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miR-193a as a potential mediator of WT-1/synaptopodin in the renoprotective effect of losartan on diabetic kidney

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1 **miR-193a as a potential mediator of WT-1/synaptopodin in the renoprotective**
2 **effect of losartan on diabetic kidney**

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14 **Running title: miR-193a improves kidney via WT-1/ synaptopodin**

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Abstract

Diabetic nephropathy (DN) is the most common complication of diabetic patients, and has become a global healthcare problem. In this study, we used diabetic mice to evaluate the effect of Losartan on diabetic nephropathy, in which the experimental animals were divided into three groups: non-diabetic mice (db/m group), untreated-diabetic mice (db/db group), and Losartan-treated diabetic mice (db/db-losartan). Next, immunohistochemistry and immunofluorescence were used to detect WT-1 and synaptopodin expression, respectively. Protein levels of WT-1, synaptopodin, claudin1, and Pax-2 were assessed by Western blotting and real-time PCR. The miR-193a mRNA levels were quantitated by real-time PCR. The results showed that albuminuria was increased in diabetic mice compared with control animals and was significantly ameliorated by treatment with Losartan. In addition, Losartan significantly upregulated the immunopositive cell numbers of WT-1, the expression of WT-1 and synaptopodin in renal tissue. By contrast, expression of claudin1 and Pax-2 in renal tissue were decreased in db/db-losartan group. Besides, expression of miR-193a was decreased significantly in db/db-losartan group compared to the untreated diabetic group. Thus, Losartan has renoprotective effects on the control of tissue damage possibly by inhibiting the expression of miR-193a, thereby promoting the repair of podocyte injury in mice with diabetic nephropathy.

Keywords: Diabetic nephropathy; Losartan; Podocytes; Synaptopodin; miR-193a

43

44 **Introduction**

45 Diabetic Nephropathy (DN) is one of the most common microvascular complications
46 in diabetic patients and the main cause of End-Stage Renal Disease (ESRD) worldwide
47 (Park, 2014). The recent epidemiological data showed that the chronic kidney disease
48 related to diabetes has exceeded chronic glomerulonephritis since 2011 in China (Zhang
49 et al., 2016). With the changes in Chinese dietary structure, the incidence of diabetes
50 has gradually increased, and the incidence of diabetic complications including diabetic
51 nephropathy has also increased. Previous study has shown that diabetes is especially
52 related to diabetes. Complications significantly increase the economic burden of
53 diabetic patients (Wu et al., 2019). The onset of diabetic nephropathy is implicit, and
54 patients often have no sense of it, however symptom becomes severe when ESRD
55 occurs. Moreover, the pathogenesis of diabetic nephropathy is complicated, and there
56 is no effective way to prevent the progression of diabetic nephropathy.

57 In the past two decades, microRNA (microRNA, miRNA) has been extensively studied,
58 which is a non-coding single-stranded microRNA composed of 18-22 nucleotides,
59 regulating gene expression after transcription (Ambros, 2004). More and more studies
60 have found that miRNAs are closely related to tumors, kidney diseases, cardiovascular
61 diseases, diabetes and other diseases (Fujii, 2018, Mendell and Olson, 2012, Zhu et al.,
62 2016). In the kidneys, studies have found that miRNAs are involved in the formation
63 and development of the kidneys, pathophysiological mechanisms, diabetic nephropathy,

hypertensive nephropathy, glomerulonephritis, and focal segmental glomerular sclerosis (FSGS), lupus nephritis and other primary and secondary nephropathy are closely related (Smeets and Moeller, 2012). Recent study discovered that the down-regulated expression of microRNA-193a (miR-193a) in human parietal epithelial cells (PEC) could induce PEC to express the podocyte marker Wilms tumor protein 1 (WT-1), and promote the podocytes transdifferentiation (Mishra et al., 2018). Moreover, downregulated miR-193a could reduce the formation of crescents and reduce the production of proteinuria in the nephrotoxic nephritis mouse (Kietzmann et al., 2015). However, the mechanism of miR-193a in diabetic nephropathy injury still remains unclear.

