

# Branched Chain Amino Acids Protects Rat Mesangial Cells from High Glucose by Modulating TGF- $\beta$ 1 and BMP-7

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**Aim:** Branched-chain amino acids (BCAAs) have been reported owning curative effects in early diabetic nephropathy. However, the mechanisms of its action have not been elucidated. The aim of this study is to investigate the effect of possible mechanism(s) of BCAAs on cultured rat mesangial cells (RMCs).

**Methods:** RMCs were treated with high glucose (30 mmol/L) and BCAAs (10 mmol/L) respectively. Cell proliferation was detected using an MTT assay. Expression of transforming growth factor (TGF)- $\beta$ 1 and gremlin mRNA was detected by semiquantitative reverse-transcription (RT) PCR. TGF- $\beta$ 1 and fibronectin (FN) protein levels were measured using enzyme-linked immunosorbent assays (ELISAs). Gremlin, bone morphogenic protein (BMP)-7, and Smad2/3 proteins were detected by immunofluorescence. Smad1/5/8 and phosphorylated (p)-Smad1/5/8 were detected by Western blotting.

**Results:** The proliferation rate of the RMCs in the high glucose group alone was 1.45-times of cells in the CON group, and it was reduced by 32% upon co-treatment with BCAAs. The expression of TGF- $\beta$ 1, gremlin, p-Smad2/3 and FN mRNA or protein in the HG group was higher than that in the CON group. In the BCAAs group, the corresponding levels were lower than that in HG group. The expression of BMP-7 and p-Smad1/5/8 were significantly lower in the HG group than in the CON group. Moreover, the expression of BMP-7 and p-Smad1/5/8 were higher in the BCAAs group than in the HG group.

**Conclusion:** BCAAs showed an antidiabetic effect via reducing TGF- $\beta$ 1-Smad2/3 pathway and Gremlin expression and upregulating BMP-7-Smad1/5/8 pathway in rat mesangial cells, consequently lessening ECM deposition in renal tissue.

**Keywords:** diabetic nephropathy, branched chain amino acids, TGF- $\beta$ 1, BMP-7, Smad

## Introduction

Diabetic nephropathy (DN) is one of the most serious complications of diabetes mellitus. It is an important reason for end-stage kidney disease. Abnormal proliferation of mesangial cells is the key pathological change involved in DN. Mesangial cells can secrete extracellular matrix (ECM) components, including fibronectin (FN), collagen IV, and laminin. Accumulation of ECM components in the kidney leads to glomerular mesangial expansion and fibrosis, which, in turn, is characteristic of DN.<sup>1,2</sup>

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) plays an important role in the physiological functions and pathological states of the kidney, and it is recognized as both a fibrogenic and an inflammatory cytokine.<sup>3</sup> Previous studies have demonstrated that the induction of TGF- $\beta$ 1 in diabetes accelerates fibrosis of renal mesangial cells. Some

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reports showed that renal mesangial cells (RMCs) exposed to high glucose stress respond by increasing TGF- $\beta$ 1 and FN expression *in vitro*.<sup>4</sup> Smad proteins participate in TGF  $\beta$  cell signal transduction. Smad proteins include BMP-smad (Smad1/5/8) and TGF- $\beta$ /activin-smad (Smad2/3). TGF- $\beta$ 1 binding to its receptor can activate its major downstream mediators Smad2/3 to exert its biological functions.<sup>5</sup>

Bone morphogenic protein (BMP)-7 is considered as a protective factor in DN.<sup>6,7</sup> A number of studies show that BMP-7 plays protective roles in cultured renal cells. Maintenance of BMP-7 activity may result in blockade of ECM accumulation in mesangial cells.<sup>8</sup> Some study showed a reversibility of TGF- $\beta$ -induced epithelial-to-myofibroblast transition (EMT) by BMP-7 in proximal tubular cells.<sup>9</sup>

