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Original Paper

The Long Noncoding RNA 150Rik Promotes Mesangial Cell Proliferation via miR-451/IGF1R/p38 MAPK Signaling in Diabetic Nephropathy

Yajuan Zhang[a,b] Yan Sun[a] Rui Peng[c] Handeng Liu[a] Weihao He[d] Luyu Zhang[a] Huimin Peng[a] Zheng Zhang[a]

[a]Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing, [b]Department of Medical Genetics Center, Southwestern Hospital, Chongqing, [c]Department of Bioinformatics, Chongqing Medical University, Chongqing, [d]Chongqing Nankai Middle School, Chongqing, China

Key Words
lncRNA • miR-451 • Cell proliferation • Diabetic nephropathy

Abstract
Background/Aims: Diabetic nephropathy (DN) as the primary cause of end-stage kidney disease is a common complication of diabetes. However, the initiating molecular events triggering DN are unknown. Recently, long noncoding RNAs (lncRNAs) have been shown to play important roles in DN. ***Methods:*** The expression level of lncRNA 1500026H17Rik (150Rik for short) was measured by qRT-PCR (quantitative real-time PCR). Cell proliferation ability was detected by 5-Ethynyl-2′-deoxyuridine (EdU). The relationship between 150Rik and microRNA 451 (miR-451) was examined by luciferase assay and RNA immunoprecipitation (RIP) assay. Finally, the effect of 150Rik on cell proliferation through the miR-451/insulin-like growth factor 1 receptor (IGF1R)/mitogen-activated protein kinases (p38MAPK) pathway was detected by EdU, flow cytometry analysis, western blot. ***Results:*** We found that 150Rik, an evolutionarily conserved lncRNA, was significantly upregulated in renal tissue of db/db DN mice and in mesangial cells (MCs) cultured under a high glucose condition. Further, overexpression or knockdown of 150Rik was found to regulate cell proliferation in MCs. Moreover, 150Rik was found to interact with miR-451 in both a direct and argonaute-2 (Ago2)-dependent manner. Results also revealed that overexpression of 150Rik inhibited cell proliferation through the miR-451/IGF1R/p38MAPK pathway in MCs under the high glucose condition, while knockdown of 150Rik increased cell proliferation via the miR-451/IGF1R/p38MAPK pathway. ***Conclusion:*** Taken together, these results provide new insight into the association between 150Rik and the miR-451/IGF1R/p38MAPK signaling pathway during DN progression.

Y. Zhang and Y. Sun contributed equally to this work.

Dr. Zheng Zhang
Molecular Medicine and Cancer Research Center, Chongqing Medical University
No.1 Medical College Road, Chongqing 400016 (China)
Fax +86 23 68485555, E-Mail zhangzheng92@163.com

Introduction

Diabetic nephropathy (DN) is one of the main microvascular complications of diabetes. The pathogenesis of DN is closely related to a variety of factors, including the presence of glomerular mesangial lesions, the proliferation of mesangial cells (MCs) and the proliferation of extracellular matrix [1-4]. However, the mechanism of MC proliferation is not fully elucidated.

Long non-coding RNAs (lncRNAs) are defined as a large class of pervasive transcriptions in the genome with poor or no protein-coding ability; lncRNAs are longer than 200 nucleotides. Growing evidence indicates that lncRNAs are key modulators of various physiological and pathological processes in disease states [5-8], including cell proliferation and apoptosis and invasion; they are also markers of cell fate and are involved in reprogramming of induced pluripotent stem cells and chromatin modification [9]. In the past decade, rapid development of high-throughput sequencing technologies and bioinformatic analytical methods has enabled researchers to identify lncRNAs that play major roles in DN. Researchers have found that lncRNA PVT1 regulates proliferation of MCs under the high glucose condition in DN [10, 11]. In another study, lncRNA TUG1 and ASncmtRNA-2 were reported to participate in the occurrence and development of DN [12]. Moreover, our previous study indicated that lncRNA GM4419 regulates inflammation in DN [13].Recent reports have also demonstrated that lncRNAs may act as competing endogenous RNAs (ceRNAs) for miRNAs, adjusting the expression of their target genes in certain diseases [14]. In a previous study, we found that miR-451 can regulate mesangial hypertrophy and inflammation in DN [15, 16]. To investigate the potential relationship between miRNA and lncRNA, in this study we searched ncRNAs that were associated miR-451 among the dysexpressed ncRNAs in renal tissue of db/db DN mice and MCs cultured under a high glucose condition. LncRNA 1500026H17Rik (150Rik for short) was one of the related lncRNAs and the expression of 150Rik was most obviously changed in MCs cultured with high glucose. The novel lncRNA 150Rik, a 3027bp lncRNA, has not been reported until now. Therefore, the function of 150Rik and its molecular mechanisms in DN are unknown.

The aim of the present study was to investigate whether lncRNAs are mechanistically involved in MC proliferation in DN. As discussed above, our data led to the identification of 150Rik as a likely regulator of MC proliferation. Further, we found that 150Rik acts as a ceRNA to regulate miR-451-targeted insulin-like growth factor 1 receptor (IGF1R) in DN and can promote MC proliferation via the miR-451/IGF1R/p38MAPK pathway. Our results provide novel insight into the mechanism underlying the proliferation of MCs in DN and could serve as a stepping stone for the development of intervention and prevention approaches for DN.

Materials and Methods

Animals

Naturally developed diabetic nephropathy in mice with genetic defects in the leptin receptor (db/db) is a well-established model for type 2 diabetes, obesity and insulin resistance. Moreover, db/db mice are widely used as diabetic nephropathy model mice because they display elevated serum creatinine, urinary albuminuria and proteinuria, as well as increased fibrosis in the proximal tubule and mesangial matrix expansion; all of these characteristics are reminiscent of the human disease. Four-week old male db/db mice with C57BL/BKS background and age-matched male genetic control db/m mice were purchased from NBRI (Nanjing, China). At eight weeks of age, db/db mice were considered to be at the early stage of DN [17]. After this time, the db/db-miR-451 group and the db/db-miR-NC (Normal Control) group were injected every second day via the tail vein with either an miR-451 agomir (RiboBio, Guangzhou, China) or an agomir NC (RiboBio, Guangzhou, China) at a dose of 10 mg/kg/day for four weeks until the urinary microalbumin level of the db/db-miR-451 group was lower than that of the untreated db/db group [16]. The mice were divided into four groups: CTR (Control) group (db/m, n=3), DN group (db/db, n=3), miR-NC-treated DN group (DN-miR-NC, n=3) and miR-451-treated DN group (DN-miR-451, n=3). All procedures were in accordance with

the institutional guidelines for the care and use of laboratory animals at Chongqing Medical University. Ethics approval was obtained from the Ethics Committee of Chongqing Medical University. The renal tissues of mice in the CTR and DN group were further used for RNA sequencing and qRT-PCR and the renal tissues of mice in the DN-miR-NC group and DN-miR-451 group were further used for RNA sequencing.

Cell culture of MCs

Mouse glomerular MCs were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in DMEM with 20% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37°C. Based on previous research [18], the mouse glomerular MCs were excitedwith5.5 mmol/L glucose 19.5 mmol/L (low glucose group, L-MC) or 25mmol/L glucose (high glucose group, H-MC). The high glucose condition simulates the growth environment of mouse glomerular MCs under the state of DN and the low glucose condition simulates the normal growth status.

RNA sequencing

Total RNA from each group of mice was isolated using TRIzol® Reagent (Life Technologies) in accordance with the manufacturer's instructions. Firstly, we measured RNA quality and quantity with an Agilent 2100 Bioanalyzer (Agilent Technologies). Then, the RNA samples were used for constructing a cDNA library using the TruSeq™ RNA Library Preparation Kit v2 protocol (Illumina); sequencing was performed with an Illumina HiSeq2000 sequencer from Beijing Genomics Institute (BGI, Shenzhen, China). The raw reads were first filtered by removing adaptor sequences, low quality reads containing >50% bases with quality ≤15, and reads with >2% undefined nucleotides [19]. To phat (v2.0.13) was used to align the clean reads with the Ensembl Mouse Genome. Cufflinks (v2.2.1) was used to assemble and quantify the transcripts. The fragments per kilobase of transcript per million mapped reads (FPKM) values were used to quantify the assembled transcripts. Cufflinks was also used to detect differential expression. A gene or transcript was considered differentially expressed if the status was OK and q-value was less than 0.05. The biological characteristics of the differentially expressed genes were obtained by Ensembl.

Plasmid construction and transfection

The full length of 150Rik was found and cloned into the pcDNA3.1 expression vector in order to construct a stable 150Rik overexpression plasmid. All the recombinant plasmids were verified by gel electrophoresis and sequencing. Then, in the light of the manufacturer's protocol, Opti-MEM and lipofectamine 3000 reagents (Invitrogen, CA, USA) were used in transfections. The mouse glomerular MCs in the low glucose group that were transfected with 150Rikoverexpression plasmid were regarded as 150Rik(+) and the mouse glomerular MCs in the low glucose group that were transfected with empty plasmid wereregardedaspcDNA3.1.qRT-PCR was used to test the transfection efficacy.

