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ARTICLE

MicroRNA-26a inhibits TGF-β-induced extracellular matrix protein expression in podocytes by targeting *CTGF* and is downregulated in diabetic nephropathy

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Abstract
Aims/hypothesis The accumulation of extracellular matrix (ECM) is a characteristic of diabetic nephropathy, and is partially caused by profibrotic proteins TGF-β and connective tissue growth factor (CTGF). We aimed to identify microRNAs (miRNAs) targeting *CTGF* on podocytes in diabetic nephropathy.
Methods We investigated miRNAs targeting *CTGF* on podocytes with miRNA array analysis and identified a candidate miRNA, miR-26a. Using overexpression and silencing of miR-26a in cultured podocytes, we examined changes of ECM and its host genes. We further investigated glomerular miR-26a expression in humans and in mouse models of diabetic nephropathy.
Results miR-26a, which was downregulated by TGF-β1, was expressed in glomerular cells including podocytes and in tubules by in situ hybridisation. Glomerular miR-26a expression was downregulated by 70% in streptozotocin-induced diabetic mice. Transfection of miR-26a mimics in cultured human podocytes decreased the CTGF protein level by 50%, and directly inhibited *CTGF* expression in podocytes, as demonstrated by a reporter assay with the 3′-untranslated region of the *CTGF* gene. This effect was abolished by a mutant plasmid. miR-26a mimics also inhibited TGF-β1-induced collagen expression, SMAD-binding activity and expression of its host genes *CTDSP2* and *CTDSPL*. Knockdown of *CTDSP2* and *CTDSPL* increased collagen expression in TGF-β-stimulated podocytes, suggesting that host genes also regulate TGF-β/SMAD signalling. Finally, we observed a positive correlation between microdissected glomerular miR-26a expression levels and estimated GFR in patients with diabetic nephropathy.
Conclusions/interpretation The downregulation of miR-26a is involved in the progression of diabetic nephropathy both in humans and in mice through enhanced TGF-β/CTGF signalling.

Keywords CTGF · Diabetic nephropathy · MicroRNA · Podocytes · Streptozotocin

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Abbreviations

CCN	Cysteine-rich, angiogenic inducer, 61, CTGF, nephroblastoma overexpressed
CTDSP	Small carboxy-terminal domain phosphatase
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
eGFR	Estimated GFR
ISH	In situ hybridisation
miRNA	MicroRNA
NC	Negative control
SBE	SMAD binding element
STZ	Streptozotocin
UTR	Untranslated region

Introduction

Diabetic nephropathy is a major microvascular complication of diabetes mellitus and the leading cause of end-stage renal disease worldwide [1]. The condition is characterised by microalbuminuria, glomerular hypertrophy, mesangial expansion and an accumulation of extracellular matrix (ECM) proteins [2]. The accumulation of ECM proteins, such as collagen, is one of the main hallmarks of diabetic nephropathy [3]. Evidence has shown that TGF-β plays a key role in these pathological changes [4]. Podocyte injury and loss are found in the very early stages of diabetic nephropathy and may be a major starting point of glomerular injury in the disease [5]. Metabolic and haemodynamic dysregulation in diabetes can cause structural and functional changes in podocytes, leading to podocyte injury or loss [5]. Several cytokines or growth factors, such as TGF-β and connective tissue growth factor (CTGF, also known as CCN2) [6, 7], have been proposed to be involved in podocyte injury. The precise mechanisms of their role, however, remain unclear.

CTGF belongs to the CCN family of cysteine-rich growth factors, which consists of six related genes with four conserved domains [8]. Under normal conditions, *CTGF* mRNA is mainly expressed in podocytes and detected in some parietal epithelial cells of the glomeruli in the kidneys of humans and rats [9, 10]. Under the diabetic milieu, *CTGF* expression is markedly upregulated in podocytes, mesangial cells and tubulointerstitial cells, which are associated with fibrotic lesions [6, 9, 10]. *CTGF* gene expression is strongly induced by TGF-β in a SMAD3/SMAD4-dependent manner [11], and recombinant CTGF potentially enhances the synthesis of ECM proteins, such as collagen or fibronectin, in cultured podocytes [12], mesangial cells [13], tubular epithelial cells [14] and fibroblasts [15]. In addition to a direct fibrogenic effect, CTGF can exacerbate TGF-β-induced fibrosis by activating TGF-β and SMAD signalling through promoting the association of TGF-β with its receptor [16]. TGF-β-induced collagen production is attenuated by a CTGF neutralising antibody or antisense oligonucleotide targeting *CTGF* [17–19]. We demonstrated that in streptozotocin (STZ)-induced diabetes, the podocyte-specific CTGF transgenic mice had marked worsening of proteinuria and mesangial expansion accompanied by podocyte loss compared with wild-type mice [7]. Although these observations are consistent with CTGF as a key mediator of diabetic nephropathy progression, the precise regulation of CTGF in glomeruli in the presence of diabetes, especially in podocytes, remains elusive.

MicroRNAs (miRNAs) are small (approximately 22-nucleotide long), noncoding RNAs that downregulate gene expression by modulating the stability and/or translation of target mRNAs. These molecules play critical roles in various cellular functions. Dysregulation of miRNAs is involved in numerous pathological conditions, including renal disease [20, 21]. miR-192 [22], miR-200 [23], and miR-215 [24] are reported to be involved in the TGF-β-dependent pathogenesis of diabetic nephropathy. The mechanism of how they modulate the disease seem very complex and further investigations are required to clarify the role of these miRNAs in diabetic nephropathy [25]. A number of studies have investigated the regulation of *CTGF* expression at the promoter level [11, 26], but few have examined the post-transcriptional regulation of *CTGF* by miRNAs. miRNA targeting *CTGF* in diabetic nephropathy has not been reported. The present study was performed to identify miRNAs targeting *CTGF* and to investigate the role of miRNAs in the progression of diabetic nephropathy.

Methods

Human biopsy samples and laser microdissection This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Ethics Committee on Human Research at Kyoto University Graduate School of Medicine (Kyoto, Japan). Patients with type 2 diabetes who underwent renal biopsy were enrolled in the study under informed consent. Renal biopsy samples were obtained from 11 patients with diabetic nephropathy, as diagnosed by renal biopsy. The estimated GFR (eGFR) was calculated using the equation adopted by the Japanese Society of Nephrology [27].

Two to ten glomeruli on a slide were microdissected and collected using the Leica 6500 Laser Microdissection System (Leica Microsystems, Wetzlar, Germany) and then RNA was extracted (for further details, please refer to the electronic supplementary material [ESM] Methods).

Diabetic animal models All animal experiments were approved by the Animal Experimentation Committee of Kyoto University Graduate School of Medicine. Twelve 8-week-old

male C57BL/6J mice were used in this study (Clea Japan, Tokyo, Japan). Mouse models of diabetes were generated by intraperitoneal injection of STZ (100 mg/kg body weight for three consecutive days [7]; for further details, please refer to the ESM Methods).

In situ hybridisation In situ hybridisation was performed as described previously [18] (for further details, please refer to the ESM Methods).

