Inhibition of the lncRNA 585189 prevents podocyte injury and mitochondria dysfunction by promoting hnRNP A1 and SIRT1 in diabetic nephropathy

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Research Article

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Abstract

Podocyte damage is a crucial cause for diabetic nephropathy and end-stage renal disease. Moreover, mitochondria play an indispensable role in diabetic nephropathy and high glucose-associated podocyte damage. Through RNA sequencing, we firstly discovered that long non-coding RNA (lncRNA) ENST00000585189.1 (lncRNA 585189), was up-regulated in the plasma of patients diagnosed with DN, accompanied with higher albumin/creatinine ratios. Additionally, RNA-FISH in the tissues and immortalized human podocytes identified that the variation of lncRNA 585189 was mainly located in podocytes. In podocytes under the high glucose condition, we found that the silence of lncRNA 585189 expression not only decreased the production of ROS, rescued mitochondrial morphology and the alteration of the mitochondrial membrane potential, but also restored the podocyte damage, reversing the aberrant expression of ZO-1 and Desmin. Through bioinformatics analysis, lncRNA 585189 was predicted to combined with hnRNP A1 at the nt224 motif, which was demonstrated by RIP, pull-down and EMSA. Meanwhile, under high glucose condition, declined hnRNP A1 can be rescued by repressing lncRNA 585189. With the treatment of cloheximide (CHX) and MG-132, we also verified that lncRNA 585189 depressed the stability of hnRNPA1. Intriguingly, hnRNP A1 oppositely promoted the expression of lncRNA 585189. Moreover, RIP, pull-down, co-IP and actinomycin D (ActD) verified that hnRNP A1 simultaneously bound with the mRNA and protein of SIRT1 and promote the stability of SIRT1. Besides, lncRNA 585189 repressed SIRT1 via hnRNPA1, impeding SIRT1 recuperating mitochondrial abnormity and podocyte damage induced by high glucose. Collectively, our findings revealed that lncRNA 585189 combined with hnRNPA1 at nt224 and depressed the expression of hnRNP A1 and subsequently decreased SIRT1 in transcriptional and translational level, leading to mitochondria dysfunction and podocyte injury in DN.

Introduction

Diabetic nephropathy (DN), as a prevalent microvascular complication of diabetes, remains the primary cause of end-stage renal disease in developed countries and the primary contributor to morbidity and mortality of diabetes(Kopel et al., 2019). According to previous research, approximately 50% of all the patients with diabetes are suffering from renal insufficiency, and the elevated urinary albumin secretion is a primary sign of DN in clinical (Pyram et al., 2012; VR et al., 2019). Pathologically, a large collection of evidences have shown that the detachment and apoptosis of podocytes are central drivers to the destruction of the glomerular filtration membrane and the loss of filtration slits, which heavily contribute to the occurrence of albuminuria(Zhang et al., 2020b). However, the specific cellular and molecular mechanisms between high glucose and podocyte dysfunction are yet to be utterly illustrated.

Long non-coding RNAs (lncRNAs), featured with a lack of coding potential, belong to a kind of non-coding RNAs with > 200 nt in length. It has been reported that lncRNAs are responsive to the progress of diabetes and in connection with high glucose-associated podocyte damage(Loganathan et al., 2020). With RNA sequencing, we firstly revealed lncRNA ENST00000585189.1 (lncRNA 585189 for short) to be aberrantly increased in renal tissues of DKD. However, the underlying mechanisms for lncRNA 585189
mediated signaling is still unclear, and the further study of its effection in diabetic kidney is required to be accomplished.

Most of Long non-coding RNAs (lncRNAs) require to interact with one or more RNA-binding proteins (RBPs)(Ferrè et al., 2016). Recent studies highlighted the that function of RNA binding proteins (RBP) in regulating pathophysiological reaction to hyperglycemia(Cui et al., 2019; Wang et al., 2022). Based on the bioinformatics analysis, hnRNP A1 was screened out from a series of RBPs and predicted to bind with lncRNA 585189 in the tissue of diabetic kidney. In kidney tissues, hnRNP A1 plays an indispensable role in diabetic nephropathy induced renal interstitial fibrosis via exosomal sorting(Liu et al., 2021). In the previous study, there has been reported that hnRNP A1 controls mitochondrial dynamics by post-transcriptional regulation of Drp1, indicating that hnRNP A1 is essential to the integrity of mitochondrial morphology and function(Park et al., 2015). However, the precise mechanism by which hnRNP A1 participates in podocyte injury and mitochondrial regulation in DN has not been fully elucidated.

In our study, we focused on the function and value of the newfound lncRNA 585189 in the cultured human podocytes. As a consequence, we continued to investigate the interplay between lncRNA 5851891 and hnRNP A1, and their potential functions in podocytes might provide a valuable insight into the regulation of DN.