Moreover, three small interfering RNAs (siRNAs) (siRNA-709, siRNA-916, siRNA-1081) were used to silence the expression of 150Rik; these siRNAs as well as the control siNC were designed and compounded by Sangon Bio Technology Co. Ltd. (Shanghai, China). The transfection was conducted using opti-MEM and lipofectamine 2000 reagents (Invitrogen, CA, USA) according to the manufacturer's protocol. SiRNA-709, which had the best silencing effect among the three siRNAs, was selected by qRT-PCR. The mouse glomerular MCs from the high glucose group that were transfected with siRNA-709 were regarded as the si150Rik group while the mouse glomerular MCs from the high glucose group that were transfected with control siRNA were regarded as the siNC group. Additionally, miR-451 mimics, the inhibitor and their negative controls were synthesised and purified by Shanghai GenePharma Co. (Shanghai, China). The mouse glomerular MCs from the high glucose group that were transfected with miR-451 mimics were regarded as miR-451 mimics, the mouse glomerular MCs from the high glucose group that were transfected with the miR-451 mimics control were regarded as the mimics NC group, the mouse glomerular MCs from the high glucose group that were transfected with the miR-451 inhibitor were regarded as the miR-451 inhibitor group and the mouse glomerular MCs from the high glucose group that were transfected with the miR-451 inhibitor control were regarded as the inhibitor NC group. QRT-PCR was used to test the transfection efficacy. The sequences of the siRNAs, miR-451 mimics, miR-451 inhibitor and their controls were used in this study as shown in Table 1.

FISH (Fluorescence in site hybridization)

The subcellular distribution of 150Rik was detected in the high or low glucose condition MCs using the 150Rik fluorescence FISH probe kit (Ribo Bio Tech, Guangzhou, China). The MCs cultured with high glucose that were not submitted to the fluorescence probe of 150Rik were taken as the control group. The MCs were fixed with 4% paraformaldehyde at room temperature for 10 min and were then washed twice with pre-cooled PBS containing 0.5% Triton X-100 for 5 min. MCs were hatched with 150Rik probe hybridisation overnight at 37°C and were then washed with SCC buffer. The cover-slips were tinted with DAPI for 10 min and were detected by laser scanning confocal microscopy.

Table 1. The sequences of siRNAs, miR-451 mimics, inhibitor and their controls

Plasmid name	Strand	Sequence
150Rik siRNA	Sense	5′-AATCATTTTCCTTACTTCCCACCA-3′
	Anti-sense	5′- UUGUCAUGAAGUUCGUUCCTT-3′
siRNA NC	Sense	5′- UUCUCCGAACGUGUCACGUTT-3′
	Anti-sense	5′- ACGUGACACGUUCGGAGAATT-3′
miR-451 mimics	Sense	5′-AAACCGUUACCAUUACUGAGUU-3′
	Anti-sense	5′-CUCAGUAAUGGUAACGGUUUUU-3′
miR-451 mimics NC	Sense	5′-UUCUCCGAACGUGUCACGUTT-3′
	Anti-sense	5′-ACGUGACACGUUCGGAGAATT-3′
miR-451 inhibitor		5′-ACGGUUCUUGCUGCUCCCA -3′
miR-451 inhibitor NC		5′-CAGUACUUUUGUGUAGUACAA-3′

Table 2. Primers used for qRT-PCR

Gene ID	Primer	Sequence
150Rik	Forward	5′-CAGAAAACCAAACCAAAGCCCT-3′
	Reverse	5′-CTCCTCTCACACATCCCCATCC-3′
Defb42	Forward	5′-CCCAACTCAAATCCCTCTTCG-3′
	Reverse	5′-GGGCTGGTTTTCTCTTGCTCAT-3′
Snora73a	Forward	5′-GTCACTCTCCCCGGGCTCT-3′
	Reverse	5′-TTCCTGCATGGTTTGTCTCCTC-3′
Snora74a	Forward	5′-GTGGTGCCCGAGATCGTGTT-3′
	Reverse	5′-CACCCAGACCAGGACCAACTC-3′
1700007L15Rik	Forward	5′-CCGAGAAGTGAAAAATGGGGA-3′
	Reverse	5′-GGAAAAAGAGGAGAGGCTAGGTAAG-3′
2610203C20Rik	Forward	5′-TGTGGCTGTTACTCCCCTCG-3′
	Reverse	5′-AAAACCCCTCAACCCCTGG-3′
Dab1	Forward	5′-GTTCCAAGGGAGAACACAAACA-3′
	Reverse	5′-ATGTCCTTCGCAATGTAGGAAAT-3′
β-actin	Forward	5′-ATATCGCTGCGCTGGTCGTC-3′
	Reverse	5′-AGGATGGCGTGAGGGAGAGC-3′
U6	Forward	5′-CTCGCTTCGGCAGCACA-3′
	Reverse	5′-AACGCTTCACGAATTTGCGT-3′
Mmu-miR-451	RT	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAACCAG-3′
	Forward	5′-CCGAAACCGTTACCATTAC-3′
	Reverse	5′-GTGCAGGGTCCGAGGT-3′

QRT-PCR

The total RNA of MCs was extracted using RNA TRIzol (Invitrogen, US) according to the manufacturer's instructions. Reverse transcription of RNA to cDNA was performed with the primeScript RT reagent kit (Takara, Dalian, China) under the following conditions: 37°C for 15 min, 85°C for 5 s. Gene amplification was performed with SYBR®Premix Ex Taq™. Relative expressions were normalized to the expression of U6 or β-actin and were calculated using the $2^{-\Delta\Delta CT}$method. All experiments were conducted at least three times. All primers used for qRT-PCR (Table 2).

Flow cytometry analysis

MCs were cultured in six-well plates after being transfected for 48 hours, washed with PBS three times, collected and resuspended in 0.5 ml PBS and fixed in 70% ethylalcohol for at least 6 h at 4°C. Then, flow cytometry (BD Biosciences, Franklin Lake, NJ, USA) was used to detect the distribution of the cell cycle. The percentages of cells in each cell cycle stage were analysed using Cell Quest acquisition software (BD Biosciences).

Cell proliferation assay

Cell proliferation analysis was performed with an EdU labelling/detection kit (RiboBio, Guangzhou, China). MCs were cultured in 24-well plates; labelling medium was added into each well for 2h at 37°C under 5% CO_2. Samples were fixed with 4% paraformaldehyde for 30 min at room temperature and were then permeated in 0.5%Triton X-100 at 4°C for 10 min; following this, samples were washed with PBS. Then, cells were stained with Hoechst33342 for 30 min at room temperature. Finally, the images were obtained with a microscope (Olympus, Tokyo, Japan) and were analysed with Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). The EdU incorporation rate was calculated as the ratio of EdU positive cells (red cells) to total Hoechst33342 positive cells (blue cells).

Dual-luciferase reporter assay

The 150Rik cDNA was amplified with primers 5′-GGCGGCTCGAGCTTGTATGGACCTTTGGTA-3′(forward) and 5′-AATGCGGCCGCGATTTAATGGGAGATGTGG-3′ (reverse) by PCR and cloned into a pmiR-RB-REPORT™ dual-luciferase vector (Guangzhou RuiBio Corp., Guangzhou, China) with restriction endonucleases *Xho I* and *Not I* to generate a pmiR-RB-REPORT™-150Rik wild-type, regarded as 150Rik-wt. The QuikChange™ Site-Directed Mutagenesis kit (Stratagene) was used to construct the mutant without

predicting binding sequences of miR-451; this was then cloned into the pmiR-RB-REPORT™ vector and is labelled as the 150Rik-mut. The MiR-451-inhibitor oligo was cloned into the GP-miRGLO dual-luciferase vector (GenePharma Co., Shanghai, China) to form a GP-miRGLO-miR-451-inhibitor. Restriction enzyme digestion and DNA sequencing were verified for all the recombinant plasmids. 293T cells were cultured in a 24-well plate to reach approximately 50% confluence and were then transfected with 150Rik-wt, 150Rik-mut, miR-451 mimics or miR-451 mimics NC with lipofectamine2000. After 48h, the cells were tested using a Dual-Luciferase® Reporter Assay System (Promega, WI, USA).

The annealing method was performed to obtain a pair of the most stable IGF1R oligos (sense strand: 5'-AAAA CTCGAG TGGAGATGCCTGGTATTCCCT-3', antisense strand: 5'-AAAA GCGGCCGC CATGTTTCTTGGGATCTCCTGG-3') and a pair of IGF1R mutant oligos (sense strand: 5'-AGTGGACACGACTGGTAATACTCAAAGGTGATTACGAACATAGTTTTGT-3', antisense strand: 5'-TTGAGTATTACCAGTCGTGTCCACTGGTGAATTTTATACTGGCATGACT-3'). All the sequence fragments were cloned in GP-miRGLO dual-luciferase vector (GenePharma Co., Shanghai, China) to produce a GP-miRGLO-IGF1R wild-type named IGF1R-wt and a GP-miRGLO-IGF1R mutant named IGF1R-mut. Restriction enzyme digestion and DNA sequencing verified all the recombinant plasmids. In total, 293 cells were transfected with IGF1R-wt, IGF1R-mut, miR-451 mimics or miR-451 mimics NC with lipofectamine 2000. Forty-eight hours later, cells were submitted to the Dual-Luciferase® Reporter Assay System (Promega, WI, USA). Firefly luciferase activity was normalized to renilla luciferase activity.

