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Nestin Protects Mouse Podocytes Against High Glucose-Induced Apoptosis by a Cdk5-Dependent Mechanism

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ABSTRACT

Podocyte apoptosis contributes to the pathogenesis of diabetic nephropathy (DN). However, the mechanisms that mediate hyperglycemia-induced podocyte apoptosis remain poorly understood. Recent findings indicate that the disruption of the cytoskeleton is related to the podocyte apoptosis. In the present study, we investigated the involvement of nestin, an important cytoskeleton-associated class VI intermediate filament (IF) protein, in the high glucose (HG)-induced podocyte apoptosis. Our data showed that HG decreased the expression level of nestin, either mRNA or protein, in a time-dependent manner in cultured podocytes. Also, through knockdown of nestin expression by miRNA interference, the HG-induced podocyte apoptotic rate was significantly increased. The expression of cleaved caspase-3 was also markedly elevated. Considering that nestin is a substrate of cyclin-dependent kinase 5 (Cdk5), we further assessed the expression of Cdk5 in HG-treated podocytes. The results showed that HG stimulation increased the protein and mRNA expression of Cdk5 in a time-dependent manner in cultured mouse podocytes. The protein activator of Cdk5, p35, was also increased in a time-dependent manner by HG stimulation, and downregulation of Cdk5 by miRNA interference attenuated the nestin reduction in HG-treated podocytes; the HG-induced podocyte apoptosis, the increased cleaved caspase-3 expression and the Bax/Bcl-2 ratio were all effectively attenuated. These data suggested that nestin, which is dependent on Cdk5 regulation, plays a cytoprotective role in HG-induced podocyte apoptosis. J. Cell. Biochem. 113: 3186–3196, 2012.

KEY WORDS: NESTIN; PODOCYTE; APOPTOSIS; HIGH GLUCOSE; CYCLIN-DEPENDENT KINASE 5

Podocytes are terminally differentiated and highly specialized cells, these cells are one of the major cell types in the glomerulus and they form a critical part of the glomerular filtration barrier. Podocyte loss leads to areas of denuded glomerular basement membrane that culminates in proteinuria and in the development of glomerulosclerosis [Kriz et al., 1998], which is considered to be a potential early pathological marker for diabetic nephropathy (DN) [Steffes et al., 2001; Wolf et al., 2005; Toyoda et al., 2007]. Apoptosis and cell detachment from the glomerular basement membrane are two main factors involved in the process of podocyte loss [Pagtalunan et al., 1997; Li et al., 2007]. Taking into account that the numbers of podocytes per glomerulus gradually declines throughout the course of DN [Pagtalunan et al., 1997; Steffes et al., 2001; Susztak et al., 2006], podocyte apoptosis seems to precede the development of DN and determines at least in part kidney function in diabetic patients [Pagtalunan et al., 1997; Riedl et al., 2011].

However, the mechanisms that contribute to hyperglycemia-induced podocyte apoptosis are not completely understood. Recently, several studies have shown that cytoskeleton disruption is related to podocyte apoptosis [Schwartz et al., 1999; Bijian et al., 2005; Yang et al., 2005; Peters et al., 2006; He et al., 2011]. Nestin is a cytoskeleton-associated class VI intermediate filament (IF) protein, which was first described as a marker for neuroepithelial stem cells

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Wei Liu and Yue Zhang contributed equally to this work.

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[Lendahl et al., 1990]. Nestin was also identified in progenitor cells from various non-neuronal tissues [Kachinsky et al., 1994, 1995; Hunziker and Stein, 2000; Li et al., 2003; Uchugonova et al., 2011]. Recently, nestin has been reported to be stably expressed in the podocytes of mature glomeruli in the adult kidney [Chen et al., 2006], and to play an important role in maintaining the normal morphology and function of podocytes. For example, in cultured mouse podocytes, knockdown of nestin by siRNA significantly reduced the number of podocyte processes [Chen et al., 2006]. Clinical studies showed that nestin expression of glomerular podocytes in nephropathic patients with proteinuria was significantly reduced compared with healthy subjects and patients without proteinuria; this improvement in the proteinuria following medical management was accompanied by an increase in nestin expression in glomerular cells of the patients [Su et al., 2007]. However, little is known about the underlying mechanisms of nestin function in the glomerular podocytes and whether the cytoskeleton protein is involved in the diabetic glomerular damage has not yet been established.

Cyclin-dependent kinase 5 (Cdk5) is a key regulator of neuronal development and normal neuronal function, and it is activated by the specific protein activator, p35 [Dhavan and Tsai, 2001]. Several studies have demonstrated that nestin is a substrate for Cdk5 [Sahlgren et al., 2003], and that inhibition of Cdk5 activity can reduce nestin degradation, which indicates that Cdk5 is involved in regulation of nestin turnover [Sahlgren et al., 2006]. It has also been shown that Cdk5 modulates cell maturation, differentiation, migration, and apoptosis in various tissues [Paglini and Cáceres, 2001; Weishaupt et al., 2003; Sharma et al., 2004; Rosales and Lee, 2006]. Within the kidney, glomerular expression of Cdk5 is limited to podocytes. However, under the high glucose environment, the effects of Cdk5 on nestin expression and podocyte apoptosis have not been studied.

In the present study, we examined the expression of nestin in mouse podocytes with HG treatment, and the effects of miRNA interference, causing knockdown of nestin expression, on HG-induced podocyte apoptosis. Considering the relationship between Cdk5 and nestin, we also detected the effects of Cdk5 knockdown on nestin expression and apoptosis in HG-treated podocytes. Our results showed that high glucose induced cell apoptosis and decreased nestin expression in a time-dependent manner in podocytes in vitro. Knockdown of nestin by miRNA interference sensitized podocytes to HG-induced apoptosis. Moreover, knockdown of Cdk5 also attenuated the decrease in nestin expression and podocyte apoptosis induced by HG stimulation. These data indicate that nestin expression, which is regulated by Cdk5, is related to the sensitivity of podocytes to HG-induced apoptosis, and displays a distinct cytoprotective effect.

MATERIALS AND METHODS

CONDITIONALLY IMMORTALIZED MOUSE PODOCYTES IN CULTURE

Conditionally immortalized mouse podocytes purchased from the Cell Culture Center (PUMC, CAMS, Beijing, China) were cultured as previously described [Mundel et al., 1997]. In this cell line, a temperature-sensitive SV40 large T-cell antigen (tsA58 Tag) is controlled by a γ-interferon inducible H-2K^b promoter. To induce proliferation, cells were grown on collagen I-coated plastic culture bottles (BD Biosciences, Bedford, MA), at 33°C in RPMI 1640 culture medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, USA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), and 100 μg/ml streptomycin (Invitrogen, USA), to which recombinant mouse γ-interferon 10 U/ml (Pepro Tech, USA) was added (growth permissive conditions). To induce quiescence and the differentiated phenotype, podocytes were grown at 37°C and deprived of γ-interferon (growth restrictive conditions) in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin. Unless otherwise specified otherwise, all studies were performed on days 10–14 for cells grown under restrictive conditions.

