

# Endoplasmic reticulum stress and proteasome pathway involvement in human podocyte injury with a truncated *COL4A3* mutation

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## Abstract

**Background:** Collagen type IV (COL4)-related nephropathy includes a variety of kidney diseases that occur with or without extra-renal manifestations caused by *COL4A3-5* mutations. Previous studies revealed several novel mutations, including three *COL4A3* missense mutations (G619R, G801R, and C1616Y) and the *COL4A3* chr:228172489delA c.4317delA p.Thr1440ProfsX87 frameshift mutation that resulted in a truncated NC1 domain (hereafter named *COL4A3* c.4317delA); however, the mutation mechanisms that lead to podocyte injury remain unclear. This study aimed to further explore the mutation mechanisms that lead to podocyte injury.

**Methods:** Wild-type (WT) and four mutant *COL4A3* segments were constructed into a lentiviral plasmid, then stably transfected into human podocytes. Real-time polymerase chain reaction and Western blotting were applied to detect endoplasmic reticulum stress (ERS)- and apoptosis-related mRNA and protein levels. Then, human podocytes were treated with MG132 (a proteasome inhibitor) and brefeldin A (a transport protein inhibitor). The human podocyte findings were verified by the establishment of a mus-Col4a3 knockout mouse monoclonal podocyte using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology.

**Results:** Our data showed that *COL4A3* mRNA was significantly overexpressed in the lentivirus stably transfected podocytes. Moreover, the *COL4A3* protein level was significantly increased in all groups except the *COL4A3* c.4317delA group. Compared to the other test groups, the *COL4A3* c.4317delA group showed excessive ERS and apoptosis. Podocytes treated with MG132 showed remarkably increased intra-cellular expression of the *COL4A3* c.4317delA mutation. MG132 intervention improved higher ERS and apoptosis levels in the *COL4A3* c.4317delA group. Mouse monoclonal podocytes with *COL4A3* chr:82717932insA c.4852insA p.Arg1618ThrfsX4 were successfully acquired; this NC1-truncated mutation suggested a higher level of ERS and relatively remarkable level of apoptosis compared to that of the WT group.

**Conclusions:** We demonstrated that excessive ERS and ERS-induced apoptosis were involved in the podocyte injury caused by the NC1-truncated *COL4A3* mutation. Furthermore, proteasome pathway intervention might become a potential treatment for collagen type IV-related nephropathy caused by a severely truncated *COL4A3* mutation.

**Keywords:** Collagen type IV-related nephropathy; *COL4A3* mutation; Podocyte injury; Proteasome pathway; MG132; Endoplasmic reticulum stress

## Introduction

Collagen type IV (COL4)-related nephropathy mainly includes Alport syndrome (AS) and thin basement membrane nephropathy, which is one of the most common hereditary glomerular diseases. Pathogenic gene mutations in the type IV Collagen  $\alpha3/\alpha4/\alpha5$  chains that code for collagen type IV alpha 3/collagen type IV alpha 4/collagen type IV alpha 5 (*COL4A3*/*COL4A4*/*COL4A5*, *COL4A3-5*, respectively) cause these diseases.<sup>[1]</sup> COL4 is a basement membrane collagen that exists in a variety of tissue basement membranes in a unique heterotrimeric superhelical chain structure ( $\alpha1\alpha1\alpha2$ ,  $\alpha3\alpha4\alpha5$ ,  $\alpha3\alpha3\alpha4$ , and  $\alpha5\alpha5\alpha6$ ).<sup>[2,3]</sup> The glomerular basement membrane (GBM) COL4  $\alpha3/\alpha4/\alpha5$  chain is synthesized and secreted only by podocytes as the main components of a mature GBM.<sup>[4]</sup> A

significant reduction or deletion of normal *COL4A3-5* caused by abnormal genetic mutations leads to an unstable GBM mechanical structure, abnormal GBM substitution components (eg, COL4  $\alpha1/\alpha2$  chain), abnormal interaction with podocytes and extra-cellular matrix receptors, and eventually podocyte injury.<sup>[5]</sup> COL4 is normally glycosylated and folded in the endoplasmic reticulum, and had been a previously reported mutation in COL4 caused by endoplasmic reticulum stress (ERS)<sup>[6]</sup>. ERS is well known to be closely related to apoptosis.<sup>[7]</sup>

A number of studies have also shown that *COL4A3* and *COL4A4* are new pathogenic genes for familial focal segmental glomerulosclerosis (FFSGS), including previous work that found *COL4A3* missense mutations in 12.5% (5/40) of FFSGS cases.<sup>[8,9]</sup> In 2018, the Alport Syndrome

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Classification Working Group published a new classification scheme that categorizes collagen IV  $\alpha 3$ - $\alpha 5$  genetic diseases as varieties of AS.<sup>[10]</sup> To date, there are more than 1000 records of *COL4A3* mutation types, including research identifying new *COL4A3* mutations in Chinese FFSGS and AS.<sup>[8]</sup> The molecular mechanisms of how *COL4A3* mutations cause podocyte injury remain unclear; further studies are required to provide additional therapeutic options, especially for cases of severe genotype-phenotype truncating *COL4* mutation.

In this study, in order to further explore the mutation mechanisms that lead to podocyte injury, we constructed an overexpressed podocyte model of four typical mutations, including three *COL4A3* missense mutations, G619R, G801R, and C1616Y, and one truncated mutation, *COL4A3* p. Thr1440ProfsX87.<sup>[8]</sup> We detected possible podocyte injury indicators (ERS and apoptosis), then applied a proteasome inhibitor (MG132) to reverse the decreased expression of *COL4A3*. In order to compensate for the disadvantage of the overexpressed cell model, a *COL4A3* knockout mouse monoclonal podocyte model was established for additional verification. Our work might provide a theoretical basis for new therapeutic treatments for kidney diseases.