Moreover, recent study shows that BMP-7 binding to its receptor can affect Smad1/5/8 phosphorylation and plays function in the pathogenesis of hepatocellular carcinoma.<sup>10</sup> Gremlin is reported to be the only one of the three BMP-7 antagonists and the expression of which is increased in kidneys of diabetic rats,<sup>11</sup> moreover, gremlin has been reported regulating TGF- $\beta$  in diabetic nephropathy.<sup>12</sup>

Branched-chain amino acids (BCAA: leucine, isoleucine and valine), are essential amino acids and presently widely used in clinical treatment, such as liver disease.<sup>13</sup> Recently, Tajiri K found that BCAAs could promote endothelial dysfunction.<sup>14</sup> In our preliminary work, we found that BCAAs could protect streptozotocin-induced insulin secretion in pancreatic beta cells.<sup>15</sup> The present study show BCAAs could inhibit the TGF- $\beta$  induced damage in HepG2 cells, suggesting a potential pharmacological significance of BCAAs supplementation to the patients with hepatic failure.<sup>16</sup> Branched-chain amino acids ( BCAAs) have been reported owning curative effects in early diabetic nephropathy, acting on metabolic signal pathways directly or indirectly. In our previous study, we have proved that branched-chain amino acids could attenuate early kidney injury in diabetic rats.<sup>17</sup> However, there were few studies about the effect of BCAAs on the function of the renal mesangial cells. Therefore, the aim of this present study was to address whether BCAAs could protect RMCs from high-glucose-induced stress and also to elucidate the possible underlying mechanisms.

## Materials and Methods

### Materials

Trizol reagent, the reverse transcription (RT) kit, and semi-quantitative real-time PCR kit were purchased from Takara (Takara, Japan). Rabbit polyclonal antibodies specific for

BMP-7 (ab56023, Abcam, USA) and gremlin1 (ab140010, Abcam, USA), Smad1/5/8 (sc-7965, Santa Cruz, Canada), phosphorylated (p)-SMAD1/5/8 (SMAD1, 5-phospho-Ser463/465, and SMAD8 phospho-Ser 426/428 antibodies; 13820, Cell Signaling Technology, Canada), and p-Smad2/3 (YP0362, Immunoway Biotechnology, China) were used in this study. Secondary antibodies were obtained from Jackson (Jackson, USA). The TGF- $\beta$ 1 and FN enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster (EK0515, Boster, Wuhan, China). Low-glucose Dulbecco's modified Eagle medium (DMEM) was purchased from Hyclone (Hyclone, USA). Penicillin/streptomycin and trypsin were obtained from North China Pharmaceutical (NCPC, China). BCAAs were purchased from Sigma-Aldrich Company (USA). Glucose was purchased from China Otsuka Pharmaceutical Company (Otsuka, China). Mannitol was purchased from Shandong JinYang Pharmaceutical (Shandong JinYang Pharmaceutical, China).

### Cell Culture

RMCs were purchased from the China Center for Type Culture Collection (Wuhan, China), and were grown in low-glucose DMEM containing 10% fetal calf serum (FCS) and 100 Units/mL of penicillin/streptomycin. RMCs ( $10^4$  cells/mL) were plated in 60-mm culture dishes. After 3 days of culture, the cells reached 80% confluence, whereby they were trypsinized, collected, centrifuged at 1000 rpm/min for 5 min. The supernatant was discarded, complete medium was added, and a cell suspension was prepared. The cell suspension was placed in new culture dishes.

### BCAAs Processing

The BCAAs were diluted with distilled water to a final concentration of 10 mmol/L. The filtered solution was stored at 4°C until use.

### Cell Processing

After RMCs adhered to the culture dishes (about 24 h), the medium was exchanged with low-glucose DMEM without FCS. After 24 h, the cells were divided into the following groups: The control (CON) group (cultured in complete medium containing 24.5 mmol/L mannitol), high-glucose (HG) group (cultured in complete medium containing 24.5 mmol/L glucose), and the BCAAs group (cultured in complete medium containing 24.5 mmol/L glucose and 10 mmol/L BCAAs). After 48 h, the cells were collected for assessments.

