


# Epigallocatechin Gallate Induces the Demethylation of Actinin Alpha 4 to Inhibit Diabetic Nephropathy Renal Fibrosis via the NF-KB Signaling Pathway In Vitro

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## Abstract

Actinin alpha 4 (*ACTN4*) is expressed in the kidney podocytes. *ACTN4* gene methylation in patients with diabetic nephropathy (DN) remains high. Underlying mechanism of epigallocatechin-3-gallate (EGCG) inducing *ACTN4* demethylation, and its inhibitory effect on DN renal fibrosis remains unclear.

**Methods:** Human podocyte cell line, HPC, was treated with high glucose to establish model of DN. The levels of cytokines, vascular endothelial growth factor (VEGF) and interleukin (IL)-8, and fibrosis markers, alpha smooth muscle actin ( $\alpha$ -SMA) and fibronectin (FN), were determined using enzyme-linked immunosorbent assay. HPC cells were treated with EGCG, and cell viability was determined by MTT assay, *ACTN4* gene methylation was analyzed by MSP. mRNA and protein expression levels were measured using RT-qPCR and Western blotting, respectively.

**Results:** Actinin alpha 4 gene promoter was hypermethylated in the high glucose-treated groups. EGCG reversed the hypermethylated status of *ACTN4*, along with the upregulation of *ACTN4* levels and downregulation of DNA methyltransferase I (DNMT1), NF- $\kappa$ B p65, p-NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , VEGF, IL-8,  $\alpha$ -SMA, and FN levels ( $P < .05$ ).

**Conclusion:** Epigallocatechin-3-gallate reduced hypermethylation of *ACTN4* in HPC cells by downregulating DNMT1 expression and restoring *ACTN4* expression, contributing to the upregulation of the NF- $\kappa$ B p65, p-NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , VEGF, IL-8,  $\alpha$ -SMA, and FN levels ( $P < .05$ ).

## Keywords

epigallocatechin gallate, actinin alpha 4, methylation, NF-KB signaling pathway,, diabetic nephropathy

## Introduction

Diabetic nephropathy (DN) is a common refractory complication that increases the mortality of patients with diabetes.<sup>1</sup> Early morphological abnormalities are characterized by glomerular hypertrophy, thickening of the glomerular basement membrane, and production of mesangial extracellular matrix, which develops into glomerulosclerosis and tubulointerstitial fibrosis, eventually leading to kidney failure.<sup>2,3</sup> Therefore, determining the key mechanism of DN development and developing an effective treatment method for delaying or halting DN progression is crucial. Recently, mounting evidence has demonstrated that the mechanism of DN is related to genetic abnormalities and epigenetic changes. DNA

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methylation is one of the most common epigenetic changes, and numerous studies have shown that gene hypermethylation promotes the occurrence of DN.<sup>4-7</sup> The number of differentially methylated regions (DMRs) in DN is significantly higher than that in diabetes without nephropathy.<sup>8</sup> Therefore, reducing the hypermethylation status represents a novel approach for DN treatment.

The current management for reducing the hypermethylation status focuses on DNA methyltransferase (DNMT) inhibitors. DNMTs play an important role in DNA methylation, while DNMT inhibitors reduce hypermethylation and restore the expression of silenced genes.<sup>9</sup> However, the available DNMT inhibitors, such as 5-Aza-2'-deoxycytidine, lack target specificity, have high side effects, and are toxic to healthy cells.<sup>10</sup> Compared with these synthetic inhibitors, natural products exhibit low toxicity and fewer side effects and are cost-effective, emerging as targeted epigenetic modulators.<sup>11</sup> Green tea is a natural drink that is popular worldwide. The major chemical component of green tea, epigallocatechin-3-gallate (EGCG), can inhibit various inflammatory, toxic, proliferative, and angiogenic effects.<sup>12,13</sup> EGCG forms hydrogen bonds with the GLU1265, Arg1310, Arg1311, and Lys1482 amino acid residues in the catalytic site of DNMT, which directly inhibits the function of DNMT and reduces the hypermethylation status of genes.<sup>14</sup>

Actinin alpha 4 (*ACTN4*) belongs to the actin family and is highly expressed in podocytes and the vascular wall of the kidney.<sup>15</sup> Mutations in human *ACTN4* gene cause autosomal dominant focal segmental glomerulosclerosis.<sup>16</sup> A high glucose environment can decrease the expression of *ACTN4* and lead to the development of proteinuria.<sup>17</sup> Furthermore, the expression levels of *ACTN4* in patients with DN were significantly lower than those in healthy individuals.<sup>18</sup> The methylation levels of *ACTN4* were higher in patients with DN than in those without DN; in particular, DMR in the promoter region of *ACTN4* exhibited high methylation status in patients with DN, indicating that differential methylation of *ACTN4* is involved in the occurrence and development of DN.<sup>5</sup> In this study, we constructed a DN cell model to investigate whether EGCG could induce the demethylation of *ACTN4* and inhibit renal fibrosis in DN, and to determine the underlying molecular mechanisms.

## Methods

### Cell Culture

The human podocyte cell line, HPC, was obtained from the National Collection of Authenticated Cell Cultures. Cells were maintained in 1640 medium (Cat. No. 11875101; Gibco, USA) containing 10% fetal bovine serum (Cat. No. 10439016; Life Technologies/Thermo Fisher Scientific; USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Cat. No. 15140122; Gibco, USA) in 5% CO<sub>2</sub> at 37°C in a humid environment.