## Methods

### *COL4A3* coding sequence mutations

Four *COL4A3* coding sequence (CDS) mutations associated with FFSGS or autosomal recessive Alport's syndrome (ARAS) patients have been previously reported.<sup>[8]</sup> G801R and C1616Y mutations were identified in two FFSGS families, G619R and *COL4A3* p. Thr1440ProfsX8 were identified from patients with ARAS. According to the Human Genome Variation Society nomenclature method,<sup>[11]</sup> the complicated frameshift mutation was named *COL4A3* chr:228172489delA c.4317delA p. Thr1440ProfsX87 (the first changed amino acid was the site 1440 threonine, and a frameshift caused early termination of the 87 amino acids located after site 1440); however, in this paper, it is hereafter referred to as *COL4A3* c.4317delA for ease of understanding.

Two missense heterozygous mutations (G801R and C1616Y) were identified from the FFSGS family, and one homozygous G619R missense mutation was identified from a patient with ARAS. One *COL4A3* c.4317delA frameshift mutation in the NC1 domain that produced the truncated *COL4A3* protein was identified from the FFSGS family.

Detailed information regarding these mutations is summarized in Table 1 and Figure 1A.

### Lentiviruses contained wild-type (WT) or mutant *COL4A3* CDS

Polymerase chain reaction (PCR) amplification (KOD-Plus-Neo PCR enzyme, Toyobo, Japan) was used to acquire a full-length WT Human (Hum) *COL4A3* CDS sequence, Hum-*COL4A3* forward primer (5'-3'): ATGAGCGCCCG-GACCG; Hum-*COL4A3* reverse primer (5'-3'): TCAG TGTCTTTTCTTCATGCACACCTGAC. Four mutant *COL4A3* sequences in G619R, G801R, C1616Y, and c.4317delA, respectively, were obtained by scaffold bridge PCR amplification (PrimeSTAR HS DNA Polymerase, TAKARA, Japan). Using a ClonExpress II One Step Cloning Kit (Vazyme Biotech, China), these sequences were then inserted into the multiple cloning site downstream cytomagalovirus (CMV) promoter in a pCDH-CMV-ATG2S3F-IRES-Blast lentiviral plasmid (Institute Pasteur of Shanghai, China); the CMV promoter would lead to high expression of the inserted target gene. After target gene sequence confirmation by Sanger DNA sequencing (GENEWIZ, China), the constructed plasmids were then transfected into 293T cells combined with envelope protein plasmid pCMV-VSV-G (Addgene Plasmid #8454) and packaging plasmid pCMV-dR8.2 dvpr (Addgene Plasmid #8455) to produce a lentivirus with either overexpressed WT or mutant *COL4A3* (G619R, G801R, C1616Y, and c.4317delA). The successfully packaged lentivirus was stored at  $-70^{\circ}\text{C}$  until use.

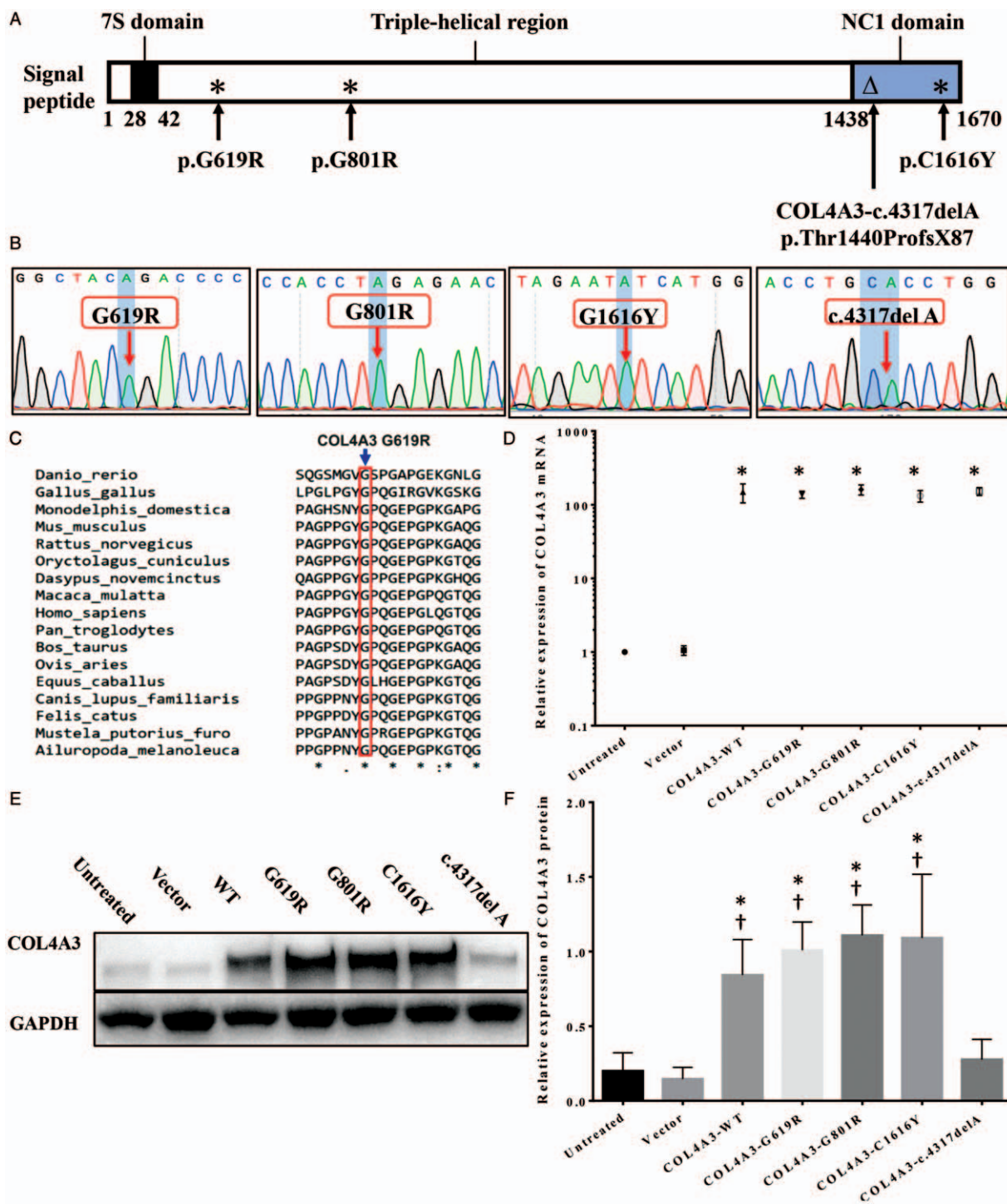
### Human podocytes stably overexpressed WT or mutant *COL4A3*