Results

LncRNA 585189 is significantly up-regulated in the patients with DN and high-glucose induced podocytes

A number of studies have demonstrated that lncRNA is an increasingly important research orientation in the procession of diabetic nephropathy(Chen et al., 2021; Xu et al., 2022). In our current studies, we collected renal tissues of DKD and the paracarcinoma renal tissues to screen aberrant expressed lnc RNAs. Compared with non-diabetic patient, RNA sequencing and data analysis revealed that the expression of lncRNA 585189 was significantly up-regulated in the patients with diabetic nephropathy (Fig. 1A). To further verify the result above, we examined the expression level of lncRNA 585189 in serums with qRT-PCR (Fig. 1B). During the inclusion period, plasma samples were collected from patients with diabetic nephropathy confirmed by renal biopsy and healthy volunteers. According to the albumin/creatinine ratio (ACR) levels, plasma samples were divided into three groups, including patients with microalbuminuria (30 mg/g < ACR ≤ 300 mg/g), macroalbuminuria (ACR > 300 mg/g) and the normal control group (ACR ≤ 30 mg/g)(Table.S1-2). Spearman's correlation analysis indicated that there was a nonnegligible positive correlation between the expression level of lncRNA 585189 and ACR (R = 0.513, P ≤ 0.001) (Fig. 1C), suggesting that lncRNA 585189 may play a noteworthy role in albuminuria. Moreover, accompanying by the robust increase of lncRNA 585189, there was a significant variation in the renal functional indicators including plasma albumin (R = -0.409, p = 0.003), serum BUN (R = 0.301, p = 0.004) and serum creatinine (R = 0.416, p = 0.002)( Fig. 1D-F).
Meanwhile, RNA fluorescence in situ hybridizations (RNA-FISH) were respectively implemented in the paraffin-embedded human renal tissues and the human immortalized podocytes (Fig. 1G). Compared with normal control group, lncRNA 585189 was observably increased in the renal tissues of patients with DN, while there was limited alteration in patients with minimal change disease or membranous nephropathy (Fig. 1J). Meanwhile, ImageJ analysis verified that lncRNA 585189 and Synaptopodin was colocalized, especially in DN(Fig. 1H-I). In podocytes, we discovered lncRNA 585189 was simultaneously located in the cytoplasm and nucleus, but primarily in the nucleus under the normal condition(Fig. 1K). 24 hrs of high glucose was sufficient to rise the in situ signals without resulting in any dislocation of lncRNA 585189 Fig. 1L-N . It is also interesting to note that fluorescence intensity of lncRNA 585189 raised under the high-glucose (30 mmol/L) condition, with the decline of Synaptopodin, indicating lncRNA 585189 may exert a critical effect to glucose-induced dysfunction of podocytes. Consistent with the analyses in tissue and plasma samples, high glucose expose led to a remarkable up-regulation of lncRNA 585189 in cultured human podocytes after 24 h of incubation with high-glucose (P < 0.001) (Fig. 10).

**Lncrna 585189 Regulates Podocyte Damage Under High-glucose Condition**

As RT-PCR and western-blot analysis revealed the level of ZO-1 was reduced under the high-glucose conditions, while expression of Desmin was increased, indicating that podocytes were injured with the stimulation of hyperglycemia(Fig. 2A-C). However, it is noteworthy that the aberrant expressions of Desmin and ZO-1 can be partially reversed by the knockdown of lncRNA 585189 in the RNA and protein levels (Fig. 2D-I). At the same time, Real-time PCR, western-blot and immunofluorescence staining also confirmed that the elevation of lncRNA 585189 significantly aggravated podocytes damage caused by high glucose, leading to the greater abnormal change in the expression of Desmin and ZO-1 (Fig. 2K-O). Besides, the transfection efficiency of small interfering RNA (siRNA) lncRNA 585189 and overexpression plasmid were confirmed in podocytes with real-time PCR (Fig. 2D and 2J).

**Lncrna 585189 Regulates Mitochondrial Function In Vitro**

Researchers have reported the hyperglycemia was one of the inducing factors of aberrant and we demonstrated it in the podocyte with ROS and Mito Tracker (Fig. 3A.B). Given the pathological roles of some lncRNAs have be verified in mitochondrial damage(Trewin et al., 2022), TMRM and Mito Tracker were employed to measure mitochondrial function with lncRNA 585189(Fig. 3C and 3D). Furthermore, with the stimulation of high glucose, the overexpression of lncRNA 585189 led to more downregulating of ROS, accompanying with the greater cutting down of the fluorescence intensity in TMRM (Fig. 3E and 3F). Moreover, ROS and TMRM staining suggested that high-glucose caused the loss of mitochondrial membrane potential and the alteration of pathological characters could be reversed by the knockdown of lncRNA 585189 (Fig. 3G-I). In a word, the favorable evidences showed that lncRNA 585189 was essential
in high glucose-induced podocyte mitochondrial deterioration, and knock-down of lncRNA 585189 manifested a protective effect on function of mitochondria in podocytes.