### Construction of a Cell Model of DN

HPC cells were cultured in 5.5 and 30 mmol/L glucose medium, and when the cell growth density reached 80–90%, the cells incubated with high glucose were used to establish a cell model of DN. The construction of the cell model was evaluated by determining the levels of vascular endothelial growth factor (VEGF), interleukin (IL)-8, alpha smooth muscle actin ( $\alpha$ -SMA), and fibronectin (FN) using enzyme-linked immunosorbent assay (ELISA). The cells were then treated with EGCG at different concentrations (25, 50, 75, 100, and 150 µg/mL) and the levels of VEGF, IL-8,  $\alpha$ -SMA, and FN were determined using ELISA.

### Cell Viability

HPC cells at a density of  $5 \times 10^4$  cells were seeded into a 96-well plate and treated with EGCG at various concentrations (25, 50, 75, 100, and 150 µg/mL) for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (20 µL/well) was used to incubate the cells at 37°C for 2 h. Then, the supernatant was aspirated after centrifugation at 1000 rpm for 10 min and washed once with phosphate-buffered saline, and 150 µL dimethyl sulfoxide was added to dissolve the formazan crystals. The optical density value was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA), which was used to calculate the relative cell viability.

### Isolation of DNA

Total genomic DNA was isolated using a TIANamp Genomic DNA Kit (Cat. No. DP304; TIANGEN, China), according to the manufacturer's protocol, to determine the promoter methylation profile of *ACTN4*. The purity and amount of the extracted genomic DNA were evaluated using a Nanodrop microspectrophotometer (Thermo Fisher, USA).

### Actinin Alpha 4 Promoter Methylation

Methylation-specific polymerase chain reaction (MSP) was performed to analyze the methylation profile of the promoter regions of *ACTN4* in HPC cells. MethPrimer program (<http://www.urogene.org/methprimer/index1.html>) was used to analyze the presence of CpG islands in the promoter regions of *ACTN4* and to design the primers for MSP. Nucleotide sequences of *ACTN4* gene promoter regions were obtained from the National Center for Biotechnology Information database. The sequences of methyl-specific primers of *ACTN4* were: F: 5'-AGTTTGGTTAATATGGTGAAATTTTC-3' and R: 5'-CAAACATAAAATACAATAACGCGAT-3'

The sequences of the non-methyl-specific primers of *ACTN4* were: F: 5'-TTTGGTTAATATGGTGAAATTTTGT-3' and R: 5'-TCTCCCAAACATAAAATACAATAACACA-3'.

**Table 1.** The Primers Used in RT-qPCR.

Gene	Up-stream primer	Down-stream primer
ACTN4	AGCAAATGCAGGAGTTCCGA	GCCAAGGGAGTCTGTTCAGG
DNMT1	AAGAATGGTGTGTCTACCGAC	CATCCAGGTTGCTCCCCTTGGATGG
NF- $\kappa$ B p65	CATGCCAGTGAGAATGTATGCCAT	ACGCAGGAGACGGAAGAATAAAT
I $\kappa$ B $\alpha$	TGACCATGGAAGTGATTGGTCAG	GATCACAGCCAAGTGGAGTGGGA
VEGF	TGGCAAAACTGCACCTTCACA	TGGCAAAACTGCACCTTCACA
IL-8	CCAGGAAGAAACCACCGGAAG	TGGCAAAACTGCACCTTCACA
$\alpha$ -SMA	TCTTGACCTGGCTGGACG	TCTCCTTGATGTCTCGCACA
FN	GCCATTTGCTCCTGCA	CAATTGGGCAATTAACATTA
IL-10	CACCTACTCCCAGCCAACC	TCAGCAGAGACTCACTCA
BMP-7	AGACGCCAAAGAACCAAGAG	GCTGTGCTCGAAGTAGAGGA
$\beta$ -actin	TGGCACCCAGCACAAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

EZ-96 DNA methylation gold (Cat. No. ZD5023; Zymo Research, USA) was used to bisulfite-treat the isolated genomic DNA. After sodium bisulfite conversion, the parameters of amplification of PCR reaction were applied as follows: preliminary denaturation for 3 min at 95°C, 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, extension for 30 s at 72°C, and final extension for 4 min at 72°C. A 1.5% agarose gel was prepared and PCR products were detected via electrophoresis.

### Reverse Transcription-Quantitative PCR

Various concentrations of EGCG (25, 50, 75, 100, and 150  $\mu$ g/mL) were used to treat the HPC cells for 48 h. TRIzol Reagent (Cat. No. 15596026; Thermo Fisher Scientific) was used to isolate the nuclear and cytoplasmic RNA, which was then reverse transcribed into cDNA using the iScript cDNA synthesis kit (Cat. No. 170889017; Bio-Rad, Carlsbad, CA, USA), according to the manufacturer's protocol. SYBR premix Ex Taq (Cat. No. RR820A; Takara) was used to perform quantitative PCR on an ABI 7900HT real-time PCR system. The mRNA of  $\beta$ -actin was used as the internal control for normalization. Primer 5 software (Premier; USA) was used to design the primers. The primers used for PCR are listed in Table 1. The relative RNA expression was determined using the  $2^{-\Delta\Delta C_t}$  method.

### Western Blotting

Cells were harvested, washed with PBS, and dissolved in the radioimmunoprecipitation assay buffer (Cat. No. P0013C; Beyotime, China), and bicinchoninic acid protein assays were performed to measure the protein concentrations. Total cellular protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred from the gel onto polyvinylidene fluoride (PVDF) membranes using an electroblotting apparatus. PVDF membranes were then incubated with Tris-buffered saline with Tween (TBST) containing 5% skim milk at 25°C for 1 h. The membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies used in this study were

mouse monoclonal anti-ACTN4 (Cat. No. sc-390205; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-DNMT1 (Cat. No. sc-271729; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-nuclear factor (NF)- $\kappa$ B p65 (Cat. No. sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-p-NF- $\kappa$ B p65 (Cat. No. sc-13579; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-inhibitor  $\kappa$ B (I $\kappa$ B)- $\alpha$  (Cat. No. sc-1643; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-VEGF (Cat. No. sc-57496; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-IL-8 (Cat. No. sc-376750; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti- $\alpha$ -SMA (Cat. No. sc-53142; Santa Cruz Biotechnology), and mouse monoclonal anti-FN (Cat. No. sc-8422; Santa Cruz, USA) antibodies. Membranes were incubated with primary antibodies overnight at 4°C and then washed with TBST, followed by incubation with a secondary antibody at room temperature for 2 h. ECL detection reagent (Cat. No. 32106; Thermo Fisher Scientific, USA) was used to visualize the protein bands. Image Lab software 1.8.0 (Bio-Rad, USA) was used for densitometric quantification.