RIP assay

The relationships between 150Rik or miR-451 and the key RNA-induced silencing complex (RISC complex) elementAgo2 were tested by RIP assay. The EZMagna RIP kit (Millipore, Billerica, MA, USA) and the Ago2 antibody (ab32381, Abcam, Cambridge, MA, USA) were used to perform the RIP assay according to the operating instructions. The expression levels of 150Rik and miR-451 were measured by qRT-PCR. The protein expression of Ago2 was detected by Western blot. Mouse IgG (Millipore, Billerica, MA, USA) was used as negative control.

Western blot analysis

Ice-cold RIPA lysis buffer (Beyotime, Shanghai,China) was used to extract the total protein from the MCs.Protein was then subjected to10% SDS-PAGE (Beyotime, Shanghai, China), shifted onto 0.22μm PVDF membranes (Millipore, Temecula, CA) and then blanked in 10% nonfat milk. Then, the PVDF membranes were hatched overnight with specific primary antibodies. The primary antibodies used in this study were: IGF1R (Proteintech, USA, 1:1000), cyclin D1 (Proteintech, USA, 1:1000), IGF1 (Sangon,China, 1:500), p38MAPK (Proteintech, USA, 1:500), p-p38MAPK (Proteintech, USA, 1:500) and GAPDH (Glyceraldehyde-3-phosphate Dehydrogenase) (Abcam, USA, 1:5000). The GAPDH antibody was used as the control. Image Lab software was used to analyse the gray value of the protein bands.

Immunofluorescence assay

The MCs were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilised in PBS containing 0.1% Triton X-100 on ice for 10 min, sealed in 5% goat serum solution (Beyotime, Nantong, China) and then hatched overnight with primary antibodies IGF1 (Sangon, China, 1:500), p38MAPK (Cell Signaling Technology, Proteintech, USA, 1:500) or p-p38MAPK (Cell Signaling Technology, Proteintech, USA, 1:500) at 4°C.Subsequently, FITC (Fluorescein Isothiocyanate)-labelled secondary antibody (Proteintech, USA, 1:1000) was added for 2h at room temperature. The cells were then tinted with DAPI for 10 mins. Finally, the images were viewed with QImaging Micro-publisher™ 5.0 RTV (Olympus Corporation, Japan) and quantified by Image-Pro Plus (Medium Cybemetics, Bethesda, MD, USA).

Statistical analysis

Statistical analysis of the data was performed usingSPSS19.0. Data are expressed as mean± SD. Student's t-tests and one-way ANOVAs (no less than three groups) with Tukey's multiple comparisons tests were performed to analyse the significance of differences between groups. $P<0.05$ was regarded as statistically significant. GraphPad Prism 6 statistical package (GraphPad Software, SanDiego, USA) was used to conduct data.

Results

150Rik is a DN-related lncRNA in vivo and in vitro

We have previously reported that miR-451has a regulatory role in DN [16]. Based on this finding, we sought to continue to explore the possible role of other noncoding RNAs in MC proliferation in DN. Therefore, we examined dysexpressed ncRNAs in renal tissue of db/db DN mice and controls using RNA sequencing ($q<0.05$) (Fig. 1A). Moreover, RNA sequencing was used to examine the expressions of ncRNAs inmiR-451-treated DN mice relative to untreated mice($q<0.05$) (Fig. 1B).The results showed that the expressions of seven ncRNAs (Dab1, Defb42, Snora73a, Snora74a, 2610203C20Rik, 1700007L15Rik and 150Rik) were correlated with miR-451. We identified a panel of seven putative ncRNA candidates. Using qRT-PCR, these seven candidate ncRNAs were verified in renal tissues of db/db DN mice. Data showed that five ncRNAs exhibited expression consistent with the results of RNA-seq (Fig. 1C). Further, the expressions of these candidate ncRNAs were detected in MCs under high (H-MC) or low glucose (L-MC) using qRT-PCR. Data showed that, among these candidate ncRNAs, lncRNA 150Rik showed the most upregulated expression in the H-MC group (Fig. 1D). Moreover, our data showed that 150Rik displayed the highest conservative factor on the sequence compared with the homologous sequence in humans (Table 3). Therefore, we focused on lncRNA 150Rik for further study.

To explore the function of 150Rik in DN, the subcellular location of 150Rik in cells was detected by FISH and qRT-PCR. Results showed that 150Rik was expressed both in the nucleus and cytoplasm of L-MCs and H-MCs, but the expression of 150Rik was markedly higher in the cytoplasm when compared with that in the nucleus (Fig. 1E and F). This suggests that 150Rik is predominantly distributed in the cytoplasm of MCs. Moreover, 150Rik expression was significantly higher in the cytoplasm of H-MCs than in the cytoplasm of L-MCs ($p<0.05$). Therefore, it appears that 150Rik may play a role in the cytoplasm of MCs.

150Rik promotes cell proliferation in MCs

To research the role of 150Rik in cell proliferation in MCs, We first successfully constructed a stable 150Rik overexpressed plasmid [150Rik (+)] and 150Rik siRNAs (si150Rik). Furthermore, EdU assay and quantitative analysis revealed that cell proliferation in the L-MC 150Rik (+) group was significantly increased compared with that of the L-MC pcDNA 3.1 group and the L-MC group. On the other hand, cell proliferation in the H-MC si150Rik group was clearly decreased compared with that in the H-MC siNC group and the H-MC group (Fig. 2A). The flow cytometry analysis revealed that, in the150Rik (+) group, there was a decrease in cells in the G_0/G_1 phase and an increase of cells in the S phase, while si150Rik was able to block cells during the G_0/G_1 phase (Fig. 2B). Furthermore, we detected expression of cyclin D1 which serves as a key sensor and integrator of extracellular signals of cells in the G_0 and early G_1 phases [20]. Cyclin D1 up regulation can induce cells from passing through the G_0/G_1 checkpoint and vice versa. Results revealed that expression of cyclin D1was increased in the 150Rik (+) group while it was decreased when the expression of 150Rik was knocked down in the si150Rik group (Fig. 2C). Therefore, these data suggest that 150Rik can regulate the G_0/G_1 phase of the cell cycle to affect MC proliferation.

150Rik directly interacts with miR-451

We predicted the miRNAs that target 150Rik using bioinformatics analysis (miRbase, RNAhybrid, miRCode, miRanda). Results revealed miR-451 showed the lowest ΔG and a good conservation binding site in the 150Rik transcript among the species (Fig. 3A). Moreover, qRT-PCR revealed that the expression level of miR-451 was significantly reduced in MCs cultured with high glucose; this was opposite to the 150Rik expression in DN. Further, 150Rik knockdown increased the expression of miR-451 compared with that in the other two H-MC groups, whereas overexpression of 150Rik knockdown elevated miR-451 expression in cells under the low glucose condition (Fig. 3B). In addition, the miR-451 mimics or inhibitor were used to up- or down regulate the expression of miR-451 in MCs