Cell culture Conditionally immortalised human podocytes were developed by transfection with the temperature-sensitive SV40 T-gene, as described previously [28]. Cells were stimulated with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA; for further details, please refer to the ESM Methods).

MicroRNA microarray Differentiated podocytes were treated with 5 ng/ml TGF-β1 (R&D Systems) for 24 h in RPMI 1640 medium under serum starvation. Labelled samples were hybridised to the Human miRNA Microarray Kit 8x15K V2 (Agilent Technologies, Palo Alto, CA, USA) containing probes for 723 mature human miRNAs. The microarray images were analysed using Feature Extraction Software (Agilent Technologies). Data are uploaded on Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, Accession no. GSE66133; for further details, please refer to the ESM Methods).

Real-time RT-PCR analysis Real-time RT-PCR was performed as described previously [7] (for further details, please refer to the ESM Methods).

Transfection Differentiated podocytes were transfected with miR-26a mimics or mimic negative control (NC; 30 nmol/l), miR-26a inhibitors or inhibitor NC (30 nmol/l), and siRNA for *CTGF*, *CTDSP2* and *CTDSPL* (5 nmol/l) by a pulse of electroporation with Nucleofector (Amaxa Biosystems, Cologne, Germany) using the Basic Nucleofector Kit for Primary Mammalian Epithelial cells (Amaxa Biosystems) [29] (for further details, please refer to the ESM Methods).

Immunocytochemical study Immunocytochemical analyses of SMAD4 was performed with the stimulation of TGF-β1 with miR-26a mimic or NC transfection (for further details, please refer to the ESM Methods).

Western blotting Western blotting was performed as previously described [30] (for further details, please refer to the ESM Methods).

Plasmid construction and luciferase assay The pMirTarget-*CTGF* 3′-untranslated region (UTR) vector was purchased from OriGene Technologies (Rockville, MD, USA). Mutant constructs were generated by replacing the seed regions of the miR-26a binding sites (two to seven) with 5′-TTGGTT-3′ (Fig. 5f, ESM Table 1) by PCR-based, site-directed mutagenesis [31]. The SMAD binding element (SBE4)-luciferase plasmid contains four copies of SBE (taaGTCTAGACggcaGTCT AGACgtac) in the pBV-luciferase plasmid (Addgene, Cambridge, MA, USA) [32] (for further details, please refer to the ESM Methods).

Statistical analyses Data are expressed as the mean±SE. Statistical analysis was performed using one-way ANOVA or Student's unpaired *t* test as appropriate. Spearman's correlation coefficients were used to examine the correlation between two variables. A p value <0.05 was considered statistically significant. All data were analysed using StatView 5.0 software (SAS Institute, Cary, NC, USA) or JMP7.1 (SAS Institute).

Results

Downregulation of miR-26a by TGF-β stimulation in cultured human podocytes CTGF is a major downstream mediator of TGF-β that promotes ECM accumulation and fibrosis by directly interacting with TGF-β in diabetic nephropathy. To identify miRNAs regulating *CTGF* downstream of TGF-β, we analysed expression profiles of 723 miRNAs in immortalised human podocytes after TGF-β1 (5 ng/ml) stimulation using microRNA array analysis. We identified 46 miRNAs in which expression levels were altered by >1.5-fold between PBS- and TGF-β1-treated podocytes (Fig. 1a, Table 1). Of these 46 miRNAs, a microRNA database (http://microrna.org, last accessed 20 January 2015) identified miR-26a as being the only microRNA targeting *CTGF*. miR-26a has a putative target site in the 3′- UTR of the *CTGF* gene (Fig. 1b). The seed regions of miR-26a are highly conserved across species (Fig. 1c). To validate microarray data, we further examined the expression of miR-26a in TGF-β1-treated podocytes by real-time RT-PCR. Stimulation with TGF-β1 decreased miR-26a expression on podocytes in a dose-dependent manner (Fig. 1d). Expression of another internal control, small nuclear RNA U6B, did not change following stimulation with TGF-β1 (ESM Fig. 1).

Expression and localisation of miR-26a in glomeruli of diabetic mice We analysed miR-26a expression in glomeruli of diabetic mice because podocytes are thought to be injured at a very early stage in diabetic nephropathy. First, we studied the localisation of miR-26a in the kidney by ISH. miR-26a expression was detected in glomerular cells, including podocytes and in tubules and collecting ducts of control mice (Fig. 2a, b). Next, we examined miR-26a expression in the