Human podocytes (Icahn School of Medicine at Mount Sinai, New York, NY, USA) were cultured in 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (30 min at  $56^{\circ}\text{C}$ ), 1% (vol/vol) insulin-transferrin-selenium (Thermo Fisher Scientific). To produce podocytes that stably overexpressed either WT or mutant *COL4A3*, the cells were infected with lentiviruses that contained WT *COL4A3* or mutant *COL4A3* (G619R, G801R, C1616Y, or *COL4A3* c.4317delA), respectively. An empty lentiviral infection was used as a control. The cells were continuously screened with 5 ng/mL blasticidin antibiotic (Thermo Fisher Scientific), approximately 60% to 80% of the podocytes were killed between days 3 and 7. The surviving podocytes obviously multiplied after 1 week, then the stable lentivirus infection and virus replication were maintained by sustained

**Table 1: The mutation from our previous original work<sup>[8]</sup>.**

ID	Renal biopsy	Mutation	Reference	Alteration	Type
FS1	FFSGS	C1616Y	G	A/G	Het
FS2	FFSGS	G801R	G	A/G	Het
AP1	ARAS	chr:228172489delA c.4317delA p. Thr1440ProfsX87	CAA	C (Del A) A	Het
AP4	ARAS	G619R	G	A	Hom

ARAS: Autosomal recessive Alport's syndrome; FFSGS: Familial focal segmental glomerulosclerosis; Het: Heterozygote; Hom: Homozygote.



**Figure 1:** COL4A3 expression in WT and COL4A3-G619R, -G801R, -C1616Y, and c.4317delA (p.Thr1440ProfsX87) mutant transfected human podocyte cells. (A) Specific position of each COL4A3 mutation site in the COL4A3 protein that indicates the c.4317delA mutation was different from the others; it was a frameshift mutation at the DNA level that caused a truncated mutation in the protein level; (B) COL4A3 mutants constructed by lentivirus were confirmed using Sanger sequencing; (C) Evolutionary conservation analysis of the COL4A3 G619R mutation site. (D) Quantitative PCR detected COL4A3 mRNA expression levels in human podocyte cells stably transfected with different COL4A3 lentiviruses (\* compared to the empty vector group,  $P < 0.05$ ); (E) Western blotting evaluated COL4A3 protein expression in human podocytes after transfection by different COL4A3 lentiviruses. The expression level of the COL4A3 c.4317delA (truncated mutation) group had an obviously lower COL4A3 protein level, unlike the WT, G619R, G801R, and C1616Y groups. (F) Three independent repetitions of COL4A3 protein expression experiment in different groups confirmed that the COL4A3 levels were differed significantly between the WT and c.4317delA groups (\* compared to the WT group, † compared to the c.4317delA group,  $P < 0.05$ ). PCR: Polymerase chain reaction; WT: Wild-type.

5 ng/mL blasticidin dosing. Until 3 to 4 weeks after transfection, the stably transfected podocytes and the untreated group were synchronously analyzed by Western blotting and real-time PCR. All transfections and experiments were performed in triplicate.

### Mouse podocytes expressed NC1-truncated COL4A3

Referring to the practical protocol,<sup>[12,13]</sup> the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) gene-editing system was applied to establish a modified mouse podocyte that

expressed NC1-truncated *COL4A3*. Single guide (sgRNA) was designed to target the NC1 domain in the mouse *COL4A3* No. 52 exon using the <http://crispr.mit.edu> website (sgRNA sequence [5'-3']: AGTTACATGTCCCTCGTCCA). The specific sgRNA was inserted downstream of the U6 promoter in the pSK-U6-gRNA plasmid. Mouse podocytes (Icahn School of Medicine at Mount Sinai) were transfected with pCDH-CMV-Cas9-GFP-Puro plasmid and pSK-U6-gRNA using Opti-MEM medium and Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After 24 h, puromycin (0.5 µg/mL; Thermo Fisher Scientific) was added for up to 48 h for screening to kill most of the mouse podocytes. The remaining viable mouse podocytes were resuspended in 0.05% Trypsin-EDTA (Thermo Fisher Scientific). A single mouse podocyte was cultivated in each well of a 96-well plate using the limited dilution method and a manual cell counting instrument. After approximately 2 to 3 weeks, monoclonal mouse podocytes began to grow in the 96-well plate. Until approximately 4 weeks, confluent monoclonal mouse podocytes were digested by 0.05% Trypsin-EDTA and re-cultivated into new 24-well plates. DNA was extracted from the cultured cells from 24-well plate for Sanger DNA sequencing to confirm the mouse *COL4A3* DNA modification.

### MG132 and brefeldin A interference in stably transfected human podocytes

Human podocytes that stably overexpressed either WT or mutant *COL4A3* were cultured on a 6-well plate. When they reached 80% confluence, 2.5 µmol/L MG132 (Sigma-Aldrich, Merck KGaA, Germany) and 10 mmol/L brefeldin A (Thermo Fisher Scientific) were added and left to stimulate the culture for 12 h. Total protein was extracted from each stable infected podocyte strain to detect the change in *COL4A3* expression by Western blotting.

