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ORIGINAL ARTICLE

Basic Study

Nicotinamide mononucleotide protects against diabetic nephropathy *via* IL-6/Rab5-mediated crosstalk between proximal tubular epithelial cells and podocytes

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Abstract

BACKGROUND

Diabetic nephropathy (DN) is a leading cause of chronic kidney disease and end-stage renal disease, and is a significant global healthcare burden. Although proximal tubular epithelial cells (PTECs) and podocytes are involved in DN progression, the specific molecular interactions between these cells are not well understood.

AIM

To elucidate the role of interleukin-6 (IL-6)/Rab5 signaling in mediating crosstalk between PTECs and podocytes, and to evaluate the protective effects of nicotinamide mononucleotide (NMN) against DN progression.

METHODS

We utilized *in vitro* and *in vivo* models to investigate the pathogenesis of DN. *In vitro*, human PTECs and murine podocytes were cultured under high-glucose conditions, and IL-6 neutralizing antibodies or NMN treatments were applied. Podocyte injury was assessed by measurements of nephrin endocytosis, Rab5 activity, cytoskeletal organization, cell adhesion, and cell-spreading assays. *In vivo*, DN was induced in mice using streptozotocin, and mice then received NMN, insulin, or both treatments over an 8-week period. Renal tissues were analyzed histologically, ultrastructurally, and immunochemically, and urinary albumin excretion was measured to assess renal function. Statistical analyses were conducted using one-way ANOVA and Tukey's test.

RESULTS

High-glucose conditions induced the epithelial-mesenchymal transition (EMT) in

PTECs, increased IL-6 secretion, and activated Rab5 signaling in podocytes, leading to increased nephrin endocytosis and podocyte injury. Blocking IL-6 significantly attenuated these effects. NMN treatment of diabetic mice markedly reduced podocyte injury, glomerular hypertrophy, foot-process effacement, and urinary albumin excretion. Mechanistically, NMN suppressed the EMT and IL-6 secretion by PTECs, inhibited Rab5 activation in podocytes, and prevented nephrin endocytosis, thereby preserving the cytoskeletal integrity and function of podocytes.

CONCLUSION

Our findings reveal a novel pathogenic mechanism of DN in which IL-6 released from glucose-stressed PTECs activates Rab5 signaling in podocytes, followed by nephrin endocytosis and structural injury of podocytes. Importantly, NMN treatment effectively disrupted this pathological pathway of intercellular communication, and provided significant protection against DN progression. These results suggest that NMN supplementation and targeting the IL-6/Rab5 signaling axis has promise as a therapeutic strategy for managing DN.

Key Words: Diabetic nephropathy; Podocyte injury; Interleukin-6; Rab5 signaling; Nicotinamide mononucleotide; Proximal tubular epithelial cell

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Core Tip: This study identified a novel mechanism in diabetic nephropathy (DN), in which high glucose-induced interleukin-6 (IL-6) secretion by proximal tubular epithelial cells activated Rab5 signaling in podocytes, followed by nephrin endocytosis and structural damage. Importantly, nicotinamide mononucleotide (NMN) effectively interrupted this pathogenic IL-6/Rab5-mediated intercellular crosstalk, thereby preserving podocyte integrity, reducing glomerular injury, and decreasing albuminuria. These findings highlight NMN as a potential therapeutic strategy for mitigation of DN by targeting IL-6/Rab5 signaling.

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INTRODUCTION

Diabetic nephropathy (DN) is a major cause of chronic kidney disease and end-stage renal disease, and these diseases have a significant global health burden[1-3]. Recent advancements have led to an improved understanding of the pathogenesis of DN and the development of potentially effective treatments. Earlier studies of DN focused on glomerular injury, including injury of mesangial cells, podocytes[4], and endothelial cells[5], but more recent studies have shown that injury of proximal tubular epithelial cells (PTECs) also contribute to the progression of DN[6-8]. Despite our increased knowledge of the pathogenesis of DN, there are still limited interventions that can successfully slow disease progression. The main therapeutic strategies currently used for treatment include control of hyperglycemia and blood pressure, and inhibition of the renin-angiotensin system (RAS)[9]. There has been less focus on interventions that target the molecular crosstalk of signaling pathways in glomerular cells and PTECs, processes that play critical roles in the progression of DN. Consequently, a more complete understanding of all the pathogenic pathways that contribute to DN may facilitate the development of more effective therapies and therapies that can be applied earlier during the course of disease.

The kidney is a structurally complex organ, and the two main regions, the cortex and medulla, contain many types of cells, including glomerular cells, PTECs, mesangial cells, endothelial cells, and podocytes[10]. Thus, DN results from the complex interactions of multiple types of cells[11]. Analyses of the expression of different mRNAs and proteins in DN have identified alterations in multiple pathways, and also showed that alterations in the intercellular communication among different types of glomerular cells may contribute to disease progression[12]. Mesangial cells provide structural support for glomerular capillary loops and also secrete growth factors, such as PDGF-β and its receptor PDGFR-β, which interact with endothelial cells[13,14]. Chemokines produced by mesangial cells, such as CXCL12, bind to receptors on podocytes (CXCR4). Other chemokines derived from podocytes, such as (CCL19 and CCL21, bind to receptors on mesangial cells (CCR7), and promote the migration and attachment of these cells to the basement membrane[15]. Molecular signaling between podocytes and endothelial cells is essential for preserving the function of the glomerular filtration barrier[16]; damage to the endothelial cell layer disrupts the molecular interactions with podocytes and leads to impaired glomerular filtration[17]. Thus, glomerular cells participate in complex molecular interactions and communicate *via* gap junctions with different signaling pathways. Moreover, there is bidirectional signaling among different glomerular cell populations within an interconnected network.

Injury of podocytes, highly specialized pericyte-like cells that surround the glomerular capillaries, is a characteristic feature of proteinuric kidney diseases and contributes to the progression of DN[18]. Recent research also highlighted the complex effects of podocytes in DN[19]. Notably, altered communication between podocytes and physically distant PTECs occurs in kidney diseases, including DN[20]. Proteins that leak from injured podocytes enter and damage the tubules by triggering the accumulation of reactive oxygen species and various cytokines, manifesting as proteinuria[21]. Likewise, metabolic changes in the PTECs lead to changes in podocyte function during DN. Hasegawa *et al*[20] demonstrated that disruption of the interactions between PTECs and podocytes was associated with decreased levels of Sirt1 and nicotinamide mononucleotide (NMN) in proximal tubules and with a decreased level of Sirt1 but an increased level of claudin-1 in podocytes. They also showed that these changes significantly contributed to the progression of diabetic albuminuria. Additionally, PTECs altered nicotinic acid metabolism, and this affected the podocyte epigenome, a mechanism they described as "proximal tubule-podocyte communication"[20].

NMN (the precursor of NAD^+) protects against age-related physiological alterations in diverse tissues and organs, including the liver, adipose tissue, muscle, pancreas, kidney, retina, and central nervous system[22]. A study of age-induced diabetes in mice showed that NMN alleviated obesity, insulin resistance, and mitochondrial dysfunction in muscle, primarily by activating sirtuins, a group of HDACs that regulate metabolism[23]. Another study of a mouse model of DN found that early intervention with NMN provided reno-protective effects by increasing the expression of Sirt1 and stimulating the NAD^+ salvage pathway, suggesting a persistent beneficial effect of NMN[24]. However, upregulation or overexpression of Sirt1 does not reverse DN and normalize kidney function[25], indicating that Sirt1 signaling along with additional mechanisms are responsible for the reno-protective effect of NMN. Additionally, studies of rat glomerular mesangial (HBZY-1) cells showed that NMN protected against cellular fibrosis by inhibiting the expression of Nampt, the rate-limiting enzyme in the synthesis of NMN, and this indirectly suppressed NF-κB p65-mediated inflammatory pathways[26]. Collectively, these findings suggest that NMN may function as a compensatory reno-protective factor because it decreases apoptosis and inflammatory fibrosis, processes that are associated with DN[27]. Although previous studies have identified interactions of PTECs and podocyte interactions and the effect of NMN on DN, much remains unknown about these complex interactions.

The present study of *in vitro* and *in vivo* models examined the novel molecular mechanisms that underlie the communication between PTECs and podocytes and examined the effect of NMN on DN and the potential mechanism by which NMN regulates the interactions of PTECs and podocytes.

MATERIALS AND METHODS

Reagents

Rabbit anti-nephrin (NBP1-30130) and rabbit SM22 alpha (NBP1-33003) polyclonal antibodies (pABs) were from Novus Biologicals (Littleton, CO, United States). Mouse anti-nephrin monoclonal antibody (mAb; sc-376522), mouse anti-β-actin mAb (sc-47778), and *Rab5* siRNA (sc-36344) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Mouse anti-Rab5-GTP mAb (Cat. No.26911) and rabbit anti-Rab5 pAB (Cat. No. 21151) were from Wuhan NewEast Biosciences (Wuhan, Hubei Province, China). Rabbit Ve-cadherin (D87F2) XP mAb (#2500) was from cell signaling technology (Danvers, MA, United States). Mouse vimentin (ab8978) was from Abcam (Cambridge, United Kingdom). Dynasore (3324413), MβCD (C4555), TRITC-phalloidin (P1951), and a mouse plasma IL-6 ELISA kit (RAB0308) were from Sigma-Aldrich (St Louis, MO, United States). NMN (N3501) was from Merk KGaA (Darmstadt, Germany). The *GFP-Rab5CA* (Q79 L; #35140) and *GFP-Rab5DN* (S34N; #35141) vectors were from Addgene (MA, United States). The Albuwell M Test kit (# 1011) was from Exocell, Inc. (Philadelphia, PA, United States). Rab5 activity was assessed using the Rab5 Activation Assay Kit (#83701; NewEast Biosciences). Mouse IL-6 neutralizing Ab (MAB406) was from R&D Systems (Minnesota, United States). HRP-conjugated secondary Ab was from Abbkine (Wuhan, Hubei, China).

