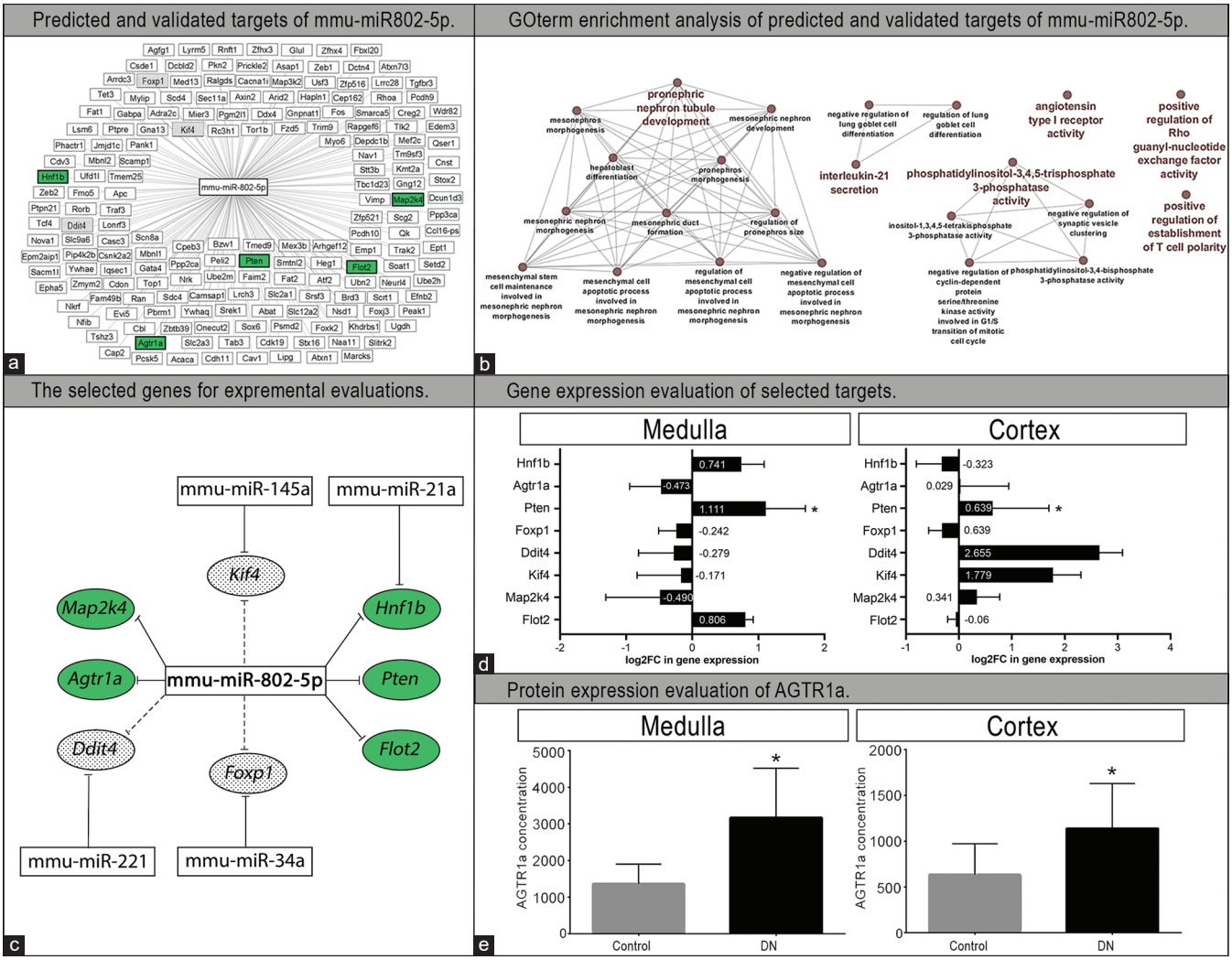

Figure 2: Predicted and validated identified targets of mmu-miR-802-5p demonstrated by white and green boxes, respectively (a). GO enrichment analysis of these targets suggests that miR-802 is involved in embryonic kidney development, inflammatory processes, and the renin–angiotensin system. The * sign indicates an adjusted $P < 0.05$. (b) In addition to the five validated targets, three predicted targets (dotted boxes) that are also targeted by other DKD-associated miRNAs were selected for further assessment. (c) The expression of these target genes was assessed by qPCR in the kidney cortex and medulla of DKD mice compared to controls. (d) The expression of Agtr1a was further assessed by ELISA. (e) The * sign denotes a $P < 0.05$. GO = Gene ontology, DKD = Diabetic kidney disease, ELISA = Enzyme-Linked Immunosorbent Assay, miRNA = micro RNA

reached a statistically significant threshold in the medulla. In addition, this receptor was found to be more abundant in the medulla compared to the cortex.