In the present study, we utilized db/db mice as *in vivo* experimental models of DN to investigate the functions and molecular mechanisms of miR-193a in the pathogenesis of DN. Furthermore, we found that Losartan reduces miR-193a expression to up-regulate WT1, and then inhibits claudin, pax-2 and enhances synaptopodin, resulting in the alleviation of podocyte apoptosis in DN progression.

Materials and methods

Patients

The study was performed with the approval of the Ethics Committee of University of Zhengzhou and written informed consents were obtained from all participants prior to experiments. The kidney tissues were collected by kidney biopsies from 15 DN patients

(for diagnostic purpose) and 15 age-matched control patients from the Department of Nephrology, the Affiliated Hospital of University of Zhengzhou from January 2018 to April 2018. The kidney tissue of the control group was derived from the normal tissue adjacent to the tumor surgically removed by patients with kidney tumors. These patients were required to have normal urine tests and normal renal function, without endocrine and metabolic diseases. The first-degree relatives in the family history had no history of diabetes; The kidney tissue of the diabetic nephropathy group is derived from the tissue of renal biopsy. It is required for patients with type 2 diabetes. The pathological diagnosis of renal biopsy is diabetic nephropathy and excludes other podocyte diseases, such as minor lesions, membranous nephropathy, FSGS, lupus nephritis. At the same time, the control group and the diabetic nephropathy group are required to be free of infectious diseases and no associated breast cancer, colon cancer, acute myeloid leukemia, Parkinson's disease, and schizophrenia-related diseases.

Sample collection

After the kidney tumor patients have surgically removed the tissue, the normal tissue next to the tumor is quickly separated and placed on ice, and the kidney tissue with a thickness of about 4 mm is removed and placed in 4% neutral formaldehyde buffer Fixation, and paraffin-embedded section, later used for PAS staining and immunofluorescence detection of kidney tissue; another 1 mm thick kidney tissue was cut and fixed in 2.5% glutaraldehyde fixative solution, and later used for renal tissue electron microscopy detection ; the remaining kidney tissue is cut into small pieces, placed in a cryotube, and

quickly placed in liquid nitrogen for temporary storage, and then stored in a -80 °C refrigerator for RNA extraction. The specimens of renal biopsy were divided into 3 parts in total, and the kidney tissues wrapped in wet gauze were embedded with OCT embedding agent. Due to the need for clinical diagnosis of the patient, after the frozen section is cut by the professor of the kidney pathology department, the kidney tissue embedded in the remaining OCT embedding agent is quickly placed in liquid nitrogen for temporary storage, and then stored in a refrigerator at -80 °C for extraction RNA. The rest of the tissues were sent to the kidney pathology room and electron microscope room for related treatment according to the normal procedures of the department. After the tissue processing was completed, the paraffin tissue sections were cut out from the kidney pathology room for relevant staining and observed and photographed in the electron microscope room.

Animals and protocols

40 five-week-old male type 2 diabetic mice db/db mice (weight, 22-26g), 20 five-week-old male healthy db/m mice (weight, 14-18g) were purchased from Model Animals Research Center of Nanjing University. The animals were acclimatized for 1 week before study and housed under a 12 h light: 12 h darkness cycle with free access to water and chow. All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at University of Zhengzhou and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

After acclimatizing for 1 week, all the animals were divided into following groups (n=20

per group): 20 db/m mice were used as normal control group, and 40 db/db mice were randomly divided into 2 groups: untreated db/db group (n=20) and db/db-losartan treatment group (n=20). The body weights and blood glucose levels of all animals were measured every four weeks from the 6 weeks of age, and timed 24 h urine was collected using individual metabolic cages. At 10 weeks of age, 20 db/db rats, which were randomly divided, were administered Losartan (MSD inventing company, Hangzhou, China) in their drinking water at 10 mg/kg per day for another 12 weeks (Figure 2A). The animals in each group were killed by exsanguination under anesthesia on the 10, 14, 18, 22 weeks of age (five animals each), and hearts and kidneys were rapidly dissected and collected for analysis. The heart was used for testing the creatinine levels in each sample. The right kidney was frozen in N₂ and stored at -80°C for mRNA and protein analysis. Half left kidney was fixed in 10% PBS-formalin, then paraffin embedded for light microscopy; the remaining tissue was embedded in optimal cutting temperature compound and snap-frozen in N₂.