### RNA isolation and real-time PCR

The mRNA levels of *COL4A3*, *CHOP*, and *sXBP1* in human podocytes stably infected by a lentivirus containing either WT or mutant *COL4A3* sequences were detected by real-time PCR. Total RNA was manually extracted using Trizol reagent (Thermo Fisher Scientific). According to the manufacturer's protocol, 0.5 mg of total RNA was reverse transcribed into cDNA utilizing the RT system (Promega, Madison, WI, USA). Real-time PCR was performed on an ABI System using SYBR Green Real-time PCR Master Mix (Vazyme, China). The amplification primers for the target mRNA are shown in Table 2. Relative expression of the target genes was analyzed by normalization to the *GAPDH* housekeeping gene using the  $2^{-\Delta\Delta C_t}$  method.

### Immunoblotting

Total protein was extracted from each stable infected podocyte strain using RIPA buffer containing a protease inhibitor (Bimake, China) on a six-well plate. The target proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting using polyvinylidene fluoride (PVDF) membranes (Merck KGaA, Germany). After blocking with 5% fat-free milk, the membranes were incubated with one

**Table 2: Fluorescence quantitative PCR primers of human podocyte genes.**

Genes	Primer sequences (5'-3')
<i>GAPDH</i>	
Forward	AAAGGGTCATCATCTCTG
Reverse	GCTGTTGTCATACTTCTC
<i>CHOP</i>	
Forward	AGAACCAGGAAACGGAAACAGA
Reverse	TCTCCTTCATGCGCTGCTTT
<i>sXBP1</i>	
Forward	TTGAGAACCAGGAGTTAAG
Reverse	CTTAACCTCCTGGTTCTCAA
<i>COL4A3</i>	
Forward	AATTCCAGGTCATCAAGG
Reverse	CCAATCACTCCATCTTCA

PCR: Polymerase chain reaction.

of diluted anti-COL4α3 (1:2000 dilution; Novusbio, Centennial, CO, USA), -CHOP (1:2000 dilution; Cell Signaling Technology), -Grp78 (Bip) (1:2000 dilution; Cell Signaling Technology), -PERK (1:2000 dilution; Cell Signaling Technology, Danvers, MA, USA), -Grp94 (1:2000 dilution; Cell Signaling Technology), -eIF2α (1:2000 dilution; Cell Signaling Technology), -phospho-eIF2α (-p-eIF2; 1:2000 dilution; Cell Signaling Technology), -Bcl-2 (1:1000 dilution; ABclonal Technology, China), -cleaved caspase 3 (1:2000 dilution; Cell Signaling Technology), or anti-GAPDH (1:10000 dilution; Proteintech, China) primary antibodies. Then, the membranes were incubated for 1 h with 1:5000 diluted horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson Laboratory, Bar Harbor, ME, USA) and visualized using an ECL Western blotting system (Thermo Fisher Scientific).

### Statistical analysis

Sequencher v 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA), Snapgene v 2.3.2 (GSL Biotech LLC, Chicago, IL, USA), and DNASTAR Lasergene v 7.1 (DNASTAR, Madison, WI, USA) were applied to analyze all of the plasmids and DNA sequences. Evolutionary protein conservation analysis was performed using the ClustalW2 program (EMBL-EBI, United Kingdom). SgRNA was designed using the <http://crispr.mit.edu> website. Data were presented as the mean ± standard deviation. Differences between two groups were analyzed by Student's *t* test. Comparisons among three or more groups were analyzed by one-way analysis of variance. A *P* value less than 0.05 was considered statistically significant. Expression graphs were analyzed using GraphPad Prism v 5.04 software (GraphPad Software, San Diego, CA, USA) and statistical analyses were performed by using SPSS 22.0 (IBM, Armonk, New York, USA).

## Results

### *COL4A3* overexpression in stably transfected human podocytes

All three missense point mutations, G619R, G801R, and C1616Y, were evolutionarily conserved [Figure 1C]. Since

*COL4A3* c.4317delA p. Thr1440ProfsX87 caused an early termination of *COL4A3*, it was not conserved. Analysis of the human podocytes that overexpressed either *COL4A3*-WT or G619R, G801R, C1616Y, or *COL4A3* c.4317delA mutations [Figure 1A and 1B] showed that the CMV promoter in the lentiviral vector led to a remarkably higher expression level of *COL4A3* mRNA in all cells with *COL4A3*-WT or the four mutations than that in the podocytes transfected with empty lentivirus ( $P < 0.05$ ) [Figure 1D]; this indicated that all transfections were stable and successful. At the same time, podocytes transfected with the three *COL4A3* missense point mutations (G619R, G801R, or C1616Y) showed significant overexpression of *COL4A3* protein by Western blotting. However, the expression level of the *COL4A3* c.4317delA group was close to that of the basal level of human podocytes in the untransfected control and empty vector transfected groups, and significantly lower than those in the cell lines with *COL4A3*-WT or the other three missense mutations ( $P < 0.05$ ) [Figure 1E and 1F]. All groups that were stably transfected by the lentiviral vector and screened with blasticidin showed similar results over three experimental repetitions. These results suggested that the truncated *COL4A3* mutation in the NC1 domain (c.4317delA) used a specific mechanism to inhibit truncated *COL4A3* expression at the protein level.

#### ***COL4A3* c.4317delA overexpression induced higher ERS- and apoptosis-related proteins in human podocytes**

The previous findings demonstrated that ERS and the apoptosis pathway played a role in *COL4A3* mutation-associated injury. So, we further detected ERS- and apoptosis-related protein expression in human podocytes that stably overexpressed WT or mutant *COL4A3*. Compared to the vector control and WT groups, both ERS- and apoptosis-related proteins in the G619R, G801R, and C1616Y groups did not differ significantly, although the mRNA levels of *CHOP* and *sXBP-1* were significantly increased in cells with the C1616Y mutation [Figure 2]. However, the protein expression of ERS-related proteins, GRP78, GRP94, PERK, and apoptosis-related proteins, CHOP, and cleaved caspase 3, were obviously increased in the *COL4A3* c.4317delA group ( $P < 0.05$ ) compared to the WT group. However, apoptosis-related Bcl-2 protein expression decreased in the *COL4A3* c.4317delA group compared to the WT group.