## Cell Proliferation Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to measure cell proliferation. Briefly, cells were seeded at  $10^4$  cells/well in 96-well plates. After starvation for 24 h in low-glucose medium, the cells were treated with DMEM contain 10% FCS in the presence of mannitol, high glucose, or high glucose with BCAAs (5 wells for each group). The optical density (OD) of the samples at wavelength of 490 nm was measured using a microplate reader. The viability of cells incubated in control medium was considered as 100% and the values for the other samples were normalized to that of the cells in the control medium.

## Semiquantitative Real-Time PCR

Total RNA was extracted from cells (collected as described in section 2.4) using Trizol reagent and processed with RNase-Free DNase before use in PCR. The RNA was reverse transcribed and semiquantitative RT-PCR was conducted using the Superscript RT-PCR kit (Invitrogen) for 40 cycles with the following

primers: TGF- $\beta$ 1-F: 5'-CTGCTGACCCCACTGATAC-3' and TGF- $\beta$ 1-R: 5'-CTGTATTCCGTCTCCTTGGTTC-3'; gremlin-F: 5'-CACCGCACTATCATCAATCGCTTCTCGA AAGAAGCGATTGATGATAGTGC-3' and gremlin-R: 5'-AAAAGCACTATCATCAATCGCTTCTTCGAGAAGC-GATTGATGATAGTGC-3'; FN-F: 5'-TGGAGAGACAGG AGGAAATAGC-3' and FN-R: 5'-CAGTGACAGCATACA GGGTGAT-3'; and GAPDH-F: 5'-ACCACAGTCCATGCC ATC-3' and GAPDH-R: 5'-TCCACCACCCTGTTGCTGTA -3' using the LightCycler 480 system (Roche, USA). The  $2^{-\Delta\Delta Ct}$  quantitative analysis method was used to analyze the results: GAPDH was used as the internal reference gene, and the expression of TGF- $\beta$ 1, FN, and gremlin mRNA in the control group was used to calculate the relative levels of TGF- $\beta$ 1, Gremlin, and FN mRNA in the HG and BCAAs groups. The  $\Delta\Delta Ct$  value of the specific query genes was calculated as = (Ct value of query gene - Ct value of GAPDH) in the treatment group - (Ct value of query gene - Ct value of GAPDH) in the control group, with the relative amounts of the query mRNA =  $2^{-\Delta\Delta Ct}$ .

## ELISA

Protein levels of TGF- $\beta$ 1 and FN were determined using the ELISA kits, which employ the semiquantitative enzyme immunoassay technique. The absorbance of the resulting yellow product was measured at 450 nm.

## Western Blotting

The cell samples (collected as described in section 2.4) were homogenized in radio immunoprecipitation assay buffer containing protease and phosphatase inhibitors, and the protein concentration was determined by the Bradford method. The proteins (30  $\mu$ g per sample) were resolved on a 10% sodium dodecyl sulfate –polyacrylamide gel and transferred to a Hybond-P polyvinylidene difluoride membrane. The membranes were blocked in 5% nonfat milk at room temperature for 1 h and incubated with the primary antibodies overnight at 4°C. The primary antibodies were used at the following dilutions: BMP-7(1:500), Smad1/5/8(1:200),  $\beta$ -actin (1:10,000), and p-Smad1/5/8 (1:500). The membranes were washed with Tris-buffered saline containing Tween-20 (TBST) and incubated with either anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The membranes were washed again with TBST before visualizing the protein-antibody complexes using enhanced chemiluminescence (Pierce Bio, USA).