Body weights, Blood glucose, Creatinine and urinary albumin

Body weights of all animals were measured at 10, 14, 18, 22 weeks of age. Before killing, blood samples were taken from the tail vein at 10, 14, 18, 22 weeks of age, and glucose levels were measured using a glucometer (Johnson & Johnson, US). Creatinine levels of animals in all groups on the 10, 14, 18, 22 weeks of age (five animals each) were measured using the Creatinine Assay Kit (BioAssay Systems, US) in samples obtained from the heart. One day before killing, all animals from each group were housed in

metabolic cages in order to collect 24 h urine samples. Urinary albumin concentration was measured by the Rat Albumin ELISA Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions.

Histological

The renal tissues were fixed in 10% neutral buffered formalin, followed by embedding in paraffin wax. Periodic acid-Schiff (PAS, purchased from Solarbio life science company, Beijing) staining was performed to assess the histological morphology of the renal tissues. Measurements were photographed using a microscope (Nikon, Japan).

Immunohistochemistry detection of WT-1

Immunohistochemical analysis was performed as described previously (Tunçdemir and Ozturk, 2008). The renal tissues were fixed in 10% neutral buffered formalin, followed by embedding in paraffin wax. Three micrometer thick sections were placed onto gelatin-coated slides, then deparaffinized and rehydrated. Immunoperoxidase staining was performed using rabbit monoclonal WT-1 (Abcam, USA; 1:300 dilution) antibody. Immunostaining procedures were carried out following the manufacturer's instructions. WT-1 staining was evaluated in 35 randomly selected glomeruli in all fields of kidney sections. Immunostaining was evaluated using a microscope (Nikon, Japan), and the number of WT-1 positive cells under the microscope was counted. The ratio of the number of positive cells to the number of globules was used to show the expression of WT-1.

168 ***Immunofluorescence staining detection of synaptopodin***

169 The kidney paraffin sections were cut into three micrometer thick sections, then
170 deparaffinized and rehydrated using for immunofluorescence staining. The sections were
171 incubated overnight at 4°C mouse monoclonal synaptopodin antibodies (Santa Cruz
172 Biotechnology, USA, 1:200 dilution), followed by goat anti-mouse IgG (H+L) secondary
173 antibody (Invitrogen Corporation, USA, 1:200 dilution) for 1h at room temperature.
174 Sections were photographed using a Zeiss LSM710 Meta confocal microscope (Carl
175 Zeiss AG, Cologne, Germany).

176 ***Western blotting***

177 Total kidney was homogenized in radioimmunoprecipitation assay buffer containing
178 RIPA buffer (Beyotime Institute of Biotechnology, China), ProE, PMSF, and protease
179 inhibitors. Tissue samples were centrifuged at 12,000 x g for 5 min at 4°C, and the protein
180 concentration was measured using BCA kit (wanleibio, China). Total protein lysed
181 extracts (50 µg lane) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel
182 electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (EMD
183 Millipore; Billerica, MA, USA) processed for immunostaining with primary antibodies
184 WT-1 (sc-7385, Santa Cruz Biotechnology, 1:100 dilution), Synaptopodin (sc-515842,
185 Santa Cruz Biotechnology, 1:100 dilution), claudin1 (ab15098, Abcam, 1:100 dilution),
186 and Pax-2 (71-6000, Zymed, 1:100 dilution) for overnight incubation at 4°C. With Tris
187 Buffered Saline Tween employed to rinse the mixture for three times, the goat anti-rabbit
188 IgG (WLA023, wanleibio, 1:5000 dilution) and goat anti-mouse IgG (A0216, Beyotime