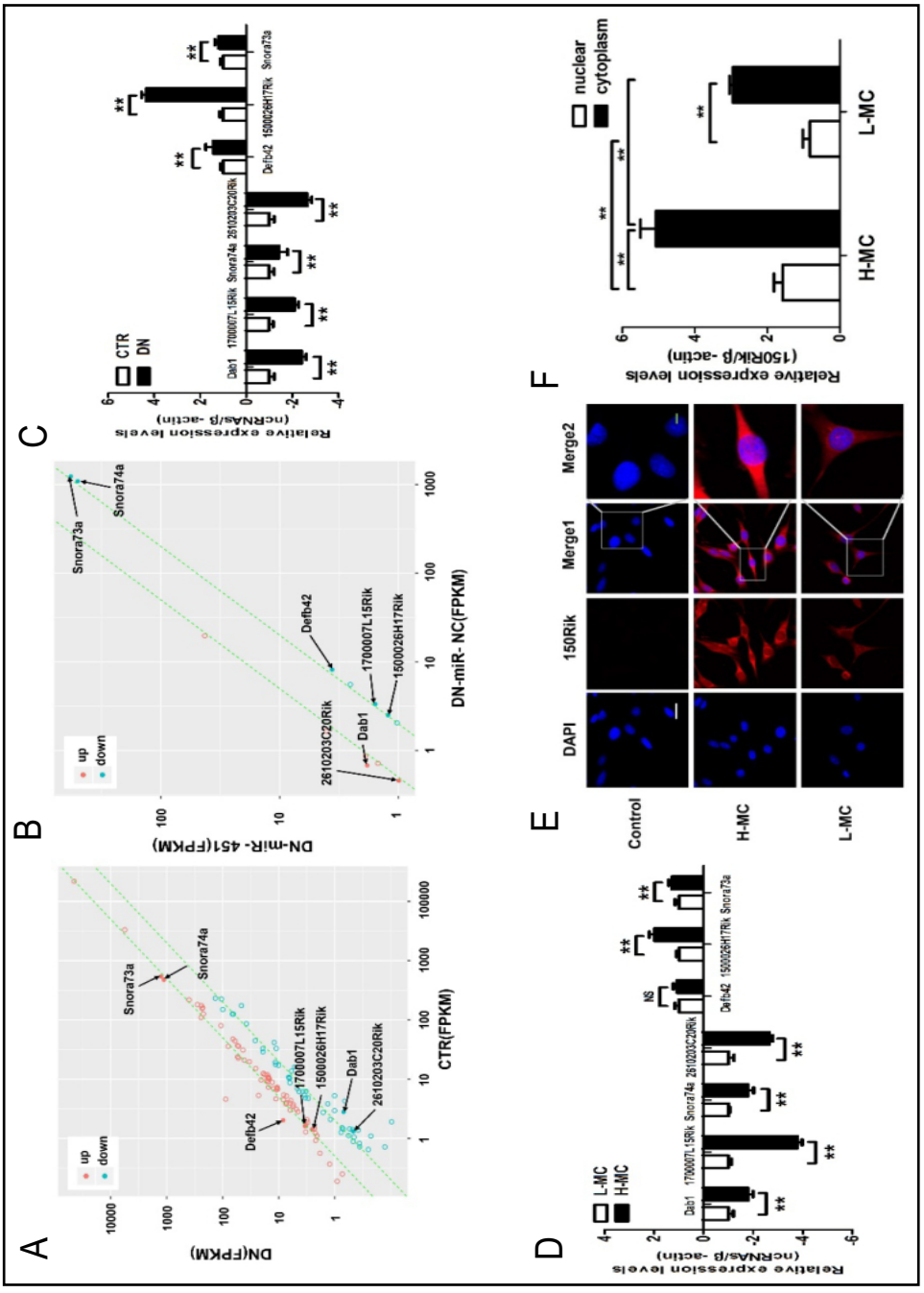

Fig. 1. 150Rik was up-expressed in vivo and in vitro and subcellular distributed in MCs. A, The expressions of ncRNAs were detected in renal tissues of db/db DN mice and controls by RNA-seq (n=3). B, The expressions of ncRNAs were detected in renal tissues of DN mice treated with or without miR-451 by RNA-seq (n=3). C, The expressions of seven miR-451 related ncRNAs were verified in renal tissues of db/db DN mice and controls by qRT-PCR (n=3). D, The expressions of seven miR-451 related ncRNAs were verified in MCs under high glucose or low glucose by qRT-PCR. E, Expression of 150Rik was showed with confocal FISH images and its mainly cytoplasmic localization in MCs and highly expressed in MCs culture with high glucose medium. White scale bar, 50μm. Green scale bar, 25μm. F, The RNA levels of 150Rik were tested in the cytoplasm and nuclear of MCs by qRT-PCR. The data were representative of the results three independent experiments, and the data were presented as means±SD. (**P<0.01, NS, no significant).

Table 3. Characteristics of candidate ncRNAs

Name	Gene ID	Transcript ID	Biotype	Locus (chr)	Conservative factor	db/db DN mice	db/m mice	Average FPKM db/db DN mice	Average FPKM miR-451treated DN mice
150Rik	ENSMUSG00000097383	ENSMUST00000181598.1	bidirectional promoter lncRNA	10:89686370-89700866	51%	1.41735	2.56977	2.51412	1.23195
1700007L15Rik	ENSMUSG00000097318	ENSMUST00000083715.1	bidirectional promoter lncRNA	16:33379854-33380727	43%	1.609	3.40984	3.3354	1.57403
Defb42	ENSMUSG00000054763	ENSMUST00000142140.7	Processed transcript	14:63034086-63058149	42%	2.02799	8.35442	8.17153	3.63008
Snora73a	ENSMUSG00000064387	ENSMUST00000082453.1	snoRNA	4:132331918-132353686	28%	542.406	1265.39	1238.06	575.386
2610203C20Rik	ENSMUSG00000074415	ENSMUST00000180622.1	lincRNA	9:41580740-41617772	3%	1.31964	0.468568	0.45846	0.994272
Snora74a	ENSMUSG00000065649	ENSMUST00000083715.1	snoRNA	18:35553409-35558316	N/A	474.222	1118.61	1094.41	502.802
Dab1	ENSMUSG00000028519	ENSMUST00000146078.7	Nonsense mediated decay	4:103619358-104744844	N/A	2.7982	0.698194	0.682993	1.84001

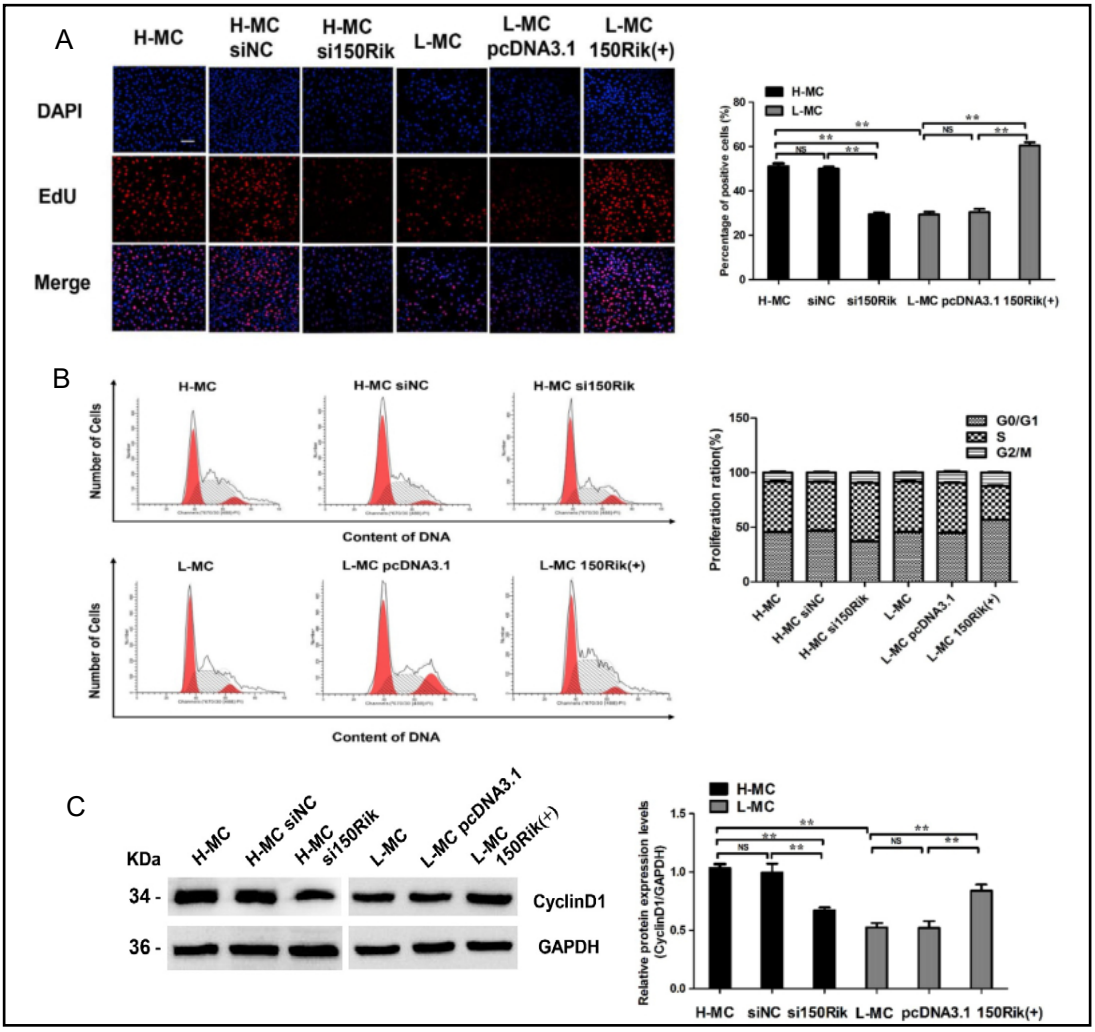

Fig. 2. 150Rik promoted mesangial cell proliferation. A, The cell proliferation of MCs was measured by EdU and quantitative analysis. Scale bar, 200μm. B, Flow cytometric assay was performed to analyze the effect of 150Rik on the cell cycle in MCs transfected with 150Rik (+), si150Rik and their matched controls, respectively. The percentage of cells in the G_0/G_1, S and G_2/M phases of the cell cycle were calculated. C, The protein expression of cyclinD1 was detected by Western blot and quantitative analysis. The data were representative of the results three independent experiments, and the data were presented as means±SD. (**P<0.01, NS, no significant).

cultured under high or low glucose conditions. The results revealed that up regulation of miR-451 decreased the expression of 150Rik in cells under the high glucose condition while down regulation of miR-451 increased the expression of 150Rik in cells under the low glucose condition (Fig. 3C). Therefore, the reciprocal relationship between 150Rik and miR-451 may play a role in DN.