When podocytes were 80% confluence under growth restrictive conditions, they were washed once with serum-free RPMI 1640 medium, and then growth-arrested in serum-free RPMI 1640 medium for 24 h to synchronize the cell growth. Cells were stimulated with normal glucose (NG, 5.6 mM) or high glucose (HG, 30 mM). In some experiments, a third group of podocytes was exposed to NG plus mannitol (24.4 mM) as an osmotic control.

PLASMID TRANSFECTION

Artificial miRNAs were designed and generated by Invitrogen to target nestin or Cdk5. Each miRNA was cloned into the BLOCK-iT Pol II miR RNAi expression vector according to the instructions of the manufacturer (Invitrogen, USA) and then sequenced. A scrambled vector was used as a control. Stable transfections of mouse podocytes with pcDNA6.2-GW/miR containing miRNA specific for either nestin or Cdk5 were performed with FuGENE® HD Transfection Reagent (Promega) according to the manufacturer's instructions.

Briefly, podocytes were cultured in six-well plates and the medium was changed the following day until 80% confluence was achieved. FuGENE® HD Transfection Reagent was premixed with the various plasmids at a ratio of 4.0 μg DNA to 12 μl transfection reagent in serum-free RPMI 1640 and incubated for 15 min before the mixture was added to the cells. After 6 h, the medium was replaced by normal RPMI 1640 with 10% FBS for culturing 24 h. The transfection efficiency was detected using Western blot analysis and real-time PCR.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE–MEDIATED dUTP NICK END LABELING (TUNEL) STAINING

Apoptotic cells were identified using the TUNEL technique according to the manufacturer's instructions (Roche Applied Science, Hangzhou, China). Hoechst staining was used to identify the cells in the field, and labeled podocytes were analyzed with a fluorescent microscope. For quantification of positive TUNEL-positive (apoptotic) cells, a minimum of 200 cells were counted per group, and the percentage of the positively labeled cells was calculated.

ANNEXIN V AND PROPIDIUM IODIDE STAINING ASSAY

Apoptotic cells in different groups were determined using an Annexin V/PI apoptosis detection kit according to manufacturer's

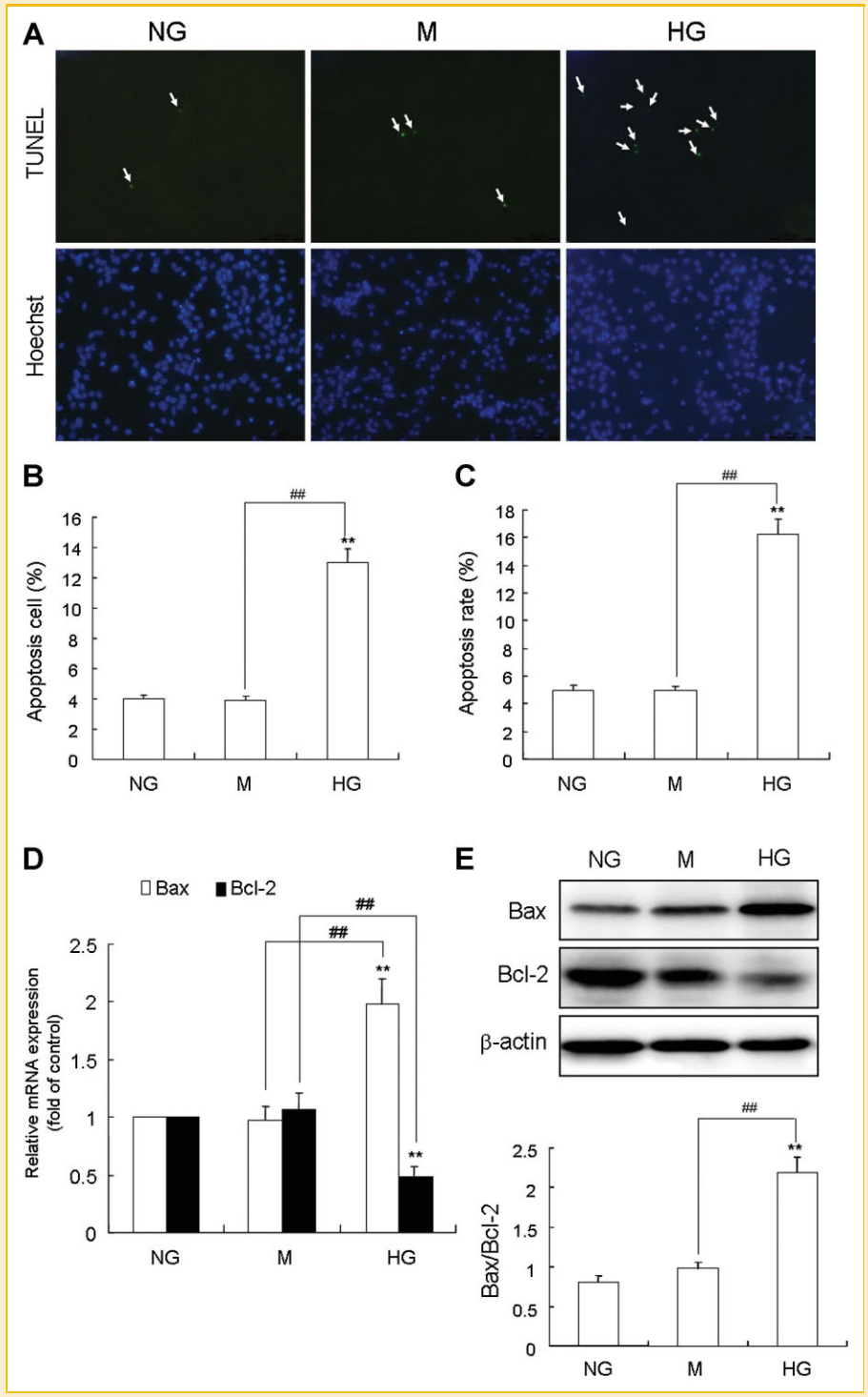

Fig. 1. Effects of HG on podocyte apoptosis. Podocytes were incubated with normal glucose (NG, 5.6 mM), NG + 24.4 mM mannitol (M) or high glucose (HG, 30 mM) for 48 h. A: Apoptosis in podocytes was detected using the TUNEL method, and also using the nuclear dye, Hoechst, to identify the cells in the field. Arrows indicate TUNEL-positive (apoptotic) cells. B: TUNEL-positive (apoptotic) cells were counted out of a total of more than 200 cells over six random fields. The results were expressed as apoptosis cell (%). C: Apoptosis were detected using flow cytometry, and the results were expressed as apoptosis rate (%). D: The mRNA levels of Bax and Bcl-2 were examined using qRT-PCR analysis, and β-actin mRNA was used as an internal control. E: The protein levels of Bax and Bcl-2 were analyzed using Western blot analysis ($n = 6$). The ratio for Bax/Bcl-2 protein was based on a densitometry reading, compared to β-actin. Values are expressed as the mean ± SD. $^{**}P < 0.01$, HG versus NG; $^{\#\#}P < 0.01$, HG versus M. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