Biotechnology, 1:5000 dilution) secondary antibodies were added to implement another 1h incubation at room temperature. Equal protein loading and the protein transfer were confirmed by immunoblotting for determination of GAPDH protein using a polyclonal GAPDH antibody (WL01114, wanleibio, 1:500 dilution) on the same (stripped) Western blots. Detection was performed using enhanced chemi-luminescence kit (Beyotime Institute of Biotechnology, China), and visualized on a Gel-Doc system (Tanon company, Shanghai, China). Finally, the gray scale of bands was semi-quantified based on Image J software (Tanon company, Shanghai, China).

Quantitative real-time RT-PCR

Total RNA was extracted from kidney tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the instructions specified by the manufacturer. First-strand cDNA was synthesized with the RNA Superscript real-time RT (Invitrogen), and oligo (dT) primer was used according to the manufacturer's instructions. Real-time PCR was performed in TaqMan real-time PCR using predeveloped TaqMan reagents (Applied Biosystems, USA). The sequences of primers are as follows: for murine miR-193a sense: 5'-TGGGTCTTTGCGGGCAAGA-3', antisense: 5'-GTGCAGGGTCCGAGGTATTC-3'; for murine 5sRNA sense: 5'-CTAAAGATTTCCGTGGAGAG-3', antisense: 5'-TGGTGCAGGGTCCGAGGTAT-3'. PCR was performed under conditions of (1) 50°C for 2 min, (2) 95°C for 10 min, and (3) 50 cycles of 95°C for 15 s and 60°C for 1 min, in the light of procedures enlisted in the TaqMan microRNA assay kit (Applied Biosystems, USA). Fluorescence for each

cycle was analyzed quantitatively and gene expression normalized relative to the expression of GAPDH. Eventually, expressions of target genes were analyzed on the ABI Prism 7000 SDS software (Applied Biosystems, USA), and they were quantified based on the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All measurement data in this experiment were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$), statistical analysis was performed using SPSS 17.0 software, single-variance analysis of variance was used for comparison between groups, and non-parametric test was applied to data with irregular variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of enrolled patients

The characteristics of the study population are shown in Table 1. DN patients were divided into two groups according to the presence of DN. Patients with DN did not differ from any of the anthropometric parameters studied; namely LDL, HDL, and hemoglobin ($p > 0.05$). Regarding biochemical determinations, differences were observed for fasting glucose, HbA1c, total cholesterol and triglycerides between T2D patients with DN and those without complications ($p < 0.05$).

miR-193a /WT-1 expressions in DN patients

PAS staining and transmission electron microscopy were used to observe the changes

231 of glomerular morphology in patients with diabetic nephropathy. Compared with the
232 results of the control group, the results of PAS staining showed that the glomerular
233 hypertrophy in patients with diabetic nephropathy was accompanied by an increase in
234 mesangial matrix, and there may be proliferation of mesangial cells. The glomerulus
235 and Bowman's capsule basement membrane thickened significantly. Electron
236 microscopy results showed that the glomerular foot process was widely fused, the
237 basement membrane was thickened diffusely, and no electron dense deposits were seen
238 (Figure 1A and 1B). Immunofluorescence was used to detect the expression of WT-1
239 in glomeruli, and the RNA expression levels were measured through qRT-PCR. The
240 expression of podocyte marker WT-1 in glomeruli of patients with diabetic nephropathy
241 were reduced, which were consistent with the RNA expression WT-1, respectively
242 (Figure 1C and 1D). In addition, the relative expression level of miR-193a was detected
243 in the kidney tissue of the patient, showing expression level of miR-193a was
244 significantly increased in patients with diabetic nephropathy (Figure 1E). Several
245 studies have suggested that there is a regulatory effect between miR-193a and WT-1
246 (Mishra et al., 2018, Wang et al., 2019), thus, we consider whether there is also a
247 correlation in patients with diabetic nephropathy. After detecting the relative expression
248 levels of miR-193a and WT-1 in kidney tissue by qRT-PCR method, we analyzed the
249 correlation expression levels of miR-193a and WT-1 (Figure 1F). The results showed
250 that the expression of miR-193a in kidney tissue was negatively correlated with the
251 expression level of WT 1 ($r_s = -0.563$ $p = 0.029$).