Animal experiments

Specific pathogen-free male mice (8-weeks-old) were obtained from Hubei BIONT Biological Technology Co., LTD. This study was approved by the Experimental Animal Welfare Ethics Committee Zhongnan Hospital of Wuhan University, Wuhan, China (No. ZN2023015) and performed in accordance with the guidelines of the National Health and Medical Research Council of China. Mice were randomly assigned into four groups (8 per group): Control, diabetes mellitus (DM), DM with NMN (DM + NMN), and DM with NMN and insulin (DM + NMN + insulin). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg, dissolved in sodium citrate buffer); control animals received an equivalent volume of sodium citrate buffer alone by intraperitoneal injection. Mice with blood glucose concentrations exceeding 16.67 mmol/L were considered diabetic and included in subsequent experiments. NMN (120 mg/kg) was administered by oral gavage every other day for eight consecutive weeks; control animals were given an equivalent volume of sterile phosphate buffered saline (PBS) by oral gavage. Mice in the DM + NMN + insulin group received insulin each day *via* subcutaneous injection throughout the study period. Urine samples were collected from all mice prior to euthanasia at the end of the 8-week experimental period.

Cell culture and treatments

Normal human PTECs [HK-2 (CRL-2190™); ATCC] were maintained in Keratinocyte Serum Free Medium (K-SFM, Invitrogen; #17005-042) that was supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor. Conditionally immortalized murine podocyte cells, generously provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York), were cultured in RPMI 1640 medium (Gibco, United States)

that was supplemented with 10% heat-inactivated fetal calf serum (Gibco, United States), 100 U/mL penicillin G, and 100 μg/mL streptomycin. Podocytes were first cultured at 33 °C with 10 U/mL of recombinant mouse interferon-γ to induce proliferation and then transferred to 37 °C and cultured without interferon-γ to induce differentiation. Differentiated podocytes (passages 19-29) were used for all experiments. HK-2 cells were treated with high-dose glucose (HG; 30 nmol/L) for 48 hours and the resulting conditioned medium was collected for culture of podocytes. Podocytes were treated with an IL-6 neutralizing Ab (1 μg/mL) or NMN (1 mmol/L) for 48 hours.

Construction of pcDNA3.1-Rab5 plasmids

The *Rab5a* primers used for amplification were: Sense: 5' TGTTGGCAAATCAAGTCTGGT 3'; Antisense: 5' TGCTAAGTCAGCCTTGTTTCCT 3'. The PCR products were analyzed by agarose gel electrophoresis, digested with restriction enzymes, and ligated into the pcDNA3.1 vector. Bacterial transformation, selection, plasmid extraction, and sequence verification were conducted following standard protocols.

Transfection

Podocytes were transfected with the pcDNA3.1-*Rab5*, *Rab5*CA, *Rab5*DN plasmids; or a control vector for 48 hours at 37 °C using the X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Transfected cells were selected using G418 (genticin; Sigma-Aldrich).

Rab5 siRNA was transfected into podocytes using the Santa Cruz transfection reagent following the manufacturer's protocol. Briefly, 6 × 10^5 cells per dish were transfected with 20 nmol/L *Rab5* siRNA or scrambled siRNA (control) and incubated at 37 °C for 48 hours.

Cytoskeleton staining

Podocytes were washed with precooled PBS (4 °C), fixed in 4% paraformaldehyde containing 0.1% Triton X-100 for 30 minutes at 4 °C, and stained with TRITC-phalloidin (5 μg/mL) overnight at 4 °C or for 2 hours at 37 °C. Nuclei were counterstained with DAPI for 5 minutes in the dark at room temperature. Images were acquired using a confocal microscope (LSM900; Zeiss, Germany).

Immunofluorescence staining and co-localization imaging

Podocytes were initially fixed in 4% formaldehyde, followed by blocking with 5% bovine serum albumin (BSA), and incubation overnight at 4 °C with the primary Ab (1:100 dilution). The cells were then treated with Cy3-labeled goat anti-mouse IgG and FITC-labeled goat anti-rabbit IgG secondary antibodies (1:200 dilution) for 30 minutes at room temperature. Immunofluorescence images were obtained using a confocal microscope (LSM 900, ZEISS, Germany).

Western blotting

Cells were lysed using RIPA buffer, and the protein concentration of each lysate was determined using the BCA protein quantification assay. Samples with equal amounts of protein were separated *via* SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary antibodies overnight at 4 °C. After washing, the membranes were incubated with an HRP-conjugated secondary Ab and visualized using enhanced chemiluminescence. Protein bands were quantified relative to β-actin using ImageJ software.

Co-Immunoprecipitation

Cells were lysed in 1 × assay/Lysis buffer, and supernatants were incubated with a mAb against Rab5 overnight at 4 °C. Protein A/G agarose beads were then added, followed by incubation for 1 hour at 4 °C. The immunoprecipitated complexes were then washed, eluted, and analyzed by western blotting.

Measurement of albuminuria

Urinary albumin levels were measured using a murine microalbuminuria ELISA kit (Exocell, Inc. Philadelphia, PA, United States). Urinary creatinine levels were measured using the QuantiChrom™ Creatinine Assay Kit (BioAssay System, Hayward, California, United States). These data were analyzed using SoftMax Pro 6.4 software.

Electron microscopy

Renal cortex samples were fixed using 2.5% glutaraldehyde, followed by dehydration and embedding in araldite resin. Ultrathin sections were subsequently stained with 3% uranyl acetate and lead citrate and then examined using transmission electron microscopy.

Immunohistochemistry

Kidney tissue sections were embedded in paraffin, deparaffinized, rehydrated, and subjected to antigen retrieval by microwaving for 5 minutes. After blocking non-specific binding sites with 5% BSA for 30 minutes, endogenous peroxidase activity was neutralized with 3% hydrogen peroxide for 15 minutes. Sections were then incubated overnight at 4 °C with an anti-Rab5-GTP Ab (dilution 1:100), and exposed to a biotin-labeled secondary Ab for 30 minutes. Immunoreactivity was visualized by adding 3,3'-diaminobenzidine for 3 minutes, counterstaining with hematoxylin for 2-3 minutes, and visualization by light microscopy.

Cell adhesion assay

Podocytes (2 × 10^5 cells) were washed 3 times and fixed with 4% paraformaldehyde. Cells were stained with 0.1% crystal violet, lysed in 10% acetic acid, and absorbance was then measured at 570 nm. The percentage of adherent podocytes was calculated as the absorbance ratio of each experimental group (including controls) to unwashed control cells.

Cell spreading assay

Podocytes (2 × 10^5 cells) were digested with pancreatin and seeded into 6-well plates that were coated with fibronectin (10 μg/mL). Podocyte morphology was then analyzed under an inverted microscope after 5 hours. Cells that had extended cellular processes were classified as "spread" and those that retained a round morphology were classified as "unspread". The percentage of "spread" podocytes was calculated.

Statistical analysis

All results are expressed as mean ± SEM. Statistical analyses were performed using SPSS 19.0 software. Groups were compared using a one-way analysis of variance (ANOVA), and Tukey's HSD test was applied for multiple comparisons. A *P* value below 0.05 was considered significant.

RESULTS

HG induces nephrin endocytosis and podocyte damage

We first examined the effect of HG treatment on nephrin endocytosis and structural damage of conditionally immortalized murine podocytes. Western blotting demonstrated that HG increased the level of cytoplasmic nephrin (C-nephrin), and decreased the level of membrane-nephrin, but did not change the level of total nephrin (T-nephrin; Figure 1A and B). Moreover, the addition of MβCD (a lipid raft-mediated endocytosis inhibitor) or Dynasore (a clathrin-mediated endocytosis inhibitor) blocked the effect of HG. In agreement, confocal microscopy revealed severe cytoskeletal disorganization in the HG group (disrupted actin filaments and reduced structural integrity) but amelioration of this effect by MβCD and Dynasore (Figure 1C). Similarly, quantitative assessments of cell adhesion (Figure 1D) and cell spreading (Figure 1E and F) showed that HG significantly decreased podocyte adhesion and spreading, but that pharmacological inhibition of nephrin endocytosis increased adhesion and spreading. These results suggest that nephrin internalization contributes to podocyte damage.