#### ***MG132 remarkably reversed lower basal intra-cellular COL4A3 expression and improved higher ERS and apoptosis in COL4A3 c.4317delA stably transfected human podocytes***

We further explored the effect of proteasome degradation and endoplasmic reticulum transport pathways in human podocytes that stably overexpressed WT or mutant *COL4A3* using a proteasome inhibitor (MG132), and an endoplasmic reticulum transport inhibitor (brefeldin A), respectively. The results showed that *COL4A3* protein expression was slightly elevated in WT, G618R, G801R, G1616Y, and c.4317delA groups by brefeldin A intervention, while the WT group showed significantly higher expression. When *COL4A3* was synthesized in the endoplasmic reticulum, the matured *COL4A3* should

have been transported to the Golgi apparatus for further assembly with *COL4A4* and *COL4A5*; as this is the specific site where brefeldin A would interfere, this is where the effect was observed. Interestingly, the truncated protein (*COL4A3* c.4317delA group) level was significantly increased by MG132 treatment. However, WT, and G618R, G801R, and G1616Y mutation groups showed no obvious effect when treated with MG132. Therefore, the protein degradation pathway may have played a role in inhibiting truncated *COL4A3* mutation expression in the NC1 domain (c.4317delA) at the protein level. Further analysis showed MG132 intervention significantly decreased GRP78, CHOP, and cleaved caspase 3 protein levels, and increased the Bcl-2 protein level in the *COL4A3* c.4317delA group [Figure 3C and 3D].

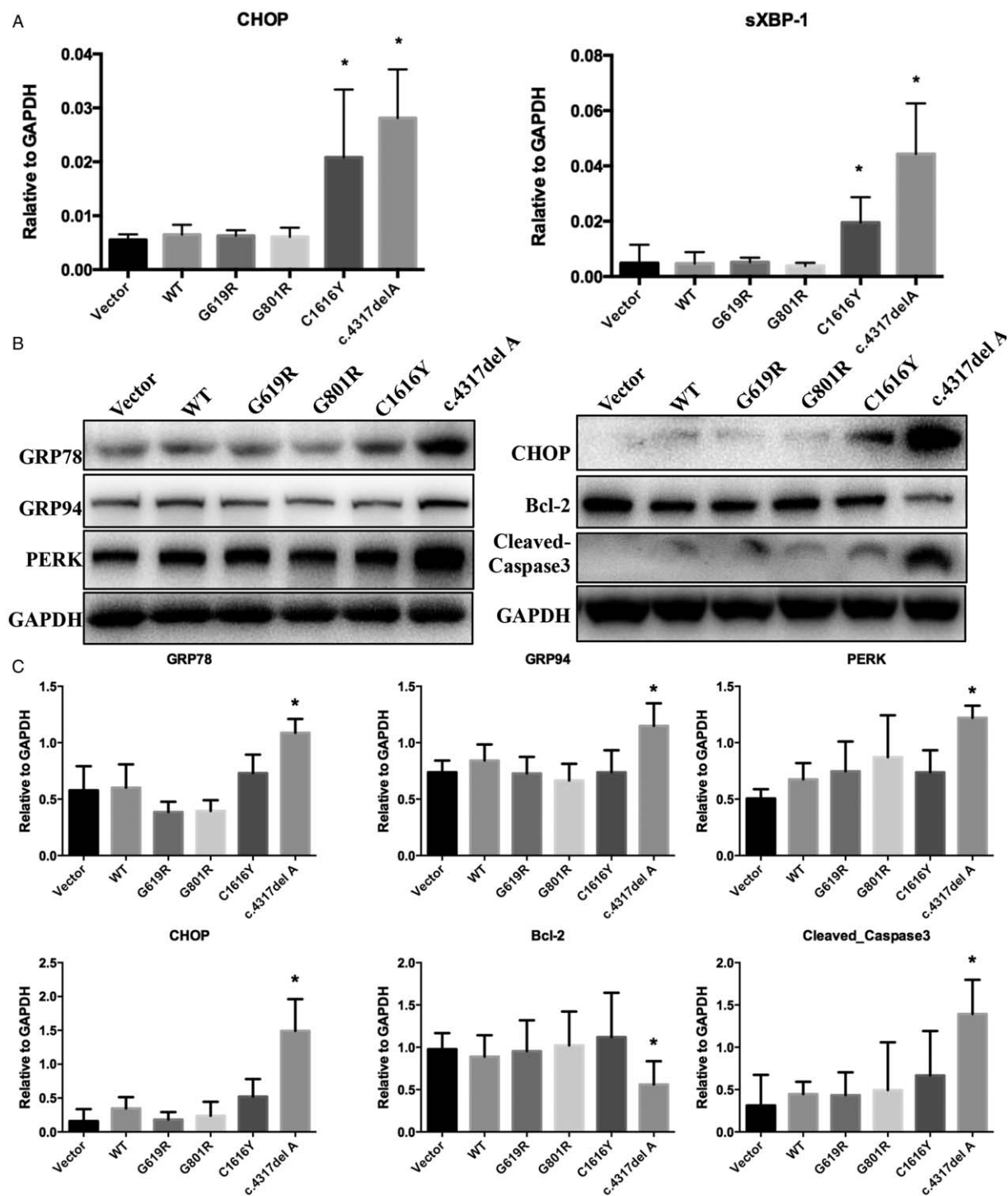
#### ***Mus COL4A3 knockout mouse monoclonal cell line showed ERS and apoptosis upregulation***

The overexpressed podocyte model was not the best cell model to study mutated protein function, owing to the fact that the basal normal gene expression and strong CMV promoter produced excessive target protein levels beyond that of the normal physiological need. To establish a better cell model, we tried to construct a new *COL4A3* podocyte model by applying the CRISPR/Cas9 system as described above.