### Statistical Analysis

All experiments were repeated at least three times, and data are presented as the mean  $\pm$  standard deviation. A group t-test was used for two-sample mean comparison, while differences among multiple groups were compared using one-way analysis of variance. SPSS 17.0 software was used to perform all statistical analyses.  $P < .05$  was considered to be statistically significant ( $*P < .05$ ).

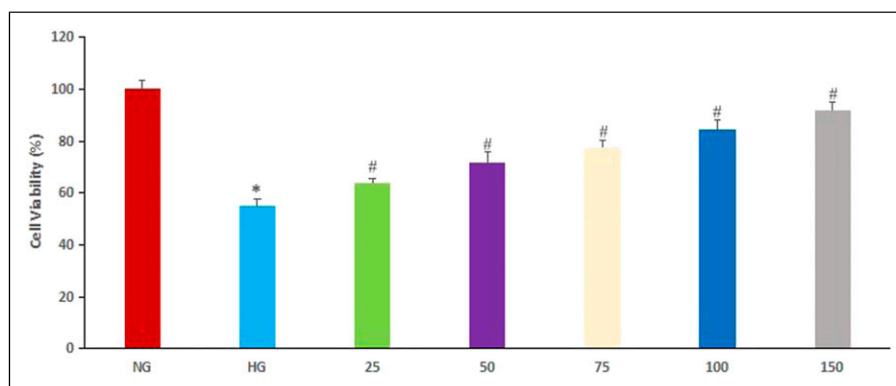
## Results

### Secreted Levels of VEGF and IL-8 in HPC Cells Under High Glucose Exposure and EGCG Treatment

The secreted levels of serum fibrosis markers,  $\alpha$ -SMA and FN, and inflammatory cytokines, VEGF, and IL-8, were

**Table 2.** The Secretion Levels (ng/L) of VEGF and IL-8 in the HK-2 Cells (Mean  $\pm$  SD. n = 3).

	$\alpha$ -SMA	FN	VEGF	IL-8
NG	534 $\pm$ 46	3865 $\pm$ 103	110 $\pm$ 10	6046 $\pm$ 184
HG	1747 $\pm$ 120	6762 $\pm$ 214	478 $\pm$ 16	12,975 $\pm$ 593
EGCG (25 $\mu$ g/mL)	1567 $\pm$ 88	6409 $\pm$ 180	404 $\pm$ 16	11,246 $\pm$ 594
EGCG (50 $\mu$ g/mL)	1324 $\pm$ 120	5961 $\pm$ 259	370 $\pm$ 13	9887 $\pm$ 403
EGCG (75 $\mu$ g/mL)	1038 $\pm$ 74	5604 $\pm$ 186	326 $\pm$ 25	8787 $\pm$ 426
EGCG (100 $\mu$ g/mL)	884 $\pm$ 80	4978 $\pm$ 211	264 $\pm$ 19	7695 $\pm$ 455
EGCG (150 $\mu$ g/mL)	664 $\pm$ 78	4527 $\pm$ 163	162 $\pm$ 25	6596 $\pm$ 401

**Figure 1.** Effect of epigallocatechin-3-gallate (EGCG) on cell viability. Normal glucose (NG), cells treated with 5.5 mmol/L glucose; High glucose (HG), cells treated with 30 mmol/L glucose, 25: 25  $\mu$ g/mL EGCG, 50: 50  $\mu$ g/mL EGCG, 75: 75  $\mu$ g/mL EGCG, 100: 100  $\mu$ g/mL EGCG, 150: 150  $\mu$ g/mL EGCG. (\* $P$  < .05, # $P$  < .01 vs NG group).

significantly increased in the high glucose-treated HPC cells (HG group) compared to those in the normal glucose-treated HPC cells (NG group), suggesting that the cell model of DN was successfully constructed. The levels of  $\alpha$ -SMA, FN, IL-8, and VEGF were found to be significantly decreased in the HG group after EGCG treatment (Table 2).

#### Treatment with EGCG Influences HPC Cell Viability

As shown in Figure 1, cell viability in the HG group was lower than that in the NG group. However, various concentrations of EGCG increased the cell viability of HPC cells in the HG group in a concentration-dependent manner compared to the cell viability of HPC cells treated with HG but without EGCG ( $P$  < .05).