**LncRNA 585189 RNA binds with hnRNP A1 and depresses the stability of hnRNP A1 in podocytes**

Given the nuclear and cytoplasmic distribution of lncRNA 585189 in podocytes, we wondered whether lncRNA 585189 regulated mitochondrial dysfunction and podocyte damage by interacting with RNA-binding proteins (RBPs). Therefore, we turned to performing bioinformatics analysis. In our study, reliable data were collected from RBPDB, which was a database of RNA-bound protein information and constructed by the University of Toronto, Canada. By the means of analyzing and classifying domains of the proteins from RBPDB data and searching the literature review from Pfam database, we extracted, analyzed, and determined the motif sequences of proteins, calculating the RNA binding proteins of lncRNA 585189 through systematic comparison. Finally, the hnRNP A1 was predicted to combine with lncRNA 585189(Fig.S1). To further verify the interaction between hnRNP A1 and lncRNA 585189, a series of tests and analyses were performed in podocytes. Primarily, RNA-FISH suggested the similar subcellular locations of lncRNA 585189 and hnRNP A1, and the reduce of hnRNP A1 is synchronous with the rise of lncRNA 585189 under high glucose condition (Fig. 4A-D). Additionally, RNA pull-down assays and western blot were performed and the biotinylated lncRNA 585189 probes specifically pulled down hnRNP A1 protein (Fig. 4E). Furthermore, we also demonstrated this interaction by RNA-binding protein IP (RIP) assays (Fig. 4F). In contrast with the control IgG sample, the level of lncRNA 585189 was notably enriched in podocytes. To further explore the binding, we also predicted that lncRNA 585189 harbors two candidate hnRNP A1 binding motifs, including the hexamers beginning at nucleotides nt224 and nt237 respectively. As it was shown in (Fig. 4G), there was an electrophoretic mobility shift for the hnRNP A1 protein at nt224 in a dose-dependent manner, in keeping with the relatively higher prediction score for this motif. In other word, these results supported that hnRNP A1 bounded to lncRNA 585189 at the nt 224 motif (TAGGGA).

Furthermore, we observed that the expression of hnRNP A1 was decreased in high glucose induced podocytes, while the knockdown of lncRNA 585189 markedly promoted the hnRNP A1 in the levels of protein and mRNA, rescuing the high-glucose repressed hnRNP A1 to some extent (Fig. 4H-J). Intriguingly, hnRNP A1 was noticed to facilitate the expression of lncRNA 585189 in the podocyte, indicating the mutual regulation between hnRNP A1 and lncRNA 585189 (Fig. 4K and 4L). Meanwhile, protein synthesis inhibitory cloheximide (CHX) and the proteasome inhibitor MG-132 were used to determine whether lncRNA 585189 reduced hnRNP A1 protein stability. As shown in Fig. 4M, we treated vector and overexpression plasmid of lncRNA 585189 podocytes with CHX at the same time, and the results demonstrated that the degradation rate of hnRNP A1 in overexpressed lncRNA 585189 podocytes was dramatically accelerated compared with the control group. In addition, overexpressed lncRNA 585189 triggered the downregulation of hnRNP A1, while MG-132 partly restrained the downregulation, which indicated that proteolysis of hnRNP A1 in overexpressed lncRNA 585189 podocytes is mediated by proteasomes (Fig. 4N).
Hnrmpa1 Binds With Sirt1 Through Multiple Approaches And Promotes The Stability Of Sirt1

In previous researches, hnRNPA1 has been reported to rise the expression of SIRT1, upregulating the function of mitochondria (Gui et al., 2020; Wang et al., 2016). As a result, we determined to explore the interaction of hnRNPA1 and SIRT1 in podocyte. Firstly, we adopted a biotinylated hnRNPA1 probe to carry out an RNA pull-down assay, followed by western blot. And the obtained result showed that SIRT1 could apparently combine with the hnRNPA1 (Fig. 5A). Similarly, we performed RIP assay by incubating anti-hnRNPA1 antibody with total RNA extracts from podocytes. Unsurprisingly, real-time PCR specific showed the present in the hnRNPA1 RIP sample was at a drastically higher level, compared with negative control sample (Fig. 5B). Notably, co-IP testified that hnRNPA1 also bond with SIRT1 in the protein level (Fig. 5C).

Under the high-glucose condition, we demonstrated SIRT1 was decreased at both translational and transcriptional levels, while the decline of SIRT1 can be reversed by overexpressed hnRNPA1, implying the effect of hnRNPA1 on SIRT1 in podocyte (Fig. 5D-G). Generally speaking, hnRNPA1 facilitated SIRT1 expression relying on its binding with SIRT1 mRNA and protein. Besides, to further test the interactive effect, we performed actinomycin D (ActD) exposure to hnRNPA1 RNA synthesis in podocytes (Fig. 5H). Analysis and assessment of data implied that the binding between SIRT1 and hnRNPA1 markedly extended SIRT1 mRNA half-life in the podocytes.

SIRT1 alleviates podocyte damage and mitochondria dysfunction

On account of SIRT1 has been reported to protect mitochondria from oxidatively injury, we tested the role of SIRT1 in mitochondrial morphological and functional abnormalities and podocyte damage (Yacoub et al., 2014). On the one hand, Mito Tracker, TMRM and ROS proved that, the mitochondrial damage decreased in the podocytes with raised SIRT1. (Fig. 6A-C). On the other, we noticed the increase of SIRT1 could alleviate the podocyte injury induced by high-glucose (Fig.6D-H). In a word, SIRT1 were veried to reverse podocyte damage and mitochondria dysfunction brought out by high-glucose stimulation.