protocol (MultiSciences Biotech, Hangzhou, China). Briefly, the cell pellet was resuspended in 1× binding buffer followed by incubation with 5 μl of Annexin V (conjugated with FITC) and 10 μl of PI in the dark for 5 min. Cell fluorescence was then analyzed using a flow cytometer (Epics-XLII, Becman Coulter). This test discriminates intact cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V^+/PI^+).

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To perform immunostaining on cultured cells, podocytes were planted on cover slides in six-well plates. After stimulation, cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After pre-treatment with 0.3% Triton X-100 for 20 min at 37°C, cells were blocked with goat serum for 30 min at 37°C. Then, the cells were incubated with mouse anti-nestin antibody (Abcam, Cambridge, UK, 1:500) or rabbit anti-Cdk5 antibody (Santa Cruz, CA, 1:200) overnight at 4°C. After three washes with PBS, cells were incubated with a polymer helper and polyperoxidase-anti-mouse/rabbit IgG at 37°C for 30 min, and the cells were then stained with diaminobenzidine. A negative control was performed by replacing the primary antibody with PBS buffer.

TOTAL PROTEIN EXTRACTION AND WESTERN BLOT

The different groups of cells were harvested and homogenized in ice-cold homogenization buffer (1% Nonidet P-40, 0.1% Triton X-100, 30 mM sodium phosphate, PH 7.4, containing 1 mM sodium orthovanadate, 100 mM NaCl, 2.5 mM Tris–HCl, pH 7.5, and protease inhibitors), followed by centrifugation at 20,000*g* for 20 min at 4°C. The supernatant was collected to characterize the relative levels of protein expression by Western blot assays. The protein concentrations were quantified with the Bio-Rad protein colorimetric assay (Bio-Rad).

Whole cell extracts (100 μg of protein/lane) were loaded, separated by 10% SDS–PAGE, and transferred to PVDF membranes (Millipore, MA). After blocking with 5% skimmed milk in Tris-buffered saline/Tween buffer (TBST buffer) for 2 h, the membranes were incubated overnight at 4°C with anti-nestin antibody (1:1,000), cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, 1:1,000), Cdk5 (1:500), Bax (Proteintech, 1:1,000), Bcl-2 (Proteintech, 1:500) and β-actin (Proteintech, 1:2,000) antibodies. Subsequently, the membranes were incubated with goat anti-rabbit or mouse IgG horseradish peroxidase conjugate, and then exposed to X-ray film using an enhanced chemiluminescence system. The intensity of the bands was measured using LabWorks 4.5.

RNA ISOLATION AND qRT-PCR

Total RNA was extracted with TriZol Reagent according to the manufacturer's instructions (Invitrogen). Complementary DNA was synthesized from the total RNA (0.5 μg) using the PrimeScript™ RT regent Kit following the instructions provided by the manufacturer