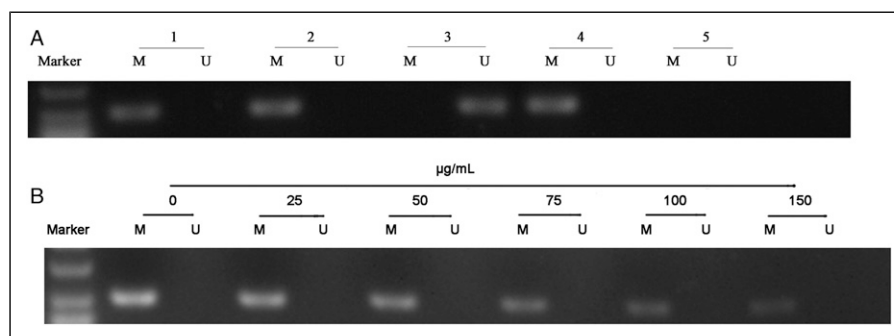
#### Methylation of the ACTN4 Gene Promoter

Genomic DNA was extracted from HG and NG groups using a methylation kit. PCR amplification was performed using the extracted genomic DNA as templates, and electrophoresis was performed to analyze the *ACTN4* gene promoter. We observed significantly high *ACTN4* promoter CpG methylation in the cell model of DN in the NG group (Figure 2A). To further explore the possibility that EGCG could reverse the methylation of the *ACTN4* gene promoter in the DN cell model, we

tested the methylation status of *ACTN4* in the HG and NG groups. As shown in Figure 2B, EGCG reversed the hypermethylated status of *ACTN4* in HPC cells of HG group in a dose-dependent manner. The gray scan results of PCR are shown in Table 3.

#### mRNA Expression Levels of *ACTN4*, *DNMT1*, *NF- $\kappa$ B p65*, *p-NF- $\kappa$ B p65*, *I $\kappa$ B $\alpha$* , *VEGF*, *IL-8*, *$\alpha$ -SMA*, *FN*, *IL-10*, and *Bone Morphogenetic Protein (BMP)-7*

To assess *ACTN4* mRNA expression in the cell model of DN, we extracted total RNA and used quantitative fluorescence PCR to detect the expression of *ACTN4* mRNA. The results showed *ACTN4* mRNA expression was decreased in the cell model of DN (.673  $\pm$  .086), but not in 5.5 mmol/L glucose-treated HPC cells (1.021  $\pm$  .056). These results suggest that *ACTN4* is commonly expressed in the DN cell model and is inversely associated with promoter region methylation. To further explore the possibility that EGCG influences the expression of *ACTN4* mRNA and its underlying mechanism, we used various concentrations of EGCG to analyze the mRNA expression levels of *ACTN4*, *DNMT1*, *NF- $\kappa$ B p65*, *p-NF- $\kappa$ B p65*, *I $\kappa$ B $\alpha$* , *VEGF*, *IL-8*,  *$\alpha$ -SMA*, *FN*, *IL-10*, and *BMP-7*. The results showed that the mRNA expression levels of *ACTN4*, *IL-10*, and *BMP-7*



**Figure 2.** Methylation status of actin alpha 4 (*ACTN4*) in HPC cells and *ACTN4* gene demethylation induced by EGCG. M: Methylation-specific PCR. U: Unmethylation-specific PCR. (A): Methylation status of *ACTN4* in HPC cells. Line 1: Methylation status of *ACTN4* in HPC without HG treatment. Line 2: Methylation status of *ACTN4* in the diabetic nephropathy (DN) cell model. Line 3: Unmethylated positive control. Line 4: Methylated positive control. Line 5: Negative Control. (B): *ACTN4* demethylation induced by EGCG.

**Table 3.** Gray Scan Results of MSP (Mean  $\pm$  SD. n = 3).

	Methylation-specific	Unmethylation-specific
Line1 (Figure 2A)	16,697.7 $\pm$ 156.3	0
Line2 (Figure 2A)	15,420 $\pm$ 381.1	0
Line3 (Figure 2A)	0	14,552.7 $\pm$ 125.2
Line4 (Figure 2A)	16,687.7 $\pm$ 82.6	0
HG (Figure 2B)	18,593.3 $\pm$ 491.9	0
EGCG (25 $\mu$ g/mL) (Figure 2B)	17,425.3 $\pm$ 256.3	0
EGCG (50 $\mu$ g/mL) (Figure 2B)	16,411.7 $\pm$ 266.6	0
EGCG (75 $\mu$ g/mL) (Figure 2B)	15,268.7 $\pm$ 215.8	0
EGCG (100 $\mu$ g/mL) (Figure 2B)	14,346.3 $\pm$ 269.6	0
EGCG (150 $\mu$ g/mL) (Figure 2B)	13,726.7 $\pm$ 395.2	0

were significantly higher, while those of *DNMT1*, *NF- $\kappa$ B p65*, *p-NF- $\kappa$ B p65*, *I $\kappa$ B $\alpha$* , *VEGF*, *IL-8*,  *$\alpha$ -SMA*, and *FN* were downregulated in the DN cell model compared with the NG group ( $P < .05$ , Figure 3).