First, we successfully established a *COL4A3* knockout mouse monoclonal cell line, which was confirmed as *COL4A3* chr:82717932insA c.4852insA p.Arg1618ThrfsX4 (the first changed amino acid was a site 1618 Arginine and caused early termination of the fourth amino acid after site 1618, hereafter referred to as *COL4A3* c.4852insA) by Sanger sequencing [Figure 4A and 4B]. We chose this cell model because it is also frameshift mutation in the NC1 domain of *COL4A3* that produced the truncated *COL4A3* protein. The experiment produced two monoclonal mouse podocytes with the same mutation site from two independent transient transfection experiments (named Clone 1 and Clone 2). Compared to those of the WT group, the protein expression levels of PERK, GRP94, CHOP, and phospho-eIF2 $\alpha$  increased obviously (eIF2 $\alpha$  was unchanged), together with increased expression of cleaved-caspase 3, and downregulation of Bcl-2. These findings suggested a higher level of ERS and relatively remarkable level of apoptosis [Figure 4C].

#### **Discussion**

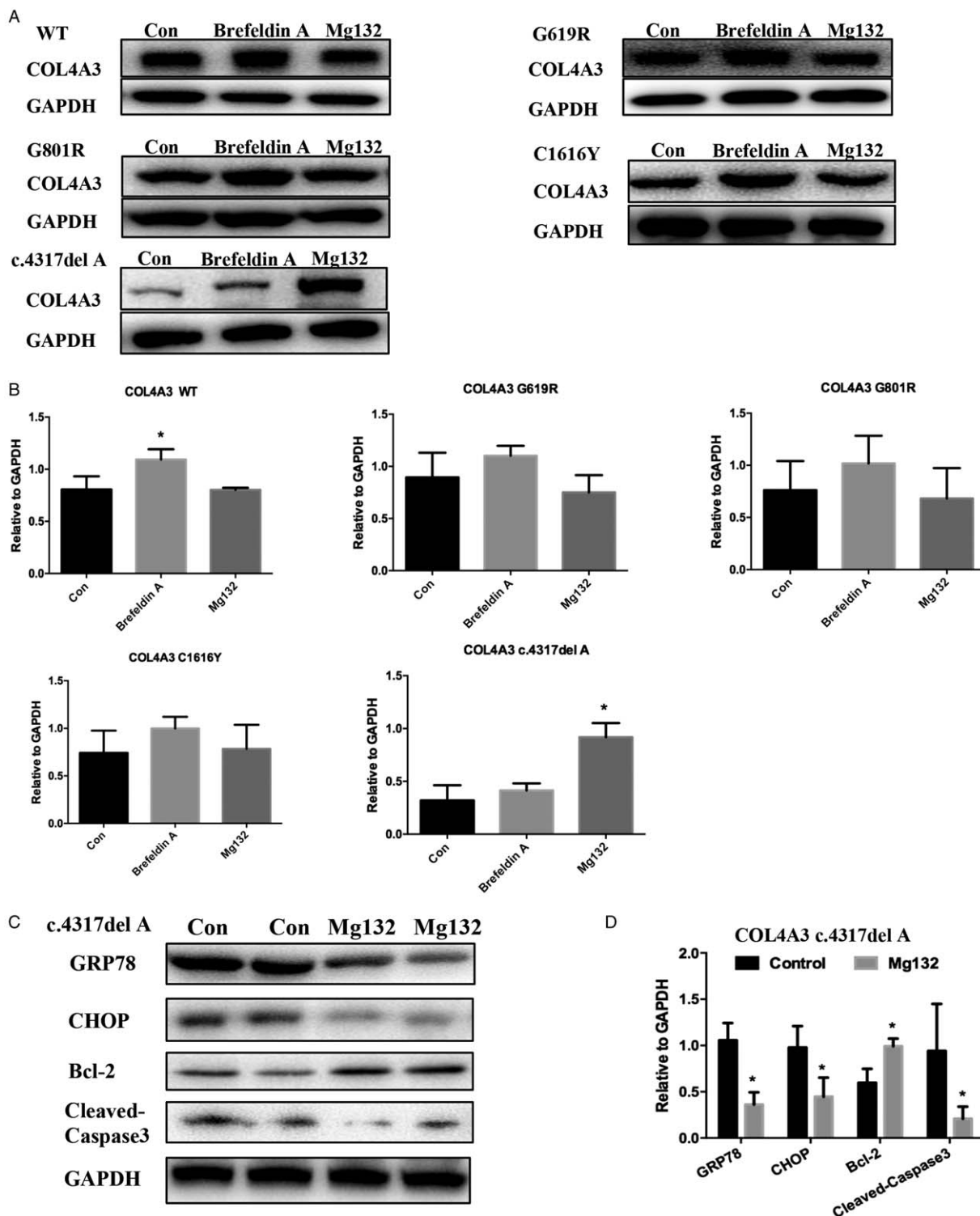
Podocytes are non-renewable and susceptible to damage from a variety of factors, including immune system disorders, genetic defects (gene mutation), and metabolic disorders.<sup>[14]</sup> Persistent or further aggravated injury can cause an imbalance in the stress and repair mechanisms, resulting in the activation of apoptosis-related genes that are related to the pathology and prognosis of glomerular diseases.<sup>[15]</sup> Podocyte injury is commonly observed in COL4-related nephropathy caused by *COL4A* mutations, and genotype-phenotype correlation in COL4-related nephropathy is relatively well documented.<sup>[16]</sup> Missense mutations usually exhibit milder phenotypic changes compared to those of truncating mutations; however, the underlying mechanism was unclear.