Rab5 mediates podocyte damage by increasing nephrin endocytosis

Rab5 is a GTPase that plays a crucial role in the regulation of endocytosis. We therefore used western blotting to investigate the role of Rab5 in the HG-induced endocytosis of nephrin by podocytes by silencing of *Rab5* (transfection with *Rab5* siRNA plasmid) or upregulation of *Rab5* (transfection with pcDNA 3.1-*Rab5* plasmid). As expected, HG alone increased the level of Rab5, *Rab5* overexpression further increased the level of Rab5, and silencing of *Rab5* decreased the level of Rab5 (Figure 2A and B). Our measurements of nephrin endocytosis demonstrated that *Rab5* knockdown decreased the level of C-nephrin, and that HG and *rab5* overexpression increased the level of C-nephrin (Figure 2C and D). Moreover, measurements of cell adhesion, cell spreading, and the podocyte cytoskeleton showed that *Rab5* overexpression led to impaired podocyte adhesion, spreading, and cytoskeleton integrity, but that *Rab5* knockdown improved podocyte adhesion, spreading, cytoskeletal integrity (Figure 2E-H).

Rab5-mediated nephrin endocytosis requires Rab5 activation

Rab5 can exist as a GTP-bound active state or a GDP-bound inactive state. We therefore examined the possible mechanism by which Rab5 mediates nephrin endocytosis by transfection of podocytes with a *Rab5*DN plasmid (dominant-negative) or *Rab5*CA plasmid (constitutively active). The results show that HG treatment significantly increased the level of active Rab5, *Rab5* overexpression further increased the level of active Rab5 in HG-treated podocytes, but *Rab5* silencing decreased the level of active Rab5 (Figure 3A and B). Co-localization imaging analysis and co-immunoprecipitation assays demonstrated that a greater level of active Rab5 was associated with an increased interaction of Rab5 with nephrin (Figure 3C-F). This interaction facilitated nephrin endocytosis under HG conditions, as indicated by an increased ratio of C-nephrin to T-nephrin (Figure 3G and H). Conversely, transfection with *Rab5*DN led to a lower level of active Rab5, a decreased interaction between nephrin and Rab5, and decreased nephrin endocytosis (Figure 3C-H).

IL-6 release by PTECs under HG conditions exacerbates podocyte injury

We then investigated possible interactions between HK-2 cells (PTECs) and podocytes under HG conditions. Western blotting indicated that HG treatment decreased the expression of an epithelial marker (E-cadherin) and increased the expression of two mesenchymal markers (vimentin and α-SMA) in HK-2 cells, signifying development of the epithelial-mesenchymal transition (EMT; Figure 4A and B). HG treatment also stimulated the secretion of the inflammatory cytokine IL-6 by these cells (Figure 4C). We then investigated the effect of growing podocytes on conditioned medium from HK-2 cells that lacked HG (control), had HG alone, or had HG with an IL-6 neutralizing Ab (NAb; Figure 4D). Cytoskeletal staining revealed that podocytes grown in conditioned medium with HG had cytoskeletal disorganization (Figure 4E). Functional assays also demonstrated that podocytes grown in conditioned medium with HG had decreased adhesion and spreading (Figure 4F–H). However, the addition of an IL-6 NAb improved cytoskeletal integrity (Figure 4E), enhanced podocyte adhesion (Figure 4F), and restored cell spreading (Figure 4G and H).

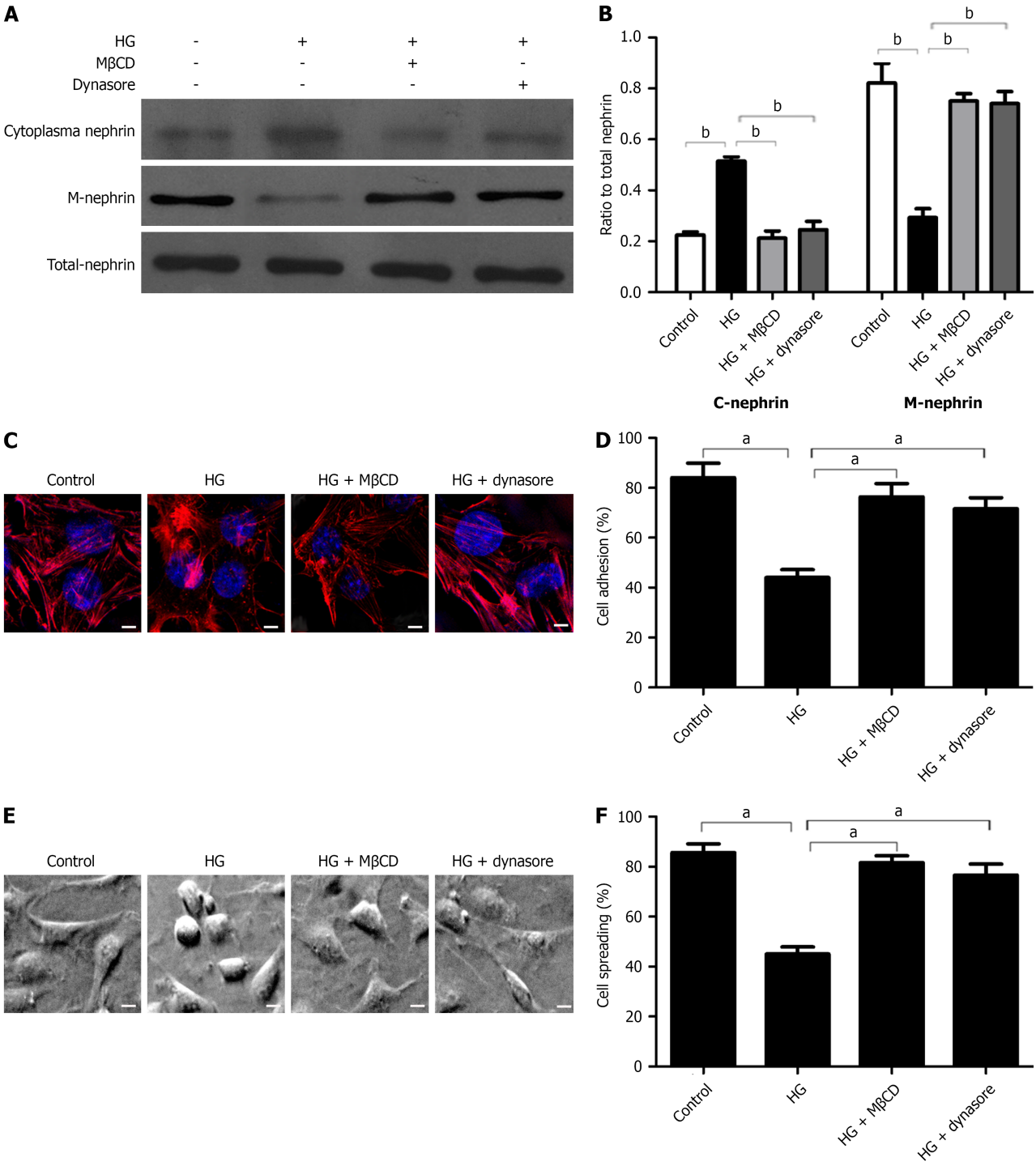
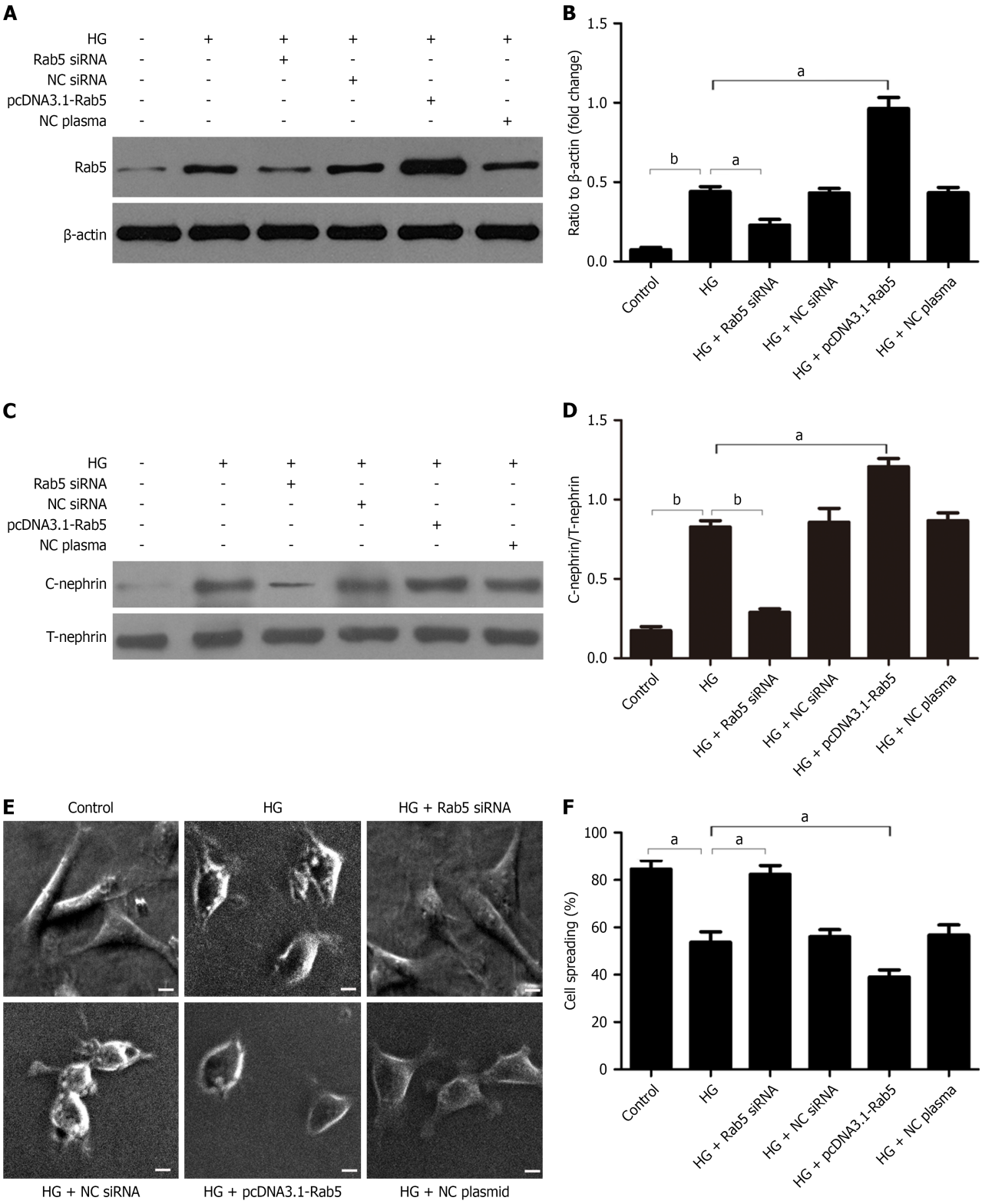


