RESEARCH

C-peptide prevents SMAD3 binding to alpha promoters to inhibit collagen type IV synthesis

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Abstract

Activation of transforming growth factor β 1 (TGF β 1)/SMAD3 signaling may lead to additional synthesis of collagen type IV (COL4), which is a major contributor to extracellular matrix (ECM) accumulation in diabetic nephropathy (DN). C-peptide can attenuate fibrosis to have unique beneficial effects in DN. However, whether and how C-peptide affects TGF β 1/SMAD3-activated COL4 synthesis is unclear. In this study, pathological changes, expression of COL4 α 1– α 5 chains (*Col4a1–a5*), COL4 distribution and protein and TGF β 1 and SMAD3 protein were first assessed in a rat model of diabetes. Then, rat mesangial cells were treated with high glucose (HG) and/or C-peptide to investigate the underlying mechanism. *Col4a1–a5* expression, COL4 protein and secretion, TGF β 1 protein, SMAD3 nuclear translocation and binding of SMAD3 to its cognate sites in the promoters of *Col4a1a2*, *Col4a3a4* and *Col4a5* were measured. It was found that C-peptide attenuated glomerular pathological changes and suppressed renal *Col4a1–a5* mRNA expression, COL4 protein content and TGF β 1 protein content. C-peptide had a dose-dependent effect to inhibit *Col4a1–a5* mRNA expression, COL4 protein content and secretion, in HG-stimulated mesangial cells. In addition, the HG-induced increase in TGF β 1 protein content was significantly reduced by C-peptide. Although not apparently affecting SMAD3 nuclear translocation, C-peptide prevented SMAD3 from binding to its sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters in HG-stimulated mesangial cells. In conclusion, C-peptide could prevent SMAD3 from binding to its sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters, to inhibit COL4 generation. These results may provide a mechanism for the alleviation of fibrosis in DN by C-peptide.

Key Words

- ▶ C-peptide
- ▶ collagen type IV
- ▶ TGF β 1
- ▶ SMAD3

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Introduction

Diabetic nephropathy (DN), a serious microvascular complication of diabetes, remains the leading cause of chronic kidney disease and end-stage renal disease (Fan *et al.* 2017, Flyvbjerg 2017). Excess extracellular matrix (ECM) deposition, leading to glomerular basement membrane thickening, mesangial expansion and then

glomerulosclerosis, is a feature of DN (Kato *et al.* 2016, Marshall 2016). Mesangial cells are believed to be a major source of ECM, and their dysfunction may lead to ECM accumulation in DN (Donegan *et al.* 2016, Sheu *et al.* 2017). The underlying mechanism of this ECM deposition is under active investigation (Al-Waili *et al.* 2017).

Transforming growth factor $\beta 1$ (TGFB1)/SMAD signaling is the key pathway that promotes fibrosis in DN (Chung *et al.* 2010, Gao *et al.* 2016). In diabetes, TGFB1 binds to its receptor, which activates downstream signaling, especially via SMAD3, to promote renal fibrosis (Chung *et al.* 2010). It has been shown that TGFB1 can trigger excess synthesis of collagen type IV (COL4), which is the most abundant protein in ECM (Turner *et al.* 2015, Peng *et al.* 2016, Dimas *et al.* 2017). COL4 has a triple -helical structure composed of three α chains, which are encoded by six genes (Robertson *et al.* 2014, Sugiyama *et al.* 2015). The genes expressing $\alpha 1$ – $\alpha 6$ have two lines head to head, such that $\alpha 1$ and $\alpha 2$ share the same promoter, as do $\alpha 3$ and $\alpha 4$, and $\alpha 5$ and $\alpha 6$. The established COL4 heterotrimers are $\alpha 1\alpha 2\alpha 1$ and $\alpha 3\alpha 4\alpha 5$, and the predicted SMAD3-binding sites have also been found in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters (Robertson *et al.* 2014). Although excess TGFB1/SMAD3-stimulated COL4 synthesis has been established to be a major contributor to ECM accumulation in DN, there is still no effective therapy.