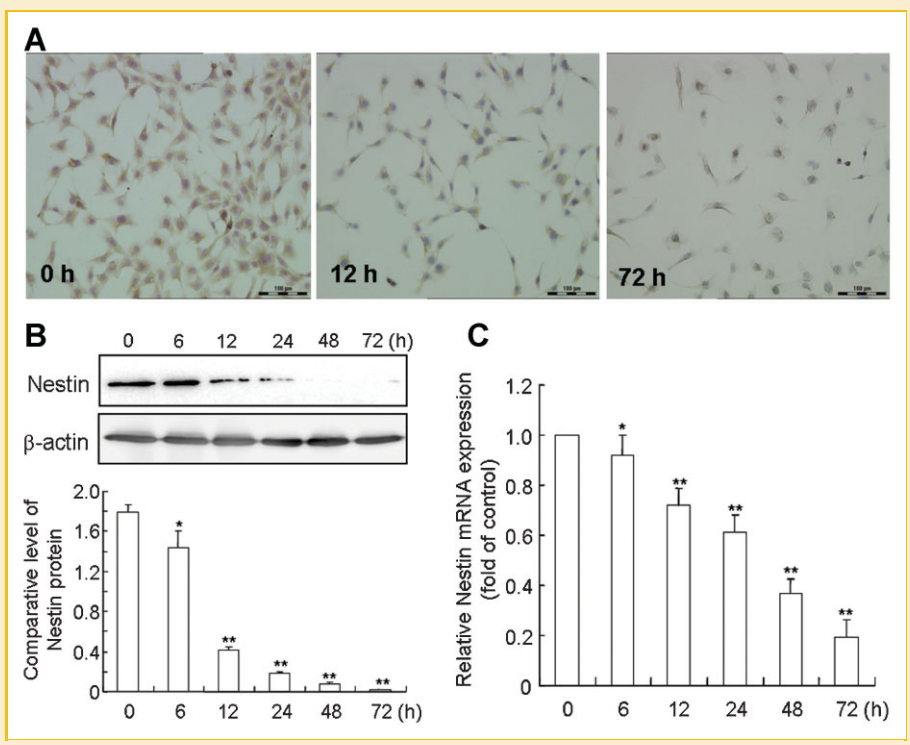

Fig. 2. Effects of HG on nestin protein and mRNA levels in podocytes. Podocytes were incubated with high glucose (HG, 30 mM) from 0 to 72 h. A: Nestin positive expression was detected using immunocytochemical staining (200×). B: Expression of nestin protein was analyzed using Western blot. The level of nestin protein was quantified by densitometric analysis and normalized to the level of β-actin. C: Expression of nestin mRNA was analyzed using qRT-PCR, and β-actin mRNA was used as an internal control. Values are expressed as the mean ± SD. $^*P < 0.05$ versus control (0 h), $^{**}P < 0.01$ versus control (0 h). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(Takara Biotechnology, Dalian, China). Subsequently, the cDNA was subject to quantitative RT-PCR (qRT-PCR) using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Each real-time PCR reaction consisted of 2 μl diluted RT product, 10 μl SYBR Green PCR Master Mix (2×) and 250 nM forward and reverse primers in a total volume of 20 μl. Reactions were carried out on 7500 qRT-PCR System (Applied Biosystems) for 40 cycles (95°C for 15 s, 60°C for 45 s) after an initial 10 min incubation at 95°C. The primers used for real-time PCR were as follows: nestin, forward primer, 5′-TAC ATC AGT CCA GAC CAT GTG C-3′, reverse primer, 5′-TTG ACC CTT GGT ATA TGT CGT G-3′, giving 114 bp PCR product; Cdk5, forward primer, 5′-TAG GCT CTC TGA ACC CCA GT-3′, reverse primer, 5′-ATC CCA CAC CCG ACT CTT-3′, giving 198 bp PCR product; Bax, forward 5′-AGA CAG GGG CCT TTT TGC TAC-3′, reverse 5′-AAT TCG CCG GAG ACA CTC G-3′, giving 137 bp PCR product; Bcl-2, forward 5′-ATG CCT TTG TGG AAC TAT ATG GC-3′, reverse 5′-GGT ATG CAC CCA GAG TGA TGC-3′, giving 120 bp PCR product; β-actin, forward 5′-GGC TGT ATT CCC CTC CAT CG-3′, reverse 5′-CCA GTT GGT AAC AAT GCC ATG T-3′, giving 154 bp PCR product. The fold change in expression of each gene was calculated using the △△Ct method, using β-actin mRNA as an internal control.

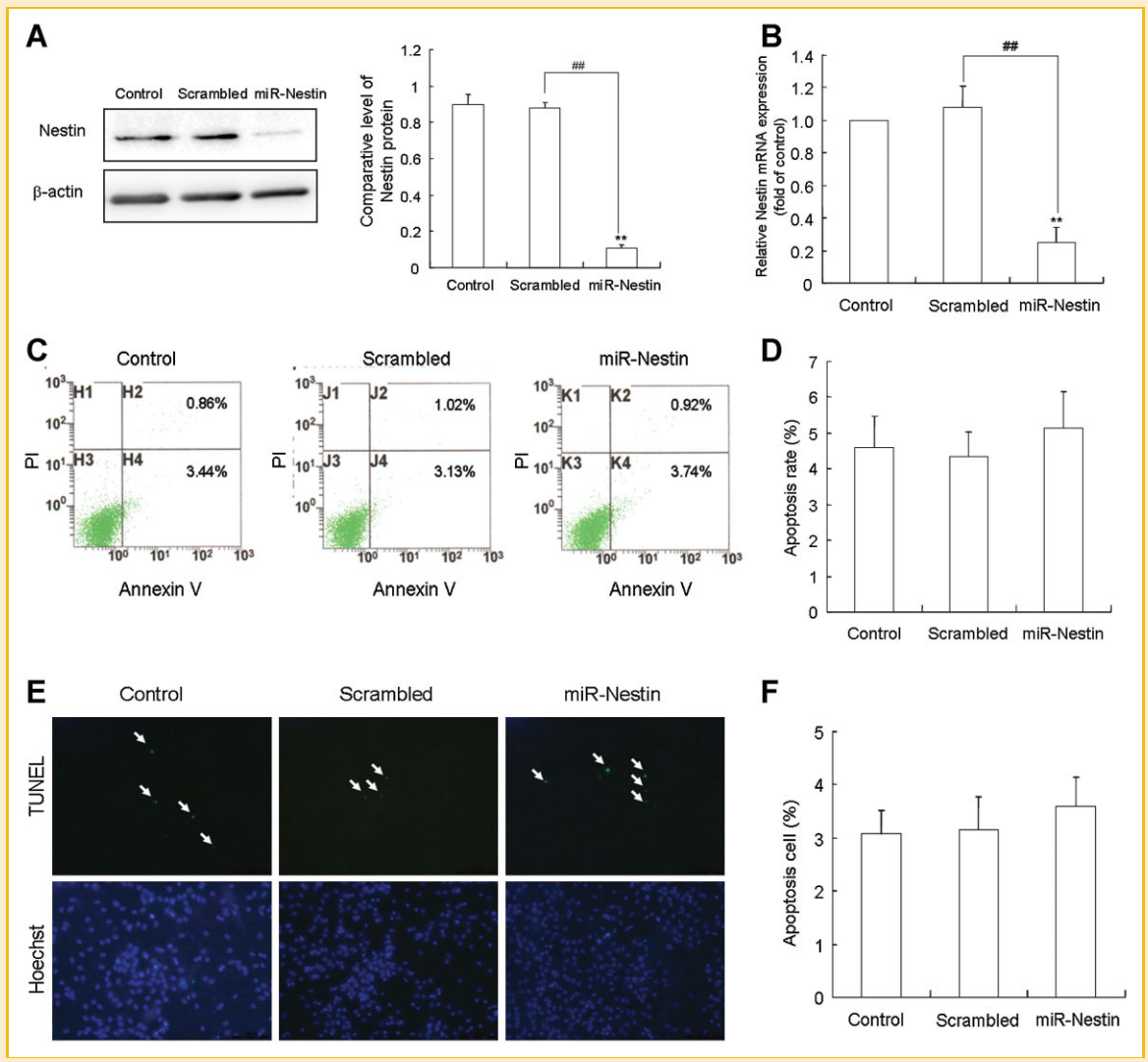

Fig. 3. Effects of nestin depletion on podocyte apoptosis with NG treatment. Podocytes were transfected with a miRNA vector for nestin and a scrambled vector was used as a control. Transfected podocytes were treated with normal glucose (NG, 5.6 mM) for 48 h. A: The protein levels of nestin were analyzed using Western blot to ensure transfection efficiency, quantified by densitometric analysis, and normalized to the level of β-actin. B: The mRNA levels of nestin in transfected podocytes were detected using qRT-PCR, and β-actin mRNA was used as an internal control. C: Podocytes were stained with Annexin V/PI for flow cytometry analysis ($n = 6$). Apoptotic cells were divided into two stages: early apoptotic (Annexin V^+/PI^-) and late apoptotic (Annexin V^+/PI^+) cells. D: The total apoptosis rates examined using flow cytometry, including the early and late apoptosis rate, were quantified and are shown using a histogram. E: Podocytes were detected using the TUNEL method to identify apoptotic cells, and the nuclear dye, Hoechst, to identify the cells in the field. Arrows indicate TUNEL-positive (apoptotic) cells. F: TUNEL-positive (apoptotic) cells were counted out of a total of more than 200 cells over six random fields. The results were expressed as apoptosis cell (%). Values are expressed as the mean ± SD. Control: untransfected cells; Scrambled: transfected with a scrambled vector; miR-Nestin: transfected with a miRNA vector for nestin. $^{**}P < 0.01$, miR-Nestin group versus Control group; $^{\#\#}P < 0.01$, miR-Nestin group versus Scrambled group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

STATISTICAL ANALYSIS

Statistical analysis was performed using a one-way ANOVA. The results are presented as the mean ± SD. Statistical significance was defined as $P < 0.05$.