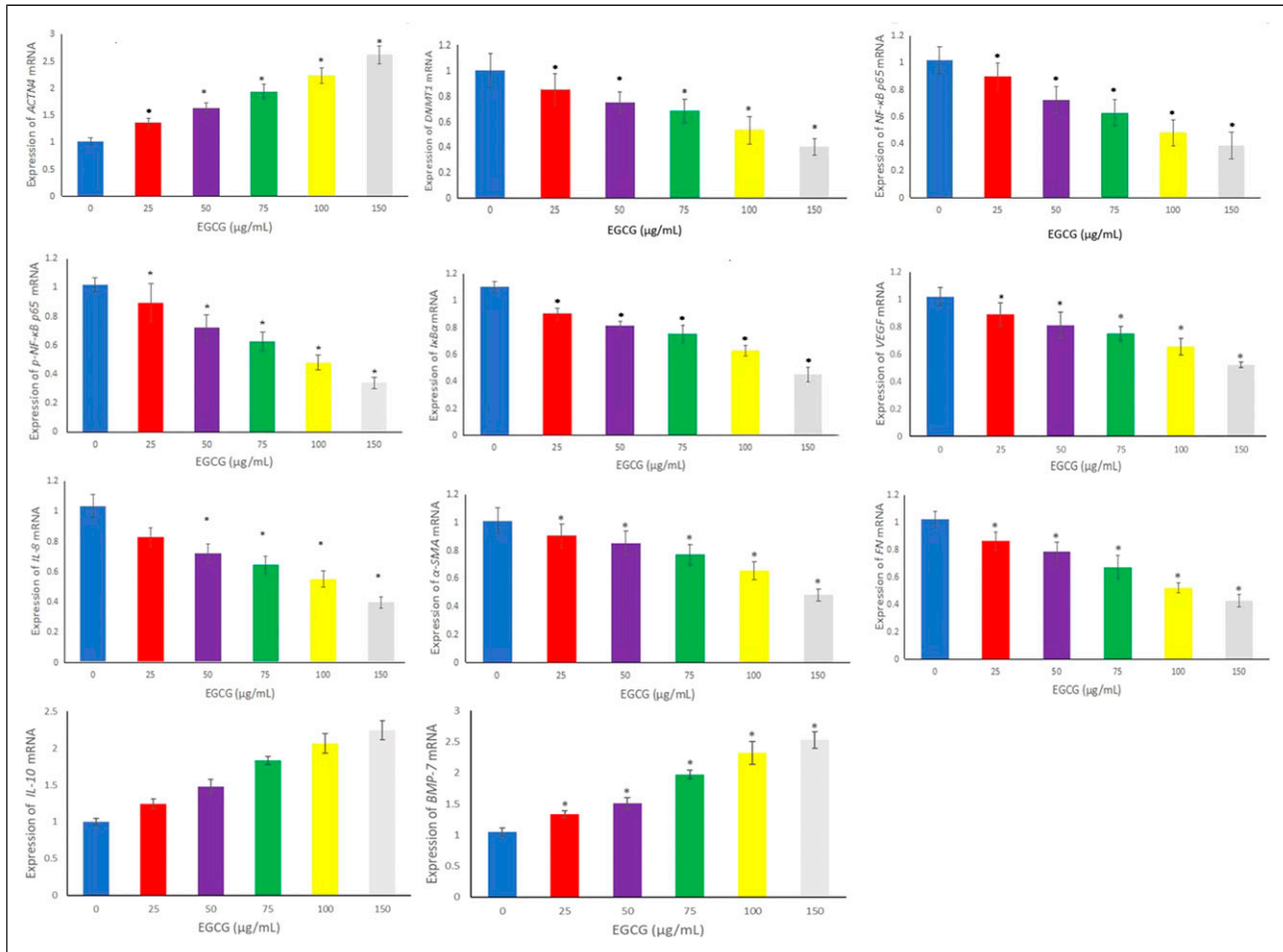
### Protein Expression Detected via Western Blotting

Western blotting was performed to determine the protein expression in HPC cells treated with or without various concentrations of EGCG. Protein levels of *ACTN4* were increased with EGCG treatment in the DN cell model compared with the NG group at 150  $\mu$ g/mL EGCG (Figure 4). In contrast, *DNMT1*, *NF- $\kappa$ B p65*, *p-NF- $\kappa$ B p65*, *I $\kappa$ B $\alpha$* , *VEGF*, *IL-8*,  *$\alpha$ -SMA*, and *FN* protein levels were moderately decreased in the DN cell model treated with EGCG, and the protein expression levels were downregulated in a dose-dependent manner ( $P < .05$ ). After HPC cells were treated with 20  $\mu$ mol/L ammonium 1-pyrrolidinedithiocarbamate (PDTC), an inhibitor of *NF- $\kappa$ B*, the protein expression levels of *NF- $\kappa$ B p65*, *p-NF- $\kappa$ B p65*, *I $\kappa$ B $\alpha$* , *VEGF*, *IL-8*,  *$\alpha$ -SMA*, and *FN* were decreased in the cell model of DN, and the combination of EGCG and PDTC exhibited a significant reduction in protein expression compared with PDTC treatment alone (Figure 5).

### Discussion

Diabetic nephropathy is a fatal complication of diabetes and the main cause of end-stage renal disease (ESRD).<sup>19</sup> DN is one of the most challenging health problems worldwide. Although the therapeutic strategies for DN have remarkably improved in recent years, nearly half of the patients inevitably develop ESRD.<sup>20</sup> Renal fibrosis is a common pathway of ESRD in the vast majority of chronic kidney diseases. Various factors, such as hypertension, inflammation, hyperglycemia, hyperlipidemia, and drug damage, cause renal fibrosis.<sup>21</sup> However, the molecular mechanisms underlying high glucose-induced renal fibrosis remain unclear. Therefore, studying this mechanism is expected to provide a theoretical basis for identifying effective treatment targets for DN.

Epigenetic regulation plays a crucial role in DN occurrence.<sup>5</sup> As one of the most important epigenetic modifications, DNA methylation contributes to the pathogenesis of renal fibrosis and causes DN via phenotypic alterations and modifications in functional gene expression.<sup>6</sup> DNA methylation alteration induced by high glucose levels plays an important role in the pathogenesis of DN by altering peripheral leukocyte DNA or salivary cell DNA in patients with DN.<sup>7</sup> DNA methylation refers to a chemical reaction in which the cytosine