C-peptide links the A and B chains of proinsulin, but has been found to have unique beneficial effects on DN (Shaw *et al.* 2015, Wahren & Larsson 2015). In animal models or in humans with type 1 diabetes, C-peptide can reduce glomerular hyperfiltration and albuminuria (Brunskill 2017). When administered to diabetic rodents in physiological concentrations, C-peptide attenuates mesangial expansion and ECM accumulation (Wahren 2017). The underlying mechanisms of its protective effects have not been fully elucidated, although various candidate mechanisms have been proposed (Lindahl *et al.* 2010, Garcia-Serrano *et al.* 2015, Kolar *et al.* 2017). However, these mechanisms are not specific to DN and do not account for the unique effect of C-peptide to attenuate ECM accumulation in DN. We have previously shown that C-peptide dynamically localizes to the nucleus in high glucose (HG)-stimulated mesangial cells, providing a model to further investigate these protective mechanisms (Li *et al.* 2013b). TGFB1 signaling, the major pro-fibrotic stimulus, has been found to be indirectly antagonized by C-peptide (Hills *et al.* 2010a,b). However, the effects of C-peptide on excess TGFB1/SMAD3-stimulated COL4 synthesis in DN are unknown.

In this study, the effects of C-peptide on pathological changes, *Col4a1-a5* mRNA expression and COL4 protein, and TGFB1 and SMAD3 protein, were first assessed in a rat model of diabetes. Then, the effects of C-peptide on *Col4a1-a5* mRNA expression, COL4 protein and secretion, TGFB1 and thrombospondin-1 (TSP-1) proteins, SMAD3

nuclear translocation and binding of SMAD3 to its sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were measured in HG-stimulated mesangial cells.

Materials and methods

Animals

A total of 40 healthy male Sprague–Dawley rats (252.05 $\pm$ 27.17 g, about 2 months old) were provided by the Department of Experimental Animals of Hebei Medical University. Experiment protocol followed the guidelines established by the Ethics Review Committee for Animal Experimentation of Hebei Medical University. Rats were randomly divided into three groups: diabetes mellitus (DM) group ($n=15$), DM+C-peptide (DM+CP) group ($n=15$) and control (Con) group ($n=10$). The former two groups were injected intraperitoneally with streptozotocin (40 mg/kg, dissolved in 0.1 mol/L citrate buffer, pH 4.4, freshly made, 10 mg/mL), to induce diabetes. Rats in DM+CP group were injected subcutaneously twice daily with 130 nmol/kg C-peptide (Ido *et al.* 1997, Sun *et al.* 2010), and other rats were given equivalent saline. Body mass and fasting blood glucose were measured at 3 days and 12 weeks of established diabetes. At 12 weeks, renal cortex was collected for hematoxylin-eosin (HE) staining, and a segment of renal cortex was used for detections of *Col4a1-a5* mRNA expressions, COL4 protein and immunohistochemical staining and TGFB1 and SMAD3 protein.

Cells and treatments

Rat mesangial cells (HBZY-1) were obtained from China Center for Type Culture Collection and cultured in DMEM containing 5 mM glucose and 10% fetal bovine serum. Cells were treated with 20, 25, 30, 35 and 40 mM HG for 24 h, and then 35 mM glucose plus 0.1, 0.5 and 0.9 nM C-peptide for 24 h, based on our previous study (Li *et al.* 2013b). Cells were collected for detection of *Col4a1-a5* mRNA expression. And after 48-h treatment, cells were collected for detection of COL4 protein content and culture medium for detection of COL4 secretion. Then, cells were divided into low glucose (LG) group (5 mM glucose), HG group (35 mM glucose) and CP group (35 mM glucose + 0.5 nM C-peptide). High L-glucose (L-HG) with the concentration of 35 mM L-glucose was used as a hypertonic control. After cells were pretreated with HG and subsequent HG plus C-peptide for 24 h, SMAD3 nuclear translocation and binding of SMAD3 to

its sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were measured. The protein contents of TGF β 1, TSP-1 and SMAD3 were evaluated after 48-h treatments. In addition, the localization of C-peptide was detected.

Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol reagent (TaKaRa) and reverse transcribed into cDNA using RevertAid First Strand cDNA synthesis Kit (Fermentas, Shanghai, China), followed by real-time PCR amplification using specific primers (Table 1). Actin primers were used as an internal standard. The comparative Ct method was used to calculate the relative abundance of the mRNA compared with that of actin.