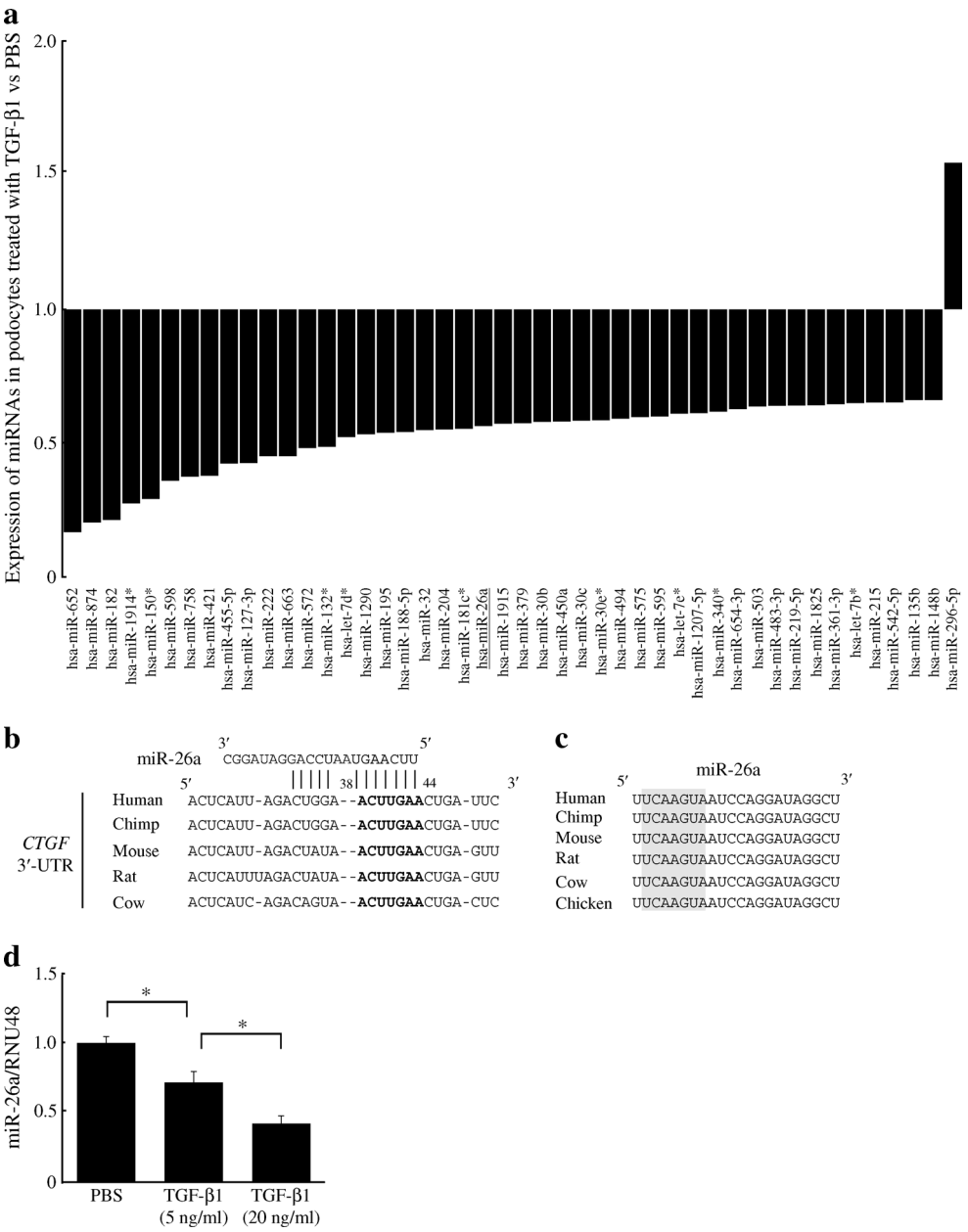

Fig. 1 miR-26a is downregulated by TGF-β1 stimulation in human podocytes. (**a**) Differentiated podocytes were treated with TGF-β1 (5 ng/ml) or PBS for 24 h. The expression profiles of 723 miRNAs were analysed by microRNA array, and 46 miRNAs showed >1.5-fold differences in the expression levels between PBS- and TGF-β1-treated podocytes. miR-26a is underlined. (**b**) miR-26a binding sites in the 3′-UTR of the *CTGF* transcripts of various species. Seven nucleotides in the 5′ region of miR-26a contain a perfect match with 3′-UTR of *CTGF* sequences. The miRNA seed regions are in bold. (**c**) The mature sequences of miR-26a are conserved across various species. The seed sequences are highlighted by grey shading. (**d**) The expression of miR-26a in 5 or 20 ng/ml TGF-β1-treated podocytes was examined by real-time RT-PCR at 24 h after stimulation. RNU48 was used as an internal control. $n=5$ for each group. *$p<0.05$. Data are mean±SE

glomeruli of STZ-induced diabetic mice. Compared with control mice, glomerular miR-26a expression was significantly lower (by 70%) at 3 weeks after STZ administration (Fig. 3a). By contrast, glomerular *Tgfb1*, *Ctgf* and *Col1a1* mRNA expressions were increased in diabetic mice compared with controls (Fig. 3b–d). In addition, the expression of host genes encoding miR-26a, *Ctdsp2* and *Ctdspl*, was increased (Fig. 3e, f). Thus, miR-26a is expressed in the kidney, including in podocytes, and is downregulated in diabetic nephropathy.

Glomerular miR-26a expression level is associated with eGFR in renal biopsy specimens from patients with diabetic nephropathy We investigated glomerular miR-26a expression in optimal cutting temperature compound-embedded, laser-microdissected renal biopsy specimens with diabetic nephropathy by real-time RT-PCR (Fig. 4a). Table 2 summarises the clinical characteristics of the patients. We examined the association between glomerular miR-26a expression and clinical variables at the time of renal biopsy. Figures 4b and c show representative views of renal biopsy specimens with diabetic nephropathy at the early and the advanced stages, respectively. Of note, glomerular miR-26a expression in human diabetic nephropathy positively correlated with eGFR (Fig. 4d; $p=0.025$; $r=0.659$). Glomerular miR-26a expression did not correlate with proteinuria (Fig. 4e).

Table 1 Fold change in miRNA expression in 5 ng/ml TGF-β1-treated cultured human podocytes compared with vehicle-treated cells

Name	miRBase ID	Fold change
hsa-miR-296-5p	MIMAT0000690	1.544
hsa-miR-148b	MIMAT0004699	0.664
hsa-miR-135b	MIMAT0000758	0.664
hsa-miR-542-5p	MIMAT0003340	0.655
hsa-miR-215	MIMAT0000272	0.654
hsa-let-7b*	MIMAT0004482	0.652
hsa-miR-361-3p	MIMAT0004682	0.648
hsa-miR-1825	MIMAT0006765	0.644
hsa-miR-219-5p	MIMAT0000276	0.643
hsa-miR-483-3p	MIMAT0002173	0.643
hsa-miR-503	MIMAT0002874	0.640
hsa-miR-654-3p	MIMAT0004814	0.630
hsa-miR-340*	MIMAT0000750	0.621
hsa-miR-1207-5p	MIMAT0005871	0.615
hsa-let-7e*	MIMAT0004485	0.613
hsa-miR-595	MIMAT0003263	0.603
hsa-miR-575	MIMAT0003240	0.600
hsa-miR-494	MIMAT0026607	0.595
hsa-miR-30e*	MIMAT0026607	0.588
hsa-miR-30c	MIMAT0000244	0.587
hsa-miR-450a	MIMAT0001545	0.584
hsa-miR-30b	MIMAT0000420	0.583
hsa-miR-379	MIMAT0000733	0.578
hsa-miR-1915	MIMAT0007891	0.576
hsa-miR-26a	MIMAT0000082	0.567
hsa-miR-181c*	MIMAT0004559	0.557
hsa-miR-204	MIMAT0000265	0.554
hsa-miR-32	MIMAT0000090	0.552
hsa-miR-188-5p	MIMAT0000457	0.545
hsa-miR-195	MIMAT0000461	0.542
hsa-miR-1290	MIMAT0005880	0.537
hsa-let-7d*	MIMAT0004484	0.526
hsa-miR-132*	MIMAT0004594	0.490
hsa-miR-572	MIMAT0003237	0.485
hsa-miR-663	MIMAT0003326	0.455
hsa-miR-222	MIMAT0004569	0.455
hsa-miR-127-3p	MIMAT0000446	0.429
hsa-miR-455-5p	MIMAT0003150	0.428
hsa-miR-421	MIMAT0003339	0.382
hsa-miR-758	MIMAT0022929	0.379
hsa-miR-598	MIMAT0026620	0.364
hsa-miR-150*	MIMAT0004610	0.296
hsa-miR-1914*	MIMAT0007890	0.279
hsa-miR-182	MIMAT0000259	0.218
hsa-miR-874	MIMAT0026718	0.209
hsa-miR-652	MIMAT0022709	0.173

*A less predominant form of miRNA

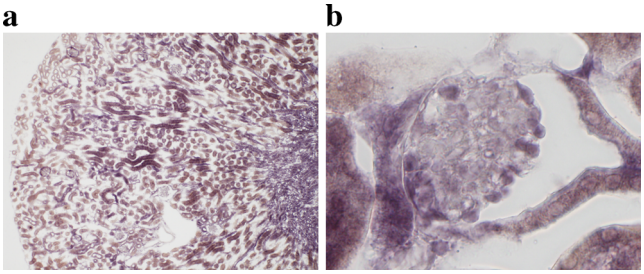

Fig. 2 Localisation of miR-26a in the kidney. ISH for miR-26a in the kidneys of control mice at (**a**) lower magnification (×40) and (**b**) higher magnification (×400). miR-26a is detected in glomerular cells, including podocytes, and in tubules and collecting ducts of control mice