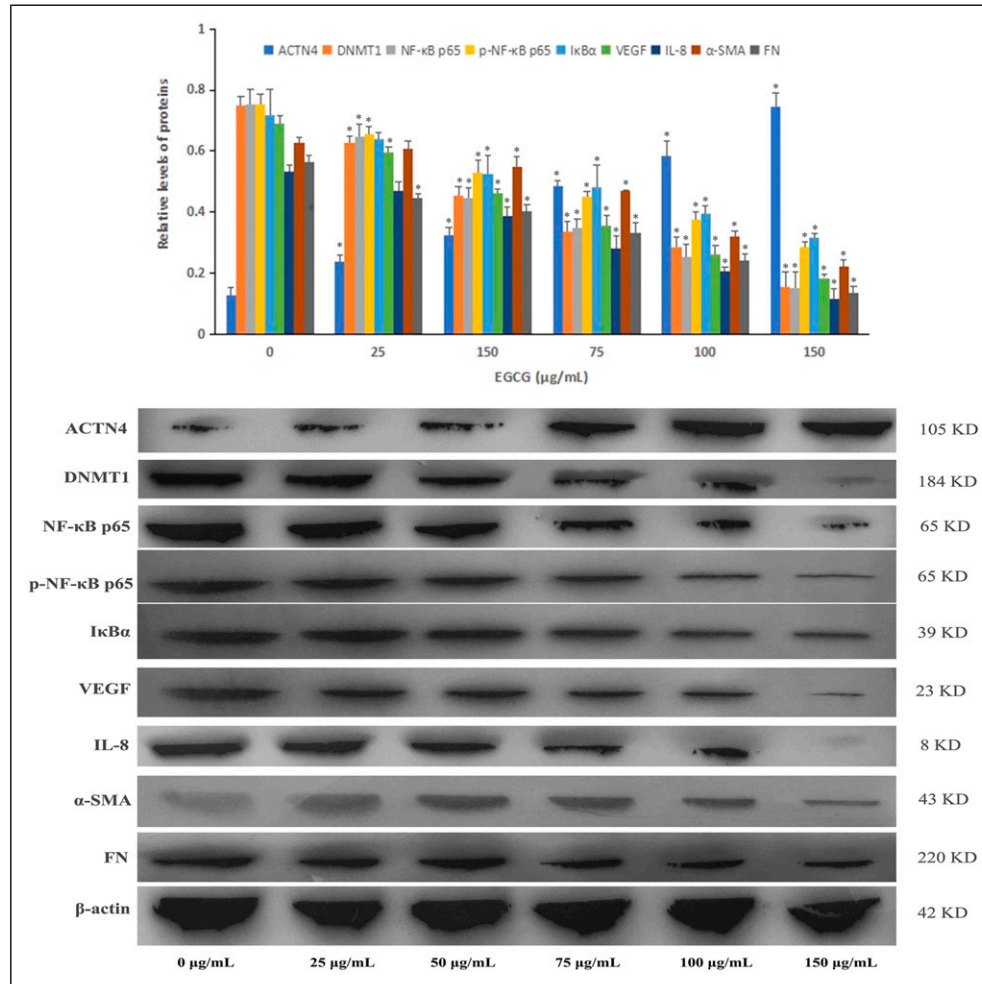


**Figure 3.** mRNA expression levels of *ACTN4*, DNA methyltransferase I (*DNMT1*), nuclear factor (*NF*)- $\kappa$ B *p65*, *p*-*NF*- $\kappa$ B *p65*, inhibitor  $\kappa$ B (*I* $\kappa$ B)- $\alpha$ , vascular endothelial growth factor (*VEGF*), interleukin (*IL*)-8, alpha smooth muscle actin ( $\alpha$ -*SMA*), fibronectin (*FN*), *IL*-10, and bone morphogenetic protein (*BMP*-7) were determined by RT-qPCR in the cell model of DN treated with EGCG (\* $P < .05$  vs Control group,  $n = 3$ ).

(C) in CpG dinucleotides is methylated into 5-methylated cytosine (5<sup>-MC</sup>) with methyl groups from S-adenosine methionine via DNMTs.<sup>10</sup> The methylation status of eukaryotic DNA is related to gene transcription regulation. During DN formation, DNA methylation patterns, including partial hypermethylation of genes, change significantly.<sup>7</sup> A study revealed that the methylation status of 6246 genes in patients with DN was significantly increased compared to that in diabetic patients without DN, while the methylation status of 3680 genes in DN was higher than that in control groups.<sup>22</sup> The overall CpG methylation level in DN patients was 72.62%, which was higher than that in diabetic patients without DN and the control group, and Maghbooli et al<sup>23</sup> obtained similar results. Dayeh et al demonstrated that the ATP binding cassette subfamily G member 1 (*ABCG1*) gene methylation was associated with a 9% increased risk of future diabetes morbidity, and silencing the expression of *ABCG1* via hypermethylation caused an accumulation of cholesterol, leading to atherosclerosis, glucose-stimulated insulin secretion impairment, and inflammation of islet beta cells.<sup>24</sup>

Marumo et al also revealed that kinesin family member 20B, claudin 18, and solute carrier organic anion transporter family member 1A1 exhibited hypermethylation in DN mice compared to the control groups.<sup>25</sup>

Actinin alpha 4 plays a crucial role in kidney diseases.<sup>26</sup> In several kidney diseases, including DN, the protein expression levels of *ACTN4* are reduced in kidney tissues, which have been reported in some animal and clinical studies. *ACTN4* downregulation is closely associated with the development of diabetes.<sup>27</sup> *ACTN4* can alleviate inflammatory and fibrosis impairment in DN.<sup>28</sup> In this study, we obtained similar results; the protein expression of *ACTN4* was decreased in the cell model of DN induced by high glucose conditions. Moreover, *ACTN4* gene hypermethylation was observed in diabetic patients with DN and DMR in the promoter region was highly methylated, suggesting that *ACTN4* aberrant methylation is associated with the pathogenesis and progression of DN. Therefore, reducing hypermethylation may be a new strategy for the treatment of DN.