Immunohistochemistry

Distribution and content of glomerular COL4 were detected by immunohistochemistry. The procedures were as follows: renal cortex embedded in paraffin was cut and dewaxed. After incubated with H₂O₂, sections were incubated with goat serum. Sections were incubated with anti-COL4 antibody (diluted 1:50, Bioworld, Nanjing, China) and then the secondary antibody (diluted 1:200, Zhongshan, Beijing, China). 3, 3'-Diaminobenzidine (DAB) solution was used for color emergence. The mean density of ten glomeruli from three rats in each group was quantified by Image Pro Plus 6.0.

Enzyme-linked immunosorbent assay

After treatment, the culture medium was collected to analyze COL4 secretion by ELISA (Jiancheng, Nanjing, China). Standards and samples were added to wells of the plate and incubated for 1 h. After the wells were washed with ELISA wash buffer, the conjugated antibody was added and incubated for 1 h. Then, the wells were washed again with the ELISA wash buffer. The substrate was added

in the wells and incubated for 15 min. Then, the stop solution was added and absorption was measured with an ELISA reader at 450 nm. The tests were carried out in duplicate.

Western blotting

The protein contents of COL4, TGF β 1, TSP-1, total and nuclear SMAD3 and phospho-SMAD3 were detected with the protocol previously described (Li et al. 2013a). The anti-COL4, anti-TGF β 1 and anti-TSP-1 antibodies (diluted 1:200, Bioworld), anti-SMAD3 antibody (diluted 1:500, CST, Shanghai, China) or anti-phospho-SMAD3 antibody (diluted 1:100, Bioworld) was used. Band intensity was quantified and calculated. Actin routinely served as a loading control.

Immunofluorescence

The localization of C-peptide and the nuclear translocation of SMAD3 were detected by immunofluorescence with the previously reported protocol (Li et al. 2013a). The used primary antibody was anti-CP (diluted 1:2000, Abcam, Shanghai, China), anti-SMAD3 (diluted 1:100, CST) and the secondary antibody was FITC conjugated (diluted 1:200, Zhongshan). The images of C-peptide were taken with a fluorescence microscope (Olympus IX51), and the images of SMAD3 were taken with a laser confocal scanning microscope (Olympus). Nuclear SMAD3-positive cells from ten visual fields at random in each group were recorded. The percentage of SMAD3 nuclear translocation was calculated. To get the exact results, controls (PBS instead of the first antibody or the second antibody) were designed.

Chromatin immunoprecipitations

The predicted SMAD3-binding sites in the shared *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were shown in

Table 1 Primers used for real-time PCR detection.

Genes	Primers		Annealing temperature (°C)	Product size (bp)
	Sense	Anti-sense		
<i>Col4a1</i>	5' GTGTCAGCAATTAGGCAGGTCAAG 3'	5' AGTCAGTCATTAGTCTGCGGATAG 3'	65	305
<i>Col4a2</i>	5' CTTCGGTGCAACACCATTTATCG 3'	5' GGGTCGGCATCTGAGGTAATAAG 3'	65	445
<i>Col4a3</i>	5' GAGACAGAGGGACACCTGCCAAC 3'	5' TGGCATTGTGGTGAATCGCTG 3'	63	209
<i>Col4a4</i>	5' CTGGTATGGACGGCTACAATG 3'	5' CCCTAAATGCTCTGGTTCTGC 3'	63	262
<i>Col4a5</i>	5' GATTACAAGTACCTCAGCGATG 3'	5' TGGGGGTACATTAGGATTAGTAGC 3'	64	246
β -Actin	5' CCGTAAAGACCTCTATGCCAAC 3'	5' GTCAAAGAAAGGGGTGTAAACGC 3'	60	303

AT, annealing temperature.

Supplementary Fig. 2 (see section on [supplementary data](#) given at the end of this article). Binding of SMAD3 to its sites was analyzed by chromatin immunoprecipitation (ChIP), performed as reported ([Li et al. 2015](#)). Briefly, anti-SMAD3 antibody (CST) or control rabbit IgG (Santa Cruz) was added, and then protein G-agarose beads (Santa Cruz) were used. Then SMAD3-binding sites in the rat *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were amplified by real-time PCR, respectively. Primers sequences used for the binding sites were in [Table 2](#).