DISCUSSION

Considering the substantial difference in the cellular architecture and molecular processes of the kidney cortex and medulla, the expression of miR-802 and its targets was separately assessed in these compartments. This miRNA showed significant upregulation both in the cortex and medulla of diabetic kidneys, which according to the functional enrichment analysis, seem to play role through modulation of inflammatory processes and the renin–angiotensin axis. The enrichment analysis also suggests that miR-802 is involved in embryonic kidney development, which is in line with a previous study showing that this miRNA contributes to the kidney development through the regulation of mesenchymal–epithelial transition.[22]

The contribution of miR-802 in the pathogenesis of kidney disorders is demonstrated in a few recent studies; in line with our study, Opazo-Ríos *et al*. have shown that miR-802 is among the most overexpressed miRNAs in the kidney cortex of a mouse model of diabetes and obesity-induced nephropathy.[23] Similarly, renal miR-802 is shown to be overexpressed in obesity-induced nephropathy, which upregulates inflammatory processes by inhibiting NF-κB-repressing factor. Silencing of this miRNA was associated with improved kidney function and reduced fibrosis and inflammation.[24] Similarly, in a canine model of X-linked hereditary nephropathy, renal miR-802 was ubiquitously upregulated in different stages of disease progression from the onset of proteinuria to advanced azotemia.[25]

Taken together, this study indicates the pivotal role of miR-802 in the pathogenesis of DKD which is in line with a few previous investigations on the contribution of this miRNA in the progression of other forms of CKD.

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Conflicts of interest

There are no conflicts of interest.

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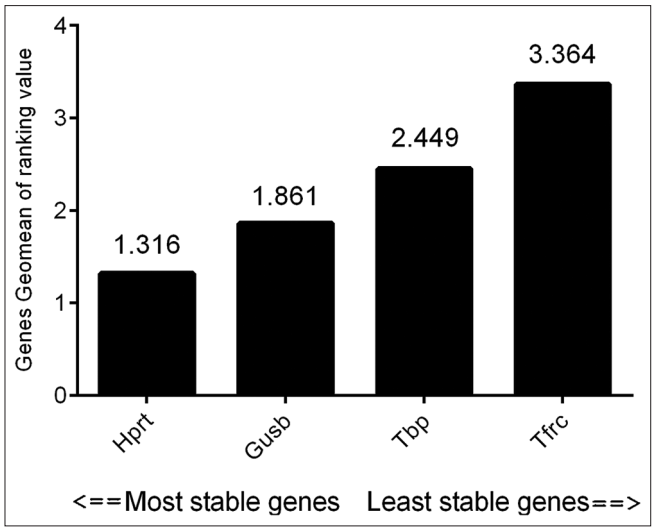

Supplementary Figure 1: Considering the sensitivity of qPCR to the appropriate selection of reference genes, four housekeeping genes were assessed and *Hprt* and *Gusb* were chosen as they were revealed to be more stable. qPCR = Quantitative polymerase chain reaction

Supplementary Table 1: The chosen primers sequences

Gene name	Sequence
Flot2	F: GTGTGGACCTTTCAAAGATACC
	R: CTGCTGGAGGCTGAGTGT
Map2k4	F: ATGATGTCCGCTCTGATGTC
	R: CTTCGTAAGGCACAAGTTGAC
Pten	F: GCAGAAAGACTTGAAGGTGTA
	R: CTCTCAGCACATAGATTGTATATC
Foxp1	F: GGCGGTTTGTGACGACTT
	R: GGCAGCTTTGGGTTCTGTAG
Kif4	F: ACCAAGCCAAGTTGAGTGA
	R: TATGCTCTGTGGATTCCTTCA
Ddit4	F: GGAAGACTCCTCATACCTGG
	R: GAGTTCCTTGCCCACCTG
Agtr1a	F: AAGCCATCACCAGATCAAGT
	R: ATATGTAACTGTGCCTGCCA
Hnf1b	F: CAGTCTCCTCTCACCTGACA
	R: GGTGTTGAGGCTCTGTGC
Gusb	F: GAGCGAGTATGGAGCAGAC
	R: CTCAGCGGTGACTGGTTC
Tfrc	F: TGCATTGCGGACTGTAGAG
	R: CCCACCAAACAAGTTAGAGAAT
miR-802-5p	RT: GTC GTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAAGGAT
	F: GGCGTCAGTAACAAAGATTC
	R: CAGCAGGGTCCGAGGT
Sno-202	RT: GTCGTATGCACAGCACGGTCCGAGGTATTCGCAGTGCATACGACCATCAG
	F: ACTTTTGAACCCTTTTCCAT
	R: CAGCACGGTCCGAGGT
Sno-234	RT: GTCGTATGCACAGCAGGGTCCGAGGTATTCGCAGTGCATACGACTCTCAG
	F: GATTTAACAAAAATTCGTCACT
	R: CAGCAGGGTCCGAGGT