Figure 1 High-dose glucose-induced nephrin endocytosis promotes podocyte injury and endocytosis inhibitors block this effect. A and B: Western blot analysis of cytoplasmic nephrin, membrane nephrin, and total nephrin in podocytes after different treatments and densitometric quantification of these results (n = 3); C: Representative confocal microscopy images of podocytes after different treatments (magnification: × 1000; scale bar: 50 μm; blue: Nuclei; red: Cytoskeleton); D: Adhesion of podocytes after different treatments (n = 3); E and F: Representative electron microscopy images of podocyte spreading after different treatments (magnification: × 400; scale bar: 20 μm) and quantification of these results (n = 3). $^{a}P < 0.05$; $^{b}P < 0.001$. HG: High-dose glucose.

IL-6/Rab5 signaling regulates nephrin endocytosis in podocytes

We further investigated the effect of IL-6 signaling on the crosstalk between HK-2 cells and podocytes by culturing podocytes in conditioned medium from HK-2 cells that received different treatments, followed by co-immunoprecipitation. The results show that podocytes cultured in conditioned medium with HG had an increased precipitation of Rab5, and that IL-6 NAb significantly decreased this effect (Figure 5A and B). Moreover, co-localization imaging and additional co-immunoprecipitation and quantitative analysis demonstrated that HG increased the interaction between nephrin and active Rab5, and that IL-6 NAb inhibited this effect (Figure 5C-E). Podocytes cultured in conditioned medium with HG also had an increased level of C-nephrin, and IL-6 NAb attenuated this process (Figure 5F). These findings suggest that the IL-6/Rab5 signaling axis plays a critical role in promoting nephrin endocytosis by podocytes


A
HG
Rab5 siRNA
NC siRNA
pcDNA3.1-Rab5
NC plasma
Rab5
β-actin
B
Ratio to β-actin (fold change)
Control
HG
HG + Rab5 siRNA
HG + NC siRNA
HG + pcDNA3.1-Rab5
HG + NC plasma
C
C-nephrin
T-nephrin
D
C-nephrin/T-nephrin
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HG + NC plasmid
F
Cell spreading (%)

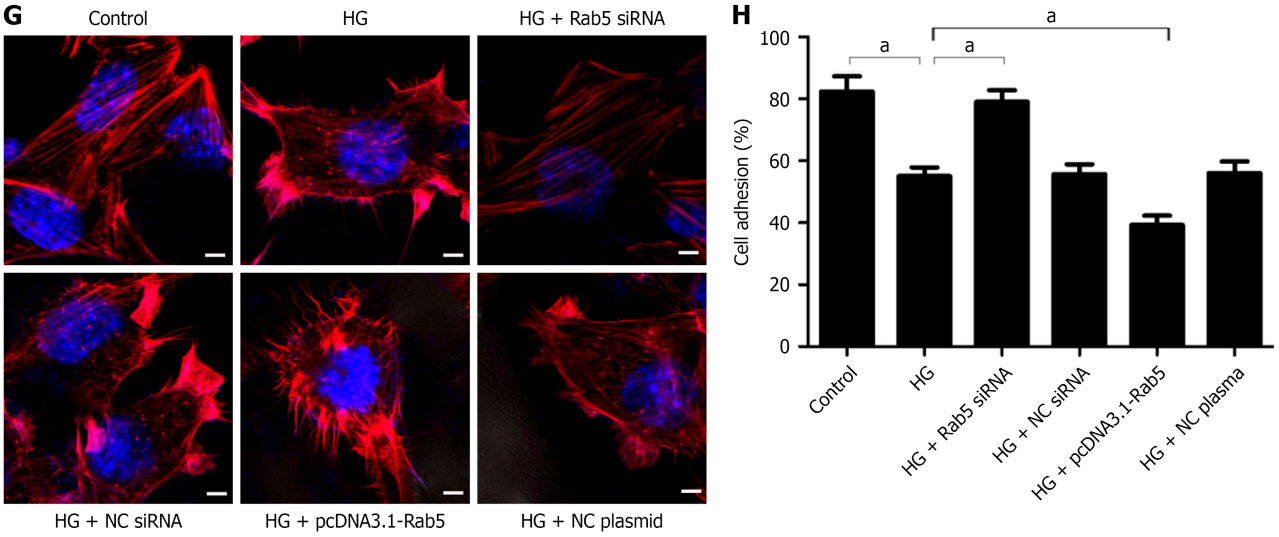
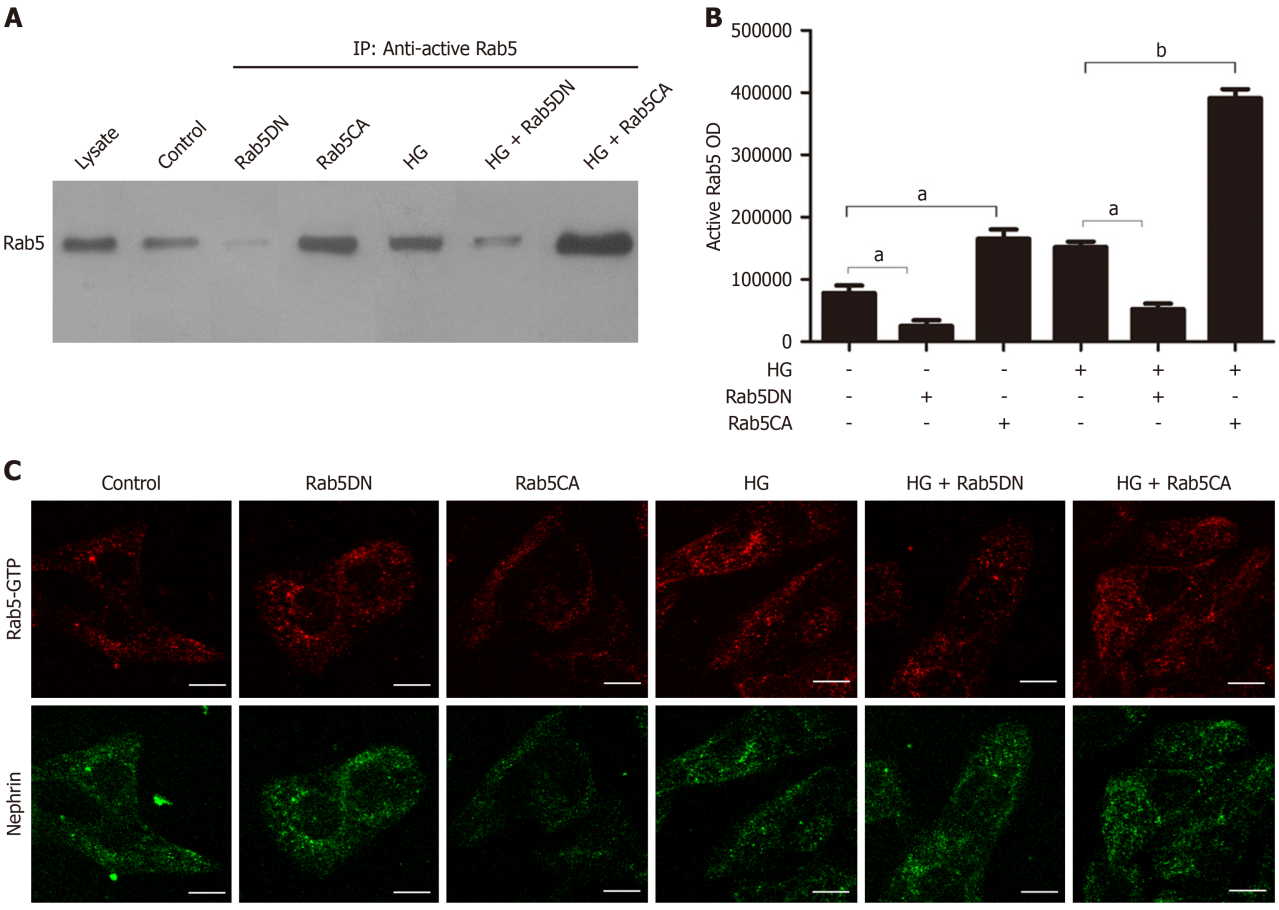