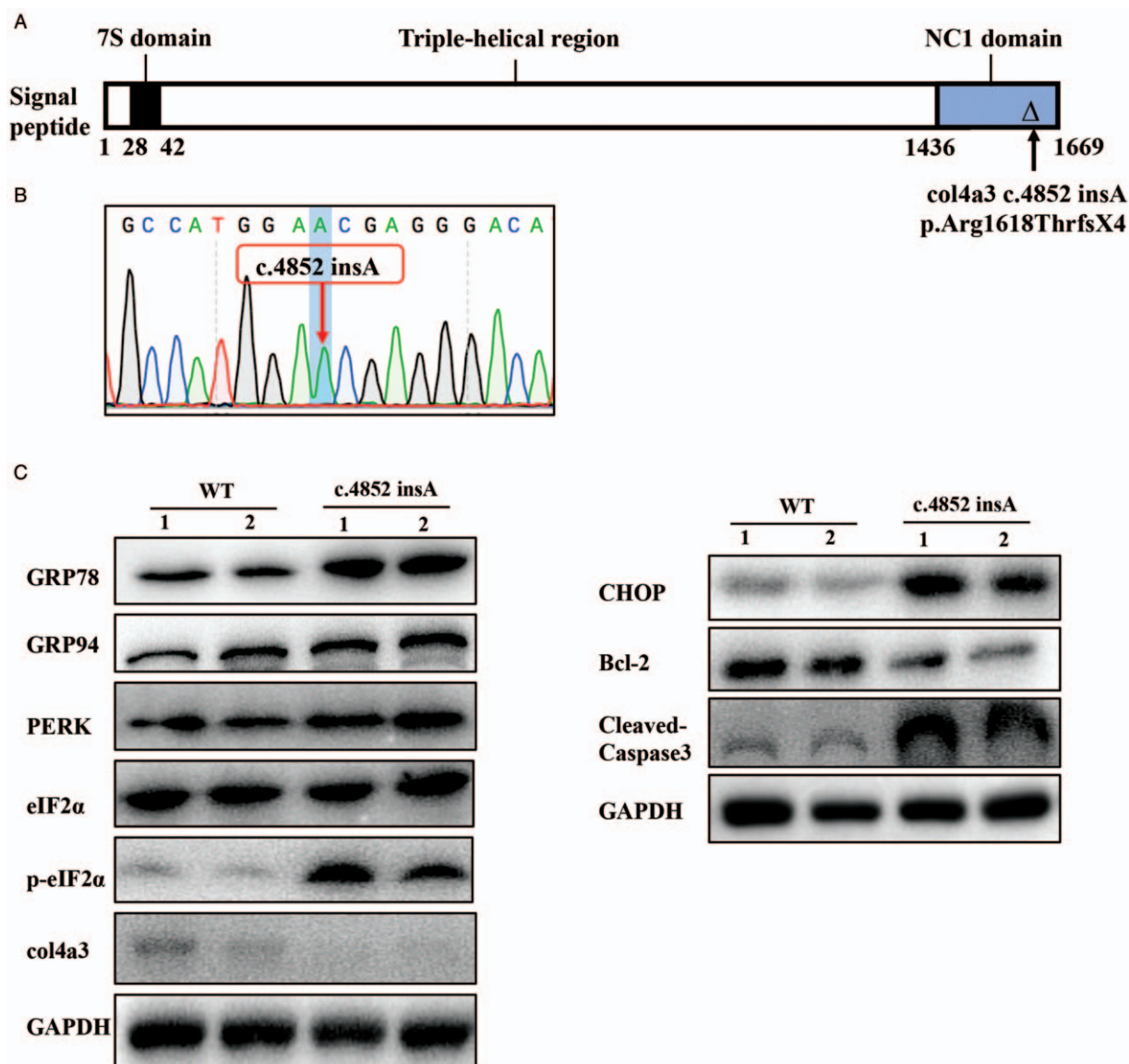
**Figure 2:** Detection of ERS- and apoptosis-related proteins in human podocytes that stably overexpressed WT or mutant *COL4A3*. (A) mRNA detection of ERS related CHOP and sXBP-1 by real-time PCR. The results showed significantly increased ERS in the COL4A3, C1615Y, and c.4317delA groups compared to those of the WT group; (B) ERS- and apoptosis-related protein detection by Western blotting showed obviously increased ERS related proteins (GRP78, GRP94, PERK, and CHOP), increased apoptosis-related protein cleaved caspase 3, and decreased apoptosis-related Bcl-2 in the c.4317delA group compared the WT group; (C) Three independent repetitions of ERS- and apoptosis-related proteins expression detection experiments in different groups (\* compared to the COL4A3-WT group,  $P < 0.05$ ). ERS: Endoplasmic reticulum stress; PCR: Polymerase chain reaction; WT: Wild-type.

COL4A3 is normally glycosylated and folded in the endoplasmic reticulum before being transported to the Golgi apparatus, and subsequently, outside of the podocyte. It has been reported that COL4A3 missense mutations have been scattered along the entire collagen domain.<sup>[17]</sup> Some COL4A3 missense mutants, such as

G1334E,<sup>[6]</sup> G871C, and G484R<sup>[18]</sup> might not be transported outside the podocyte, but instead, accumulate in the ER and cause an unfolded protein response activation. However, no significant ERS was observed in our three overexpressed missense point mutations. Our results could be explained by the fact that three novel missense



**Figure 3:** The proteasome pathway and endoplasmic reticulum transport in human podocytes stably overexpressed wild-type or mutant *COL4A3*. (A) Protein expression in each group (*COL4A3*-WT and four mutations) after drug intervention (left lane-control; middle lane-brefeldin A 10 mmol/L, 12 h; right lane-MG132 2.5 μmol/L, 12 h). (B) Three independent repetitions of *COL4A3* protein expression in different groups (\*compared to the control group,  $P < 0.05$ ). *COL4A3* protein expression is slightly elevated in G618R, G801R, and G1616Y groups by brefeldin A intervention. The WT group showed significantly higher expression. The *COL4A3* c.4317delA group showed markedly different conditions; MG132 intervention produced significantly higher *COL4A3* expression ( $P < 0.05$ ) that appeared to reverse the lower *COL4A3* expression of the untreated or brefeldin A treated groups. (C) ERS and apoptosis-related protein detection in the *COL4A3* c.4317delA group after MG132 intervention (left two lanes were duplicates – control; right two lanes were duplicates – MG132 2.5 μmol/L, 12 h). (D) MG132 intervention significantly decreased ERS related proteins (GRP78 and CHOP) and apoptosis-related protein cleaved caspase 3, and increased Bcl-2 protein expression in the *COL4A3* c.4317delA group (\*compared to the control group,  $P < 0.05$ ). CHOP: C/EBP homologous protein; ERS: Endoplasmic reticulum stress; WT: Wild-type.



