Long Noncoding RNA MEG3-205/Let-7a/MyD88 Axis Promotes Renal Inflammation and Fibrosis in Diabetic Nephropathy

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Keywords
Long noncoding RNA maternally expressed gene 3 - Diabetic nephropathy - Inflammation - Fibrosis - Myeloid differentiation primary-response protein 88

Abstract
Aim: The aim of this study was to investigate the role and mechanism of long noncoding RNA (lncRNA) maternally expressed gene 3 (MEG3)-205 in renal inflammation and fibrosis in diabetic nephropathy (DN). Materials and Methods: lncRNA microarray profiling was used to examine differentially expressed lncRNAs of kidney tissues in db/db mice compared to db/m mice. Mouse mesangial cells (mMCs) were cultured in vitro with advanced glycation end products (AGEs) via transfection with lncRNA MEG3-205 siRNAs or plasmids. The role of lncRNA MEG3-205 in vivo was examined in db/db mice treated with long-acting lncRNA MEG3-205 siRNA. The interaction between lncRNA MEG3-205 and let-7a was investigated using luciferase assay and RNA immunoprecipitation assay. Results: lncRNA MEG3-205 was markedly upregulated in renal tissues of db/db mice, DN patients, and AGEs-treated mesangial cells. Overexpression of lncRNA MEG3-205 promoted the secretion of pro-inflammatory cytokines and synthesis of extracellular matrix proteins in mesangial cells. Both lncRNA MEG3-205 and myeloid differentiation primary-response protein 88 (MyD88) could bind to let-7a, and lncRNA MEG3-205 overexpression can significantly rescue the silencing effect of let-7a on MyD88 protein expression in mMCs. Mechanistically, we identified that lncRNA MEG3-205 could act as a competing endogenous RNA by binding with let-7a and thus regulate MyD88. Knockdown of lncRNA MEG3-205 alleviated albuminuria and attenuated renal inflammation and fibrosis in db/db mice. Conclusion: These findings indicated an important role of the lncRNA MEG3-205/let-7a/MyD88 axis in regulating renal inflammation and fibrosis in DN. Targeting lncRNA MEG3-205 might present a promising therapeutic strategy for DN.

Qimei Luo, Xi Xia, Qingqing Luo, and Yue Qiu have contributed equally to this work.
**Introduction**

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease in the world [1]. Accumulation of the extracellular matrix (ECM) in the mesangial region and glomerulosclerosis are the main pathological features of DN [2]. Hyperglycemic factors such as advanced glycation end products (AGEs) can increase ECM expression and induce renal fibrosis [3]. Increasing evidence indicate that inflammation is a key pathophysiological mechanism of DN [4]. Long non-coding RNAs (lncRNAs) are defined as a class of transcripts longer than 200 nucleotides [5]. Previously, lncRNAs are reported to contribute to the occurrence and development of various diseases including cancer and kidney diseases [6, 7]. Moreover, emerging researches suggest that lncRNAs are promising biomarkers or intervention targets of diseases [8].

lncRNA maternally expressed gene 3 (MEG3) is located on chromosome 12 and chromosome 14q in the mouse and human, respectively [9]. Alternative RNA splicing has resulted in production of numerous transcript isoforms from MEG3, and isoform expression patterns are tissue and cell type-specific [10]. lncRNA MEG3 is downregulated in multiple cancers and is reported to be a tumor suppressor [11]. However, expression profiles and functions of lncRNA MEG3 in DN are controversial. Zha et al. [12] found that MEG3 was upregulated in kidneys of streptozotocin (STZ) DN rats, and mesangial cells stimulated with high glucose (HG) and MEG3 could enhance fibrosis and inflammatory response in DN. Li et al. [13] demonstrated MEG3 was elevated significantly in the serum of DN patients, and MEG3 knockdown alleviated proliferation and fibrosis and induced apoptosis of mesangial cells under HG condition. On the contrary, Che et al. [14] showed MEG3 was decreased in renal tissues of STZ DN rats and in podocytes after HG treatment, and MEG3 ameliorates podocyte injury in DN. Moreover, the transcription and function of MEG3 isoforms in DN are still unclear.