Figure 2 Rab5 promotes podocyte injury by increasing nephrin endocytosis, Rab5 silencing blocks this effect, and Rab5 upregulation promotes this effect. A and B: Western blot analysis of Rab5 in podocytes after different treatments and densitometric quantification of these results (n = 3); C and D: Western blot analysis of cytoplasmic nephrin (C-nephrin) and total nephrin (T-nephrin) in podocytes after different treatments and densitometric quantification of the C-nephrin/T-nephrin ratio (n = 3); E and F: Representative electron microscopy images of podocyte spreading after different treatments (magnification: × 1000; scale bar: 50 μm) and quantification of these results (n = 3); G: Representative confocal microscopy images of podocytes after different treatments (magnification: × 1000; scale bar: 50 μm; blue nuclei; red: Cytoskeleton); H: Adhesion of podocytes after different treatments (n = 3). $^{a}P < 0.05$; $^{b}P < 0.001$. HG: High-dose glucose; NC: Negative control.



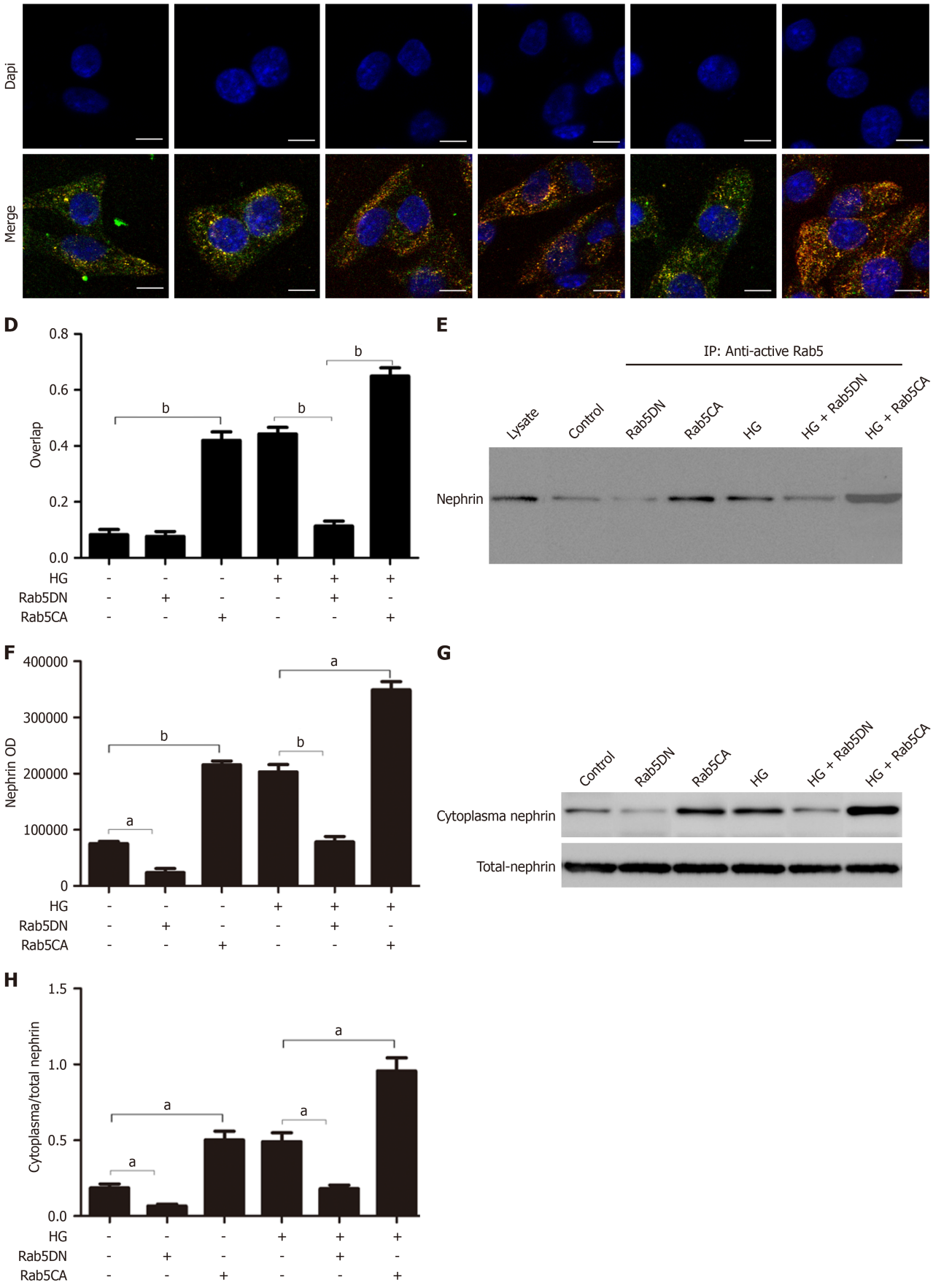


Figure 3 Rab5-mediated nephrin endocytosis by podocytes depends on Rab5 activation. A and B: Co-immunoprecipitation of active Rab5 (Rab5-GTP) in podocytes after different treatments and quantification of these results (n = 3); C and D: Representative co-localization images and quantitative of nephrin (green) and active Rab5 (red) in podocytes after different treatments (magnification: × 630; Scale bar: 20 μm); E and F: Co-immunoprecipitation of nephrin and active

Rab5 in podocytes after different treatments and quantification of these results (n = 3); G and H: Western blot analysis of cytoplasmic nephrin and total nephrin in podocytes after different treatments and quantification of the C-nephrin/T-nephrin ratio (n = 3). $^{a}P < 0.05$; $^{b}P < 0.001$. HG: High-dose glucose.

under HG conditions.

NMN alleviates podocyte damage in diabetic mice

We further evaluated the renoprotective effects of NMN in diabetic mice by focusing on its effect on the crosstalk between PTECs and podocytes. Mice in the DN group had significantly increased levels of blood glucose and HbA1c compared to the controls, but these parameters were normalized in the DN + insulin and DN + insulin + NMN groups (Figure 6A and B). The four groups had no significant differences in systolic or diastolic blood pressure (Figure 6C). PAS staining of renal tissues indicated marked glomerular hypertrophy in DN mice, characterized by an expanded glomerular surface area. This effect was substantially attenuated in the DN + insulin and the DN + insulin + NMN groups (Figure 6D and E). Electron microscopy demonstrated extensive foot process effacement in the DN group, indicative of severe podocyte injury. However, these ultrastructural changes were markedly less in the DN + insulin and the DN + insulin + NMN groups (Figure 6F). Correspondingly, urinary albumin excretion was significantly elevated in the DN group, but significantly lower in the DN + insulin and DN + insulin + NMN groups (Figure 6G). Co-localization imaging showed that the renal EMT, indicated by decreased expression of an epithelial marker (E-cadherin) and increased expression of mesenchymal markers (α-SMA and vimentin), was significantly enhanced in DN mice, but that insulin alone or insulin combined with NMN substantially prevented these changes (Figure 6H and I).

Importantly, immunohistochemical staining revealed that the glomerular level of active Rab5 (Rab5-GTP) was significantly elevated in DN mice, suggesting enhanced Rab5-mediated nephrin trafficking. However, treatment with insulin alone or insulin combined with NMN remarkably decreased this effect (Figure 6J and K). Immunofluorescence co-localization experiments confirmed an increased interaction of nephrin and Rab5 in the DN group, indicative of enhanced nephrin endocytosis. Again, treatment with insulin or insulin+NMN significantly reduced this pathological interaction (Figure 6L and M). Collectively, these *in vivo* data demonstrate that NMN was effective in alleviating podocyte injury in diabetic mice, and the protective mechanism involved suppression of the EMT and attenuation of Rab5-mediated nephrin endocytosis by modulation of crosstalk between PTECs and podocytes.

NMN protection depends on crosstalk between PTECs and podocytes

We further examined these effects by performing *in vitro* experiments with HK-2 cells. The results show that HG treatment decreased the expression of E-cadherin and increased the expression of α-SMA and vimentin, and that NMN partially reversed the effects of HG (Figure 7A and B). HG treatment of HK-2 cells also increased the secretion of IL-6 and NMN partially blocked this effect (Figure 7C). Next, we used a co-immunosuppression assay to assess the effect of conditioned medium from HK-2 cells induced by high glucose, with or without NMN, on the level of active Rab5. The results showed that the level of active Rab5 was greater in podocytes cultured in this conditioned medium, and that NMN decreased this effect (Figure 7D). Podocytes cultured in conditional medium from HK-2 cells induced by HG also displayed stronger interaction between nephrin and activated Rab5, and NMN decreased this interaction (Figure 7E and F). Nephrin endocytosis was greater in podocytes cultured in conditioned medium from HK-2 cells induced by HG, and NMN decreased this effect (Figure 7G and H). There were similar pathological effects of the HG and ameliorating effects of NMN with respect to the cytoskeleton (Figure 7I), podocyte adhesion (Figure 7J), and podocyte spreading (Figure 7K and L).