**Figure 4:** Detection of ERS- and apoptosis-related proteins in the *COL4A3* knockout mouse monoclonal cell line. (A) Schematic diagram of the specific position of each *COL4A3* mutation site in the *COL4A3* protein. (B) Sequence analysis showed the generation of a knockout *COL4A3* c.4852 insA p.Arg1618ThrfsX4 genotype in mouse podocytes using CRISPR/Cas9 technology. (C) Western blotting detection of mouse podocyte mutants (*COL4A3* c.4852 insA p.Arg1618ThrfsX4); Clone 1 showed no obvious *COL4A3* protein expression. Basal detectable *COL4A3* protein expression could be observed in the WT group. This truncated mutation group presented increased ERS-related proteins (GRP78, GRP94, PERK, p-eIF2 $\alpha$ , and CHOP), but eIF2 $\alpha$  was unchanged. Apoptosis-related protein expression (CHOP and cleaved caspase-3) was upregulated, but Bcl-2 was downregulated. Lane 1 and 2 were duplicates; similar results were observed for the same mutation in clone 2. ERS: Endoplasmic reticulum stress; PERK: PKR-like endoplasmic reticulum Kinase; p-eIF2 $\alpha$ : Phosphorylated eukaryotic translation initiation factor 2 $\alpha$ ; WT: Wild-type.

mutations (G619R, G801R, and C1616Y) were used. Additionally, the different cell overexpression models may have also contributed to the difference; previous studies all used a transiently transfected cell model,<sup>[6,18]</sup> but ours used a stably transfected lentivirus system.

Unlike the missense mutation, the truncated mutation located in the NC1 domain (*COL4A3* c.4317delA) could not be overexpressed by stably transfected lentivirus vector podocytes, and induced excessive ERS and apoptosis. Using the Cas9 modified knockout mouse podocyte model, we verified the same phenomenon; the NC1-truncated *COL4A3* mutation caused higher levels of ERS and apoptosis. We speculated that among the four mutations found in a previous study,<sup>[8]</sup> the NC1-truncated mutation

may beget severe podocyte injuries including induced ERS and increased apoptosis, whereas the three missense mutations may cause pathology mainly by changing GBM composition. This phenomenon requires further clarification.

In addition, we found that the NC1-truncated mutation could not be overexpressed in human podocytes by the stably transfected lentivirus vector. The MG132 proteasome inhibitor significantly elevated the mutated protein expression, whereas the transport protein inhibitor (brefeldin A) had little effect. These data indicated that the truncated *COL4A3* protein was mainly degraded by the proteasome pathway, and moreover, caused excessive podocyte ERS and apoptosis.



MG132 is a peptide aldehyde that covalently binds to the active site of the 20S proteasome and effectively blocks the proteolytic activity of the 26S proteasome complex; it has been used in the study of multiple diseases.<sup>[19]</sup> MG132 reversed mutated *COL4A3* expression and improved the higher ERS- and apoptosis status in the specific truncated *COL4A3* C.4317delA mutation. Our results showed a potential therapeutic effect of MG132 that might function through regulating podocyte apoptosis; previously reported MG132 protection occurred by blocking HeLa cell apoptosis induced by severe DNA damage,<sup>[20]</sup> and also against deltamethrin induced apoptosis in rat hippocampus.<sup>[21]</sup> However, further experiments in podocytes, and validation in animal models are required.

This study performed *COL4A3* gene editing using CRISPR/Cas9 technology; we established a *COL4A3* knockout mouse monoclonal cell line (*COL4A3* c.4852 insA p.Arg1618ThrfsX4) for result verification. Other studies using CRISPR/Cas9 editing have recently been published in several important studies in the field of kidney disease<sup>[22,23]</sup>; Using a CRISPR/Cas9-mediated gene knockout of *GLA* in HEK-293T cells provided a successful *in vitro* Fabry disease model,<sup>[24]</sup> and the CRISPR/Cas9 knockout of podocalyxin in podocyte-like cells<sup>[25]</sup> and conditional CRISPR/Cas9 in *Drosophila* garland cell nephrocytes found that reactive oxygen species formation might be a pathological mechanism of COQ2-nephropathy.<sup>[26]</sup> The gradual improvement of Cas9 technology could be quite useful in the advanced kidney research field. Compared to the WT group, our mutant mouse podocyte model showed excessive ERS and apoptosis-related podocyte injury. The results were consistent with our overexpression of truncated *COL4A3* in the lentivirus transfected human podocyte model.

In summary, ERS and ERS-induced apoptosis activation were involved in podocyte injury caused by the *COL4A3* truncated mutation that caused premature protein translation termination, and were not associated with three novel missense mutations (G619R, G801R, and C1616Y). These findings may partially explain the genotype-phenotype correlation in *COL4*-related nephropathy. Moreover, the proteasome pathway contributed to decreased *COL4A3* expression caused by the severe NC1-truncated mutation, improved the higher ERS and apoptosis-related protein expression, and might be a potential avenue for treatment. In addition, CRISPR/Cas9 technology was used to successfully construct a *COL4A3* knockout mouse podocyte model expressed *COL4A3* truncated protein, which verified the excessive ERS and apoptosis. The results of this study require further validation in animal models.

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### Conflicts of interest

None.

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