To further determine the relationship between 150Rik and miR-451, we constructed a luciferase reporter plasmid containing the 150Rik cDNA wild-type (150Rik-wt) or seed-sequence mutant (150Rik-mut) and then tested the luciferase activity of 150Rik using dual luciferase assays in HEK 293cells. This analysis revealed that the luciferase activity of 150Rik-wt was significantly decreased by miR-451 mimics, while miR-451 had no influence on 150Rik-Mut (Fig. 3D). These findings suggest that 150Rik is a direct target for miR-451. Additionally, since miRNAs bind to their targets to depress and/or degrade RNA through the RISC complex and Ago2 acts as a key component of the RISC complex, several lncRNAs have been found to regulate the expression of miRNAs via the Ago2-mediated RNA interference (RNAi) pathway in certain diseases [21, 22]. Therefore, to explore whether 150Rik and miR-451 are in the RISC complex, RIP was conducted using the antibody against Ago2. Results revealed that the Ago2 protein could be precipitated from the cellular extract. Further, in the RNA extracted from the precipitated Ago2 protein, both miR-451 and 150Rik could be detected with a 4∼5-fold enrichment compared to IgG (Fig. 3E). Therefore, these results suggest that 150Rik interacts with miR-451 in both a direct and Ago2-dependent manner.

150Rik and miR-451 synergistically affect cell proliferation in MCs

Given the interaction between150Rik and miR-451, we explored their synergistic effect on cell proliferation in MCs. Cell proliferation profiles were determined by EdU assay, flow cytometry and Western blot analysis. Results revealed that 150Rik knockdown and miR-451 overexpression decreased cell proliferation in MCs (Fig. 4A), caused accumulation of cells in the G_0/G_1 stage and resulted in a lower proportion of cells in the S phase, relative to the control (Fig. 4B). Western blot analysis revealed that 150Rik knockdown and miR-451 overexpression can reduce cyclin D1 expression (Fig. 4C). Importantly, these effects were enhanced when si150Rik and miR-451 mimics were applied in combination. In contrast, 150Rik overexpression and the miR-451 inhibitor increased the proportion of cells in the S phase, decreased the G_0/G_1 phase ratio (Fig. 4D) and upregulated cyclin D1 (Fig. 4C), thereby activating MC proliferation (Fig. 4A). As expected, these effects were enhanced when the 150Rik overexpression plasmid and the miR-451 inhibitor were applied in combination (Fig. 4A, B, C) . Taken together, these findings suggest that the interaction between 150Rik and miR-451 results in a synergistic effect on MCs proliferation in DN.

MiR-451 inhibits cell proliferation in MCs via targeting of IGF1R

Our research showed that miR-451 can inhibit MCs proliferation in DN both *in vitro* and *in vivo*; however, the mechanism underlying this remains unknown [15, 16]. To explore the mechanism of miR-451 in DN, miR-451 target genes were predicted by bioinformatics analysis (miRbase, TargetScan). We found the DN-related gene IGF1R which has three binding sites in the miR-451 sequence and a low ΔG value (-16.8 KJ/mol). Results revealed a highly conserved sequence within the IGF1R 3′-UTR that was complementary to the "seed sequence" of miR-451 (Fig. 5A). Further, the luciferase assay data showed a direct relationship between miR-451 and IGF1R in 293T cells. Luciferase reporter plasmids containing sites of the IGF1R wild-type (IGF1R-wt) or seed-sequence mutant (IGF1R-mut) were used. The results showed that miR-451 suppressed IGF1R luciferase reporter gene activity within the wild-type complementary site (Fig. 5B). This suggests that IGF1R is a direct target for miR-451. Moreover, Western blot results showed that the expression level of IGF1R was significantly reduced in the H-MC group when miR-451 was overexpressed, while the expression of IGF1R was increased when endogenous miR-451 was inhibited in the L-MC group with the miR-451 inhibitor (Fig. 5C). Immunofluorescence results further confirmed that miR-451 directly inhibits IGF1R expression (Fig. 5D). In summary, these data suggest that miR-451 negatively regulates the expression of IGF1R in a direct manner.

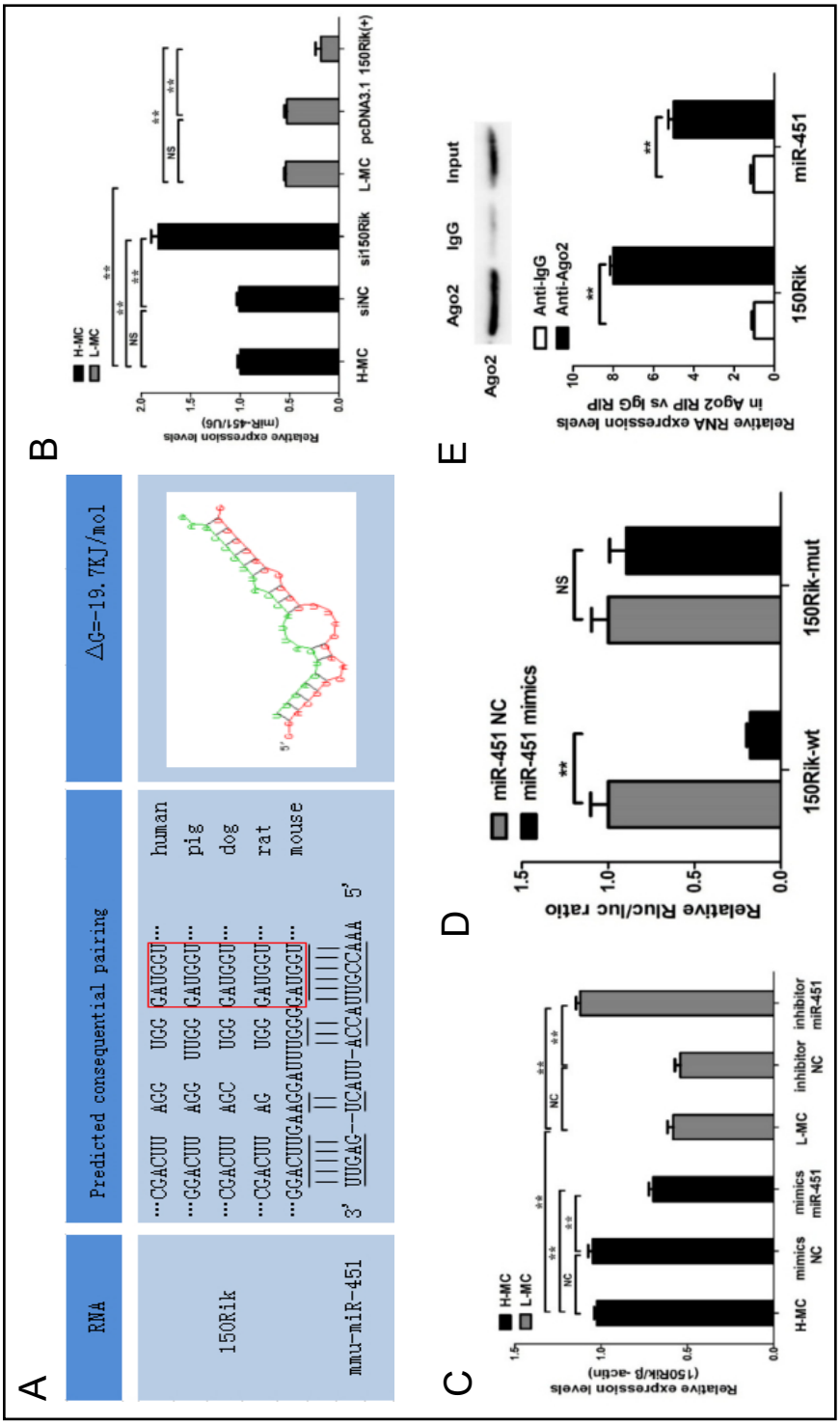

Fig. 3. 150Rik directly interacted with miR-451. A, Bioinformatics analysis predicted the relationship between 150Rik and miR-451. B, The expression of miR-451 regulated by 150Rik overexpression plasmid and si150Rik was measured by qRT-PCR. U6 was utilized as internal reference. β-actin was utilized as internal reference for normalizing 150Rik expression and U6 was used for normalizing microRNA expression. C, The expression of 150Rik regulated by miR-451 mimics or inhibitor was detected by qRT-PCR and β-actin was used as internal reference. Data suggested that 150Rik had an opposite expression comparing with miR-451 in MCs. D, Dual luciferase reporter assay was performed to measure the luciferase activity in cells co-transfected with miR-451 and luciferase reporters containing 150Rik (150Rik wt) or mutant transcript (150Rik mut). These results indicated that there was direct interaction between 150Rik and miR-451. Data were presented as the relative ratio of firefly luciferase activity to renilla luciferase activity. E, RIP assay was performed in MCs cultured in high glucose. (Top) the protein expression of Ago2 was analyzed by western blot; (Bottom) RNA levels were determined by qRT-PCR. The data were representative of the results three independent experiments, and the data were presented as means±SD. (**P<0.01, NS, no significant).