Lncrna 585189 Induces Podocyte Damage And Mitochondria Dysfunction Via Repressing Hnrmpa1 And Sirt1

As we mentioned above, Western blot and PCR demonstrated that the knockdown of LncRNA 585189 or overexpression of hnRNPA1 could respectively restore the expression of SIRT1 under high glucose (Fig. 7A and 7B. Figure 5F-G). Hence, we wonder whether the procession of LncRNA 585189 regulating SIRT1 is related to hnRNPA1, and demonstrated that the downregulate of SIRT1 caused by LncRNA 585189 can be rescued by upregulated hnRNPA1 (Fig. 7C).
In addition, SIRT1 is an indispensable regulatory element for podocyte damage and mitochondria dysfunction (Fig. 6A-C). The alteration of ZO-1 and Desmin verified that, the podocyte damage brought out by overexpressed IncRNA 585189 can also be rescued by rising hnRNPA1 (Fig. 7D-F). Besides, the increased hnRNP A1 could effectively reverse the mitochondrial injury, decrease the production of ROS, restore the alteration of the mitochondrial membrane caused by elevated IncRNA 585189, which was proved by the detection of ROS and TMRM staining (Fig. 7G-H).

In brief, we can reach a conclusion that IncRNA 585189 combine with hnRNP A1 and repress hnRNP A1, subsequently depress SIRT1 in translational and transcriptional level, regulating the homeostasis of mitochondrial and inducing hyperglycemic podocyte damage (Fig. 7I).

**Discussion**

Recently, there are a plenty of lncRNAs involving in in multifarious cellular function in the podocyte and diabetic nephropathy (Loganathan et al., 2020). To screen differential expression lncRNAs in the aspect of transcriptome, we performed RNA sequencing and firstly discovered that IncRNA 585189 was risen in the tissues of patients diagnosed with diabetic nephropathy (Fig. S1). Besides, RNA-FISH verified the IncRNA 58518 was specifically increased in patients suffering from diabetic nephropathy, rather than minimal change disease or membranous nephropathy, implying IncRNA 58518 may be one of the indicators of diabetic nephropathy. Then, we analyzed the plasma samples from the normal control group and the patients suffering from diabetic nephropathy. The increase of IncRNA 585189 in the diabetic nephropathy plasma samples was illuminated to be correlation to albumin/creatinine ratio (ACR) levels, accompanied with the abnormity in plasma albumin, serum BUN and serum creatinine, indicating the unique value of IncRNA 585189 in the field of pathophysiology and clinic.

Furthermore, studies have demonstrated the damage of podocyte has been considered as one of the major causes of proteinuria and the essential clinical sign of renal failure in diabetes (Zhang et al., 2020a). As a kind of highly specialized, terminally differentiated cells, podocytes have few abilities in self-renewing. In order to maintaining the complex structure and normal function, podocytes require quantities of healthy mitochondria to fill the energy demand (Audzeyenka et al., 2022). As a result, the disruption of mitochondrial associated pathways in the kidney is an important driving factor underlying DN. However, as far as we known, the potential mechanism of mitochondrial dysfunction in podocytes of diabetes is not fully understood. In our current study, we noticed that the elevation of IncRNA 585189 could damage mitochondrial membrane potential and podocytes integrality, releasing ROS, even dramatically influence podocyte. But the pathological transform could be reversed by the knockdown of IncRNA 585189.

It's worth noting that recent studies emphasized that lncRNA located in nucleus can regulate the downstream through interacted with RNA binding proteins (RBP) (Cao et al., 2018; Marchese et al., 2017). Therefore, we identified the subcellular location of IncRNA 585189 in the podocytes of DN, and predicted that there is a precise and intricate network of multiple RBPs behind IncRNA 585189. The single most
striking observation to emerge from the bioinformatics analysis and data comparison was hnRNP A1 can interacted with IncRNA 585189. Forceful evidences indicated that hnRNP A1 can bind to a specific hexamer of the IncRNA 585189 at nucleotides (nt) 224, which was significant to hnRNPA1 transcription. Additional, IncRNA 585189 was verified to repress the protein stability of hnRNP A1. The overexpression of IncRNA 585189 is conducive to accelerate the degradation of hnRNP A1, which is probably associated with proteasome pathway.