In this study, we used microarrays to identify differentially expressed lncRNAs and found the MEG3 lncRNA transcript MEG3-205 was significantly upregulated in vivo and in vitro in DN. We investigated the potential role of lncRNA MEG3-205 involved in the progression of renal inflammation and fibrosis and their underlying mechanisms in DN.

**Materials and Methods**

**lncRNA/mRNA Microarrays and Bioinformatics Analysis**

Kidney tissues of 6 mice homozygous for the diabetes spontaneous mutation (db/db) and six control littermates (db/m) (16-week-old, purchased from the Model Animal Research Center of Nanjing University) were collected for lncRNA and mRNA microarray analysis. Microarray profiling was performed using a Mouse LncRNA Array v2.0 (8 × 60K, Arraystar, Rockville, MD, USA) and were scanned using an Agilent Scanner (Agilent Technologies, Santa Clara, CA, USA). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies, Santa Clara, CA, USA).

Differentially expressed lncRNAs and mRNAs between kidney tissues of db/db and db/m mice models were selected out and visualized using “limma” package in R software (version 4.0.0) and listed in online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000523847). Besides, R package clusterProfiler was used to identify Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms between db/db and db/m mice according to c5 GO and c2 KEGG databases. The significance threshold was p < 0.05. Co-expression network analysis based on differentially expressed lncRNAs and mRNAs was conducted by Cytoscape (version 3.8.2). Node and edge files that revealed the coefficient factor threshold >0.85 and adjusted p < 0.05 were produced using R software. Expression correlation analysis was produced by “ggplot” in R.

**Cell Culture**

Mouse glomerular mesangial cells (the murine SV40-transfected mouse mesangial cell [mMC] line SV40 MES 13) were cultured in DMEM mixed 1:1 (vol/vol) with F12 medium (Gibco, Billings, MT, USA) containing 5% FBS (Gibco, Billings, MT, USA) at 37°C in 5% CO2. The cells were deprived of serum for 24 h before the experiments were performed. Cells were stimulated with AGE (100 μg/mL, Abcam, Waltham, MA, USA) or BSA (Sigma Aldrich, St. Louis, MO, USA) for periods of 12 h, 24 h, or 48 h in serum-free medium.

**RNA Extraction and Real-Time Quantitative PCR**

Total RNA was extracted from kidney tissues of db/m mice, db/db mice, and cultured cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and cDNA was synthesized from 1 μg total RNA using an M-MLV First-Strand cDNA Synthesis Kit (InvitrogenTM, Waltham, MA, USA) according to the manufacturer’s instructions. Real-time quantitative PCR (qRT-PCR) was performed using SYBR Premix Ex TaqTM (Takara, Kusatsu, Japan) and an Applied Biosystems 7900 Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, USA). Primers used for qRT-PCR are presented in online supplementary Table S2.

**RNA in situ Hybridization**

The probe used for detecting lncRNA MEG3-205 and human homolog of lncRNA MEG3-205 was designed and synthesized by Gszcbo Co., Ltd. (Guangzhou, China). The lncRNA MEG3-205 probe was labeled with FITC, and human homolog of lncRNA MEG3-205 was labeled with digoxigenin. The probe sequences are listed in online supplementary Table S3. Fluorescence in Situ hybridization (FISH) was conducted to detect lncRNA MEG3-205 in...
renal tissues of mice. Briefly, paraffin-embedded kidney sections (4-µm thickness) were deparaffinized and rehydrated. The sections were prehybridized in a prehybridization solution at 37°C for 2 h and then incubated with RNA probes in hybridization buffer overnight. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlington, CA, USA). Images were captured by laser-scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Goettingen, Germany). In situ hybridization (ISH) assay was carried out to examine human homolog of lncRNA MEG3-205 in human kidney tissues and used the methods previously described [15].