## Immunofluorescence Analysis

Briefly, chamber slides were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in TBS for 14 min at room temperature. After they were rinsed in TBS, the cells were permeabilized with 0.5% Triton X-100 for 3 min and then rinsed three times in TBS. The slides were incubated with primary antibodies specific for gremlin (1:50) and Smad2/3 (1:50) overnight at 4°C. The slides were rinsed and incubated with secondary antibodies conjugated to red or green (Beyotime, China) for 1 h at room temperature. Control incubation was included, with the secondary antibody alone. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Beyotime, China).

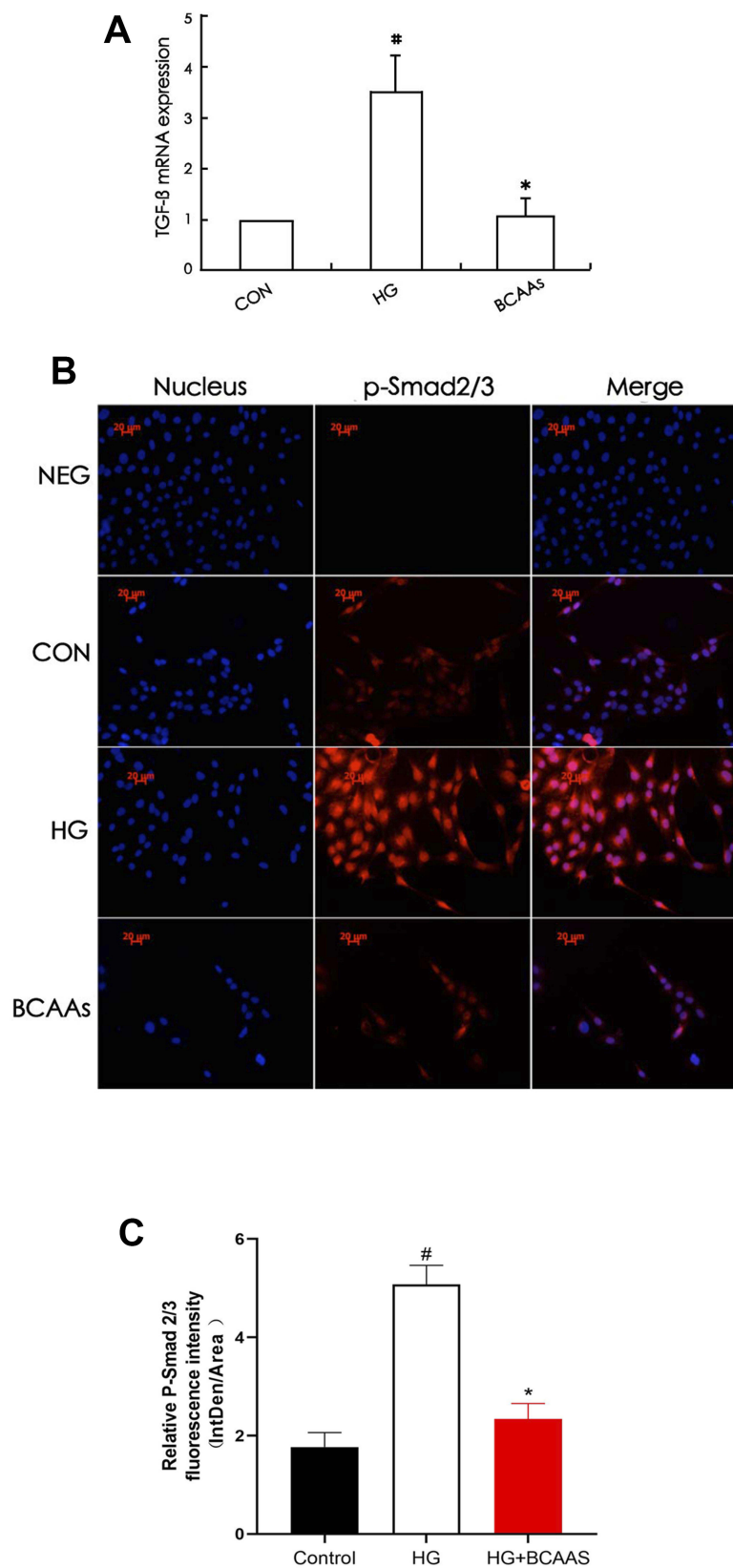
## Statistical Analysis

Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed by one-way ANOVA with F-test. P value of  $< 0.05$  was considered significant. The data were analyzed with SPSS II for Windows