RESULTS

HG INDUCED PODOCYTE APOPTOSIS

To determine the effect of HG on podocyte apoptosis, podocytes were cultured in RPMI 1640 medium containing 5.6 mM glucose (NG group), 5.6 mM glucose plus 24.4 mM mannitol (M group, as an osmotic control) or 30 mM glucose (HG group) for 48 h. Apoptotic rates were determined using the TUNEL method and flow cytometry. As shown in Figure 1A,B, the number of TUNEL-positive (apoptotic) cells was significantly increased in the HG group; there were 4% apoptotic cells in the NG group and 3.94% apoptotic cells in the mannitol group, compared to 13% apoptotic cells in the HG group. The apoptotic rate in the HG group was examined using flow cytometry, and it was also elevated compared with the NG group or the mannitol group (Fig. 1C). As expected, mannitol had no effect on

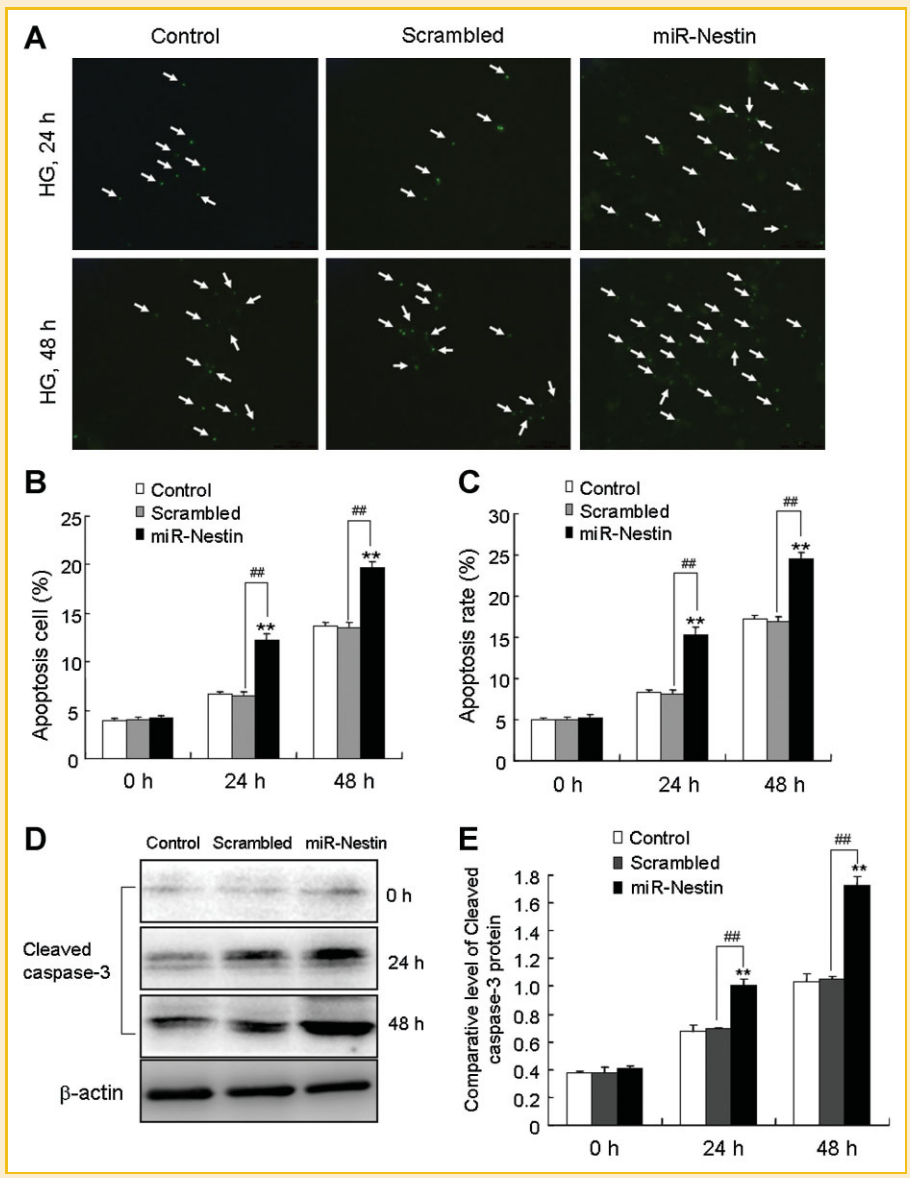

Fig. 4. Effects of knockdown of nestin expression on HG-induced podocyte apoptosis. Podocytes were transfected with the nestin miRNA vector or a scrambled vector, and then treated with high glucose (HG, 30 mM) for 24 or 48 h. A: Podocytes were detected using the TUNEL method to identify apoptotic cells. Arrows indicate TUNEL-positive (apoptotic) cells. B: TUNEL-positive (apoptotic) cells were counted out of a total of more than 200 cells over six random fields, and the results were expressed as apoptosis cell (%). C: The apoptosis rate was examined using flow cytometry. D: The expression of cleaved caspase-3 was analyzed using Western blot ($n = 6$). E: The level of cleaved caspase-3 protein was quantified by densitometric analysis and normalized to the level of β-actin. Values are expressed as the mean ± SD. Control: untransfected cells; Scrambled: transfected with a scrambled vector; miR-Nestin: transfected with a miRNA vector for nestin. $^{**}P < 0.01$, miR-Nestin group versus Control group; $^{\#\#}P < 0.01$, miR-Nestin group versus Scrambled group.

podocyte apoptosis as assessed using these two methods. We also investigated the expression of Bax and Bcl-2, which are well recognized indicators of apoptosis [Vogel, 2002; Reed, 2006]. As shown in Figure 1D, qRT-PCR analysis revealed that the mRNA expression level of Bax was significantly increased, while the mRNA level of Bcl-2 was significantly decreased in podocytes with HG stimulation. The results of Western blot analysis also indicated that the ratio of Bax/Bcl-2 was markedly increased in the HG group (Fig. 1E). Similar to the apoptosis results, mannitol had no effect on the ratio of Bax/Bcl-2.

HG STIMULATION DECREASED THE EXPRESSION OF NESTIN IN PODOCYTES

The effects of HG stimulation on nestin protein expression were examined in podocytes in vitro using immunocytochemistry and Western blot analysis. As shown in Figure 2A, nestin-positive expression was significantly decreased when HG stimulation for 12 and 72 h. Western bolt analysis revealed that HG stimulation decreased the nestin protein levels in a time-dependent manner; these protein levels were reduced after 6 h exposure to HG and almost disappeared at 48 h exposure, compared to control (Fig. 2B). We also examined the mRNA levels of nestin using qRT-PCR analysis. HG stimulation decreased the mRNA levels of nestin in a time-dependent manner in podocytes after 6–72 h exposure to HG (Fig. 2C). Collectively, these data indicate that HG stimulation decreases the expression of nestin in podocytes.