Clinical Samples
All human kidney tissues were collected at the First Affiliated Hospital of Sun Yat-Sen University, and the study was approved by the local Institutional Ethics Committee. Human DN kidney tissues were obtained from renal biopsies, and para-carcinoma normal kidney tissues were obtained from patients who underwent unilateral nephrectomy.

Cell Transfection of Plasmid, siRNA, and miRNA Mimics and Inhibitors
The cDNA encoding IncRNA MEG3-205 was amplified using PCR, subcloned into the BamHI and ECOR I sites of pSil-EP2-puro vector (kindly provided by Prof. Mengfeng Li, Sun Yat-sen University, China), and named pSil-EP2-MEG3-puro. The cDNA was also cloned into the pEXZ-MTO6 luciferase reporter plasmid (GeneCopoeia™, Rockville, MD, USA) and named pEXZ-MTO6-MEG3-205-wt. The pEXZ-MTO6-MEG3 plasmid with point mutations in the let-7a-binding site was cloned and named pEXZ-MTO6-MEG3-205-mut. Myeloid differentiation primary-response protein 88 (MyD88) mRNA 3'-UTR with the predicted binding sites of let-7a and mutants was also cloned into the luciferase reporter vector. Oligonucleotides for IncRNA MEG3-205 siRNA and let-7a mimics and inhibitor were designed and synthesized by the Ribobio Company (Guangzhou, China).

Cells were seeded in six-well plates at a confluence of approximately 70–80% before transfection. Transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Growth medium was replaced with new growth medium 6 h after transfection.

Enzyme-Linked Immunosorbent Assay
Expression of inflammatory cytokines was measured in the cellular supernatant of mMCs treated with AGES for the indicated time (12 h, 24 h, 48 h), TNF-α, IL-6, MCP-1 protein levels were blocked using 5% nonfat powdered milk, followed by overnight incubation at 4°C with the primary antibody against fibronectin (Abcam, catalog No. ab2413), collagen I (Southern Biotech, catalog No. 1310-01), MyD88 (Abcam, catalog No. ab28763), phospho-nuclear factor-kappa B (NF-kB) p65 (Cell Signaling Technology; catalog No. 3033), NF-kB p65 (Cell Signaling Technology; catalog No. 2697), IKKβ (Cell Signaling Technology; catalog No. 2370), and β-actin (CST Signaling Technology, catalog No. 4970). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG, or HRP-conjugated anti-goat IgG (Cell Signaling Technology, Danvers, MA, USA). Signals were detected using a FluoroChem E system (ProteinSimple, San Jose, CA, USA), followed by quantitative analysis using the Image J software program.

Luciferase Reporter Assay
Cells were seeded in 24-well plates at a confluence of 50–70%. Luciferase reporter plasmids or control luciferase plasmid (500 ng/μL) and 100 nmol/L normal control or microRNA mimics were co-transfected into mMCs. A Luc-Pair™ Duo-Luciferase Assay Kit (GeneCopoeia™, China) was used to measure luciferase activity at 48 h after transfection. Relative luciferase activity was normalized to Renilla luciferase activity. More than three independent experiments were performed.

RNA-Binding Protein Immunoprecipitation Assay
RNA immunoprecipitation (RIP) assay was performed using the EZ-Magna RIP kit (Millipore) according to the manufacturer’s protocol. mMCs lysate was incubated with RIP buffer containing magnetic beads conjugated to mouse anti-Ago2 antibody or negative control normal mouse IgG. The positive control used the anti-SNRNP70 antibody. Lysis samples were all treated with proteinase K, and to identify the presence in the binding target antibody, the RIP qRT-PCR assay with respective primers was used.