CTGF is a direct target of miR-26a To investigate whether or not miR-26a targets the *CTGF* gene, we transfected miR-26a mimics (30 nmol/l) into podocytes by nucleofection and then examined *CTGF* expression by real-time RT-PCR and western blotting. Transfection of miR-26a mimics did not change *CTGF* mRNA level (Fig. 5a) but significantly reduced CTGF protein level by approximately 50% (Fig. 5b, c), suggesting that miR-26a negatively regulates *CTGF* in a post-transcriptional manner. To determine whether or not *CTGF* is a direct target of miR-26a, we performed luciferase reporter assays using the pMirTarget-*CTGF* 3′-UTR plasmid, which contains the *CTGF* 3′-UTR sequence downstream of the firefly luciferase gene (Fig. 5d). Co-transfection of the reporter plasmids with miR-26a mimics significantly reduced luciferase activity by approximately 40% compared with NC (Fig. 5e), and miR-26a inhibitors significantly increased luciferase activity by 30% (Fig. 5e). These results suggest that binding of miR-26a to the 3′-UTR of *CTGF* mRNA inhibits translation. To confirm these results, we generated a corresponding miR-26a mutant plasmid by replacing the seed regions of the miR-26a-binding sites with 5′-TTGGTT-3′

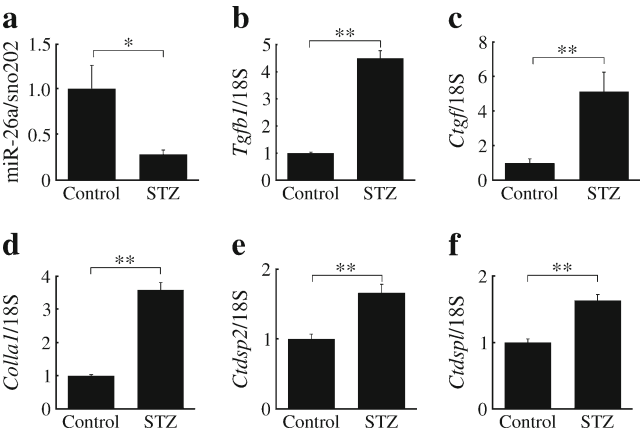

Fig. 3 miR-26a is downregulated in glomeruli of diabetic mice. Real-time RT-PCR analyses of (**a**) miR-26a, (**b**) *Tgfb1*, (**c**) *Ctgf*, (**d**) *Col1a1*, (**e**) *Ctdsp2* and (**f**) *Ctdspl* mRNA expression in glomeruli of control or STZ-treated mice (n=6 in each group) at 3 weeks after vehicle or STZ injection. *p<0.05, **p<0.01. Data are mean±SE. sno202, small nucleolar RNA MB11-202

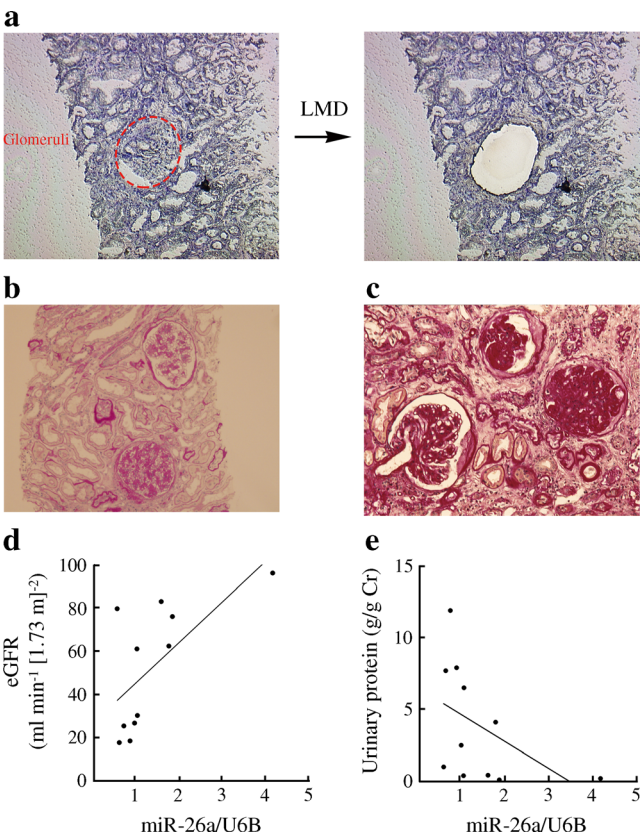

Fig. 4 Correlation between glomerular miR-26a expression and clinical variables in human biopsy samples. (**a**) Representative views of glomerular extraction in OCT-embedded renal biopsy specimens before and after laser-micro dissection (magnification ×200). Representative views of renal biopsy specimens with diabetic nephropathy at the (**b**) early stages in PAS stain (serum creatinine [Cr] level, 70.7 mmol/l; urinary protein, 0.6 g/g Cr, magnification ×100) and (**c**) advanced stages in PAS stain (serum Cr level, 256 mmol/l; urinary protein, 7.9 g/g Cr, magnification ×200). Dot plot analysis of glomerular miR-26a with (**d**) eGFR (r=0.66, p=0.025) and (**e**) proteinuria (r=−0.47, p=0.15). Line in each graph shows the regression. n=11. p values <0.05 were considered statistically significant. LMD, laser microdissection; OCT, optimal cutting temperature; PAS, periodic acid–Schiff

(Fig. 5f, ESM Table 1). Such mutation abolished the ability of miR-26a to suppress luciferase expression (Fig. 5g). These results demonstrate that *CTGF* is a direct target of miR-26a.

Inhibition of TGF-β-induced ECM accumulation and TGF-β/SMAD signalling by miR-26a We further examined whether miR-26a can inhibit TGF-β-induced ECM accumulation by inhibiting *CTGF*. Transfection of miR-26a mimics significantly decreased TGF-β1-induced upregulation of *COL1A1* and *COL4A3* expression (Fig. 6a, b). This effect was consistent with the findings that knockdown of *CTGF* by siRNA decreased TGF-β1-induced *COL1A1* and *COL4A3* expression (Fig. 6c–f). These observations demonstrate that miR-26a can inhibit TGF-β/CTGF-induced ECM accumulation. We also analysed SMAD activity in response to stimulation by

Table 2 Baseline clinical characteristics of patients at renal biopsy analysed for miR-26a expression