252 ***The amelioration of renal function and renal pathology in diabetic mice treated with***
253 ***losartan***

254 In db/db group, we observed that mice developed significant serum creatinine and
255 urinary albumin after 10 weeks (Figure 2A), which is considered to be the DN state,
256 confirming by PAS staining (Figure 2). To ensure the therapeutic effect, the losartan
257 treatment lasted for 22 weeks. As shown in Figure 2B and 2C, losartan could
258 significantly decrease the levels of serum creatinine and urinary albumin after 14 weeks
259 ($P < 0.01$). Also, losartan improved the pathological injury compared with db/db group,
260 suggesting that losartan can partially reduce the pathological changes of kidneys in
261 db/db mice, thereby protecting the glomerular filtration barrier to a certain extent
262 (Figure 2D). However, losartan could not efficiently decrease the DN mice weight and
263 blood glucose (Figure 3), suggesting losartan might directly act on kidney instead of
264 lowering blood glucose.

265 ***miR-193a /WT-1 expressions after losartan treatment in DN mice***

266 From the clinical data analysis, miR-193a and WT-1 might play an important role in
267 the DN process development. Next, we explore the relationship between losartan and
268 miR-193a and WT-1 during DN therapy. As shown in Figure 4A, losartan significantly
269 decreased the level of miR-193a after 14 weeks, while the miR-193a reached highest at
270 14 weeks. In addition, the WT-1 expression was determined by immunohistochemical
271 detection, whose result showed that the number of WT-1 immunopositive podocytes
272 were lower in the db/db group and losartan-treated diabetic group than that in the db/m

mice at the same age ($P<0.05$, Figure 4B). However, the expression level of WT1 in losartan-treated diabetic group is higher than that in db/db group from 10 to 22 weeks. All these findings were consistent with clinical results.

Effect of losartan on synaptopodin/WT-1 pathway for DN treatment

As the disease progressed, the expression of synaptopodin in glomeruli of untreated diabetic (db/db) group was lower than that of db/m mice of the same age. After losartan treatment, the expression of synaptopodin in glomeruli of losartan-treated diabetic group was increased compared to that in the untreated diabetic (db/db) group of the same age (Figure 5A). Western blot analysis showed that the protein expression levels of podocyte markers WT-1 and synaptopodin in untreated diabetic (db/db) group and losartan-treated diabetic group were lower than those in db/m mice at the same age ($P<0.05$). The protein expression levels of layer epithelial cell markers claudin1 and Pax-2 in untreated diabetic (db/db) group and losartan-treated diabetic group increased significantly compared to the db/m group at the same age ($P<0.05$). After losartan treatment, the protein expression levels of podocyte markers WT-1 and synaptopodin in losartan-treated diabetic group were higher than those in untreated diabetic (db/db) group at the same age ($P<0.05$), while the parietal epithelial cell marker claudin1 and Pax-2 were decreased significantly in losartan-treated diabetic group compared to those in untreated diabetic (db/db) group of the same age ($P<0.05$). (Figure 5B and Figure 5C).