Animal Studies
For the in vivo study, twelve male C57BLKS/J background Lep<sup>db</sup>/Lep<sup>db</sup> (8-week-old mice, 35–40 g) and six nondiabetic control Lep<sup>db</sup>/m (db/m) mice (8-week-old mice, 20–25 g) were purchased from the Nanjing University Animal Model Research Center (Nanjing, China). Animal studies were approved by Institutional Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University. The mice were housed in individual metabolism cages for urine collection. Urinary albumin and urinary creatinine were measured using Albuwell M and Creatinine Companion assay kits (Exocell, Logan Township, NJ, USA). Blood glucose measurement was performed using a OneTouch UltraSmart Blood Glucose Meter (Lifescan, Milpitas, CA, USA). Long-acting IncRNA MEG3 siRNA (Ambion, Austin, TX, USA) was used to knock down IncRNA MEG3 expression in vivo [16]. Long-acting IncRNA MEG3-205 siRNA was diluted and mixed with Invivosert 3.0 reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions.

The Invivosert 3.0-siRNA complexation (200 μL of final volume) was injected into the caudal vein at a dose of 3 mg/kg per mouse. Db/db mice were randomly divided into two groups. IncRNA MEG3 siRNA or control siRNA were administered to the db/db mice (n = 6 per group). A group of age-matched db/m mice received injections of control siRNA at the same dose. This treatment was repeated every 2 weeks. Body weight and blood glucose were monitored. Twenty-four-hour urine and serum samples were collected at 8, 16, and 24 weeks of age. All mice were sacrificed at 24 weeks of age, and their kidney tissues were separated and embedded in paraffin for morphometric studies. The paraffin sections (4-µm thick) were stained using FISH, periodic acid-Schiff (PAS), or Masson staining for subsequent histological examination.
(For legend see next page.)
IncRNA MEG3-205 in Diabetic Nephropathy

Histology and Immunofluorescence

Periodic acid-Schiff and Masson staining were performed to examine changes in renal morphology. The percent positive staining areas were quantified using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA); 10 consecutive fields (×40) were assessed. Fixed renal tissues were permeabilized in 0.1% TritonX-100 (Biosharp, Anhui, China) and incubated with blocking buffer (5% BSA in PBS). These antibodies in this study included FITC/80 (Abcam, catalog No. ab6640), MCP-1 (Abcam, catalog No. ab7202), and MyD88 (Abcam, catalog No. ab28763). Renal tissues were incubated with monoclonal rat, rabbit, or goat antibodies and followed by Alexa Fluor 488-conjugated anti-rat, rabbit, or goat IgG antibody and then stained with 4,6-diamidino-2-phenylinodole. Positive staining was captured with a laser-scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Goettingen, Germany).

Statistical Analysis

All of the cell experiments were repeated at least three times, and the data from the animal study were obtained from six mice. All quantitative data are presented as mean ± SEM. Two-group comparisons were performed using two-tailed Student’s t test. Multigroup comparisons were performed using one-way ANOVA followed by the LSD test. Statistical significance was defined as p < 0.05. Statistical analyses were performed with SPSS 20.0 for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism 7.0.

Results

IncRNA MEG3-205 Is Upregulated in DN

Kidney tissues were collected from 6 diabetic db/db mice and 6 db/m mice littermates at 16 weeks. IncRNA and mRNA expression profiles were evaluated using mouse microarrays. Hierarchical cluster analysis and volcano plot identified 1,079 lncRNAs that were significantly upregulated in the renal tissues between db/db and db/m mice (Fig. 1a, b), including 626 upregulated and 453 downregulated lncRNAs (online suppl. Table S1). Four MEG3 transcripts were remarkably upregulated (Fig. 1a, b), and qRT-PCR validated that IncRNAs MEG3-205 and MEG3-209 were overexpressed in the kidney tissues of db/db mice compared with db/m mice (Fig. 1c). In particular, MEG3-205 (Ensembl ID: ENSMUST00000129245; 1498bp) increased more than nine folds of db/m mice (p < 0.0001), indicating that MEG3-205 may play an important role in the pathogenesis of DN. The results of FISH and ISH suggested that MEG3-205 was predominantly localized in the cytoplasm of glomerular mesangial cells and renal tubular epithelial cells in kidney tissues of the db/db mice (Fig. 1d) and biopsy-proven DN patients (Fig. 1g). We also found that AGEs induced an increase of the MEG3-205 expression level in mMCs compared to the BSA control group (Fig. 1e). qRT-PCR and ISH confirmed that homolog of MEG3-205 expression in kidney tissues from patients with DN was significantly upregulated compared with that of normal kidney tissues (Fig. 1f, g). Taken together, these results indicated that IncRNA MEG3-205 was markedly upregulated in the kidney tissues of the DN mouse models and patients.