DISCUSSION

There are significant challenges in the clinical management of DN and limited efficacy of existing therapies because of the complex pathogenic mechanisms that underlie this disease[28]. Although standard clinical interventions, including control of glucose and blood pressure and inhibition of the RAS, can slow DN progression, they cannot prevent the eventual deterioration of renal function[29]. Recent research has focused on the role of intercellular communication during the progression of DN, and demonstrated that interactions among various types of renal cells, including glomerular cells and PTECs, significantly contribute to the pathological progression of DN[28]. In particular, recent evidence indicated that PTECs and podocytes exchange biochemical signals, and that each type of cell affects the health of the other[30]. Proteinuria-induced factors released by injured podocytes can induce inflammation in PTECs, and metabolic disturbances in PTECs precede and can promote podocyte injury in DN[30,31]. Hasegawa *et al*[20] elegantly demonstrated the significance of Sirt1/NMN-mediated metabolic crosstalk between PTECs and podocytes in DN, and identified key metabolic and epigenetic interactions underlying renal injury. In contrast, the current study provides a fundamentally distinct yet complementary mechanistic perspective by uncovering a novel IL-6/Rab5 inflammatory signaling axis. Specifically, we demonstrated that IL-6 secretion from glucose-stressed PTECs can directly trigger Rab5-mediated nephrin endocytosis, a new pathogenic inflammatory pathway that links tubular inflammation to structural injury of podocytes. This IL-6/Rab5 axis is thus a distinct and previously unrecognized pathway, and its identification significantly expands our understanding of the complex intercellular communication networks that drive the progression of DN and points to new opportunities for targeted therapeutic interventions (Figure 8).

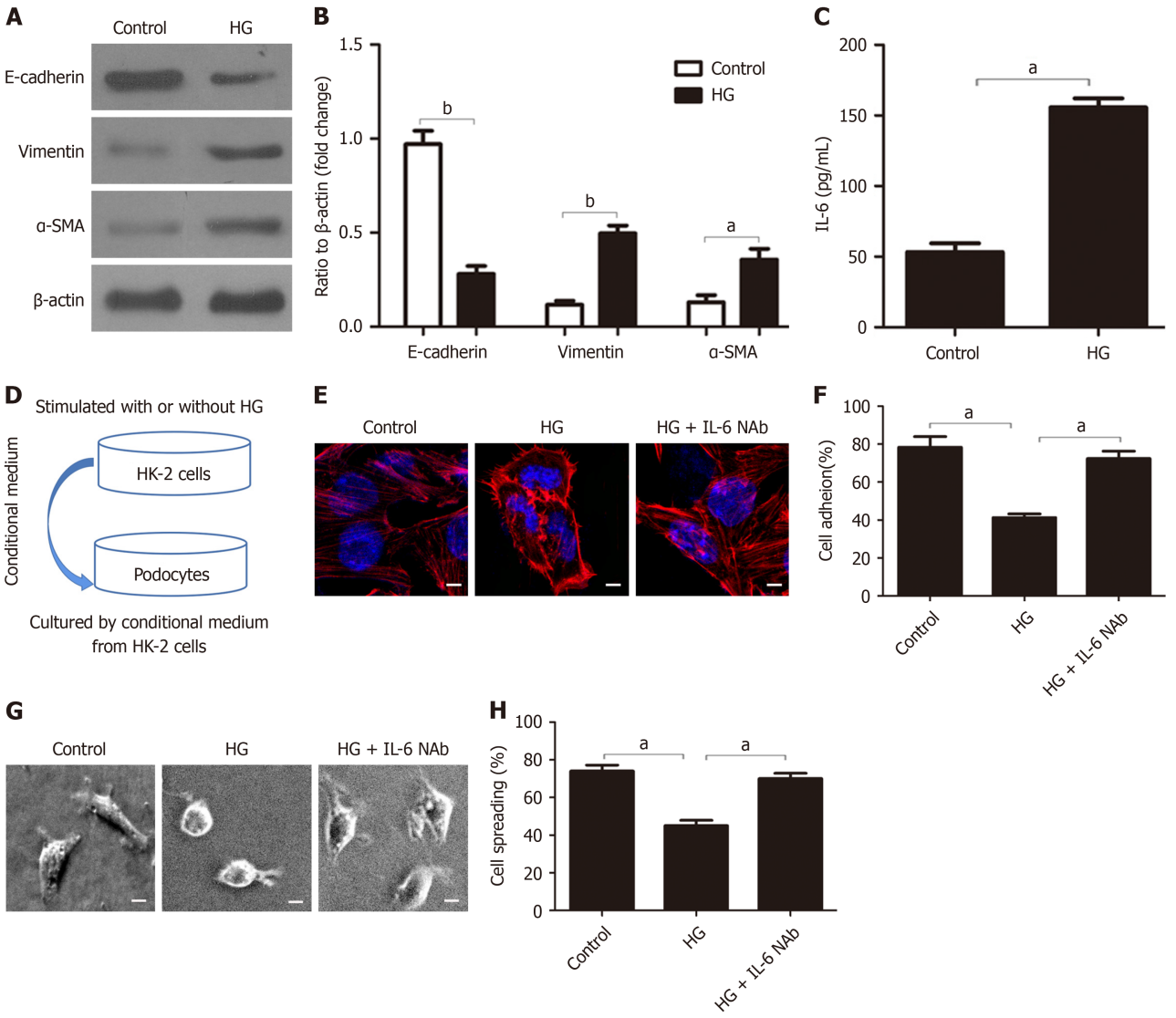


Figure 4 IL-6 secretion by HK-2 cells contributes to the HG-induced injury of podocytes. A and B: Western blot analysis of E-cadherin, vimentin, and α-SMA in HK-2 cells after different treatments and quantification of these results (n = 3); C: ELISA measurements of IL-6 after different treatments (n = 3); D: Method used to examine the effect of HK-2 conditioned medium on podocytes; E: Representative confocal microscopy images of podocytes after culture in different conditioned media (magnification: × 1000; scale bar: 50 μm; blue: Nuclei: Red: Cytoskeleton); F: Quantitative analysis of podocyte adhesion after culture in different conditioned media (n = 3); G and H: Representative electron microscopy images of podocyte spreading after culture in different conditioned media (magnification: × 400; scale bar: 20 μm) and quantification of these results (n = 3). $^{a}P < 0.05$; $^{b}P < 0.001$. HG: High-dose glucose; NAb: Neutralizing Ab.

Podocyte dysfunction is a critical event in the progression of DN[32] because it leads to persistent albuminuria and glomerulosclerosis[33]. Damage to the podocyte slit diaphragm, of which nephrin is a key structural component, disrupts the glomerular filtration barrier[34]. Under diabetic conditions, hyperglycemia accelerates the internalization and loss of nephrin from the podocyte cell surface, thereby weakening the slit diaphragm and increasing albuminuria[35]. Consistent with previous studies, we observed that HG triggered excessive nephrin endocytosis by podocytes, and this was accompanied by disruption of the cytoskeleton and impaired cell adhesion, two hallmarks of podocyte injury. We also found that pharmacological blockage of nephrin internalization prevented most of these pathological changes. These results underscore the importance of nephrin endocytosis in driving podocyte damage under hyperglycemic conditions. This interpretation is supported by other studies which showed that prevention of nephrin trafficking ameliorated podocyte injury during diabetes. For example, genetic deletion of an endocytic adaptor (Cin85) prevented nephrin endocytosis and proteinuria in diabetic mice[36].

Multiple molecular pathways contribute to nephrin endocytosis during diabetes. For example, protein kinase C-α activation in a high-glucose milieu can trigger β-arrestin-2-dependent internalization of nephrin[37], and angiotensin II can enhance nephrin endocytosis *via* a β-arrestin-mediated mechanism[38]. Adaptor proteins, such as IQGAP1[39] and CIN85[36], and regulators of membrane curvature, such as PACSIN2[40], also contribute to nephrin retrieval from the podocyte surface during hyperglycemic conditions. However, upstream signals that connect the diabetic milieu to disruption of podocyte endocytosis remain incompletely understood. The present study identified Rab5, a small GTPase, as a critical regulator of nephrin endocytosis under hyperglycemic conditions. Rab5 has an established role as an essential controller of early endosomal trafficking[41]. We found that its activity was markedly increased in podocytes exposed to