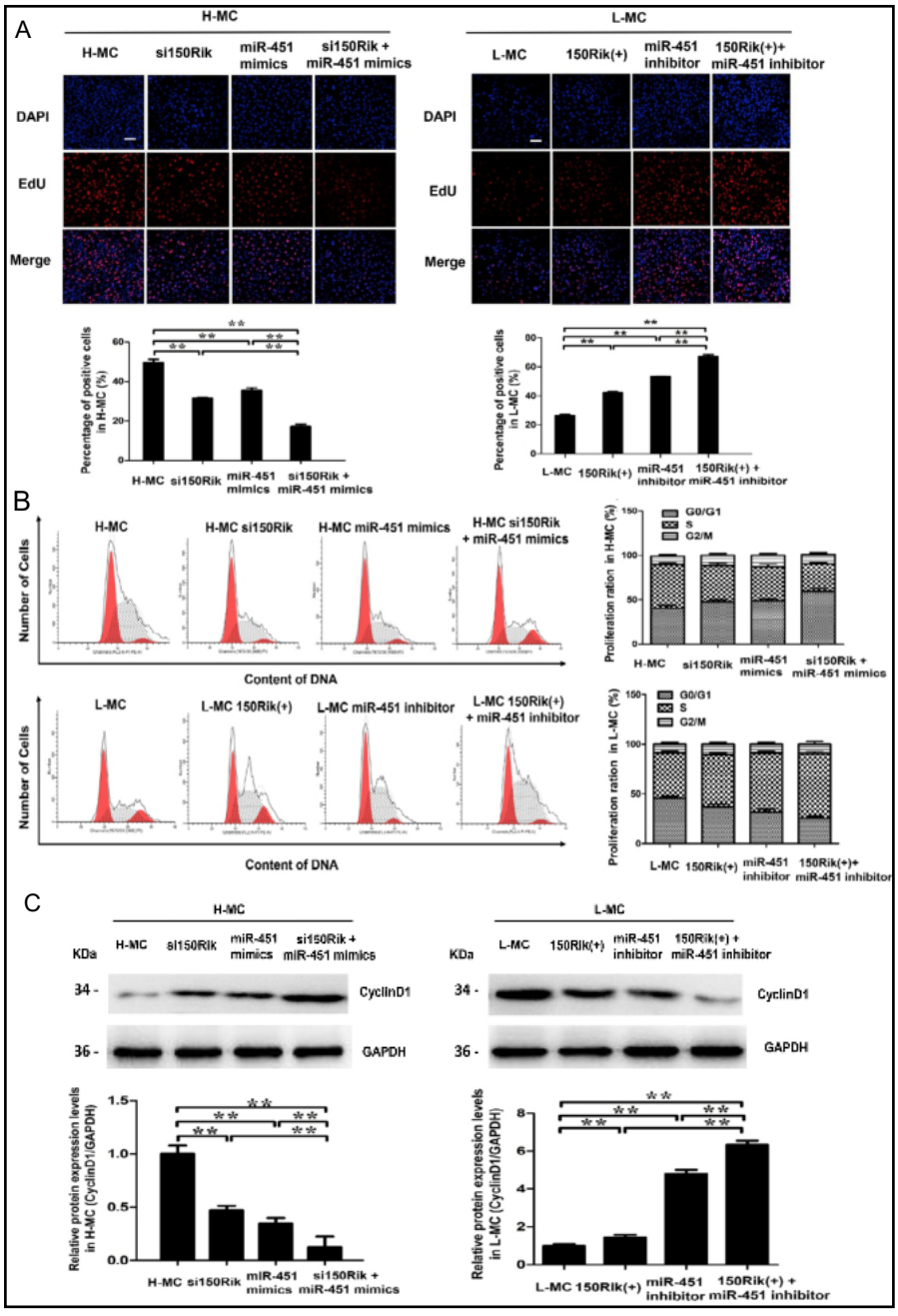

Fig. 4. The synergetic effect of 150Rik and miR-451 on cell proliferation in MCs. A, Cell proliferation of MCs transfected with 150Rik (+), si150Rik, miR-451 mimics, miR-451 inhibitor, 150Rik (+) +miR-451 inhibitor, si150Rik+ miR-451 mimics, and their matched controls was measured by EdU and quantitative analysis. Scale bar, 200μm. B, Flow cytometric assay was performed to analyze the effect of 150Rik on the cell cycle in MCs transfected with 150Rik (+), si150Rik, miR-451 mimics, miR-451 inhibitor, 150Rik (+) +miR-451 inhibitor, si150Rik+ miR-451 mimics, and their matched controls, respectively. The percentage of cells in the G_0/G_1, S and G_2/M phases of the cell cycle were calculated. C, The protein expression of cyclinD1 was detected by Western blot and quantitative analysis. GAPDH was utilized as internal reference. The data were representative of the results three independent experiments, and the data were presented as means±SD. (**P<0.01, NS, no significant).

150Rik and miR-451 synergistically regulate cell proliferation of MCs via the IGF1R/IGF1/p38MAPK pathway

Aberrant IGF1R/IGF1 signalling has been implicated in DN [23], and the p38MAPK pathway is one of the most important downstream pathways that activates IGF1R [24]. Thus, to explore the mechanism underlying the effect of 150Rik on MC proliferation in DN, we first verified the effect of 150Rik on the expression of IGF1R by Western blot. The results showed that knockdown of 150Rik reduced IGF1R levels while overexpression of 150Rik resulted in upregulation of IGF1R expression in MCs; these effects were strengthened by the