DNA pull-down assay

The contents of SMAD3 bound to the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were measured by a DNA pull-down assay, as previously reported ([Li et al. 2017](#)). The sequences of the *Col4a1a2* (Gene ID: 290905 and 306628), *Col4a3a4* (Gene ID: 363265 and 301562) and *Col4a5* (Gene ID: 363457) promoters were found. The oligonucleotides containing biotin on the 5'-end of *Col4a1a2* promoter were biotin-5'-GGGGCCCCGGCTC AGCGTCTGGCTTCTGCTGCCGCTCGCC-3' (forward) and biotin-5'-GGCGAGCGGCAGCAGAAGCCAGACGC TGAGCCGGGGCCCC-3' (reverse). The oligonucleotides containing biotin on the 5'-end of *Col4a3a4* promoter were biotin-5'-ACCCGGCCTCCAGAGTCGCACTCC CGCGCGCTCCCGC-3' (forward) and biotin-5'-GCG GGAGGCGCGGGAGTCTGCGACTCTGGAGGCCG GGT-3' (reverse). The oligonucleotides containing biotin on the 5'-end of *Col4a5* promoter were biotin-5'-AATA GTCCTAGCTCAGGTCTGTTTGTCAAAGCCTTTGATG-3' (forward) and biotin-5'-CATCAAAGCCTTTGACAAAC AGACCTGAGCTAGGACTATT-3' (reverse). The mutant oligonucleotides were shown in [Supplementary Table 1](#). Each pair of oligonucleotides was annealed following standard protocols. Nuclear protein extracts were pre-cleared with ImmunoPure streptavidin-agarose beads (Thermo, Shanghai, China), and the supernatant was incubated with 4 µg of biotinylated double-stranded oligonucleotides. Twenty microliters of streptavidin-agarose beads were added and the protein-DNA-streptavidin-agarose complex was washed, separated

and subjected to western blotting with anti-SMAD3 antibody (CST).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Data were presented as mean ± s.d. One-way ANOVA was used to analyze the differences among groups, and the differences between two groups were evaluated by the least-significant difference. Statistical significant was set at $P < 0.05$.

Results

C-peptide attenuates glomerular ECM accumulation in DN

First, body mass and blood glucose were measured in the rats after 3 days and 12 weeks of established diabetes. Although there was no difference among the groups at the beginning of the experiment ([Fig. 1A](#)), body mass was significantly lower at 12 weeks in the DM and DM+CP groups than in the Con group ([Fig. 1B](#)). Compared with the Con group, blood glucose was significantly higher throughout the experiment in the DM and DM+CP groups ([Fig. 1C](#) and [D](#)). C-peptide treatment had no obvious effect on body mass and blood glucose of the diabetic rats.

Typical glomerular changes were demonstrated by HE staining ([Fig. 1E](#)). Pathological changes were not observed in the Con group. Nephrosclerosis and broadened capsular spaces were observed in the DM group, but glomerular pathological changes were observed to a considerably lesser extent in the DM+CP group. These results indicate that C-peptide treatment could attenuate glomerular pathological changes in DN, without affecting body mass and blood glucose.

C-peptide suppresses renal COL4 synthesis in diabetic rats

Col4a1-a5 mRNA expression, and COL4 protein and distribution, were then evaluated after 12-week C-peptide

Table 2 Primers used for real-time PCR detection of Col IV gene promoters.

Gene promoters	Primers		Annealing temperature (°C)	Product size (bp)
	Sense	Anti-sense		
<i>Col4a1a2</i>	5' GACTCCCTGCGACCAAGTCC 3'	5' GTGGCTCTGAGCAGAGACCTGAG 3'	67	168
<i>Col4a3a4</i>	5' AAGACCGGCAGCAGATGGGCGAG 3'	5' GACTCCGGTGCCTAGCGGGTGC 3'	68	189
<i>Col4a5</i>	5' CTATGTGACATGTGTTGAACATGC 3'	5' AGCGGATGGATTTCAGAGTGTG 3'	60	230

Figure 1

Effects of CP on pathological changes of diabetic nephropathy. Body weight at 3 days (A) and 12 weeks (B) of established diabetes were measured. Blood glucose at 3 days (C) and 12 weeks (D) were also detected ($n=9$). * $P<0.05$ vs control (Con) group. (E) HE staining of glomerulus. CP, C-peptide; DM, diabetes mellitus; HE, hematoxylin and eosin.