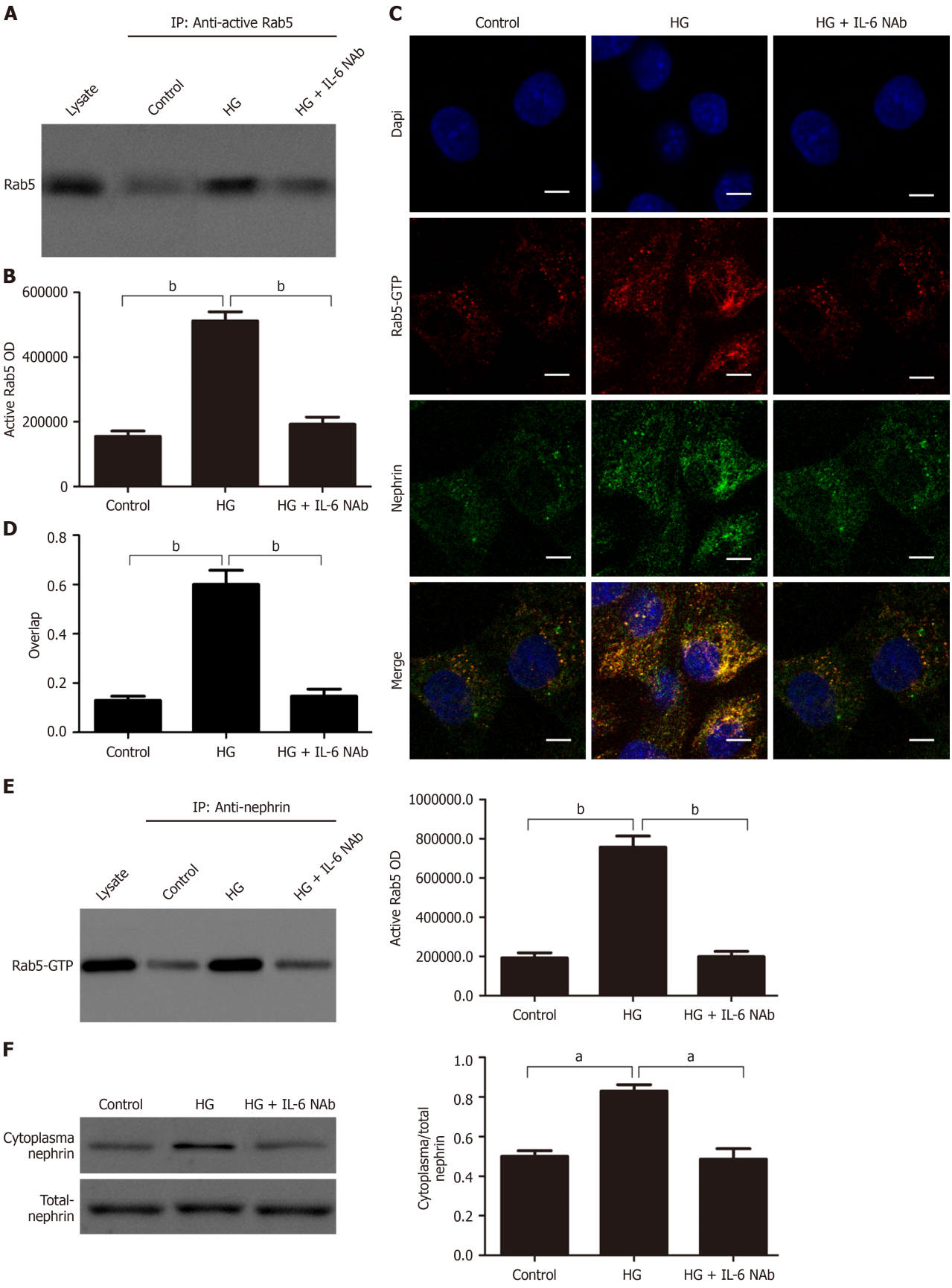


Figure 5 IL-6/Rab5 signaling mediates nephrin endocytosis in podocytes by promoting crosstalk between HK-2 cells and podocytes. A and B: Co-immunoprecipitation of active Rab5 (Rab5-GTP) in podocytes cultured in different conditioned media and quantification of these results (n = 3); C and D: Representative co-localization images and quantitative of nephrin (green) and active Rab5 (red) in podocytes grown in different conditioned media (magnification: × 630; scale bar: 20 μm); E: Co-immunoprecipitation of nephrin and active Rab5 in podocytes grown in different conditioned media and quantification of these results (n = 3; F: Western blot analysis of cytoplasmic nephrin (C-nephrin) and total nephrin (T-nephrin) after growth in different conditioned media and quantification of the C-

nephrin/T-nephrin ratio (n = 3). [a]P < 0.05; [b]P < 0.001. HG: High-dose glucose; NAb: Neutralizing Ab.

HG, and this enhanced nephrin internalization. We also showed that overactivation or overexpression of Rab5 exacerbated podocyte injury, whereas inhibition or dominant-negative suppression of Rab5 attenuated nephrin endocytosis and protected podocyte architecture. To our knowledge, this is the first demonstration that hyperglycemia-induced podocyte injury is mediated by Rab5-dependent nephrin trafficking. These findings expand our understanding of the pathogenesis of DN.

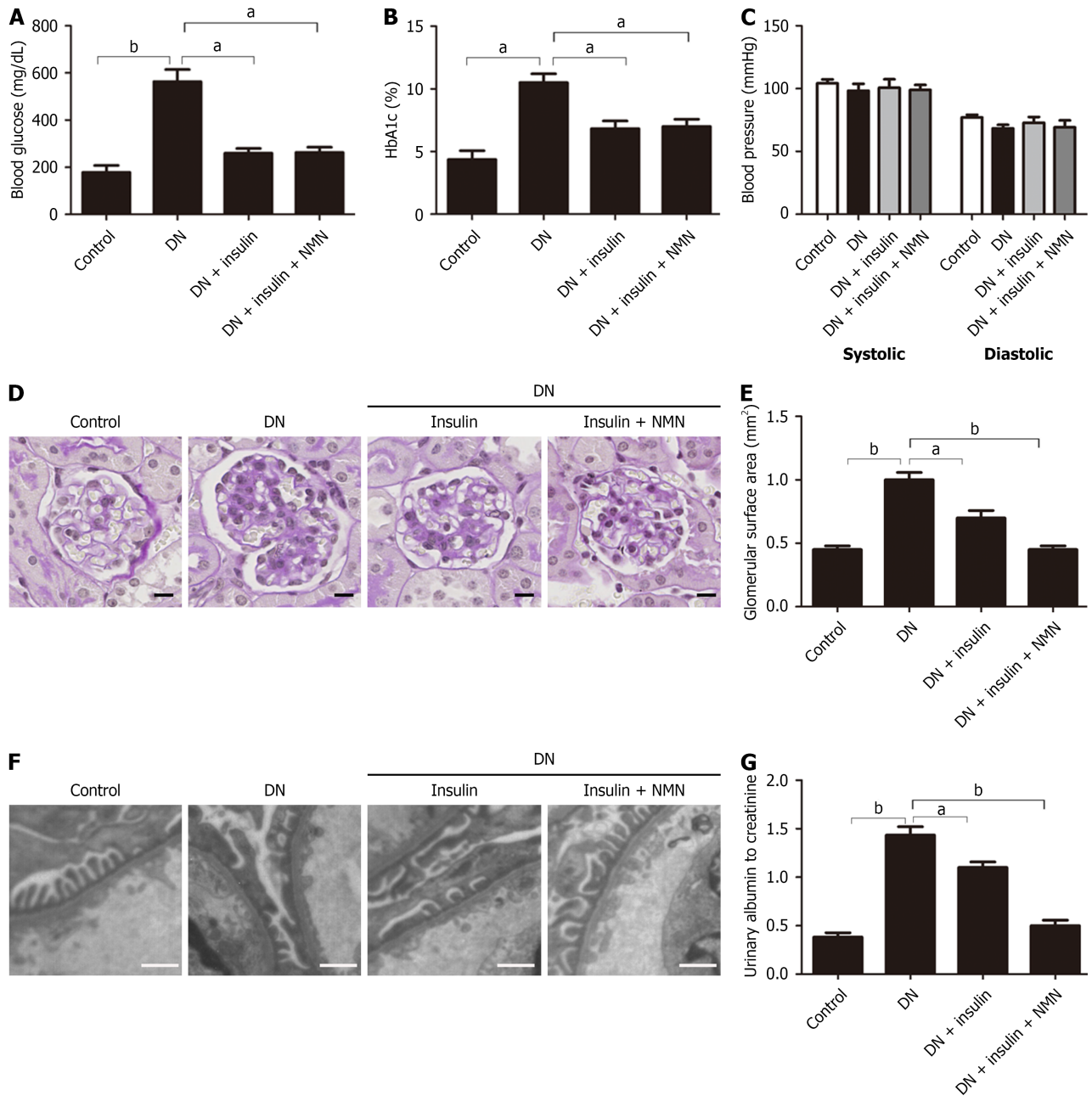
Another major finding of our study is the identification of IL-6 as a key mediator of crosstalk between PTECs and podocytes in DN. We demonstrated that HG induced the EMT in PTECs and their secretion of IL-6, creating a pro-inflammatory milieu. When podocytes were cultured in conditioned medium from HG-treated PTECs, they developed significant cytoskeletal abnormalities and loss of adhesion; neutralizing IL-6 substantially alleviated these changes. These results indicate that PTEC-derived IL-6 has an important role in driving podocyte dysfunction, and provide clear evidence for pathogenic crosstalk between PTECs and podocytes during DN. Although previous studies have emphasized the impact of podocyte injury and proteinuria on PTECs[33], we found that the secretion of IL-6 from stressed PTECs led to damage of podocytes. This mechanism is biologically plausible because the level of IL-6 is typically elevated in the diabetic kidney and IL-6 can also drive inflammation and cell injury in DN. Notably, renal resident cells, including PTECs, can produce IL-6 under hyperglycemic conditions[42], and podocytes express both subunits of the membrane-bound IL-6 receptor (IL-6Rα and gp130). Our results therefore establish IL-6 as a critical link in the intercellular crosstalk that contributes to kidney injury in DN.