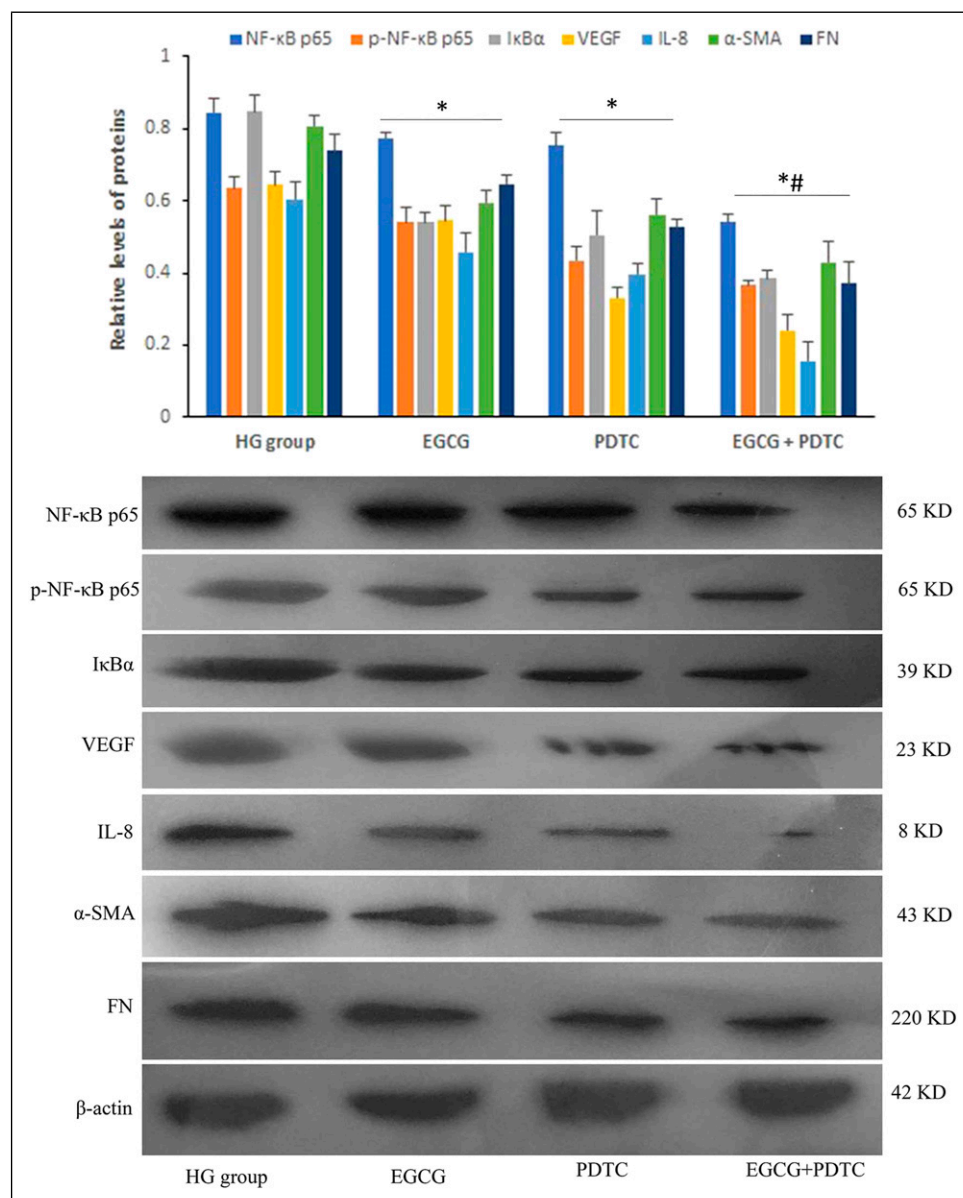


**Figure 4.** Protein expression levels of ACTN4, DNMT1, NF-κB p65, p-NF-κB p65, IκBα, VEGF, IL-8, α-SMA, and FN were determined by Western blotting in the cell model of DN treated with EGCG (\* $P < .05$  vs Control group,  $n = 3$ ).

Numerous studies have reported that 5-aza-2'-deoxycytidine reverses methylation and alleviates diabetic kidney inflammation in DN.<sup>29</sup> However, 5-aza-2'-deoxycytidine and other chemical compounds exhibit unpredictable side effects and cell toxicity.<sup>10</sup> The identification of new agents for reversing DNA methylation appears to be particularly important. Natural products that inhibit DNMT activity exhibit some advantages, such as minor side effects and low toxicity. Green tea exhibits both physiological and pharmacological effects. As the major polyphenol of green tea, EGCG is a potential demethylating agent that directly inhibits DNMT, demethylates CpG islands, and reactivates the methylation-silenced genes.<sup>12,13</sup> In 2020, Yang et al<sup>30</sup> revealed that EGCG treatment significantly reduced the levels of Klotho promoter methylation under high glucose conditions. In addition, EGCG treatment exerts a protective effect on the Klotho protein by upregulating its expression. However, the mechanism by which EGCG exerts its protective effects remains unclear.

In our study, we established a cell model of DN and detected the secreted levels of serum fibrosis markers, α-SMA

and FN, and inflammatory cytokines, VEGF, and IL-8, to evaluate the successful construction of the DN cell model. While the DN cell model was treated with EGCG, the levels of α-SMA, FN, IL-8, and VEGF were significantly decreased, indicating that EGCG plays a role in inhibiting fibrosis and inflammation in DN. In addition, EGCG increased the viability of HPC cells treated with high glucose, possibly by the alleviation of fibrosis and inflammation. *ACTN4* is rich in CpG islands, indicating that the promoter of *ACTN4* gene is closely related to DNA methylation. MSP assay revealed that the level of *ACTN4* gene methylation in the DN cell model was higher than that in normal glucose-treated cells. In the DN cell model, the protein expression of ACTN4 was downregulated, while that of DNMT1 was upregulated. However, EGCG treatment reversed these effects, indicating that EGCG reduced the expression of DNMT1, thereby demethylating the *ACTN4* gene promoter to reverse the protein expression of ACTN4. Similar to our results, Yang et al found that the protein expression levels of DNMT1 and DNMT3a were increased in diabetic kidney disease.<sup>30</sup> Moreover, Liu et al found that the