IncRNA MEG3-205 Increases Pro-Inflammatory Cytokine Production and ECM Accumulation in mMCs

Inflammation and ECM accumulation in the glomerular mesangium was the key features of DN progression. To study the role of IncRNA MEG3-205 in mesangial cells in DN, we overexpressed MEG3-205 in mMCs treated with AGEs for three indicated time (12 h, 24 h, 48 h) (Fig. 2a). Results of ELISA revealed that compared to empty vector, MEG3-205 promoted pro-inflammatory cytokines production of TNF-a, IL-6, and MCP-1 in the cellular supernatant of mMCs after AGEs stimulation for 12 h, 24 h, and 48 h (Fig. 2b–d). Western blot and following quantitative analysis indicated that overexpression of MEG3-205 promoted accumulation of ECM including fibronectin and collagen type I in AGEs treated mMCs for 12 h, 24 h, and 48 h (Fig. 2e–g). Conversely, knockdown of MEG3-205 suppressed pro-inflammatory cytokine production (online suppl. Fig. S1a–d) and reduced ECM accumulation (online suppl. Fig. S1e–g) in mMCs stimulated by AGEs. Thus, these findings showed that IncRNA MEG3-205 exerted a role in accelerating glomerular inflammation and sclerosis in DN.

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Fig. 1. IncRNA MEG3-205 was upregulated in both db/db mice and DN patients. The hierarchical clustering heat map (a) and the volcano plot of the differentially expressed IncRNAs (b) in kidney tissues of db/db compared with db/m mice (n = 6, fold change >2; p < 0.05). c Expression levels of four MEG3 transcripts were validated using qRT-PCR in mouse models of DN (db/db) and controls (db/m) (n = 6). d Expression and localization of IncRNA MEG3-205 was detected using FISH in kidney tissues of db/db and db/m mice. Scale bars, 50 μm. e qRT-PCR analysis of IncRNA MEG3-205 levels in mMCs treated with AGEs (100 μg/mL) and BSA controls for the indicated time (n = 3, 12 h, 24 h, 48 h). f qRT-PCR analysis of human homolog of IncRNA MEG3-205 levels in renal tissues of patients with biopsy-proven DN and normal controls (n = 3). g Expression and localization of human homolog of IncRNA MEG3-205 was detected using ISH in kidney tissues of patients with biopsy-proven DN and normal controls. Scale bars, 50 μm. Data were represented as mean ± SEM. Ns means p ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
(For legend see next page.)
IncRNA MEG3-205 Induces Renal Inflammation via MyD88

We next investigated the possible mechanism by which IncRNA MEG3-205 mediated the functions in DN. Bioinformatics differential analysis selected out the significantly differentially expressed mRNAs in renal tissues of db/db compared to db/m mice and showed MyD88 was one of the upregulated mRNAs (Fig. 3a; online suppl. Table S1). Thereafter, gene set enrichment analysis, KEGG analysis, and GO analysis showed that the NF-κB signaling pathway and inflammatory response were activated in kidney tissues of db/db mice compared with db/m mice (Fig. 3b–d). Furthermore, the co-expression network between differentially expressed IncRNAs and mRNAs and expression correlation analysis revealed that IncRNA MEG3-205 had high-positive correlation with MyD88 (Fig. 3e, f).