**Table I** Proliferation of RMCs. CON, HG, and BCAAs (N=5)

Groups	$\bar{x} \pm s$	Proliferation Rate
CON	0.6822 $\pm$ 0.0944	1
HG	0.9858 $\pm$ 0.2158 <sup>#</sup>	1.45
BCAAs	0.6744 $\pm$ 0.1372*	0.99

Notes: <sup>#</sup>p<0.05 vs CON group. \*P<0.05 vs HG group. Data are shown as the mean  $\pm$  SD



**Figure 1** (A) TGF- $\beta$ 1 mRNA levels were assayed using semiquantitative RT-PCR assay in CON group, HG group, BCAAs group, respectively. (B) Immunofluorescence staining for p-Smad2/3 in RMCs in CON group, HG group, BCAAs group, respectively. (C) Quantification of p-Smad2/3 fluorescence intensity (integrated density per stain area). <sup>#</sup> $p < 0.05$  vs CON group, <sup>\*</sup> $p < 0.05$  vs HG group. Data were shown as the mean  $\pm$  SD, with  $n = 5$  samples in each group.



(release 11.0.1J, SPSS Japan Inc.). All experiments were performed at least three times.

## Results

### Proliferation of RMCs

The proliferation rate of the RMCs in the high-glucose group alone was 1.45-times that of cells in the CON group ( $0.6822 \pm 0.0944$  vs  $0.9858 \pm 0.2158$ ,  $p=0.022$ ) (Table 1). This higher proliferation rate was reduced by 32% upon co-treatment with BCAAs ( $0.9858 \pm 0.2158$  vs  $0.6744 \pm 0.1372$ ,  $p=0.0264$ ). These data indicate the BCAAs restore the cell proliferation rate of high-glucose-treated cells to that of the control cells.

### Expression of TGF- $\beta$ 1 and Smad2/3

TGF- $\beta$ 1 mRNA levels in RMCs were increased (to 350%) in the presence of high glucose stimulation, compared with that in the CON cells (Figure 1A), and the expression of TGF- $\beta$ 1 protein and p-Smad2/3 protein were also significantly higher than that in the CON group (Table 2 and Figure 1B and C). TGF- $\beta$ 1 mRNA levels were reduced (69.3%) when cells were co-treated with BCAAs and high glucose, compared with that in the HG group, and the protein levels exhibited a similar change.

### Expression of BMP-7, Gremlin, and Smad1/5/8

The expression of gremlin mRNA and protein in the HG group was significantly higher than that in the CON group, and in the BCAAs group, the expression of gremlin mRNA and protein was lower than that in the HG group (Figure 2A–C). The expression of BMP-7 and p-Smad1/5/8 were significantly lower in the HG group than in the CON group, moreover, the expression of BMP-7 and p-Smad1/5/8 were higher in the BCAAs group than in the HG group (Figure 2D–F).

### Expression of FN

The expression of FN mRNA and protein in the HG group was higher than that in the CON group; In the BCAAs group, the FN mRNA and protein levels were lower than that in HG group (Table 3, Figure 3A and B).