Intriguingly, RBPs were not only regulated by IncRNA, but also mediated the localization, translation, modification and stability of IncRNA, modulating the progression of diabetic complication(Hentze et al., 2018; Sebastian-delaCruz et al., 2021). For example, as a classical RBP and a kind of nucleocytoplasmic shuttling heterogeneous nuclear ribonucleoprotein, hnRNP A1 is reported to suppressing gluconeogenesis and lipogenesis, depressing insulin resistance (Gui et al., 2020; Wang et al., 2018). Meanwhile, hnRNP A1 also participates in transcription, splicing, stabilization and translation of transcripts of RNA(Gui et al., 2020). We observed, the decline of hnRNP A1 was observed in high glucose induced podocytes and the repression of IncRNA 585189 restored the expression of hnRNPA1. Whereas, the over-expression of IncRNA 585189 facilitated the degradation of hnRNP A1, which constituted a negative feedback combined with former finding. Surprisingly, the IncRNA 585189 didn't maintain at a relatively stable level under the hyperglycemia, but prominent elevated in the tissues and plasma of DN patients and human cultivated podocytes. Following reasons can be used to explain the finding mentioned above. On the one side, the risen transcription of IncRNA 585189 may attribute to comprehensive regulation from multiple regulators, which is in keeping with our previous statement of bioinformatics analysis (Fig S1). On the other side, M6A modification was reported to play a role in the upregulation of IncRNA and promote epithelial-mesenchymal transition (Wu et al., 2019), suggesting that post-transcriptional modification can take part in the activation of Inc RNAs. Similarly, IncRNA 585189 may be posttranscriptional modified and continuous activated by the high glucose stimulation, but the mechanism needs to be further exploring and explaining, at the same time, more clinical longitudinal studies and specific grouping will be imperative to accomplished.

Several independent studies revealed that hnRNP A1 can combine with SIRT1, modulating insulin resistance and PKM2-dependent glycolytic pathway (Gui et al., 2020; Wang et al., 2016; Yang et al., 2019). However, the concrete interactive mechanism between hnRNP A1 and SIRT1 in podocytes suffering from diabetic nephropathy was limited. Hence, we proved that the hnRNP A1 could respectively specific connection with the mRNA or the protein of SIRT1 in the podocyte, and promoted the stability of SIRT1.

Furthermore, regulation targeting to SIRT1 also verified to influence podocyte injury and mitochondrial dysfunction in DN (Wang et al., 2021).Our research bear out the finding, and further determined that SIRT1 alleviated mitochondrial abnormality, restrained the overproduction of ROS, and restored the mitochondrial membrane potential. Additionally, the decline of SIRT1 induced by IncRNA 585189, can be restored by hnRNP A1. Given that the agonists of SIRT1 can diminished diabetic kidney injury via various methods and pathway, such as resveratrol, puerarin, MS417 and BF175, the drugs targeted to suppress
lncRNA 585189 may be another potential direction in treatment of diabetic kidney (Hong et al., 2018; Zhong et al., 2018).

In brief, our study revealed that lncRNA 585189 specific combined with hnRNPA1 at nt224 and depressed the expression and the stability of hnRNP A1, depressing SIRT1 in transcriptional and translational level, finally causing mitochondria dysfunction and podocyte injury in DN. Our study is helpful to enriching the knowledge in the aspect of molecular interaction and mitochondrial homeostasis, and may provide a novel mind in therapeutic methods of diabetic kidney.

Materials And Methods

Sample collection

Plasma samples in diabetic nephropathy group were collected from the Department of Nephrology in Shandong Provincial Hospital, while the samples in the normal control group were collected from the healthy volunteers in Physical Examination Center. Similarly, freshly frozen DN renal biopsy samples and normal kidney samples (> 2 cm adjacent to urological neoplasms) were also collected.

The related biochemical indexes of each group were shown in Table S1.

Cell Culture And Treatment

Conditionally immortalized human podocytes were kindly provided by Professor Saleem MA from University of Bristol at UK. In a nutshell, podocytes were fed in RPMI 1640 medium (Gibco, Grand Island, USA) with 10% vesicle-free foetal bovine serum (Gibco, Grand Island, USA) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) and cultured in a humidified incubator with 5% CO2 and 95% saturation humidity. The cells were cultured at 37 °C for 7 days to induce differentiation, after that, podocytes were cultured with RPMI 1640 containing low glucose (LG, 5.5 mmol/L D-glucose), high glucose (HG, 30 mmol/L D-glucose) and hyperosmolality (HO, 5.5 mM D-glucose plus 24.5 mM mannitol) for 24 h.

The protease inhibitor MG132 and actinomycin D (ActD) were all purchased from Sigma-Aldrich (USA), while cycloheximide (CHX) was procured by Aladdin (Shanghai, China).

Vectors Constructions And Transfection

The oeLncRNA 585189.1, oehnRNP A1, oeSIRT1, LncRNA 585189siRNAs and the NCs were constructed by Genomeditech (Shanghai, China). Transfection of podocytes was performed in a 6-well plate with the plasmids or siRNA using Lipofectamine3000 reagent (Invitrogen,USA).
Reagents

The detailed informations of the antibodies were collected in Table S3.

Real-time Reverse Transcriptase-pcr (Rt -pcr)

According to the manufacturer’s instructions, BIOG cfRNA Easy Kit (BIOG, Changzhou, China) and Trizol Reagent (Takara Biotechnology, Japan) were respectively used to extracted RNA in the plasma and cultured podocytes. The detail of the RNA reverse transcription and amplification were described as previously(Lv et al., 2013). β-actin acts as an internal reference and the consequence of the relative expression was calculated with the $2^{-\Delta\Delta CT}$ method or a log transformation after the $2^{-\Delta CT}$ method later. Every experiment was calculated three times.

The primers of target genes in our study were designed and synthesized by Biosune (Shanghai, China), which were listed in Table S4.