IncRNA MEG3-205 Targets miR-Let-7a to Promote MyD88 Expression

According to the TargetScan, MyD88 and MEG3-205 share the same miRNA response elements of mir-let-7a, whose target sequence was complementary to both MEG3-205 and MyD88 3′-UTR (Fig. 4a; online suppl. Fig. S2a). In order to confirm the above prediction analysis, dual-luciferase reporter assays were applied in mMCs. IncRNA MEG3-205 cDNA (RLuc-IncRNA-MEG3-205-wt) and mutant derivatives lacking the putative let-7a binding sites (RLuc-IncRNA-MEG3-205-mut) were subcloned into a luciferase reporter vector (Fig. 4b). The results indicated that let-7a mimics significantly reduced the luciferase activity of the MEG3-205-wt group but not the MEG3-205-mut group (Fig. 4b). Likewise, the luciferase activity of MyD88 mRNA 3′-UTR was significantly decreased by the let-7a mimic in the wild-type group, but no difference was found in the mutant group (online suppl. Fig. S2b).

Knockdown of IncRNA MEG3-205 Inhibits Renal Inflammation and Fibrosis in Db/Db Mice

IncRNA MEG3-205 promotes renal inflammation and fibrosis through the MEG3-205/miR-let-7a/MyD88 axis, suggesting that MEG3-205 may become a notably key treatment target for DN patients. Therefore, we investigated whether knockdown of IncRNA MEG3-205 can ameliorate progression of DN by delivering long-acting IncRNA MEG3-205 siRNA into 8-week-old db/db mice for 16 weeks (3 mg/kg, caudal-vein injection, every 2 weeks). At 24 weeks, compared with db/m mice, the staining intensities of MEG3-205 and MyD88 were all increased in kidney tissues of db/db mice but decreased in db/db-siRNA mice (Fig. 5a). Pathologically, the mesangial matrix and collagen accumulation were significantly increased in db/db mice and reduced in db/db-siRNA mice (Fig. 5a).

Moreover, the detection of 24-h urine and serum samples collected at 8, 16, and 24 weeks of age showed that knockdown of IncRNA MEG3-205 reduced body weight and alleviated the urine-albumin-creatinine ratio of db/db mice (Fig. 5b, d). However, MEG3-205 siRNA did not affect fasting blood glucose (Fig. 5c).

Treatment with IncRNA MEG3 siRNA inhibited MCP-1 expression and reduced the infiltration of F4/80+ macrophages, as determined by immunofluorescence (Fig. 6a). Western blot analyses showed that compared...
**Fig. 3.** lncRNA MEG3-205 in Diabetic Nephropathy

- **a** Heat map of some differentially expressed mRNAs in kidney tissues of db/db compared to db/m mice.
- **b** GSEA of upregulated genes in db/db mice for the NF-κB signaling pathway.
- **c** KEGG analysis of differentially expressed genes in db/db mice.
- **d** GO analysis of differentially expressed genes in db/db mice.
- **e** Co-expression network diagram of gene intersections of differentially expressed lncRNAs and mRNAs.
- **f** Expression correlation analysis of lncRNAs MEG3-205 and MyD88.

**Fig. 4.** IncRNA MEG3-205 upregulated MyD88 by sponging Let-7a.

- **a** Sequence alignment between Let-7a and MEG3-205-wt and MEG3-205-mut. Red color indicates the sequence of the mutated Let-7a binding site.
- **b** Relative luciferase activities were detected in mMCs after transfected with MEG3-205-wt, MEG3-205-mut, and Let-7a mimics or NC (n = 4).
- **c** Anti-AGO2 and anti-IgG RIPs were executed in mMCs, followed by qRT-PCR to detect the enrichment ability of AGO2 on MEG3-205 and Let-7a compared with IgG (n = 3).
- **d** Anti-SNRNP70 and anti-IgG RIPs were executed in mMCs, followed by qRT-PCR to detect the enrichment ability of SNRNP70 on U1 compared with IgG (n = 3).
- **e** Representative Western blot (e) and quantitative analysis (f) of MyD88 in mMCs were detected of an empty vector or MEG3-205 with Let-7a mimics or NC. Data were represented as mean ± SEM. Ns means p ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
- **f** MEG3-205-wt, MEG3-205 wild-type; MEG3-205-mut, MEG3-205 mutant; NC, negative control.
Fig. 5. In vivo delivery of long-acting lncRNA MEG3-205 siRNA ameliorated progression of DN. 