Number of patients	11
Age (years)	56±3.1
Sex (male/female)	9/2
HbA_{1c} (NGSP, %)	6.8±0.4
HbA_{1c} (mmol/mol)	51±4
Systolic BP (mmHg)	146.9±5.3
Diastolic BP (mmHg)	82.5±1.5
Urinary protein (g/g creatinine)	3.9±1.2
Creatinine (mmol/l)	124±27
eGFR (ml min^{-1} 1.73 m^{-2})	52.5±9.8
BUN (mmol/l)	9.2±1.5
Albumin (g/l)	36±3.0
Triacylglycerol (mmol/l)	1.85±0.24
HDL-cholesterol (mmol/l)	1.24±0.09
LDL-cholesterol (mmol/l)	4.10±0.34
RAS blocker (yes/no)	4/7
Global sclerosis (%)	26.2±4.9

Data are mean±SE

BUN, blood urea nitrogen; RAS; renin–angiotensin system

TGF-β1. TGF-β augmented phosphorylation of SMAD3, and this phosphorylation was significantly abolished by miR-26a (Fig. 6g). Nuclear translocation of SMAD4 induced by TGF-β was attenuated by miR-26a (Fig. 6h). The activation of SBEs by TGF-β was also significantly inhibited (by 90%) by treatment with miR-26a mimics (Fig. 6i). Interestingly, the miR-26a mimics led to an approximately 75% decrease in *TGFB1* autoinduction (Fig. 6j). Next, we examined whether miR-26a inhibited putative miR-26a-target genes, SMAD2 and SMAD4, by western blotting. SMAD2 and SMAD4 protein levels were not altered by miR-26a mimics (ESM Fig. 2). These results suggest miR-26a suppresses TGF-β-induced ECM accumulation by directly inhibiting *CTGF* in podocytes.

Reduction of CTDSP2 and CTDSPL enhances TGF-β signalling Mature miR-26a is generated from two distinct loci, miR-26a-1 and miR-26a-2, which are located in the intron of *CTDSPL* and *CTDSP2*, respectively (Fig. 7a). The targeting sequences of miR-26a for *CTDSPL* and *CTDSP2* are highly conserved among species (Fig. 7b). We examined expression levels of *CTDSP2* and *CTDSPL* at 24 h after stimulation with TGF-β1. The expression of *CTDSP2* and *CTDSPL* was significantly downregulated by TGF-β1 stimulation (Fig. 7c, d), indicating that miR-26a is expressed in parallel with its host gene. siRNA knockdown of *CTDSP2* or *CTDSPL* enhanced TGF-β1-induced ECM

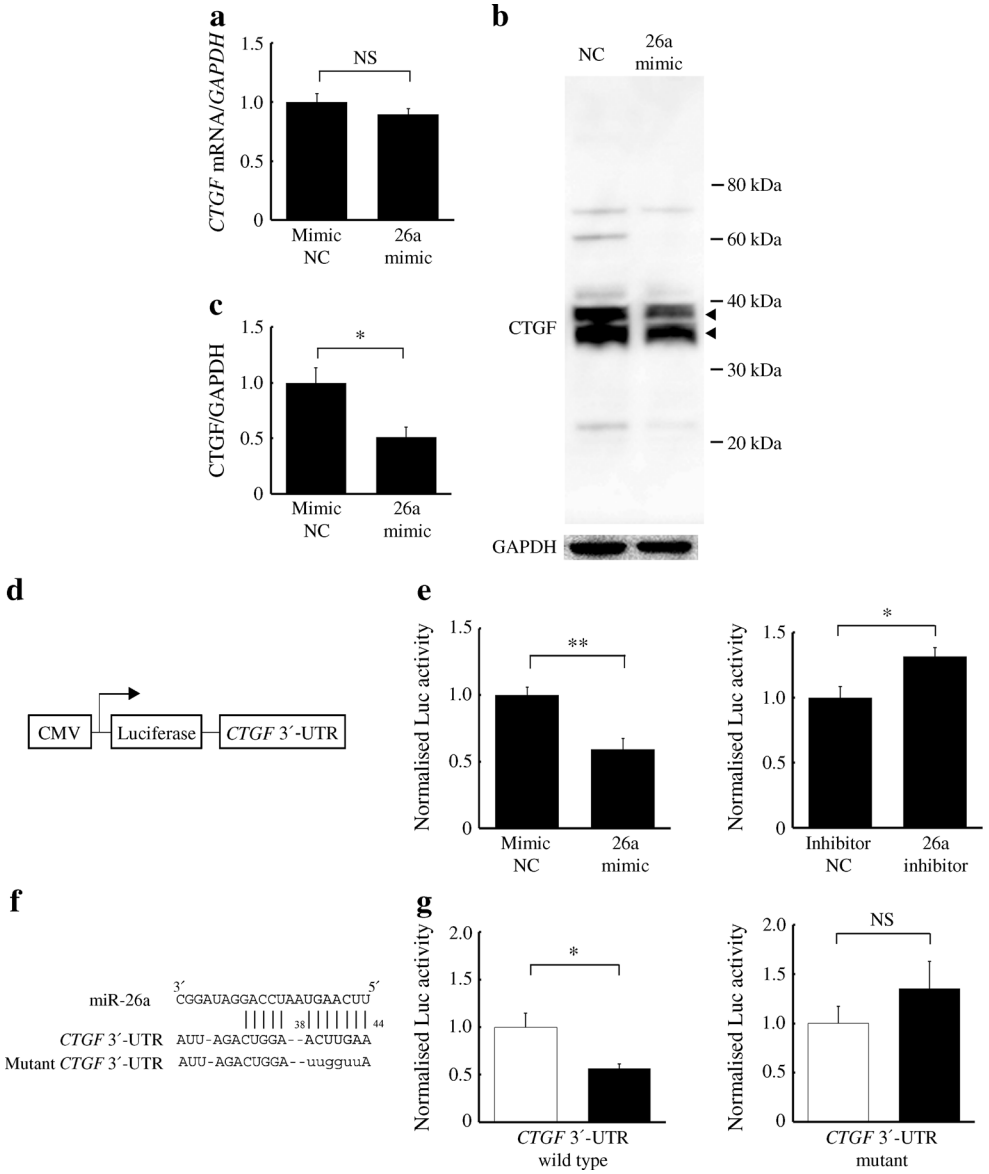

Fig. 5 *CTGF* is a direct target of miR-26a. (**a**) Real-time RT-PCR analysis of *CTGF* and (**b**, **c**) western blots of CTGF in cultured human podocytes transfected with miR-26a mimics or mimic NC at 48 h after transfection. Arrowheads indicate 36 and 38 kDa CTGF of which a larger band indicates a glycosylated form. GAPDH was used as an internal control (n=6 in each group). (**d**) Schematic representation of luciferase reporter constructs which contain *CTGF* 3′-UTR sequence downstream of the firefly luciferase gene (pMirTarget-*CTGF* 3′-UTR plasmid). (**e**) Podocytes were transfected with pMirTarget-*CTGF* 3′-UTR plasmid in the absence or the presence of miR-26a mimics or miR-26a inhibitors (30 nmol/l) and analysed at 8 h after transfection (n=6 in each group). (**f**) Site-directed mutagenesis was performed to mutate the seed regions within the *CTGF* 3′-UTR. Lower case letters indicate mutated nucleotides. (**g**) Podocytes were transfected with a luciferase reporter plasmids containing wild or mutant *CTGF* 3 -UTR and analysed at 8 h after transfection. miR-26a mimics inhibited luciferase activity, but a mutant plasmid abolished the inhibitory effect of miR-26a. White bars, mimic NC; black bars, miR-26a mimic (n=6 in each group). CMV, cytomegalovirus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Luc, luciferase. *p<0.05, **p<0.01. Data are mean±SE

accumulation in cultured podocytes (Fig. 7e–h), suggesting that CTDSP2 and CTDSPL have inhibitory effects on TGF-β/SMAD signalling in podocytes. Furthermore, miR-26a mimics suppressed *CTDSP2* and *CTDSPL* mRNA (Fig. 7i, j). These data suggest that miR-26a is involved in the complex regulation of its host gene (Fig. 8).