293 Discussion

294 Diabetic nephropathy (DN) is a serious advanced complication of diabetes that has few
295 effective treatments and is pathologically characterized by glomerular hypertrophy,
296 albuminuria, and accumulation of glomerular matrix, ultimately culminating in
297 tubulointerstitial fibrosis, glomerulosclerosis, and progressive kidney failure (Gaede et
298 al., 2008, Haneda et al., 2015). The loss of podocytes, which is indicated as an early
299 pathologic marker of diabetic nephropathy, is important in terms of causing
300 albuminuria and glomerular damage (Mifsud et al., 2001, Reddy et al., 2008). In our
301 study, miR-193a was found to be significantly elevated, and WT-1 descended in DN
302 patients. Moreover, db/db mice experiment showed similar trends, compared with the
303 clinical data. In addition, losartan was found to be improving the kidney function
304 through inhibiting miR-193a expression in db/db mice.

305 Podocytes are a major component of the glomerular filtration barrier. Injury and loss of
306 podocytes lead to albuminuria, a hallmark of diabetic nephropathy and a predictor for
307 the progression of kidney diseases. Wilm's tumor suppressor gene (WT-1) plays a key
308 role in epithelial differentiation. During renal development, WT-1 is expressed in
309 mesenchyme and glomerular visceral epithelium and it generates the conversion of
310 these cells into mature podocytes. The continuous expression of WT-1 in terminally
311 differentiated podocytes is essential for maintenance of podocyte function (Fukuzawa
312 et al., 2003, Palmer et al., 2001). Susztak et al. (2006) reported that the significant
313 decrease in podocyte number, which is detected with WT-1 immunostaining, is an early

314 indication of podocyte apoptosis. In the present study, we performed WT-1
315 immunostaining to recognize and quantify the podocytes. We detected significantly
316 decreased WT-1 immunoreactivity in podocytes with a reduction in WT-1
317 immunopositive podocyte number relative to the increase in urinary albumin levels in
318 the untreated diabetic mice compared to non-diabetic mice. With the progress of
319 diabetic nephropathy, the expression of protein and mRNA of podocyte markers WT-1
320 and synaptopodin were lower than those of db/m mice at the same age ($P<0.05$). These
321 findings were similar to previous studies in which damaged podocytes lose their
322 functions and are an early marker of diabetic nephropathy (Macconi et al., 2009, Siu et
323 al., 2006).

324 Therefore, podocyte damage and shedding will directly affect the integrity of the
325 filtration barrier (Yoder, 2007). But recent research suggests that podocytes can
326 regenerate from other sources and play an important role in glomerular damage repair.
327 There is increasing evidence that PECs have the potential to replace repaired podocytes
328 (Gaffney, 1982, Gaffney and Panner, 1981, Smeets and Moeller, 2012). A large number
329 of animal studies have confirmed that progenitor cells may be transdifferentiated to
330 podocytes in place of PECs, replacing the repair of their damage, restoring the number
331 of podocytes, and alleviating proteinuria and glomerular sclerosis. The Shankland team
332 found that in the case of kidney injury, podocyte marker protein expression was reduced,
333 while PECs marker protein expression was increased, and double positives of
334 podocytes and PECs marker proteins were detected (Ohse et al., 2010, Zhang et al.,

2012), thereby repairing damaged podocytes. As in previous studies, claudin1 and Pax-2 were considered as the markers of parietal epithelial cell (PEC) (Sakamoto et al., 2014). Thus, in our study, the protein and mRNA expression levels of claudin1 and Pax-2 were used to assess the changes of parietal epithelial cell (PEC). With the progress of diabetic nephropathy, the protein and mRNA expression of claudin1 and Pax-2 in the parietal epithelial cells increased compared with db/m mice, which also could be attenuated by losartan ($P<0.05$).