a Left, FISH of lncRNA MEG3-205, immunofluorescence staining of MyD88, PAS, and Masson staining were performed on kidney tissues of three group mice. Scale bars, 50 μm. Right, the relative fluorescence intensity of lncRNAs MEG3-205 and MyD88 and the area percentage of mesangial matrix and collagen staining were calculated and visualized (n = 6), respectively. Levels of body weight (b), FBG (c), and UACR (d) in db/m, db/db, and db/db-siRNA mice (n = 6). Db/db-siRNA mice and db/db mice treated with long-acting lncRNA MEG3-205 siRNA. Data were represented as mean ± SEM. Ns means p ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. PAS, periodic acid–Schiff; UACR, urinary-albumin-creatinine ratio; FBG, fasting blood glucose.
with db/m control mice, the protein abundance of fibrosis markers was significantly increased in db/db mice. However, treatment with IncRNA MEG3 siRNA inhibited NF-κB activation, as demonstrated by the reduction of phospho-IKKα/β and phospho-P65/RelA in db/db mice (Fig. 6b). Moreover, IncRNA MEG3 siRNA rescued the enrichment of fibronectin and collagen I (Fig. 6c). These findings suggested that knockdown of IncRNA MEG3 attenuated the progression of renal inflammation and fibrosis during DN in vivo.

Discussion

In this study, we found that the transcript of MEG3 (IncRNA MEG3-205) was significantly upregulated in diabetic db/db mice and in AGEs-induced mMCs. IncRNA MEG3-205 served as a competing endogenous RNA of let-7a to modulate the expression of MyD88 and thereby activate the NF-κB signaling pathway and finally accelerate renal inflammation and fibrosis in DN (Fig. 7).

Targeted RNA sequencing has revealed that IncRNAs can be alternatively spliced to produce differential transcripts [17]. Emerging evidence indicates that IncRNA splice variants are critical for many aspects of cell biology and molecular functions [18–21]. However, little is known about the functional implications of MEG3 transcript isoforms. Overexpression of IncRNA MEG3-4 exacerbated mouse susceptibility to lung infection by regulating IL-1β [22], and IncRNA MEG3-210 downregulation promoted endometrial stromal cells migration and invasion via interaction with Galectin-1 in endometriosis [23]. Our results revealed that 4 transcripts of IncRNA MEG3 were differentially expressed in the kidney tissues of the db/db mice, and IncRNA MEG3-205 was the top upregulated isoforms validated by qRT-PCR. However, how IncRNA MEG3 isoforms are selectively edited and transcribed remains largely unknown. Thus, the regulatory mechanism and the functional implications of these variants in human diseases deserve further investigation.

Numerous studies have implicated that IncRNAs can act as a competing endogenous RNA or a molecular sponge to modulate expression and function of micro-RNAs [24–26]. In this study, we found IncRNA MEG3-205 can sponge let-7a and act as the regulator to affect the expression of its target gene MyD88 in DN. The let-7 family members have an anti-fibrotic role in renal fibrosis [27], lung fibrosis [28], and cardiac fibrosis [29] and are involved in inflammatory processes as well [30]. It was reported that let-7a was decreased under HG conditions, and let-7a can negatively regulate transforming the growth factor-β1/Smad signaling pathway in DN [31, 32]. In our study, we demonstrated that IncRNA MEG3-205 overexpression significantly rescued the silencing effect of let-7a on MyD88 protein expression in mMCs, indicating that MEG3-205 acted as a molecular sponge for let-7a and thus regulated MyD88 expression. Renal inflammation involving the activation of Toll-like receptor 4 (TLR4) and the NF-κB signaling pathway plays a crucial role in the development and progression of DN [33, 34]. Exposure to diabetic substrates such as HG levels induced the expression of TLR4 and activated the TLR signaling cascade [35]. MyD88 is an essential adapter protein for TLR signaling, which is involved in translocation of NF-κB to the nucleus and transcription of genes that code pro-inflammatory cytokines [36]. Polymorphisms in the MyD88 gene are associated with susceptibility to type 2 diabetes mellitus in a southern Han Chinese population [37]. In particular, the level of MyD88 was increased in STZ-induced diabetic rats and rat renal tubular epithelial cells cultured under HG conditions [38], and LM8, a small-molecule MyD88 inhibitor, suppressed inflammation in tubular epithelial cells and prevented DN in experimental mice [39]. In the present study, we demonstrated MEG3-205 regulated MyD88 expression through competition for let-7a binding, and it may be an important underlying mechanism in the renal inflammation and fibrosis in DN.