## Discussion

Excess glucose and proteins become advanced glycosylation end products (AGEs), adding glaciated LDL and high glucose itself, can induce the expression of TGF- $\beta$ 1 on

mesangial cells. TGF- $\beta$ 1 is just seemed as a biochemical marker for DN development in type 2 diabetic patients.<sup>18</sup> In vitro, high glucose can induce TGF- $\beta$ 1 and its receptor expression in tubular and mesangial cells.<sup>19,20</sup> The high glucose induces serine/threonine protein kinase/protein kinase B (Akt/PKB) phosphorylation in a protein kinase C- $\beta$  (PKC- $\beta$ )-dependent manner resulting in the upregulation of TGF- $\beta$ 1 transcription.<sup>21,22</sup> TGF- $\beta$ 1 is widely thought to be the most important cytokine in the ECM glomerular pathology. It is also a key fibrogenic factor that regulates epithelial to myofibroblast transition in renal tubular cells.<sup>23,24</sup> It binds to a type II serine/threonine kinase receptor, which transphosphorylates and activates a type I receptor. This process is followed by modulation of the downstream-signaling molecules Smad, MAPK, and perhaps protein kinase A cellular pathways.<sup>25</sup> TGF- $\beta$ 1 binds to the TGF- $\beta$  receptor II (T $\beta$ RII) to result in phosphorylation of Smad2 and Smad3<sup>26</sup> to form a heterodimeric complex with Smad4, which translocate into the nucleus and regulates transcription of TGF- $\beta$ 1 target genes, such as collagen a 1 (I), PAI-1, Jun B, c-Jun, and fibronectin.<sup>27,28</sup>

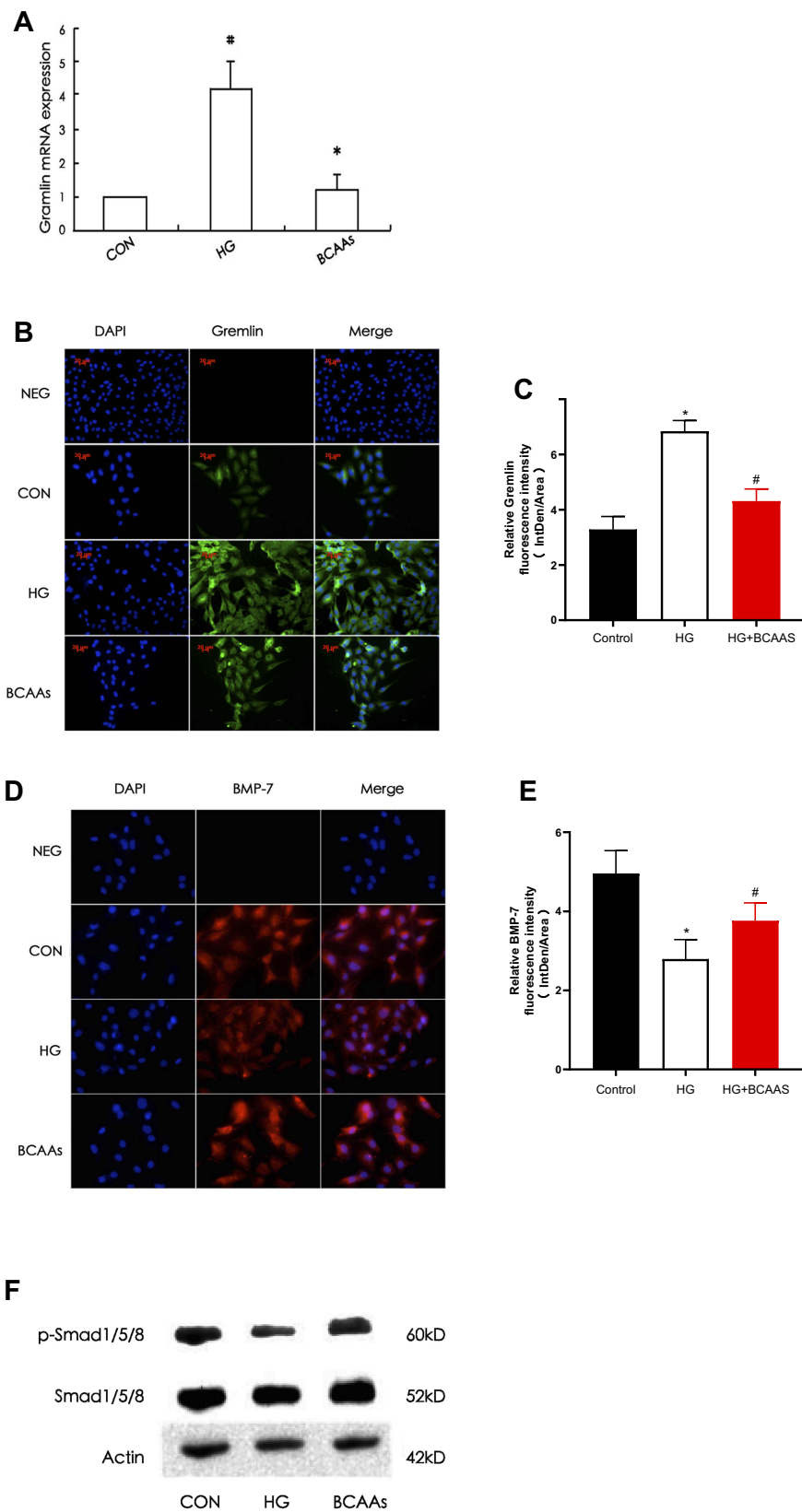
Bone morphogenetic protein-7 (BMP-7), a member of TGF- $\beta$  superfamily, could reduce glomerular and tubulointerstitial fibrosis and protected the kidney from hyperglycemia-induced oxidative stress in diabetic nephropathy.<sup>7,29–32</sup> It has the distinguishing property of inhibiting TGF- $\beta$ -dependent biological functions.<sup>33</sup> BMP-7 promotes the activating phosphorylation of Smad1/5/8. Phosphorylated Smad1/5/8 and phosphorylated Smad2/3 bind the Smad4 protein and regulate the transcription of target genes.<sup>34</sup> BMP-7-Smad1/5/8 pathway and TGF- $\beta$ -Smad2/3 pathway keep balance in healthy kidney.