MiR-193a is specifically expressed in mature PECs, and its level of dysregulation induces a series of molecular cascades that ultimately lead to glomerular damage in mice. Recent studies have found that miRNA-193a regulates the transdifferentiation of human parietal cell epithelial cells to the podocyte phenotype (Kietzmann et al., 2015). PECs express high levels of PEC-specific protein and miRNA-193a, which inhibit podocyte differentiation by down-regulating mouse WT-1. Inhibition of miR-193a expression, expression levels of PECs markers are reduced, while expression levels of podocyte markers are increased. In contrast, overexpression of miR-193a resulted in up-regulation of PECs markers and reduction in podocyte markers. In the previous research, it was found that inhibition of miR-193a expression in a mouse model of nephrotoxic nephritis causes a decrease in crescent formation and proteinuria (Kietzmann et al., 2015). However, whether miR-193a targeting WT1 participates in losartan effect on DN has not yet been explored. In this study, we demonstrated a reduction in urinary albumin, serum creatinine levels and renal hypertrophy in losartan-

356 treated diabetic group comparison to the untreated diabetic group, indicating that
357 losartan has the renoprotective effect of DN. Besides, with the treatment with losartan,
358 the protein and mRNA expression of podocyte markers WT-1 and synaptopodin in
359 db/db-losartan group increased significantly compared with the untreated group
360 ($P<0.05$). Meanwhile, the protein and mRNA expressions of epithelial cell markers
361 claudin1 and Pax-2 were decreased significantly compared with those in the untreated
362 group ($P<0.05$). It suggests that losartan shows renoprotective effects on podocyte
363 damage, promotes PECs transdifferentiation to podocytes, maintains the number of
364 podocytes, and delays the development of glomerular sclerosis. In our present study,
365 we gave an investigation of the mRNA levels of miR-193a in the experimental mice.
366 Results showed that the mRNA levels of miR-193a were significantly reduced in the
367 db/db-losartan group compared with that in the untreated group ($P<0.05$) after
368 treatment with losartan. Thus, based on the above experiment results, it can conclude
369 that after treatment with losartan, mRNA levels of miR-193a were significantly reduced,
370 and the protein and mRNA expressions of epithelial cell markers claudin1 and Pax-2
371 were decreased, whereas, the protein and mRNA expression of podocyte markers WT-1
372 and synaptopodin were increased. Thus, losartan may inhibit the expression of miR-
373 193a in the process of promoting the repair of podocyte injury, promoting PECs
374 transdifferentiation to podocytes, replacing the repair of damaged podocytes, thereby
375 delaying the progression of diabetic nephropathy. Although we have found a
376 relationship between losartan and the abnormal expressions of miR-193a by RT-PCR,

the molecular mechanism behind the regulation of miR-193a expression is still unknown. Further researches should be conducted more in-depth to clarify the relationship between miR-193a and losartan in DN.

Although losartan showed significant preventive effects against histological impairments, the hypoglycemic effect of losartan was much lower. Different sites of action, specifically related to blood glucose lowering abilities, may explain these findings. Alotaibi et al. (2019) did not notice a significant change in fasting elevated blood glucose levels in losartan -treated diabetic rats either alone or in combination with Glibenclamide. In addition, another angiotensin-II type 1 (AT₁) receptor antagonist, eprosartan, was also unable to significantly alter the 24-h plasma glucose profile in patients with type 2 DM who had previously been stabilized on glyburide (Martin et al., 1997). Thus, angiotensin-II type 1 (AT₁) receptor blockers may play an important role in progression of diabetic complications by exacerbating oxidative injury and inflammation with regulating blood glucose.

In summary, this study demonstrated the increasing expression of miR-193a in DN progression by inhibiting WT-1 expression. Thus, miR-193a high-expression to reduce WT-1 may be a new sight of revealing the pathogenesis of DN. Losartan reduces miR-193a expression to up-regulate WT1, and then inhibits claudin, pax-2 and enhances synaptopodin, resulting in the alleviation of podocyte apoptosis in DN progression. The inhibition of miR-193a to increase WT1 by losartan may be a novel strategy for the treatment of diabetic nephropathy.