treatment in the diabetic rats. Compared with the Con group, mRNA expressions of all of *Col4a1-a5* were 4.69 ± 0.72 , 4.22 ± 0.65 , 4.11 ± 0.55 , 3.94 ± 0.54 and 4.19 ± 0.42 -fold, considerably higher in the DM group, but were 2.72 ± 0.51 , 2.32 ± 0.38 , 2.02 ± 0.40 , 1.83 ± 0.42 and 2.14 ± 0.60 -fold in the DM+CP group, which were significantly reduced ($n=3$, $P<0.05$) (Fig. 2A). Compared with the Con group, the 1.61 ± 0.21 -fold increase in COL4 protein content in the DM group, however, was 1.13 ± 0.15 -fold in the DM+CP group – significantly suppressed ($n=3$, $P<0.05$) (Fig. 2B and C). In addition, there were consistent differences among the groups in COL4 distribution and content (Fig. 3A and B). Compared with the Con group (95.33 ± 9.45), distinct COL4 staining was observed in the DM group (143.33 ± 11.59) ($P<0.01$), which was attenuated in the DM+CP group (106.00 ± 13.52) ($P<0.05$).

The effects of C-peptide on renal TGF β 1 and SMAD3 protein were also assessed in diabetic rats at 12 weeks (Fig. 3C). Compared with the Con group, both TGF β 1 and SMAD3 protein contents were 3.15 ± 0.42 and 1.46 ± 0.14 -fold, significantly more abundant in the DM group, but only the amount of TGF β 1 protein was 1.93 ± 0.31 -fold, lower in the DM+CP group ($n=3$, $P<0.05$ or 0.01) (Fig. 3D

Figure 2

C-peptide suppressed COL4 generation in diabetic nephropathy. (A) The mRNA expression of *Col4a1-a5* was detected by real-time RT-PCR. The protein content of COL4 was detected by western blotting (B) and calculated (C). * $P<0.05$ vs control (Con) group; # $P<0.05$ vs DM group ($n=3$). CP, C-peptide; DM, diabetes mellitus.

and E). In addition, C-peptide had a tendency to inhibit the increased phospho-SMAD3 contents, but without significant difference (Supplementary Fig. 3A and B).

These results suggest that C-peptide can suppress renal *Col4a1-a5* mRNA expression and reduce COL4 protein level, alongside a reduction in TGF β 1 protein content, in this rat model of DM.

C-peptide inhibits HG-stimulated COL4 generation in mesangial cells

The effects of HG on *Col4a1-a5* mRNA expression, and COL4 protein and secretion, were initially measured. Compared with the 5 mM group, expressions of *Col4a1-a5* were increased after 24-h HG stimulation, especially 4.12 ± 1.05 , 4.01 ± 1.04 , 3.83 ± 0.92 , 4.32 ± 1.20 and 3.56 ± 0.75 -fold at 35 mM ($n=3$, $P<0.05$ or 0.01) (Fig. 4A). HG had similar effects on COL4 protein content and secretion at 48 h, which were 1.63 ± 0.14 and 1.79 ± 0.26 -fold at 35 mM ($n=3$, $P<0.05$ or 0.01) (Fig. 4B and C), indicating that COL4 generation can be stimulated by HG.

Then, the concentration-dependent effects of C-peptide on *Col4a1-a5* mRNA expression, COL4 protein and secretion were investigated in HG-stimulated mesangial cells. Compared with the LG group, mRNA expressions of *Col4a1-a5* were 4.11 ± 1.05 , 4.64 ± 0.95 , 3.98 ± 0.77 , 4.32 ± 0.93 and 3.64 ± 0.93 -fold, significantly higher in

Figure 3

C-peptide suppressed COL4 distribution and TGFβ1 content in diabetic nephropathy. The COL4 distribution was illustrated by immunohistochemistry (A) and calculated (B). (C) The protein contents of renal TGFβ1 and SMAD3 were detected by western blotting. (D) TGFβ1 content was obviously higher in DM group, but was significantly inhibited by C-peptide. (E) The increased SMAD3 content was not significantly inhibited by C-peptide. * $P < 0.05$ or ** $P < 0.01$ vs control (Con) group; # $P < 0.05$ vs DM group ($n = 3$). CP, C-peptide; DM, diabetes mellitus.