The rapid development in the field of noncoding RNAs has helped these RNA-based biopharmaceuticals to be novel therapeutic approach for DN [40]. Kidney-specific silencing of IncRNA Erbb4-IR and LRNA9884 with ultrasound technique can ameliorate renal injury in db/db mice [24, 41]. IncRNA ANRIL knockout mice treated with STZ have lower urine albumin levels than the wild-type diabetic animals [42]. However, the function of tar-
Targeting lncRNA MEG3 in DN was controversial in vivo. Zha et al. [12] showed that lncRNA MEG3 overexpression in STZ diabetic rats using lentiviral MEG3 injection enhanced DN development. Besides, Li et al. [13] found that knockdown of lncRNA MEG3 in db/db mice using si-MEG3 decreased blood glucose, urine protein, mRNA levels of fibronectin, and collagen IV in kidney tissues. However, lncRNA MEG3 overexpression in STZ diabetic rats using pcDNA3-MEG3 suppressed podocyte injury and attenuated kidney injury in another study [14]. In this study, we found that targeting lncRNA MEG3-205 using long-acting siRNA attenuated renal inflammation and fibrosis in db/db mice. We also found human homolog of lncRNA MEG3-205 levels was increased in kidney tissues of patients with biopsy-proven DN, which may be a potential therapy target in humans.

This study had some limitations. We were not able to investigate the role of lncRNA MEG3-205 in a kidney-

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**Fig. 7.** Schematic diagram summarizing our findings that lncRNA MEG3-205 serves as a ceRNA of let-7a to modulate the expression of MyD88 and thereby activate the NF-κB signaling pathway, which accelerates renal inflammation and fibrosis in DN. ceRNA, competing endogenous RNA.
specific IncRNA MEG3-205 knockout mouse. It was unknown whether IncRNA MEG3-205 renal expression was correlated with the severity and outcome of DN patients. In addition, we did not determine the effect of silencing MEG3-205 on podocyte in vivo. Further studies will be needed to verify the role of IncRNA MEG3-205 as biomarkers or therapeutic targets in DN patients.

In conclusion, our study findings indicate that IncRNA MEG3-205 promotes renal inflammation and fibrosis in DN by interacting with let-7a and upregulating MyD88. Our findings suggest that IncRNA MEG3-205 may serve as a potential therapeutic target in DN.

Statement of Ethics

The protocol for the use of biopsied samples and nephrectomized tissues from patients was approved by the local committee on human subjects at the First Affiliated Hospital of Sun Yat-Sen University. The patients/participants provided their written informed consent to participate in this study. All animal experiments were performed in accordance with the guidelines and regulations of the Animal Experimentation Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University. Study approval number: [2013]A-281.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Qimei Luo, Xi Xia, and Feng He designed the research. Qimei Luo, Xi Xia, Qingqing Luo, Yue Qiu, Lan Dong, Chen Zhao, Fenfen Peng, and Jing Yu performed the experiments. Qimei Luo, Xi Xia, Qingqing Luo, Yue Qiu, and Feng He analyzed and interpreted the data. Qimei Luo, Xi Xia, and Qingqing Luo wrote the draft of the manuscript. Fengxian Huang and Feng He revised the manuscript. All the authors approved the submitted version.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.
IncRNA MEG3-205 in Diabetic Nephropathy

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