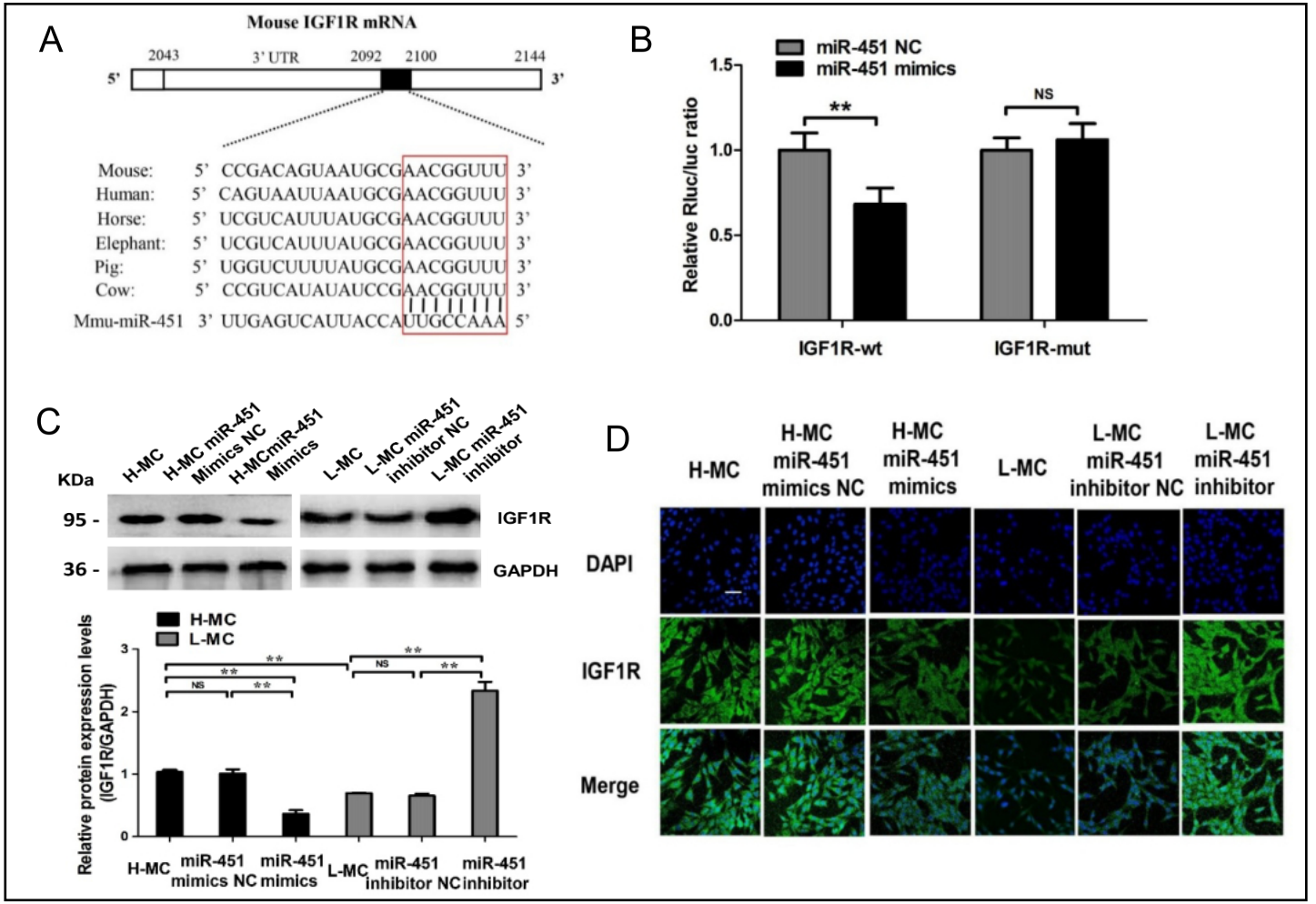

Fig. 5. IGF1R was the target gene of miR-451 and regulated by miR-451. A, Bioinformatics analysis predicted the relationship between miR-451 and IGF1R. B, Dual luciferase reporter assay was performed to measure the luciferase activity in cells co-transfected with miR-451 and luciferase reporters containing IGF1R (IGF1R-wt) or mutant transcript (IGF1R-mut). These results indicated that there was direct interaction between IGF1R and miR-451. Data were presented as the relative ratio of firefly luciferase activity to renilla luciferase activity. C, The protein expression of IGF1R was tested by Western blot when miR-451 was over-expressed and down-expressed and quantitative analysis of the gray values of the bands. D, The expression of IGF1R was tested by immunofluorescent in MCs when miR-451 was over-expressed and down-expressed. Scale bar, 200μm. The data were representative of the results three independent experiments, and the data were presented as means±SD. (**P<0.01, NS, no significant).

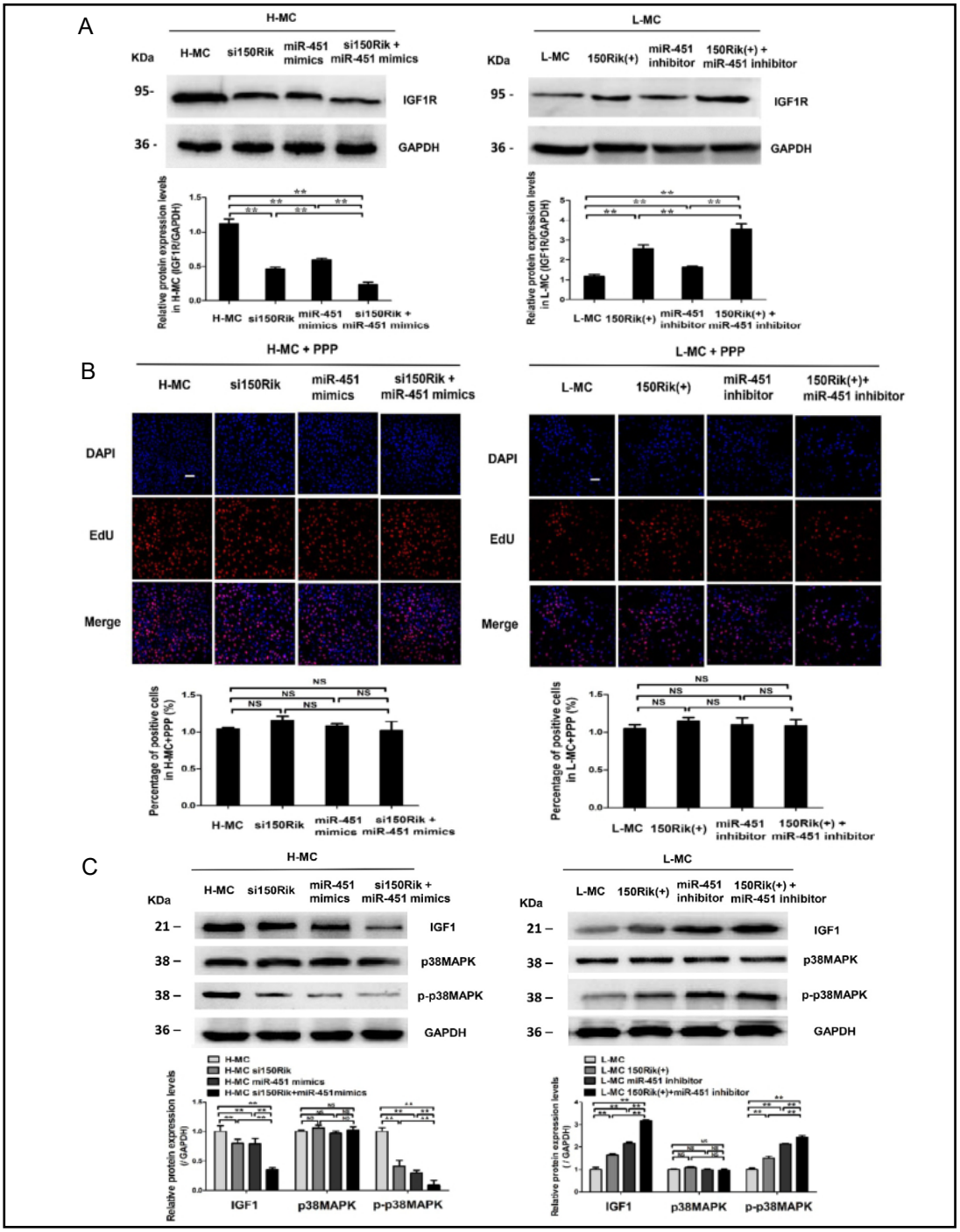

Fig. 6. 150Rik regulated mesangial cell proliferation via miR-451/IGF1R/IGF1/p38MAPK signalling pathway. A, The expression of IGF1R was measured by Western blot when 150Rik was over-expressed and down-expressed. And the gray values of the bands were quantitative analyzed. B, The cell proliferation of MCs transfected with 150Rik (+), si150Rik, miR-451 mimics, miR-451 inhibitor,150Rik (+) +miR-451 inhibitor and si150Rik+ miR-451 mimics was measured by EdU when IGF1R was specifically inhibited by picropodophyllin (PPP). Scale bar, 200μm. C, The protein expressions of IGF1, p38MAPK and p-p38MAPK were measured by Western blot in MCs transfected with150Rik (+), si150Rik, miR-451 mimics, miR-451 inhibitor,150Rik (+) +miR-451 inhibitor and si150Rik+ miR-451 mimics. And the gray values of the bands were quantitative analyzed. The data were representative of the results three independent experiments, and the data were presented as means± SD. (**P<0.01, NS, no significant).

combined application of the miR-451 mimics or inhibitor (Fig. 6A). Moreover, to rescue the experiment, picropodophyllin (PPP), an established IGF1R inhibitor [25, 26], was used. The results showed that the effect of 150Rik and miR-451 on cell proliferation was impeded by PPP (Fig. 6B).

Furthermore, our data showed that 150Rik knockdown and miR-451 overexpression can decrease the expression of IGF1, p38MAPK and p-p38MAPK, while 150Rik overexpression and miR-451 inhibition can increase the expression of IGF1, p38MAPK and p-p38MAPK. Interestingly, the inhibition effect was enhanced when 150Rik knockdown was combined