the HG group ($n = 3$, $P < 0.01$), but only slightly higher in the L-HG group at 24 h. The HG-stimulated increases in mRNA expressions of *Col4a1-a5* were 1.29 ± 0.39 , 1.56 ± 0.45 , 1.58 ± 0.49 , 1.52 ± 0.31 and 1.10 ± 0.31 -fold in the presence of 0.5 nM C-peptide and 1.70 ± 0.46 , 1.90 ± 0.52 , 2.14 ± 0.51 , 2.06 ± 0.60 and 1.33 ± 0.89 -fold in the presence of 0.9 nM C-peptide, substantially suppressed at 24 h ($n = 3$, $P < 0.01$) (Fig. 5A). Compared with the LG group, the HG-stimulated 2.20 ± 0.16 and 1.69 ± 0.15 -fold increases in COL4 protein content and secretion were inhibited by C-peptide treatment at 48 h, especially 1.71 ± 0.25 and 1.21 ± 0.12 -fold at 0.5 nM ($n = 3$, $P < 0.05$ or 0.01) (Fig. 5B and C), consistent with the changes in *Col4a1-a5* mRNA expression.

These results demonstrate that C-peptide can inhibit COL4 generation in HG-stimulated mesangial cells in a concentration-dependent manner. Furthermore, at its physiological concentration (0.5 nM), C-peptide had its most conspicuous effect, and this concentration was therefore adopted for subsequent experiments.

C-peptide has no discernible effect on HG-stimulated SMAD3 nuclear translocation

First, the effects of C-peptide on TGFβ1 and TSP-1 proteins were investigated in HG-stimulated mesangial cells. Compared with the LG group, the HG-induced 1.62 ± 0.24 and 1.66 ± 0.17 -fold increase in TGFβ1 and TSP-1 protein contents were reduced significantly by C-peptide to 1.11 ± 0.18 and 1.10 ± 0.23 -fold at 48 h ($n = 3$, $P < 0.05$) (Fig. 6A).

Subsequently, the nuclear translocation of SMAD3 was assessed using western blotting (Fig. 6B) by measuring the ratio of nuclear SMAD3 to its total content (Fig. 6C). Compared with the LG group, nuclear SMAD3 content in the HG and HG+CP groups was 2.00 ± 0.37 and 1.69 ± 0.28 -fold, significantly higher at 48 h ($n = 3$, $P < 0.05$). However, no significant difference was observed between the HG and HG+CP groups. Besides, C-peptide had a tendency to inhibit the HG-induced increase of phospho-SMAD3 contents, but without significant difference (Supplementary Fig. 3C).

Furthermore, SMAD3 nuclear translocation was visualized using immunofluorescence (Fig. 7A). Immunofluorescent staining for SMAD3 showed that it was scattered throughout the cytoplasm in the LG group, but was mainly within the nucleus in the HG group. The HG-stimulated predominantly nuclear distribution of SMAD3 was not clearly affected by C-peptide treatment at 24 h. And consistently, the percentage of SMAD3 nuclear

Figure 4

Effects of high glucose (HG) on COL4 generation in mesangial cells. After the cells were stimulated with different concentrations of HG, mRNA expression of *Col4a1-a5* was detected by real-time RT-PCR (A), COL4 protein content by western blotting (B) and COL4 secretion by ELISA (C). * $P < 0.05$ or ** $P < 0.01$ vs 5 mM group. The data were repeated three times and represented as mean $\pm$ s.d.

Figure 5

C-peptide inhibited COL4 generation in high glucose (HG)-stimulated mesangial cells. After the cells were incubated with C-peptide at different concentrations, mRNA expression of *Col4a1-a5* was detected by real-time RT-PCR (A), COL4 protein content by western blotting (B), and COL4 secretion by ELISA (C). ** $P<0.01$ vs Low glucose (LG) group; # $P<0.05$ or ## $P<0.01$ vs HG group. The data were repeated three times and represented as mean $\pm$ s.d. CP, C-peptide; L-HG, high L-glucose.

translocation was also elevated in the HG and HG+CP groups (Fig. 7B).