**Figure 5.** Protein expression levels of NF- $\kappa$ B p65, p-NF- $\kappa$ B p65, I $\kappa$ B $\alpha$ , VEGF, IL-8,  $\alpha$ -SMA, and FN were determined by Western blotting in the cells treated with EGCG and pyrrolidinedithiocarbamate (PDTC) (\* $P < .05$  vs HG group,  $n = 3$ ; ## $P < .05$  vs PDTC group).

protein expression of DNMT3b was increased in chronic diabetic kidney disease.<sup>31</sup>

Renal inflammation and fibrosis are associated with DN pathogenesis and progression. The secretion of cytokines, VEGF, and IL-8, and the expression levels of fibrosis markers,  $\alpha$ -SMA, and FN, were significantly increased in DN.<sup>19</sup> The glomeruli were hypertrophied and the mesangial matrix was broadened, eventually leading to ESRD due to the increase in  $\alpha$ -SMA and FN levels.<sup>20</sup> In contrast, some anti-inflammatory and antifibrotic cytokines have been shown to alleviate the development and progression of DN. In Wang's study, schisandrin C exerted protective effects against DN by increasing the expression of the anti-inflammatory cytokine, IL-10, in macrophages.<sup>32</sup> Meng et al revealed that lncRNA zinc finger E-

box binding homeobox 1 antisense RNA 1 (ZEB1-AS1) inhibits high glucose-induced fibrogenesis by improving the expression level of BMP7 in DN, which provides a novel target for DN therapy.<sup>33</sup> In this study, we demonstrated that EGCG significantly reduced the expression levels of inflammatory and fibrotic markers, while increasing the expression levels of anti-inflammatory and anti-fibrotic cytokines by upregulating the protein expression of ACTN4, suggesting that the reversal of ACTN4 protein expression can alleviate the cell damage induced by high glucose treatment.

However, the molecular mechanism by which EGCG regulates ACTN4 expression via demethylation to alleviate high glucose-induced cell damage needs to be further explored. ACTN4 functions as a selective regulator of RelA/p65



which is subunit of the transcription factor, NF- $\kappa$ B, and P65 inhibits the proliferation of H1299 cells, while ACTN4 overexpression regulates this inhibitory effect via RelA/p65 interacting with nuclear ACTN4.<sup>34</sup> Huang indicated that ACTN4 overexpression affects the activation of P65 in osteosarcoma cells and ultimately regulates their invasion ability via the NF- $\kappa$ B pathway.<sup>35</sup> Thus, we presume that EGCG regulates ACTN4 expression via demethylation to alleviate high glucose-induced cell damage, which may be related to the regulation of the NF- $\kappa$ B signaling pathway. Therefore, we investigated the effects of EGCG on the mRNA and protein expression levels of the major components of the NF- $\kappa$ B signaling pathway. The results showed that the mRNA and protein expression levels of NF- $\kappa$ B p65, p-NF- $\kappa$ B p65, I $\kappa$ B $\alpha$ , VEGF, IL-8,  $\alpha$ -SMA, and FN with EGCG treatment were decreased in the DN cell model compared with those in the NG group. In addition, the NF- $\kappa$ B inhibitor, PDTC, decreased the expression levels of NF- $\kappa$ B p65, p-NF- $\kappa$ B p65, I $\kappa$ B $\alpha$ , VEGF, IL-8,  $\alpha$ -SMA, and FN, suggesting that EGCG, similar to PDTC, inhibits renal inflammation and fibrosis in DN by regulating the NF- $\kappa$ B signaling pathway. Hence, these results show that EGCG induces the demethylation of *ACTN4*, suppressing ACTN4 expression, and inhibits renal fibrosis in DN via the NF- $\kappa$ B signaling pathway.

In summary, these results indicate that the methylation level of the *ACTN4* gene promoter was increased by upregulating DNMT1 expression in the cell model of DN, along with low protein expression of ACTN4. The expression levels of fibrosis and inflammatory markers were also increased in the cell model of DN compared with those in the NG group. EGCG significantly increased the viability of high glucose-treated HPC cells. EGCG treatment induced demethylation and reduced promoter methylation of *ACTN4* to reverse ACTN4 expression. Moreover, EGCG downregulated the mRNA and protein expression levels of NF- $\kappa$ B p65, p-NF- $\kappa$ B p65, and I $\kappa$ B $\alpha$ , inhibited the expression levels of fibrosis and inflammatory markers, and increased the levels of anti-inflammatory and anti-fibrotic cytokines in the cell model of DN. Furthermore, EGCG showed similar inhibition as PDTC on renal inflammation and fibrosis in DN via the suppression of the NF- $\kappa$ B signaling pathway. Hence, this study indicated that EGCG exerts its protective effects via the demethylation of *ACTN4* to reverse its expression and alleviate renal fibrosis in DN and that the NF- $\kappa$ B signaling pathway is involved in this process.

## Conclusions

Collectively, we found that EGCG reduced the hypermethylated status of *ACTN4* by downregulating DNMT1 expression and restoring ACTN4 expression, resulting in the alleviation of renal inflammation and fibrosis in DN via upregulation of the NF- $\kappa$ B signaling pathway.

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## Authors' contributions

M. W., C. H., and J. L. designed the study. Y. H., X. H., and S. S. performed the experiments. C. H., D. W., and J. L. analyzed the data. M. W. and C. H. prepared the article.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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