Discussion

We identified that miR-26a, which targets the *CTGF* gene, is a potent suppressor of TGF-β/SMAD signalling in cultured human podocytes. Overactivity of TGF-β/SMAD signalling stimulates the production of ECM and plays an important role in the pathogenesis of diabetic nephropathy [33, 34]. CTGF is

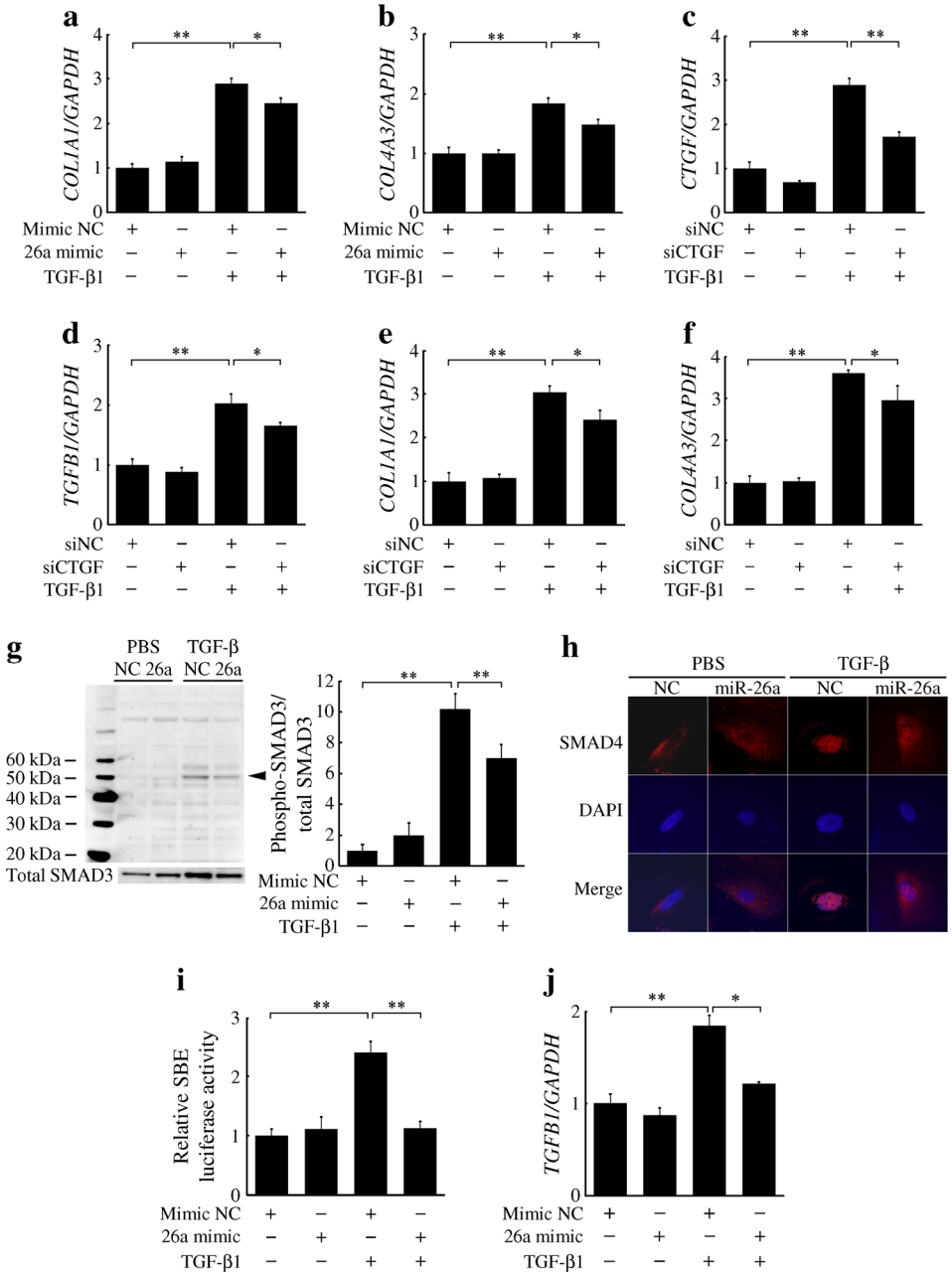

Fig. 6 miR-26a inhibits TGF-β/SMAD signalling in podocytes. Cultured human podocytes were transfected with mimics or plasmid and then stimulated with TGF-β1 (20 ng/ml) at 2 days after the transfection. The expression levels of (**a**) *COL1A1* and (**b**) *COL4A3* were examined at 24 h after stimulation with transfection of miR-26a mimics or mimic NC by real-time RT-PCR (n=3 in each group). (**c**–**f**) Human podocytes were transfected with siRNA for *CTGF* or NC (5 nmol/l). The expression levels of (**c**) *CTGF*, (**d**) *TGFB1*, (**e**) *COL1A1* and (**f**) *COL4A3* were examined (n=4 in each group). (**g**) Western blots for phospho-SMAD3 (arrow head) and total SMAD3 in cultured podocytes. Podocytes transfected with miR-26 mimics or NC were stimulated with TGF-β and then analysed at 30 min after stimulation. 26a, miR-26a mimics. (**h**) Immunocytochemical analyses of SMAD4 (red) in TGF-β-stimulated podocytes transfected with miR-26a mimics or NC (magnification ×400). DAPI, blue. (**i**) SMAD activity was measured using luciferase reporter plasmids containing the SBE. Podocytes were transfected with SBE plasmids and then stimulated with TGF-β1 (20 ng/ml) for 48 h. Luciferase activity was measured (n=6 in each group). (**j**) The expression levels of *TGFB1* were examined at 24 h after stimulation with transfection of miR-26a mimics or mimic NC (n=3 in each group). *p<0.05, **p<0.01. Data are mean±SE

a major downstream profibrotic factor of TGF-β [13, 16] and the inhibition of CTGF could be a promising therapeutic strategy against diabetic nephropathy [35, 36]. Duisters et al reported that miR-133 and miR-30 suppress *CTGF* expression post-transcriptionally in myocytes [37]. In the current study, we showed that miR-26a negatively regulated *CTGF* at the post-transcriptional level in cultured human podocytes. A previous report showed that miR-26a is highly expressed in the