These results imply that although it reduces TGF β 1 protein content, C-peptide does not seem to have an effect on HG-stimulated nuclear translocation of SMAD3 in mesangial cells.

C-peptide prevents SMAD3 from binding to *Col4a* promoters in HG-stimulated mesangial cells

Immunofluorescence showed that C-peptide could enter cell and concentrated in nucleus for HG stimulation (Supplementary Fig. 1). Based on the nuclear localization of C-peptide, the binding of SMAD3 to its cognate sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters was measured using ChIP (Fig. 8). Compared with the LG group, the quantity of SMAD3-bound site in the *Col4a1a2* promoters was significantly higher in the HG group, but this was significantly lower in the HG+CP group at 24h (Fig. 8A). The HG-stimulated increases in SMAD3-bound sites in the *Col4a3a4* and *Col4a5* promoters were also significantly inhibited by C-peptide treatment (Fig. 8B and C).

Meanwhile, the contents of SMAD3 bound to the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were measured by DNA pull-down assay (Fig. 9). The content of SMAD3 bound to the *Col4a1a2* promoters was increased in the HG group, but was significantly reduced by C-peptide treatment at 24h (Fig. 9A). Similarly, C-peptide treatment

Figure 6

Effects of C-peptide on TGF β 1 and SMAD3 protein in high glucose (HG)-stimulated mesangial cells. After the cells were treated with 0.5 nM C-peptide, TGF β 1 and TSP-1 protein contents (A) and total and nuclear contents of SMAD3 (B) were detected by western blotting. (C) The ratio of nuclear SMAD3 to total SMAD3 was calculated to show SMAD3 nuclear translocation. * $P<0.05$ vs low glucose (LG) group; # $P<0.05$ vs HG group. The data were repeated three times and represented as mean $\pm$ s.d. CP, C-peptide; L-HG, high L-glucose.

also reduced the HG-increased SMAD3 content in *Col4a3a4* and *Col4a5* promoters with significant difference (Fig. 9B and C).

These results suggest that C-peptide may prevent the HG-stimulated binding of SMAD3 to its sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters, resulting in the inhibition of *Col4a1-5* expression in mesangial cells.

Discussion

Although C-peptide is known to attenuate ECM accumulation and therefore have beneficial effects in DN, the underlying mechanism has not been fully elucidated. Activation of TGF β 1/SMAD3 signaling pathway may lead to excess production of COL4, the major contributor to ECM accumulation in DN. However, whether and how C-peptide affects TGF β 1/SMAD3-stimulated COL4 generation is unclear. In the study, we have shown that, in addition to reducing TGF β 1 content, C-peptide can prevent SMAD3 from binding to its cognate sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters, thereby suppressing COL4 generation *in vivo* and *in vitro*. We propose this as a mechanism whereby C-peptide attenuates ECM accumulation in DN.

TGF β 1 promotes ECM production and simultaneously inhibits its degradation, resulting in progressive renal

Figure 7

Effects of C-peptide on SMAD3 nuclear translocation in high glucose (HG)-stimulated mesangial cells. (A) The nuclear translocation of SMAD3 was illustrated by immunofluorescence. Scale bar 10 μm. (B) The percentage of SMAD3 nuclear translocation was calculated. CP, C-peptide; L-HG, high L-glucose.

fibrosis (Meng *et al.* 2016). In this study, we have also shown that TGFβ1 protein levels were higher in both *in vivo* and *in vitro* models. Activation of TGFβ1/SMAD3 signaling causes production of excess COL4, which is considered to be the central pathogenic process contributing to ECM accumulation in DN (Gu *et al.* 2016). However, because TGFβ1 signaling regulates various cellular processes, its inhibition could lead to undesirable side effects (Yiang *et al.* 2016), and therefore there is still no effective therapy for ECM accumulation in DN.