DOWNREGULATION OF NESTIN SENSITIZED PODOCYTES TO HG-INDUCED APOPTOSIS

To investigate the relationship between nestin and podocyte apoptosis, we employed miRNA interference to assess the effect of nestin depletion prior to subjecting the podocytes to HG stimulation. A scrambled vector was used as a control throughout the experiments. Downregulation of nestin was confirmed by Western blot and qRT-PCR analysis (Fig. 3A,B). Under normal glucose (NG) conditions in vitro, there were no significant changes in podocyte apoptosis in cells transfected with nestin miRNA vector compared with the untransfected control group or the scrambled group, as analyzed by either flow cytometry (Fig. 3C,D) or the TUNEL method (Fig. 3E,F). These results indicate that apoptosis was not affected in nestin-depleted podocytes with normal glucose levels in vitro.

We also assessed the effects of nestin depletion on HG-induced podocyte apoptosis using the miRNA interference. When podocytes were treated with 30 mM glucose, apoptotic rates were significantly

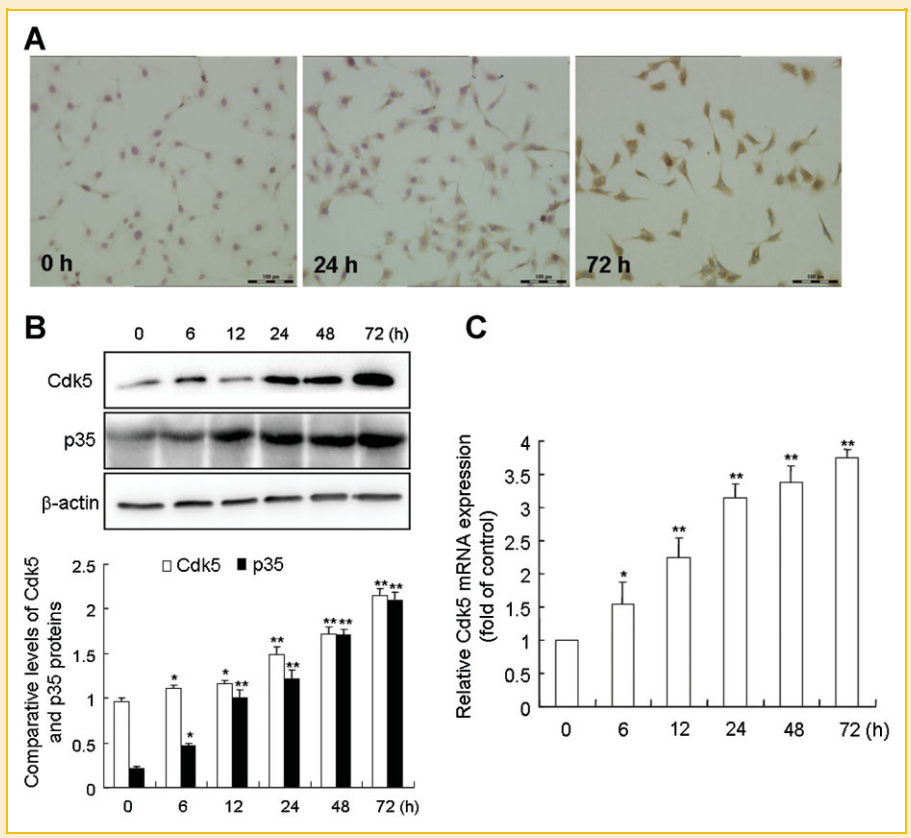

Fig. 5. Effects of HG on Cdk5 and p35 expressions in podocytes. Podocytes were incubated with high glucose (HG, 30 mM) from 0 to 72 h. A: Cdk5 positive expression was detected using immunocytochemical staining (200×). B: Expressions of Cdk5 or p35 protein were analyzed using Western blot. The levels of Cdk5 or p35 protein was quantified using densitometric analysis and normalized to the level of β-actin. C: Expression of Cdk5 mRNA was analyzed using qRT-PCR, and β-actin mRNA was used as an internal control. Values are expressed as the mean ± SD. $^{*}P < 0.05$, versus control (0 h), $^{**}P < 0.01$, versus control (0 h). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

increased by transfection of the nestin miRNA vector, as compared to untransfected cells and cells transfected with the scrambled vector (Fig. 4A–C). This increase was observed after 24 h of exposure to HG, and the apoptotic rate was further increased at 48 h. We also examined the protein expression of cleaved caspase-3. The results showed that the protein expression of the cleaved caspase-3 was significantly increased by transfection of the nestin miRNA vector in HG-treated podocytes at 24 and 48 h exposure (Fig. 4D,E). These data suggested that the downregulation of nestin enhances the HG-induced podocyte apoptosis.

HG STIMULATION INCREASED THE EXPRESSION OF CDK5 IN PODOCYTES

Considering the relationship of nestin and Cdk5, we further examined the expression of Cdk5 in HG-treated podocytes using immunocytochemistry, Western blot and qRT-PCR. As shown in Figure 5A, Cdk5 expression was significantly increased when HG stimulation was applied for 24 and 72 h. HG stimulation increased the protein levels of Cdk5 in a time-dependent manner, between 6 and 72 h, as assessed using Western blot analysis. Also, p35, the protein activator of Cdk5, was also significantly increased after 6 h of HG stimulation, and this increased level of p35 continued until 72 h (Fig. 5B). Similarly, the mRNA levels of Cdk5 were also increased in a time-dependent manner in HG-treated mouse podocytes, as detected by qRT-PCR (Fig. 5C).

DOWNREGULATION OF CDK5 ATTENUATED THE DECREASED NESTIN EXPRESSION AND PODOCYTE APOPTOSIS INDUCED BY HG STIMULATION

In order to further examine the impact of Cdk5 on nestin expression and podocyte apoptosis, the Cdk5 miRNA vector was transfected into podocytes, which then were treated with high glucose (30 mM). A scrambled vector was used as a control and downregulation of Cdk5 was confirmed by Western blot and qRT-PCR analysis (Fig. 6A,B). Figure 6C,D shows that the reduced nestin expression in the HG group was attenuated by using Cdk5 miRNA interference, indicating that the Cdk5 level and the nestin level are inversely related.