Western Blot Analysis

The protocol was described previously(Lv et al., 2013). To be specific, primary antibodies against the following target proteins: ZO-1 (1:1000), Desmin (1:1500), hnRNP A1 (1:1000), SIRT1 (1:20000), and β-actin (1:1000). The specific bands were visualized by ECL reagent (Millipore, USA) the Bio-Rad electrophoresis image analyser (Bio-Rad, Hercules, CA, USA). Each experiment was repeated 3 time.

Immunofluorescence Assay (If)

For immunofluorescence, fixed podocytes were immunostained at 4°C overnight, accompanied with primary antibodies, such as ZO-1 (1: 200), desmin (1:200), hnRNP A1 (1:100) and SIRT1(1:150). Then we choose appropriate fluorescent secondary antibody incubated the podocytes for 1–2 h at 37°C. Images were captured under afluorescence microscope (Leica, Germany) and analyzed by ImageJ 10.2 software prudently.

RNA-FISH

According to the instructions of fluorescence in Situ Hybridization kit (Ribio,Guangzhou, China), cultured podocytes in 6-well plates were fixed in 4% paraformaldehyde for 10 min. After washed and reacted with pre-chilled 0.5% Triton X-100 and treated with pre-hybridization buffer for 30 min to block non-specific binding, we discarded the pre-hybridization solution and add the preheated hybridization solution containing the probe at 37°C overnight. The followed steps would also be performed in dark conditions. Following the order in the instructions, the cells were rinsed at 42°C with Buffer I, II and III in successively.
After the stained by DAPI (1:800) for 10 minutes, cells images were observed and the images were captured under a fluorescence microscope (Olympus, Japan)

As for the tissues, the prepared paraffin sections of kidney tissues were baked, dewaxed and hydration. After preheating the slices, they were placed in the hyperthermal antigen repair solution for 3 min and immerse in PBS 3 times, 5 min each. Next, the process of permeation, pre-hybridization, hybridization, and DAPI stain were performed as above-mentioned. Finally, observe and photograph under fluorescence microscope. The experiment should be ration repeated at least 3 times.

**RIP**

The cells were used to perform RNA immunoprecipitation (RIP) experiments using a hnRNP A1 antibody (ABclonal) and the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, 17–701, USA) according to the manufacturer's instructions. The cells were lysed in complete RIP lysis buffer. A total of 100 µl of whole cell extract was incubated with RIP buffer containing protein G-agarose beads conjugated with antibodies against hn-RNPA1 or control IgG antibodies (Millipore) for 6 h at 4°C. The beads were washed with wash buffer, and then the complexes were incubated with 0.1% SDS/0.5 mg/ml Proteinase K (30 min at 55°C) to remove the proteins. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific). Finally, the immunoprecipitated RNA was analysed by qRT-PCR.

**Rna Pulldown**

The IncRNAs were transcribed in vitro using a MAXIscript™ SP6/T7 Transcription Kit (Invitrogen, AM1320, USA) and were biotinylated with a Pierce RNA 3’ End Desthiobiotinylation Kit (Thermo Fisher Scientific, 20163) according to the manufacturer’s instructions. The proteins were extracted from podocytes using Pierce IP Lysis Buffer (Thermo Fisher Scientific). Then, RNA pull-down assays were performed with a Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, 20164). Briefly, the biotinylated IncRNAs were captured with streptavidin magnetic beads and incubated with the cell lysates at 4°C overnight. Then, the beads were washed with washing buffer. The eluted proteins were detected by western blot analysis.

**Co-immunoprecipitation**

Co-IP assays were performed according to standard protocols (YJ201, Epizyme, Shanghai, China). Then, the cells were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was collected, followed by incubation with primary antibodies or isotype immunoglobulin G (IgG), gentle rocking 1h at room temperature. Then, 25 µL of Protein A/G beads were added and incubated overnight at 4°C. The next day, the mixtures were washed three times with immunoprecipitation lysis buffer and 80 µL of 1 × loading buffer were added to the final pellet and incubated at 100°C for 10 min. The supernatants were collected and proceeded to SDS-PAGE Western blot analysis.
Rna Electrophoretic Mobility Shift Assay (Emsa)

LightShift™ Chemiluminescent RNA EMSA Kit (Pierce, Rockford, IL, USA) was used to conduct the RNA EMSA assay. At the same time, all of the biotin-labeled probes, cold probes (unlabeled probes) and mutant probes, were synthesized (Beyotime, Shuzhou, China). According to the manufacturer's protocol, 50 fmol of biotin-labeled RNA probes were incubated with hnRNPA1 (Cambridge, MA, USA), and 200-fold molar excess of the unlabeled probe (cold probe) were used to perform the competition experiments. Then, the protein/RNA probe complex was separated by electrophoresis and transferred onto a PVDF membrane. Moreover, the membranes were cross-linked under a UV light, followed by incubated with streptavidin-HRP solution at room temperature. Using the chemiluminescence reagent (Beyotime), the biotin-labeled probe was visualized and then analyzed with Gel-Pro-Analyzer software.

Detection Of Intracellular And Mitochondrial Ros Production

According to the manufacturer's instructions, DCFH-DA and MitoSOX Red (Beyotime, Shanghai, China) was respectively incubated with the podocytes and analyzed under a fluorescence microscope (Leica, Germany).