C-peptide has been shown to have unique beneficial effects in DN (Hills *et al.* 2010a,b, Xu *et al.* 2015), specifically the attenuation of ECM accumulation and fibrosis (Hills *et al.* 2010a,b, Brunskill 2017, Wahren 2017). In this study, the effects of C-peptide on COL4 generation were investigated *in vivo* and *in vitro*. First, it was found that C-peptide could attenuate glomerular ECM accumulation, reduce *Col4a1-a5* mRNA expression and COL4 protein content in a diabetic rat model, without having an effect on blood glucose. In addition, C-peptide suppressed TGFβ1 content in this model. Second, C-peptide had concentration-dependent effects to inhibit *Col4a1-a5* mRNA expression, and COL4 protein content and secretion, in HG-stimulated mesangial cells. C-peptide had its most conspicuous effect at its

Figure 8

C-peptide prevented SMAD3 from binding to its sites in high glucose (HG)-stimulated mesangial cells. The bindings of SMAD3 to its sites in the *Col4a1a2* promoter (A), *Col4a3a4* promoter (B), and *Col4a5* promoter (C) were detected by ChIP. * $P < 0.05$ vs low glucose (LG) group; # $P < 0.05$ vs HG group. The data were repeated three times and represented as mean $\pm$ s.d. CP, C-peptide; L-HG, high L-glucose.

physiological concentration, indicating that it may have a physiologically relevant effect on excess COL4 synthesis in DN. However, the effect of C-peptide on TGFβ1/SMAD3-activated COL4 generation remains to be evaluated.

Several mechanisms have been reported for the protective effects of C-peptide, including receptor binding-mediated effects, interaction with proteins to affect their function, and localization to the nucleus to regulate gene expression (Lindahl *et al.* 2010, Garcia-Serrano *et al.* 2015, Kolar *et al.* 2017). However, these mechanisms are not specific to DN and do not at present explain the unique effects of C-peptide to attenuate ECM accumulation in DN. We have previously shown that C-peptide scarcely enters mesangial cells when they are cultured in medium containing a normal glucose concentration, but in the presence of HG, C-peptide might be internalized *via* activated endocytosis and dynamically localizes to the nucleus, exhibiting its protective mechanism (Li *et al.* 2013b). In this study, the effects of C-peptide on TGFβ1

Figure 9

C-peptide reduced SMAD3 bound to its sites in high glucose (HG)-stimulated mesangial cells. The contents of SMAD3 bound to the *Col4a1a2* promoter (A), *Col4a3a4* promoter (B) and *Col4a5* promoter (C) were analyzed by DNA pull-down assay. * $P < 0.05$ or ** $P < 0.01$ vs low glucose (LG) group; # $P < 0.05$ vs HG group. The data were repeated three times and represented as mean $\pm$ S.D. CP, C-peptide.

protein content and SMAD3 nuclear translocation were measured in HG-stimulated mesangial cells. Although C-peptide reduced TGF β 1 protein content, it did not reduce HG-stimulated nuclear SMAD3 content in mesangial cells. This phenomenon may be attributed to the nuclear localization of C-peptide to regulate gene expressions, including COL4 and even TGF β 1.

Then, the HG-stimulated binding of SMAD3 to its cognate sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters were also measured, and this showed that C-peptide can prevent such bindings, thereby inhibiting *Col4a1-5* mRNA expression. Lindahl *et al.* also reported that C-peptide could bind to histones to regulate gene expression (Lindahl *et al.* 2010). From the results of current study, it is speculated that the effect of C-peptide might be mediated by its transport to the nucleus, where it interacts with SMAD3 and/or histones to suppress the binding of SMAD3 to *Col4a* promoters, regulating *Col4a1-5* expressions. However, this needs further investigation in the future study, with a scrambled control to specify the mechanism of C-peptide.

This study has added information to our knowledge of the mechanism whereby C-peptide can suppress TGF β 1/SMAD3-stimulated COL4 synthesis in DN. These findings may provide a basis for the development of more effective and specific treatments for ECM accumulation in DN. Additional work should be aimed at identifying the particular region of the peptide sequence responsible for its effect.

In conclusion, C-peptide can prevent SMAD3 from binding to its sites in the *Col4a1a2*, *Col4a3a4* and *Col4a5* promoters, suppress *Col4a1-a5* mRNA expressions and reduce COL4 synthesis *in vivo* and *in vitro*. Our results provide additional details of the mechanism whereby C-peptide alleviates ECM accumulation in DN.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-18-0009>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J Q designed the experiments. Y L and Y Z performed the *in vivo* experiments. Y L, W G and X G performed *in vitro* experiments and made the figure. Y Z, H Q and K L performed the statistics and made the remaining figures. J Q wrote the manuscript and all authors reviewed and edited the manuscript.

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