We then assessed the effects of Cdk5 on HG-induced podocyte apoptosis. As shown in Figure 7A–C, the HG-induced apoptosis was markedly attenuated by using Cdk5 miRNA interference. Cdk5 miRNA interference treatment also ameliorated HG-induced

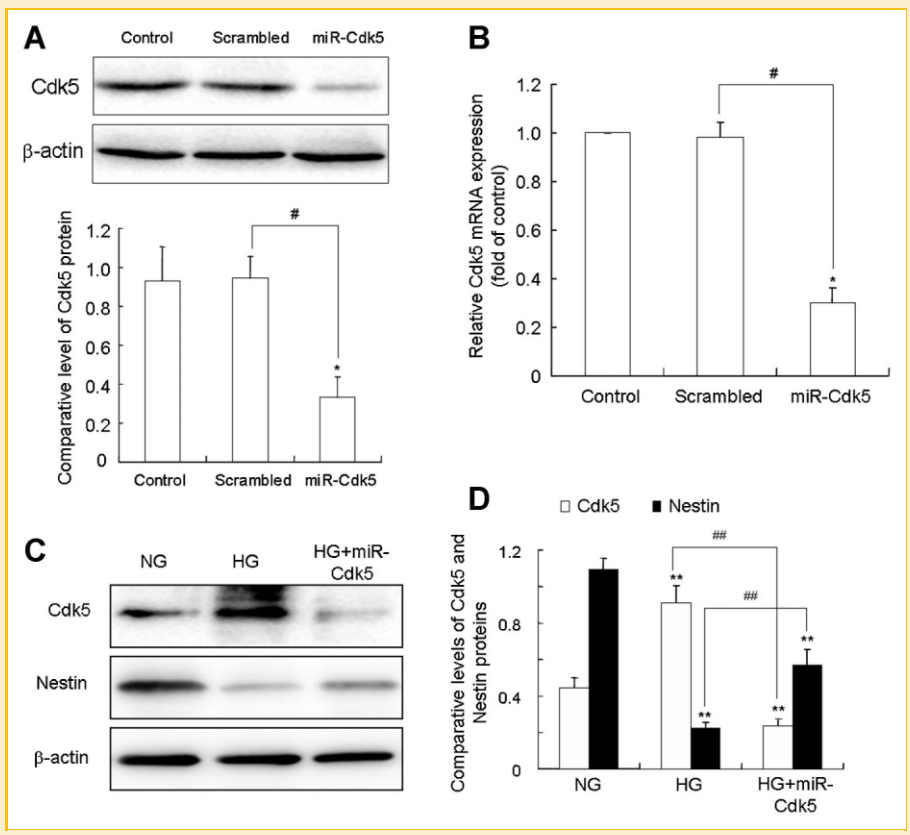

Fig. 6. Effects of Cdk5 depletion on nestin expression with HG stimulation. Podocytes were transfected with a miRNA vector for Cdk5, and a scrambled vector was used as a control. A: The protein levels of Cdk5 were analyzed using Western blot to ensure transfection efficiency, quantified by densitometric analysis and normalized to the level of β-actin. B: The mRNA levels of Cdk5 in transfected podocytes were detected using qRT-PCR, and β-actin mRNA was used as an internal control. C: Podocytes were transfected with the Cdk5 miRNA vector and treated with high glucose (HG, 30 mM) for 48 h. Expressions of Cdk5 or nestin protein were analyzed using Western blot ($n = 6$). D: The levels of Cdk5 or nestin protein were quantified by densitometric analysis and normalized to the level of β-actin. Values are expressed as the mean ± SD. $^{*}P < 0.05$, miR-Cdk5 group versus Control group; $^{\#}P < 0.05$, miR-Cdk5 group versus Scrambled group. $^{**}P < 0.01$, HG + miR-Cdk5 group versus NG group; $^{\#\#}P < 0.01$, HG + miR-Cdk5 group versus HG group.