TmrM-staining Of Mitochondrial Membrane Potential

Podocytes were incubated with medium containing 100 nM tetramethyl rhodamine methyl ester (TMRM; Invitrogen) for 40 min at 37°C, Thereafter, fluorescence microscopy were used to obtain and collect the images, accompanied with analyzing with ImageJ 10.2 software to measure the mean fluorescence densities of the regions of interest.

Statistical analysis

Data analysis was performed with SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) and all data are expressed as the means ± standard deviation. Unpaired Student t tests and One-way analysis of variance (ANOVA) were applied to estimate the means of two groups or multi-groups. P < 0.05 was considered to be statistically significant.

Declarations

Ethical Approval

Each experimental procedure of human study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University, and complied with the principles of the Declaration of Helsinki. Signed informed consents were strictly obtained from all enrolled participants or their families.
Declaration of interests

The authors declare no competing interests.

Author contributions

Huimin Chen performed the material preparation and data collection, Yue Liu conceived and drafted the review article, and data analysis. Tingwei Zhang, Tongtong Huang and Yating Lang created the model figure. Qinghao Sheng and Yingxiao Liu prepared the tables. Zhijuan Kong, Ying Gao, Shangwei Lu were involved in the compilation of the references. Meilin Yang and Yaqi Luan revised the manuscript. Among them Huimin Chen and Yue Liu contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

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Data availability

The data sets used and/ or analyzed during the current study are available from the corresponding author on reasonable request.

References


**Figures**
Figure 1

**IncRNA 585189 is significantly up-regulated in the podocytes of diabetic nephropathy**

A. Heat map for differentially expressed IncRNA in the renal tissues of DN and the control renal tissues.
B. LncRNA 585189 expression in the plasma from normal urinary albumin subjects (n = 52) and patients presenting microalbuminuria (n = 26), and macroalbuminuria (n = 26). The expression level was shown
with the log₁₀2^−ΔCT, Δ CT = CT_{objective gene} − CT_{β-actin}. **P < 0.01, **** P < 0.0001 The experiment was repeated three times independently. C. The correlation between IncRNA 585189 expression and ACR (R = 0.513, p < 0.001) (n=52). D. The correlation between IncRNA 585189 expression and BUN (R = 0.391, p = 0.004) (n=52). E. The correlation between IncRNA 585189 expression and plasma albumin (R = -0.409, p = 0.003) (n=52). F. The correlation between IncRNA 585189 expression and SCR (R = 0.416, p = 0.002) (n=52). G. RNA-FISH illustrated the distribution of IncRNA 585189 and its relative position to Synaptopodin in paraffin-embedded human sections by fluorescence microscope (n = 3). The podocytes were labeled with arrows. Scale bar, 50μm. H-H. Rectangular scans of fluorescence intensity were measured using ImageJ software, showing colocalization of IncRNA 585189 and Synaptopodin. J. RNA-FISH identified the distribution of IncRNA 585189 and its relative position to Synaptopodin in minimal change disease (MCD) and membranous nephropathy (MN) by fluorescence microscope (n = 3). The podocytes were labeled with arrows. Scale bar, 50μm. K. RNA-FISH revealed the subcellular location and expression of IncRNA 585189 under LG, HG or HO conditions in the podocytes by fluorescence microscope (n = 3). Scale bar, 50μm. LG: low-glucose; HG: high-glucose; HO: hyperosmolality. L-N. Rectangular scans of fluorescence intensity identified the subcellular colocalization of IncRNA 585189 in LG(L), HG(M) or HO(N). O. Real-time PCR detected the expression of IncRNA 585189 with the treatment of high-glucose in human podocytes. **P< 0.001 (n = 3).
Figure 2

**Effects of IncRNA 585189 on podocyte damage**

A-C. Real-time PCR and Western blot demonstrated the expression of Desmin and ZO-1 after LG, HG or HO stimulation for 48 h. *P < 0.05 **P < 0.01 (n = 3).

D. The level of IncRNA585189 after silencing of IncRNA 585189 in podocytes. ***P < 0.001 (n = 2).

E-F. The RNA levels of Desmin and ZO-1 in response
to the treatment of LG, HG, HG+si-NC, and HG+si-585189. *P<0.05, **P<0.01, ***P<0.001 (n=4). G. Western blot revealed the level of Desmin and ZO-1 with the treatment of LG, HG, HG+si-NC, and HG+si-585189 (n=4). H-I. Immunofluorescence staining showed the intensities of Desmin (H) and ZO-1 (I) with the treatment of LG, HG, HG+si-NC, and HG+si-585189 (n = 4). Scale bar, 50 μm. J. The level of lncRNA585189 after transfected with the over-expression lncRNA 585189 plasmids in podocytes. ***P<0.001 (n=2). K-M. Real-time PCR and Western blot measured the Desmin and ZO-1 after transfection of oe-585189. *P<0.05, ***P<0.001 (n=4). N-O. Immunofluorescence staining showed the intensities of Desmin (N) and ZO-1 (O) (n = 4) with the treatment of LG, HG, LG+oe-NC, and LG+oe-585189. Scale bar, 50μm.
Figure 3

Influence of IncRNA 585189 to mitochondrial dysfunction.