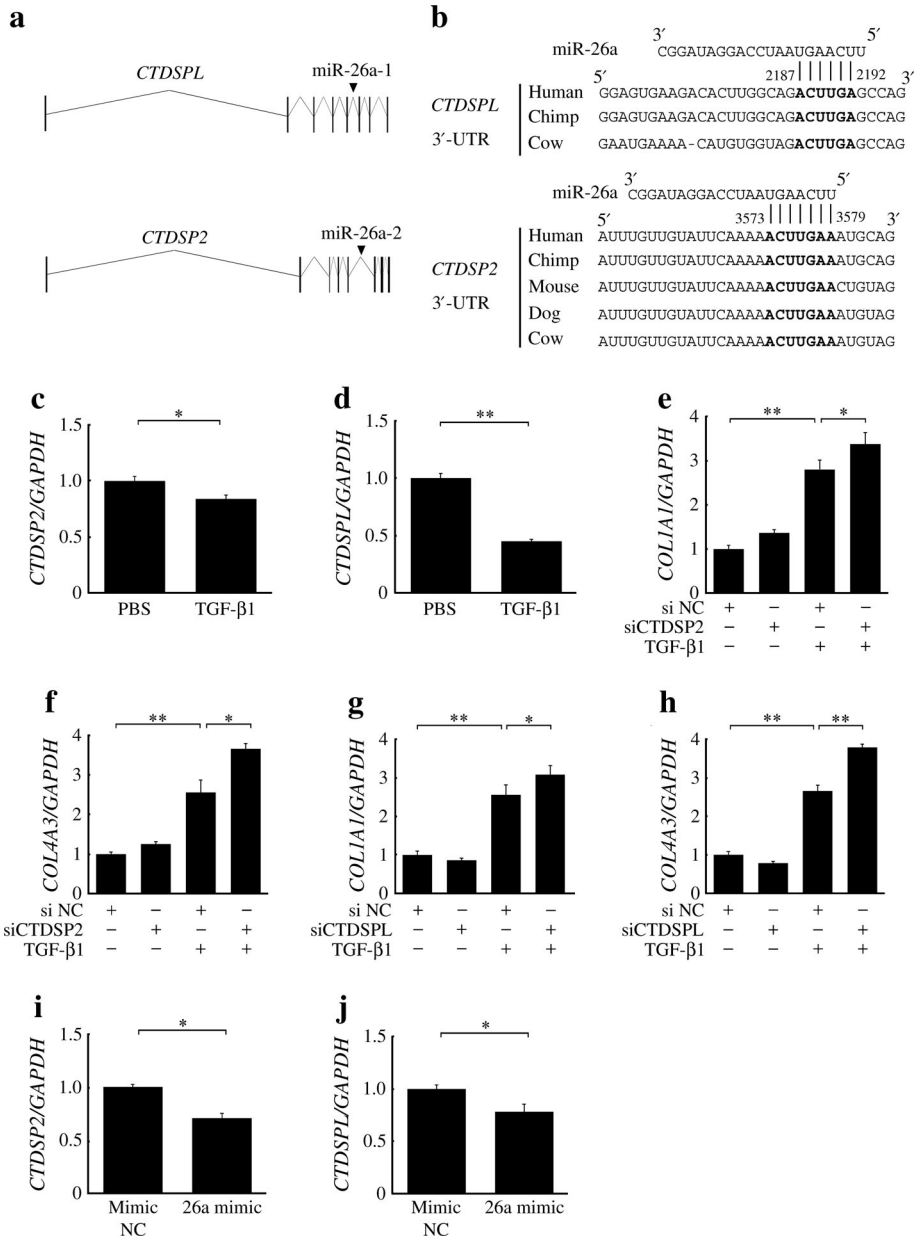

Fig. 7 Regulation and function of CTDSP2 and CTDSPL in podocytes. (**a**) Schematic genomic maps of host genes of miR-26a. Mature miR-26a is generated from two distinct loci, miR-26a-1 and miR-26a-2, which are located in the intron of *CTDSPL* and *CTDSP2*, respectively. (**b**) miR-26a binding sites in the 3′-UTR of the *CTDSP2* and *CTDSPL* transcripts across various species. Six or seven nucleotides in the 5′ region of miR-26a contain a perfect match with 3′-UTR of *CTDSP2* or *CTDSPL* sequences, respectively. The miRNA seed regions are in bold. Expression of (**c**) *CTDSP2* and (**d**) *CTDSPL* in podocytes stimulated with TGF-β1 for 24 h were examined by real-time RT-PCR. *GAPDH* was used as internal control (n=9 in each group). (**e**–**h**) Human podocytes were transfected with siRNA for 5 nmol/l (**e**, **f**) *CTDSP2* or (**g**, **h**) *CTDSPL*, and then stimulated with TGF-β1 (20 ng/ml) at 2 days after the transfection. The expression levels of (**e**, **g**) *COL1A1* and (**f**, **h**) *COL4A3* were examined at 3 days after stimulation (n=4 in each group). Real-time RT-PCR analyses of (**i**) *CTDSP2* and (**j**) *CTDSPL* in cultured human podocytes transfected with miR-26a mimics or mimic NC. (n=3 in each group). *p<0.05, **p<0.01. Data are mean±SE

kidneys of 3-week-old mice and displays a pan-glomerular pattern based on ISH [21]. We also revealed that miR-26a was highly expressed in glomeruli within podocytes, tubules and collecting ducts of kidneys from control mice. Previous studies showed that miR-26a inhibits SMAD1 and SMAD4 in smooth muscle cells [38] and that miR-26a represses TGF-β1-induced fibrogenesis in MRC-5 cells by inhibiting *CTGF* and *SMAD4* [39]. Inhibition of miR-26a causes pulmonary fibrosis in vivo and deposition of collagen I and III in vitro [39]. Wei et al reported that miR-26a, which is regulated by nuclear factor κB, inhibits both CTGF and collagen I in cardiac fibroblasts [40]. However, the mechanism underlying miR-26a regulation including its host genes has not yet been elucidated. We revealed that miR-26a decreases CTGF and ECM proteins and that phosphorylation of SMAD3 and miR-26a is expressed in parallel with its host genes, *CTDSP2* and *CTDSPL* in podocytes. However, we could not confirm that miR-26a targets SMAD2 and SMAD4 in podocytes (ESM Fig. 2). Direct evidence that *CTGF* is the candidate gene targeted by miR-26a in TGF-β1-induced ECM accumulation has not been provided in this study. However, the following points are consistent with the notion that miR-26a decreases ECM thorough CTGF inhibition: first, miR-26a mimics decrease CTGF protein levels in podocytes by 50%, which could significantly decrease ECM. Second, our study indicates that SMAD2 and SMAD4 are not the direct targets of miR-26a, but that *CTGF* is the only gene targeted by miR-26a in the