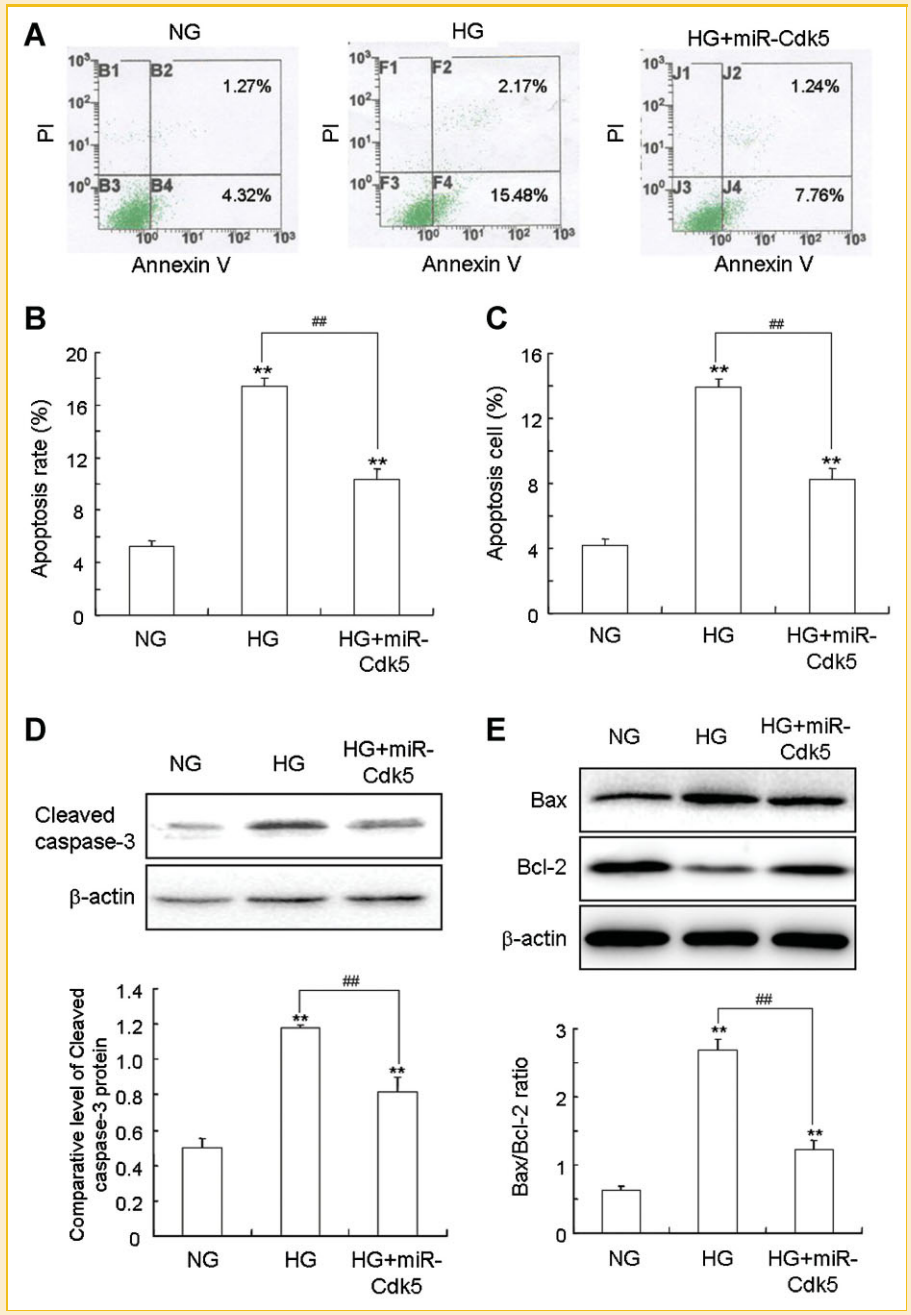

Fig. 7. Effects of the knockdown of Cdk5 expression on HG-induced podocyte apoptosis. Podocytes were transfected with Cdk5 miRNA vector, and were then treated with high glucose (HG, 30 mM) for 48 h. A: Podocytes were stained with Annexin V/PI for flow cytometry analysis ($n = 6$). Apoptotic cells were divided into two stages: early apoptotic (Annexin V^+/PI^-) and late apoptotic (Annexin V^+/PI^+) cells. B: The total apoptosis rates examined using flow cytometry, including the early and late apoptosis rate, were quantified and shown using a histogram. C: Apoptosis was detected using TUNEL staining. TUNEL-positive (apoptotic) cells were counted out of a total of more than 200 cells over six random fields. The results were expressed as apoptosis cell (%). D: The expression of cleaved caspased-3 protein was analyzed using Western blot ($n = 6$). The level was quantified by densitometric analysis and normalized to the level of β-actin. E: The expression levels of Bax and Bcl-2 proteins were analyzed using Western blot ($n = 6$). The ratio for Bax/Bcl-2 protein was based on densitometric results as compared to β-actin. Values are expressed as the mean ± SD. $^{**}P < 0.01$, HG + miR-Cdk5 group versus NG group; $^{\#\#}P < 0.01$, HG + miR-Cdk5 group versus HG group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

upregulation of cleaved caspase-3 in podocytes in vitro (Fig. 7D). Similarly, the HG-induced increase of the protein ratio of Bax/Bcl-2 was also decreased by Cdk5 miRNA interference (Fig. 7E). Taken together, these data suggested that nestin, dependent on Cdk5 regulation, contributed to HG-induced podocyte apoptosis.

DISCUSSION

An important early feature of diabetic kidney disease is glomerular podocyte injury, which contributes to the increased urinary albumin losses and accelerated sclerosis of the glomerular microvascular bed

[de Zeeuw et al., 2004]. Apoptosis has been considered a major cause of podocyte loss, and several studies have shown that podocyte apoptosis occurs early in the development of DN and correlates with its progression [Meyer et al., 1999; Steffes et al., 2001; Dalla Vestra et al., 2003; Susztak et al., 2006; Verzola et al., 2007]. In our study, we also demonstrated that high glucose (30 mM) could induce podocyte apoptosis in vitro, and along with the upregulation of cleaved caspase-3 and the ratio of Bax/Bcl-2.

Many IFs contain caspase consensus domain sites, and are thought to be involved in the execution of cellular apoptosis [Dinsdale et al., 2004; Marceau et al., 2007; Townson et al., 2010]. The anti-apoptotic effects of IFs have been investigated in some cell types, including nestin [Sahlgren et al., 2006; Marceau et al., 2007; Huang et al., 2009]. Nestin can interact with microtubules and microfilaments through its characteristic long C-terminus and can regulate the cellular structural protein organization and distribution. Nestin has also been shown to protect neural progenitor cells against apoptosis induced by oxidative stress [Sahlgren et al., 2006]. In the adult kidney, nestin expression is limited to glomerular podocytes. Recently, several studies have demonstrated that nestin plays an important role in maintaining the normal morphology and function of podocytes. Nestin expression in the kidneys of patients with IgA nephropathy and proteinuria was significantly reduced compared with that in normal kidneys [Su et al., 2007]. In cultured podocytes stimulated by puromycin aminonucleosid (PAN), knockdown of nestin expression by shRNA resulted in a significant increase in apoptosis [Wen et al., 2011]. In our study, we showed that nestin expression was decreased in a time-dependent manner in mouse podocytes treated with HG. Knockdown of nestin by miRNA interference resulted in a significant increase in podocyte apoptosis induced by HG, while the apoptosis was not affected in nestin-depleted podocytes in the absence of HG. It has been shown that the cell viability was not affected in nestin-depleted neural progenitor cells in the absence of oxidative stress, which indicated that depletion of nestin is not sufficient to induce cell death [Sahlgren et al., 2006]. Taken together, these results indicate that nestin plays a cytoprotective role in podocytes, and its downregulation was a prerequisite for induction of apoptosis during HG stimulation.

In addition, we also investigated the expression of Cdk5, which had a physical interaction with nestin, in HG-treated podocytes [Sahlgren et al., 2003]. Cdk5 has been demonstrated to phosphorylate nestin at Thr-316 during differentiation of myoblasts, and phosphorylation at this site is associated with increased nestin solubility [Sahlgren et al., 2003]. Sahlgren et al. showed that nestin could serve as a scaffolding molecule for Cdk5, and that oxidative stress in neuronal precursor cells led to the degradation of nestin and induction of apoptosis, which was associated with increased Cdk5 activity [Sahlgren et al., 2003, 2006]. It was also shown that Cdk5 was also a key regulator of podocyte differentiation, proliferation and morphology [Griffin et al., 2004]. In the present study, we showed that the expression of Cdk5 increased in a time-dependent manner in HG-treated podocytes, while nestin decreased in a time-dependent manner under the same conditions. We also investigated the level of p35, a specific activator of Cdk5, and demonstrated a trend similar to that of Cdk5. Furthermore, downregulation of Cdk5 by miRNA interference reduced the degradation of nestin and increased the level of nestin in HG-treated podocytes. The apoptotic rate, the level of cleaved caspase-3, and the Bax/Bcl-2 ratio were all reduced by knockdown of Cdk5 expression in HG-treated podocytes. These results suggested that nestin, as a substrate of Cdk5, played a cytoprotective role in HG-induced podocyte apoptosis, and that podocyte damage could be attenuated by downregulation of Cdk5 and upregulation of nestin.

In summary, our study demonstrated that HG decreases nestin expression in a time-dependent manner in cultured podocytes. Knockdown of nestin expression sensitized podocytes to HG-induced apoptosis. Also, downregulation of Cdk5 attenuated the reduction in nestin expression and podocyte apoptosis that was induced by HG stimulation, suggesting that nestin, which is dependent on Cdk5 regulation, plays a cytoprotective role in HG-induced podocyte apoptosis. Our results suggest a new rationale for understanding the underlying mechanism of DN and also suggest a potential target for therapy in DN patients.

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