A-B. ROS and Mito Tracker staining were used to detect the mitochondrial membrane potential on the high-glucose condition. (n = 3). Scale bar, 50μm.
C.D. Mito Tracker and TMRM determined the mitochondrial function with elevated lncRNA 585189 (n = 2). Scale bar, 50μm. E.F. TMRM and ROS observed mitochondria of podocyte in response to LG, HG, LG+ oe-NC, LG+oe-lnc585189 (n = 4). Scale bar, 50μm. G-I. ROS, TMRM and Mito Tracker showed mitochondrial conditions of podocytes after silencing of lncRNA 585189 (n = 4). Scale bar, 50μm.
IncRNA 585189 RNA Bind with hnRNP A1 and depresses the stability of hnRNPA1

RNA-FISH suggested the subcellular location and the expression of IncRNA 585189 and hnRNP A1 in response to the treatment of LG, HG, HO (n = 3). Scale bar, 50μm. B-D. Rectangular scans of fluorescence intensity were demonstrated the colocalization of IncRNA 585189 and hnRNP A1 after LG(B), HG(C) or HO(D) stimulation. E HnRNP A1 is pulled down by IncRNA 585189 in the podocytes (n = 3). F. RIP revealed the interaction of IncRNA 585189 and hnRNP A1 in podocytes ***P<0.001 (n = 4). G. Motif analysis. Two putative HNRNPA1 binding motifs were predicted in the HBBP1 transcript where the nt224 motif was validated by EMSA assay (n=1). H-I. Real-time PCR and Western blot revealed the level of hnRNPA1 after the knockdown of Inc585189, *P<0.05, **P<0.01 (n=4). J. Immunofluorescence showed the staining intensity of hnRNPA1 with silencing Inc585189 (n = 4). Scale bar, 50μm. K. Lnc585189 was measured by Real-time PCR after transfection of over-expressed hnRNPA1, * P<0.05 (n=4). L. RNA-FISH demonstrated the level of Inc585189 with over-expressed hnRNPA1 (n = 4). Scale bar, 50μm. M. Podocytes incubated with 20 mg/ml CHX (the protein synthesis inhibitor) at the indicated time points (0, 20, 40, 60 and 80 min) after overexpression of Inc585189. And all the collected samples were subjected to Western blot analysis(n=4). N. The blocking effect of proteasome inhibitor (MG132) on the degradation of hnRNPA1 mediated by oelnc585189 in podocytes (n=4).
**Figure 5**

HnRNPA1 binds with SIRT1 and promotes the stability of SIRT1

A. The binding between SIRT1 and hnRNP A1 was determined by RNA pulled down in the podocytes (n=3).
B. RIP suggested the interaction between SIRT1 and hnRNP A1 in podocytes *** P <0.001 (n = 4) C. Podocyte lysates were immunoprecipitated with anti-SIRT1 antibody or rabbit IgG, and immunoblotted
with anti-hnRNP A1 antibody (n=3). **E.** Real-time PCR and Western blot demonstrated the expression of SIRT1 after LG or HG stimulation for 48 h. ***P < 0.001 n = 3.** **F-G.** The level of SIRT1 upon oe-hnRNP A1 treatment, ****P < 0.0001 (n=4). **H.** hnRNP A1 overexpression prolonged SIRT1 mRNA half-life. Podocytes were transfected with oe-hnRNP A1 or oe-NC for 48 h and then exposed to actinomycin D (2 μg/ml) at 0, 1, 2, 3, 6, and 9 hours total RNAs were isolated at indicated times and subjected to real-time PCR to assess the half-life of SIRT1 mRNA. These experiments were conducted on three independent occasions. t-test, mean ± SD, **P < 0.01 (n=2).**
Figure 6

SIRT1 can be promoted by hnRNPA1 and alleviates podocyte damage and mitochondria dysfunction

A-C. TMRM, ROS and Mito Tracker measured the mitochondrial membrane potential in the podocytes with oeSIRT1. (n = 4). Scale bar, 50μm. D-E. The level of Desmin and ZO-1 after transfection of oeSIRT1 were demonstrated by Real-time PCR (n=4) *P <0.05, ** P <0.01. F-H. The protein level of Desmin and ZO-1 with oeSIRT1 were detected by Western blot and immunofluorescence. (n=4) Scale bar, 50μm.
LncRNA 585189 induces podocyte damage and mitochondria dysfunction via repressing hnRNPA1 and SIRT1

**A-B.** PCR and Western blot demonstrated SIRT1 expression after the silencing of LncRNA 585189 under high glucose n=4. **C-F.** The RNA and protein level of SIRT1, Desmin and ZO-1 with the treatment of oe-

Figure 7
vector oe-585 oe-hnmpa1 oe-hnmpa1+oe-585. n=4 **P<0.01,****P<0.0001 G-H. ROS and TMRM staining suggested the mitochondrial membrane potential on different condition. Scale bar, 50μm. I. Proposed model of lncRNA 585189 mediated regulation of mitochondria dysfunction and podocyte damage.

**Supplementary Files**

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