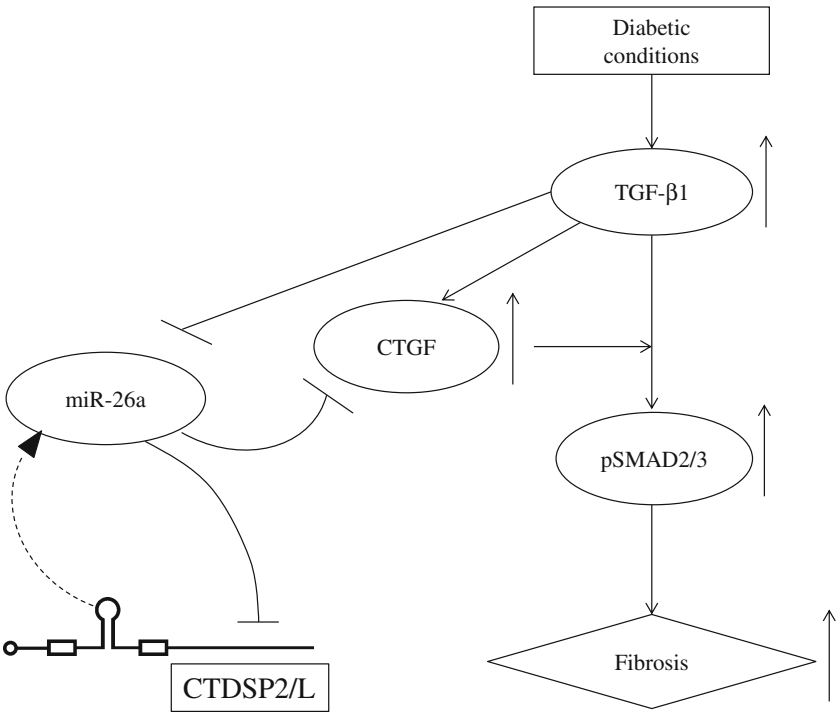

Fig. 8 Proposed mechanism of the role of miR-26a targeting *CTGF* in diabetic conditions. TGF-β downregulates miR-26a expression and the reduction of miR-26a upregulates CTGF at the post-transcriptional level, which promotes the downstream activation of TGF-β leading to ECM accumulation. miR-26a is coded in the intron of host genes, *CTDSP2* and *CTDSPL*. miR-26a targets *CTDSP2* and *CTDSPL* genes forming an autoregulatory feedback loop

pathway of TGF-β-induced ECM expression (ESM Fig. 2, www.mirbase.org, last accessed 20 January 2015). Taken together, these observations indicate that miR-26a ameliorates TGF-β-induced ECM accumulation by inhibiting CTGF in podocytes (Fig. 8).

Podocyte dysfunction plays a pivotal role in the progression of diabetic nephropathy and is partly caused by increased levels of intraglomerular TGF-β in diabetic nephropathy [41]. TGF-β upregulates α1(1) and α3(4) collagens, contributing to the thickening of glomerular basement membrane in diabetes [34]. CTGF is also reported to increase collagen IV and fibronectin in cultured podocytes [12]. We identified miR-26a targeting genes relevant to TGF-β-induced ECM accumulation. Treatment of miR-26a inhibited *COL1A1* and *COL4A3* gene expression in podocytes and decreased the expression of *CTGF*, suggesting that miR-26a works as an anti-fibrotic factor in podocytes. A recent study has shown that miR-26a regulates genes associated with podocyte differentiation and formation of the cytoskeleton, and that the level of glomerular miR-26a is lower in patients with lupus nephritis and IgA nephropathy compared with healthy controls [42]. These results are consistent with our observation that glomerular miR-26a is lower in patients with diabetic nephropathy than controls. Because the expression of miR-26a in glomeruli is not confined to podocytes by ISH, miR-26a may function in other glomerular cells such as mesangial cells.

Overactivity of TGF-β in the kidneys is observed within days of diabetes onset, and Kato et al reported that the glomerular expression level of miR-192, which suppresses TGF-β signalling, begins increasing at a very early stage in STZ mice [22]. Our in vitro study findings indicate that the downregulation of miR-26a results in the upregulation of the *CTGF* gene, leading to overactive TGF-β signalling. The downregulation of miR-26a is thus likely to be involved in the upregulation of TGF-β signalling at the onset of diabetic nephropathy. Kato et al recently reported that miR-192 expression is induced by complex mechanisms [43]; initially, by SMAD transcription factors followed by sustained expression by epigenetic histone acetylation in mesangial cells of diabetic model mice. We speculate that miR-26a expression is also regulated by several different mechanisms in different phases of the disease. Investigation of miRNA in human renal biopsy specimens, especially in glomeruli, is not fully performed in diabetic nephropathy. Although we had a limited number of biopsy specimens, we investigated miR-26a expression in glomeruli of human diabetic nephropathy and observed a positive correlation between miR-26a and eGFR; miR-26a was lower in advanced diabetic nephropathy patients. This reduction in miR-26a may lead to dysregulation in TGF-β signalling in the progression of diabetic nephropathy.

miR-26a-1, miR-26a-2 and miR-26b are coded in the introns of their host genes, *CTDSPL*, *CTDSP2* and *CTDSP1*, respectively [44]. The arrangement between miR-26a family genes and host genes is well conserved across vertebral species. In the present study, miR-26a reduced the mRNA levels of its host genes. *CTDSP2* and *CTDSPL* are widely expressed in the human tissues, including the kidneys [45, 46]. CTDSPs act as phosphatases and dephosphorylate the linker region of regulatory SMADs leading to activation of the SMAD

pathway [47]. Blockade of CTDSPs is reported to decrease the activity of TGF-β/SMAD signalling [47]. However, we observed that inhibition of CTDSPs augmented TGF-β/SMAD signalling in podocytes. miR-26a reduced the mRNA levels of its host genes. These results suggest the role of CTDSPs is cell type/context-dependent and that miR-26a autoregulates the expression of its host genes. miRNAs act as buffers against fluctuations in gene expression [48]. Our results indicate that miR-26a acts as a buffer against TGF-β/SMAD signalling by attenuating the transcriptional noise of *CTGF*. This action may confer robustness to TGF-β/SMAD signalling. Furthermore, we observed that TGF-β downregulated miR-26a. This observation suggests that TGF-β transduces its downstream signals by abolishing suppressive effects of miR-26a.

In conclusion, we determined that miR-26a targets the *CTGF* gene and attenuates TGF-β signalling and ECM accumulation in podocytes under diabetic condition. The downregulation of miR-26a was observed at the onset of diabetic nephropathy in mice and in advanced diabetic nephropathy in humans. The augmentation of miR-26a could be a novel therapeutic strategy for the treatment of diabetic nephropathy.

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Contribution statement KK, HY and MM designed the study, wrote the paper and approved its final version. KM, MK, TK, HI, AI, KPM, YK, SO, NT, KN and MY contributed to the study design, acquisition of data, interpretation of the article, revision of the manuscript and approved its final version. MAS and AS contributed materials and analysis and interpretation of data, revised the article's intellectual content and approved the final version. HY is responsible for the integrity of the work as a whole.

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