High glucose could affect BMP-7-Smad1/5/8 pathway through Gremlin, and BCAAs might decrease the expression of Gremlin and release the expression of BMP-7–Smad1/5/8. Gremlin belongs to a novel family of bone morphogenetic protein (BMP) antagonists.<sup>35</sup> Gremlin, an antagonist of bone morphogenetic protein 7(BMP-7),<sup>36</sup> it is overexpressed in adult diabetic nephropathy (DN).<sup>37</sup> Some experiments showed

**Table 2** Expression of TGF- $\beta$ 1 (N=4)

Groups	TGF- $\beta$ 1 Protein (pg/mL)	T value	P value
CON	724.62 $\pm$ 57.02		
HG	826.59 $\pm$ 21.54 <sup>#</sup>	3.3459	0.0221
BCAAs	727.76 $\pm$ 18.70*	6.9294	0.0031

Notes: <sup>#</sup> $p < 0.05$  vs CON group, \* $P < 0.05$  vs HG group. Data are shown as the mean  $\pm$  SD



**Figure 2** (A) RT-PCR was performed to evaluate the expression of gremlin mRNA in CON group, HG group, BCAAs group, respectively. (B) Immunofluorescence staining was performed to evaluate the expression of gremlin in RMCs in three groups. (C) Quantification of Gremlin fluorescence intensity (integrated density per stain area). (D) Immunofluorescence staining for BMP-7 in RMCs in three groups. (E) Quantification of BMP-7 fluorescence intensity (integrated density per stain area). (F) Western blotting for p-Smad1/5/8 and total Smad1/5/8 in three groups. <sup>#</sup> $p < 0.05$  vs CON group, <sup>\*</sup> $p < 0.05$  vs HG group.