398

399 **Acknowledgements**

400 Not applicable

401 **Competing interests statement**

402 The authors declare there are no competing interests.

403 **Contributors' statement**

404 DG and GW designed the study; YJQ did the methodology; PY, SHJ, CCY, DDD did
405 the investigation and data analysis; Writing - original draft preparation: DG, PY wrote
406 the paper; DG revised the paper. All authors read and approved the final manuscript.

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411 **Data availability statement**

412 The datasets used and/or analysed during the current study are available from the
413 corresponding author on reasonable request.

414

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Table 1. Clinical and biological data of the patients studied

Parameter	Con (n=15)	DN (n=15)	P value
Male: Female	9:6	7:8	0.464
age(year)	49.46±15.48	46.63±10.50	0.473
SBP (mmHg)	132.69±19.57	150.97±21.72	0.009
DBP (mmHg)	80.54±8.55	92.63±11.64	0.002
hemoglobin(g/L)	128.62±16.49	115.17±16.49	0.098
serum creatinine(μmol/L)	67.00(51.50, 85.00)	130.00(92.50, 176.50)	<0.001
Blood glucose(mmol/L)	4.72(4.50, 4.93)	7.14(5.19, 8.28)	<0.001
blood albumin(g/L)	43.32±2.30	36.58±7.63	0.003
TC (mmol/L)	3.75(3.51, 4.77)	5.52(4.05, 6.49)	0.037
TG (mmol/L)	1.26(1.03, 1.65)	1.84(1.40, 2.76)	0.003
LDL (mmol/L)	2.53(1.92, 3.31)	2.94(1.87, 4.28)	0.290
HDL (mmol/L)	1.17±0.30	1.23±0.53	0.683
eGFR (mL/min per 1.73m ²)	99.61±20.94	54.95±32.99	<0.001
(HbA1c) (%)	4.60(4.20, 5.15)	7.00(6.30, 8.30)	<0.001
ACR (mg/mmoL)	0.45(0.26, 0.82)	228.96(85.58, 385.91)	<0.001
24hTP(g)	0.05(0.03, 0.09)	3.93(2.10, 7.91)	<0.001

Figure Legends

Figure 1. Different indicators between Control and DN patients. Morphological changes of kidney in control group and diabetic nephropathy group (PAS (A), bar=50 μ m, EM (B), bar=1 μ m); The expression of WT-1 in immunofluorescence (C) and qRT-PCR (D) in control group and diabetic nephropathy group (bar=50 μ m, ***p<0.001); (E) The expression of miR-193a in control group and diabetic nephropathy group (***p<0.001); (F) Correlation analysis of miR-193a and WT 1 in kidney tissue of diabetic nephropathy group

Figure 2. Effect of losartan on renal function and renal pathology in diabetic mice. (A) Scheme of animal experiment; (B) Serum creatinine levels during losartan treatment; (C) Urinary albumin levels during losartan treatment; (D) PAS staining of kidney sections during losartan treatment (bar=50 μ m).

Figure 3. Mice weight (A) changes and blood glucose (B) levels during losartan treatment.

Figure 4. Effect of losartan on miR-193a /WT-1 expressions in diabetic mice. (A) The expression level of miR-193a in mice of different ages. (*p < 0.05); (B) Immunohistochemistry detection of WT-1 in mice of different ages (400 $\times$). (* compared to db/m mice at the same age, P < 0.05; # compared to db/db mice at the same age, P < 0.05)

Figure 5. Effect of losartan on synaptopodin/WT-1 pathway for DN treatment. (A) Immunofluorescence was used to detect the expression of synaptopodin in glomeruli of

542 mice of different ages (400×); (B) The protein expression levels of WT-1, synaptopodin,
543 claudin1 and Pax-2 in kidney tissues of mice at different ages. (C) Gray analysis of
544 protein expression of WT-1, synaptopodin, claudin1 and Pax-2 in kidney of different
545 ages (*compared to db/m mice at the same age, $P < 0.05$; # compared to db/db mice at
546 the same age, $P < 0.05$).

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113x33mm (300 x 300 DPI)

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131x131mm (300 x 300 DPI)