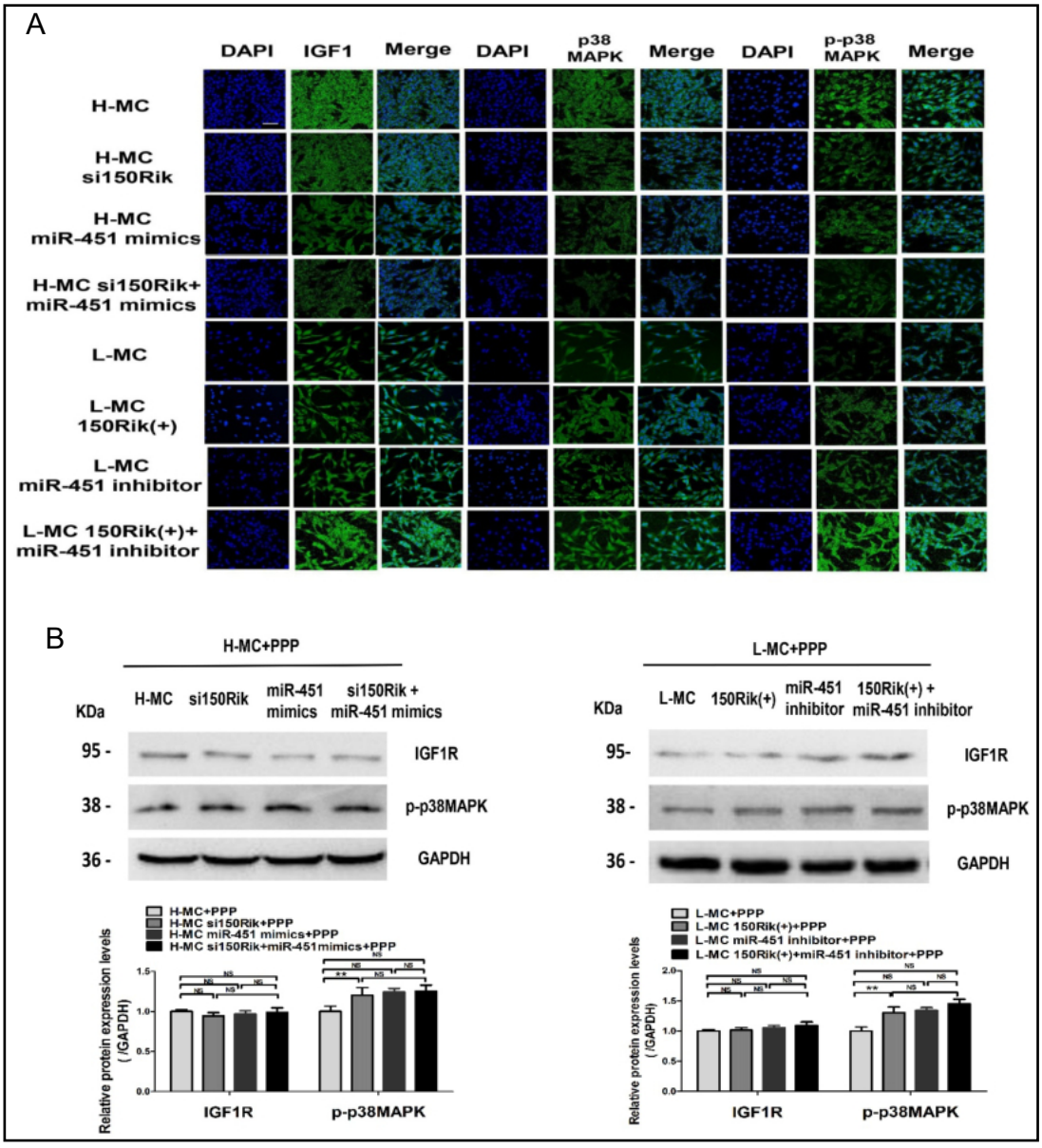

Fig. 7. 150Rik regulated mesangial cell proliferation via miR-451/IGF1R/IGF1/p38MAPK signaling pathway by immunofluorescent and Western blot. A, The protein expressions of IGF1, p38MAPK and p-p38MAPK were measured by immunofluorescent in MCs transfected with150Rik (+), si150Rik, miR-451 mimics, miR-451 inhibitor, 150Rik (+) +miR-451 inhibitor and si150Rik+ miR-451 mimics. Scale bar, 200μm. B, The protein expressions of IGF1R and p-p38MAPK were measured by Western blot in MCs transfected with 150Rik (+), si150Rik, miR-451 mimics, miR-451 inhibitor, 150Rik (+) +miR-451 inhibitor and si150Rik+ miR-451 mimics when IGF1R was specifically inhibited by PPP. The data were representative of the results three independent experiments, and the data were presented as means±SD. (**P<0.01, NS, no significant).

with miR-451 overexpression and the increasing effect was enhanced when 150Rik overexpression was combined with the miR-451 inhibitor (Fig. 6C).The immunofluorescence results were consistent with the Western bolt results (Fig. 7A). Rescue experiments further supported the finding that IGF1R plays a key role in the activation of p38MAPK through mediation of 150Rikand miR-451 (Fig. 7B). Therefore, these data indicate a synergistic effect of 150Rik and miR-451 on the regulation of MCs proliferation via the IGF1R/IGF1/p38MAPK pathway.

Discussion

To date, research on lncRNAs has focused on the role of lncRNAs in genetics, particularly in the regulation of transcription, post-transcription or epigenetics via the biological processes of histone modification, chromatin remodelling or DNA methylation [27]. Studies have also shown that lncRNAs are involved in various diseases, including DN [28-31]. Two lncRNAs, ENSMUST00000147869 and CYP4B1-PS1-001, have recently been reported to regulate MCs proliferation and fibrosis induced by DN [29, 32]. Moreover, the lncRNAs MIAT and Pvt1 have been reported to participate in renal tubular epithelial injury and extracellular matrix accumulation in DN [11, 33]. The lncRNAs ENSRNOG00000037522 and LINC01619 have been reported to participate in podocyte injury in DN [34, 35]. Further, Erbb4-IR is reportedly a Smad3-dependent lncRNA that promotes renal fibrosis in type 2 DN [36]. The lncRNA Gm4419 has been reported to regulate inflammation in DN [13]. Furthermore, single-nucleotide polymorphisms of lncRNA Pvt1 have been shown to contribute to susceptibility to DN [37]. However, the exact mechanism underlying the role of lncRNAs in DN remains unknown. An in depth understanding of the function of target lncRNAs in MC proliferation is crucial to understanding the mechanism underlying DN. Therefore, based on our previous studies on miR-451, the current study identified a new lncRNA-150Rik which is involved in regulation of MCs proliferation in DN.

LncRNA 150Rik (GenBank: NR_130956.1, Ensembl: ENSMUST00000181598.1) is located at chromosome 10. However, to date, there have been no reports implicating 150Rik in diseases. Our results show that expression of 150Rik is increased in the renal tissues of db/db DN mice and in MCs cultured with high glucose. Moreover, the overexpression of 150Rik was found to increase cyclin D1 expression, induce cells to pass through the G_0/G_1 checkpoint and raise the proportion of MCs in the S phase, thereby promoting MCs proliferation. On the other hand, 150Rik knockdown was found to decrease cyclin D1 expression and block cells in the G_0/G_1stage, thereby inhibiting MCs proliferation. Recent reports have shown that lncRNAs can act as regulators of cell proliferation. The lncRNA LINC01510 contributes to the proliferation of colorectal cancer cells [38], lncRNA HOXA11-AS is involved in proliferation in liver cancer [39], and lncRNA HCP5 promotes proliferation in follicular thyroid carcinoma [40]. Consistent with these reports, our findings suggest that the increased expression of 150Rik may represent a potential risk for renal mesangial proliferation and may contribute to DN development and progression.

To further explore the biological characteristics of 150Rik in MCs, we detected the subcellular distribution of 150Rik in MCs by FISH and qRT-PCR. The results revealed that 150Rik is mainly a cytoplasmic lncRNA in MCs, indicating that its action is in gene regulation. It is known that the functions of lncRNAs are various depending on their subcellular location. If they are located in the nucleus, they are mainly involved in the process of transcription and chromatin remodelling; when they are located in the cytoplasm, they principally participate in post-transcriptional gene regulation by forming complexes with specific proteins or acting as ceRNAs [41, 42]. Since ceRNAs are important regulators between lncRNAs and microRNAs [43-47], we focused on the ceRNA mechanism of 150Rik and miR-451 in this study. We found that miR-451 is directly targeted by lncRNA 150Rik and IGF1R is the target gene of miR-451. Furthermore, 150Rik can regulate IGF1R by directly targeting miR-451 and binding with Ago2 to promote the proliferation of MCs. Moreover, this study

revealed the synergistic effect of 150Rik and the miR-451 inhibitor on the promotion of MCs proliferation and the synergistic effect of si150Rik and miR-451 mimics on the inhibition of MCs proliferation. Thus, our results suggest that 150Rik regulates MCs proliferation by acting as a molecular sponge of miR-451 to regulate the target gene IGF1R.

IGF1R is a protein and transmembrane tyrosine protein receptor which is involved in the IGF1/MAPK signalling pathway [48, 49]. P38MAPK, an important member of the MAPK signalling pathway, can activate the MAPK signalling pathway [50-53], causing the proliferation of cells [54-56]. Moreover, our previous studies have shown that p38MAPK signalling is inhibited by miR-451 in early DN [15]. In the current study, overexpression of 150Rik was found to increase the expression of IGF1, induce phosphorylation of p38MAPK, activate the IGF1R/IGF1/p38MAPK signalling pathway, and result in MCs proliferation in DN. 150Rik knockdown exhibited the reverse effect on MCs proliferation. Furthermore, we revealed a synergistic effect of 150Rik+miR-451 inhibitor and si150Rik+miR-451 mimics on regulation of the IGF1R/IGF1/p38MAPK signalling pathway. A recent report showed that miR-451 downregulates p38 MAPK protein expression to reduce synovial fibroblast proliferation in rheumatoid arthritis [57]. Further, miR-451 was found to suppress neutrophil chemotaxis via p38 MAPK in rheumatoid arthritis [58]. Taken together, these results indicate that the IGF1R/IGF1/p38MAPK signalling pathway maybe the mechanism underlying the effect of 150Rik/miR-451 on MCs proliferation.

Conclusion

In summary, the present study identified that the interaction between the lncRNA 150Rik and miR-451 can influence MCs proliferation. Our results further revealed that IGF1R is the target gene of miR-451. Moreover, we demonstrated that 150Rik acts as an endogenous sponge RNA and inhibits miR-451 expression and activity. In addition, we identified a synergistic effect of 150Rik and miR-451 on the regulation of MCs proliferation via the IGF1R/IGF1/p38MAPK signalling pathway. The modulation of 150Rik and miR-451 may provide an intriguing approach for tackling renal MCs proliferation in DN.

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Disclosure Statement

The authors declare no conflicts of interest.

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