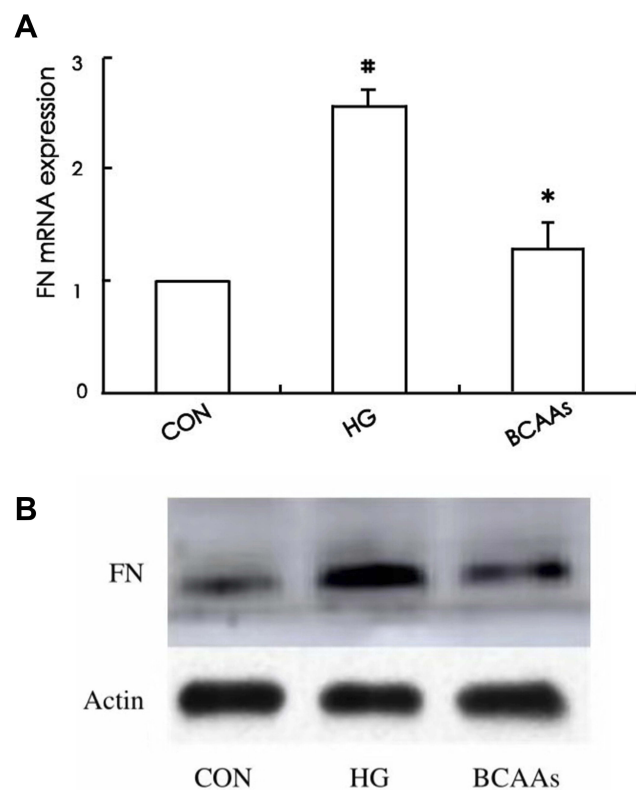
**Table 3** Expression of FN

Group	FN Protein (pg/mL)	T value	P value
CON	68.8667±3.5407		
HG	131.5317±9.2666 <sup>#</sup>	12.6312	0.0005
BCAAs	71.5733±5.5217 <sup>*</sup>	11.1168	0.0008

Notes: <sup>#</sup>p<0.05 vs CON group, <sup>\*</sup>p<0.05 vs HG group. Data are shown as the mean ± SD

that both in animals and humans, the up-regulation of Gremlin in DN has been correlated with TGF- $\beta$  expression. It has been suggested that Gremlin acting as a downstream mediator of TGF- $\beta$  play a role in EMT. Expression of Gremlin in cultured kidney mesangial cells is induced by high glucose environment and TGF- $\beta$ , so another name for gremlin is IHG-2 (induced in high glucose 2).<sup>38</sup> It plays distinct roles in the development and progression of kidney diseases.

Our experiments found that high glucose upregulated the expression of TGF- $\beta$ 1 and Gremlin while downregulate BMP-7-Smad1/5/8 pathway. It was in accordance with the mentioned findings. Our preliminary work has found that BCAAs could decrease the blood glucose level of diabetic rats and have anti-oxidative stress effect in diabetic nephropathy<sup>15,17</sup>



**Figure 3** (A) The FN mRNA expression was assayed in CON group, HG group, and BCAAs group, respectively. (B) Western blotting for FN protein expression in three groups. <sup>#</sup>p<0.05 vs CON group, <sup>\*</sup>p<0.05 vs HG group. Data were shown as the mean ± SD.

thereby alleviate diabetic kidney injury. We used rat glomerulus mesangial cells treated with high glucose model to find the possible mechanism of the BCAAs treated DN in vitro. In our experiments, we found that BCAAs could suppress the abnormal proliferation of RMCs. It also could downregulate the expression of TGF- $\beta$ 1 and Gremlin while upregulate BMP-7 and p-Smad1/5/8. In other words, high glucose disturbed the balance of TGF- $\beta$ 1 and BMP-7.

## Conclusions

BCAAs displayed an anti-diabetic effect, via attenuation of the TGF- $\beta$ 1-Smad2/3 pathway and gremlin expression and upregulating the BMP-7-Smad1/5/8 pathway in RMCs, consequently reducing ECM deposition in renal tissue.

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## Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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