We demonstrated that IL-6 signaling activated Rab5 in podocytes, based on the finding that IL-6 neutralization protected podocyte morphology and significantly reduced the level of active Rab5 in podocytes exposed to PTEC-conditioned medium. Blocking IL-6 signaling also attenuated the pathological interaction between nephrin and Rab5. Typically, IL-6 exerts its biological effects by binding to its receptor complex, specifically the membrane-bound gp130 subunit[43,44]. This leads to activation of the classical JAK/STAT3 signaling pathway, a critical inflammatory cascade implicated in various kidney injuries[45]. Activation of STAT3 downstream of gp130 could directly or indirectly influence Rab GTPase activity[46,47], thus providing a plausible mechanistic connection between IL-6 stimulation and enhanced Rab5-dependent nephrin endocytosis[48]. Consistent with this, Bhattacharya *et al*[48] previously demonstrated that IL-6 specifically upregulated Rab5, thereby altering endosomal trafficking pathways in other cell types through mechanisms that potentially include JAK/STAT signaling. Our findings extend these observations by suggesting this IL-6-mediated signaling cascade also occurs in the kidney, a novel pathway that links inflammatory signaling to structural damage of podocytes in DN.

Given the central role of IL-6/Rab5 signaling in DN, we investigated NMN as a potential therapeutic strategy to disrupt this crosstalk. NMN is a precursor of NAD^+ that enhances Sirt1 activity[49], and has broad protective effects in models of aging and metabolic diseases[50]. A previous study of kidney disease in diabetic mice showed that short-term administration of NMN conferred lasting reno-protective effects, improved the integrity of podocyte foot processes, and reduced albuminuria without affecting blood glucose or blood pressure[24]. Consistent with these previous findings, our study demonstrated that NMN treatment significantly prevented the damage of diabetic kidneys *in vivo*, in that NMN-treated diabetic mice had decreased expansion of the mesangial matrix, preservation of podocyte ultrastructure, and lower urinary albumin excretion. NMN effectively dampened the IL-6/Rab5 signaling cascade underlying the crosstalk of PTECs and podocytes. NMN also mitigated the HG-induced EMT and secretion of IL-6 in PTECs, decreased the level of active Rab5 in podocytes, inhibited the interaction of nephrin and Rab5, and prevented nephrin endocytosis. Although our study clearly identified Rab5 as a crucial mediator of nephrin endocytosis in podocytes under hyperglycemic conditions, we did not investigate specific downstream effectors, such as clathrin and caveolin-1. Both clathrin-dependent and caveolin-1-dependent pathways are critical regulators of endocytic processes[51,52], and future studies are needed to assess their roles in Rab5-mediated nephrin trafficking. Overall, NMN preserved the podocyte cytoskeleton, and maintained normal cell spreading and adhesion. These outcomes also occurred following blockage of IL-6 signaling. Notably, this mechanism aligns with previous reports that enhancement of Sirt1 activity in podocytes ameliorated diabetic kidney injury[25] as Sirt1 is a downstream effector of NAD^+. Sirt1 activation is known to suppress inflammatory cytokine production and attenuate the EMT in various models of renal injury. Thus, future experiments that use genetic knockdown or pharmacological inhibition of Sirt1 in PTECs could directly confirm whether the protective effects of NMN on IL-6 and EMT require Sirt1 activity. Elucidating this relationship will be critical for fully understanding the potential of NMN as a treatment for DN.

From a therapeutic perspective, our findings suggest that the IL-6/Rab5 axis represents an attractive adjunctive target in DN. Current standard treatments, including RAS inhibitors and SGLT2 inhibitors[53], primarily address systemic hemodynamic and metabolic stress but do not directly target the inflammatory crosstalk between kidney cells[54]. Although these approaches, together with endothelin blockade, have improved outcomes, substantial residual risk of disease progression remains, highlighting the need for additional strategies. IL-6 inhibition has already shown promise: Administration of an IL-6 receptor antibody (tocilizumab) ameliorated kidney injury in diabetic mice[55], and early clinical trials suggest that IL-6 blockade reduces inflammatory markers and proteinuria in kidney disease[56]. However, enthusiasm for systemic IL-6 inhibition has been tempered by safety concerns, including dyslipidemia and potential off-target immune effects.

In contrast, NMN offers a distinct mechanism of action. By restoring NAD^+/Sirt1 metabolism at the cellular level, NMN suppresses IL-6 production and disrupts downstream Rab5-mediated nephrin trafficking, thereby preserving podocyte integrity. This pathway is not specifically targeted by existing therapies such as SGLT2 inhibitors or GLP-1 RAs,


A
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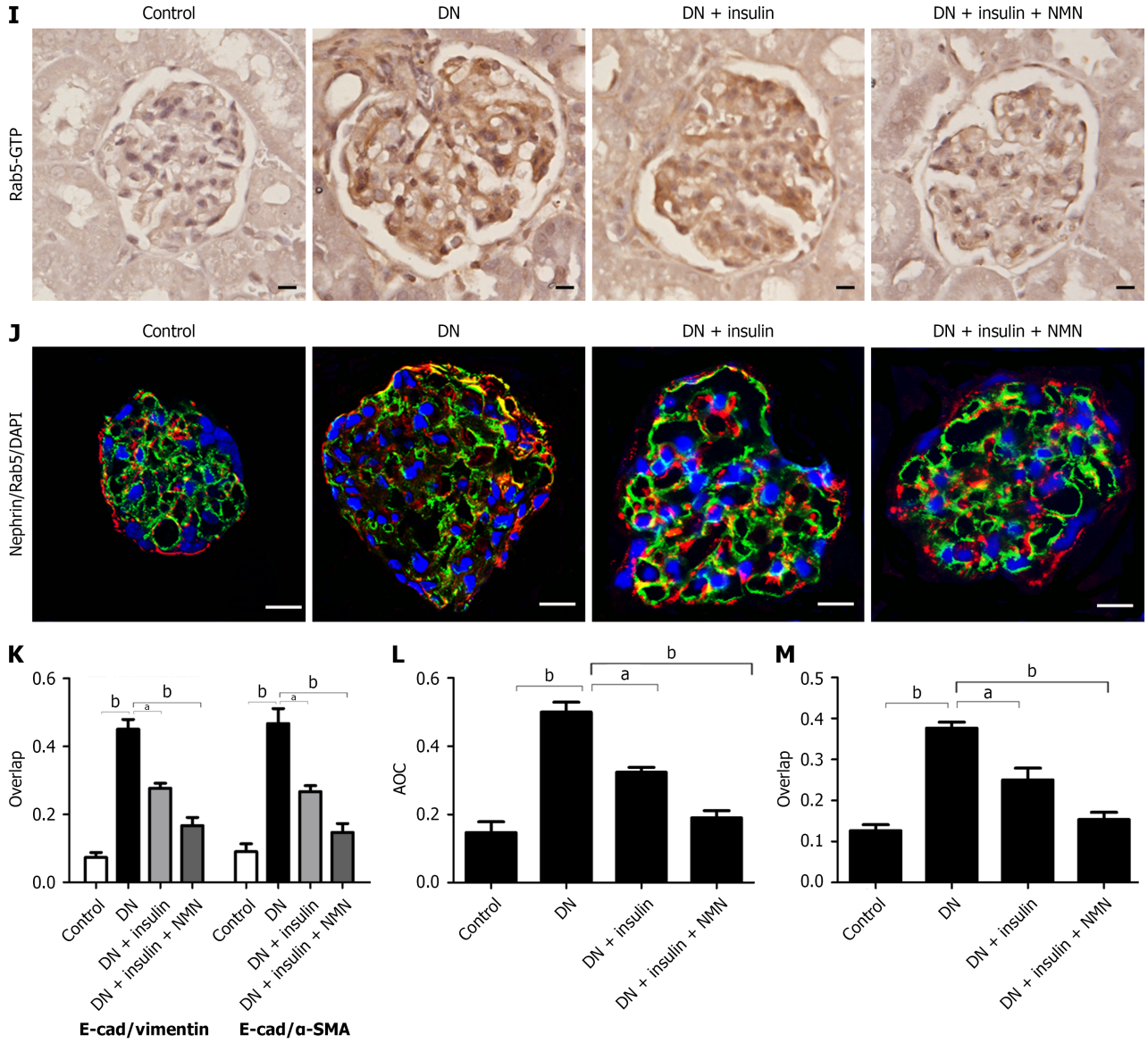

Figure 6 Nicotinamide mononucleotide alleviates podocyte injury in diabetic mice. A and B: Blood glucose and HbA1c levels in the different groups; C: Systolic and diastolic blood pressure in the different groups; D and E: Representative PAS staining (magnification × 400; scale bar: 50 μm) and quantification of the glomerular surface area in different groups (n = 10 glomeruli/group); F: Representative electron microscopy images of podocyte foot process effacement in the different groups (magnification × 10,000; scale bar: 100 μm); G: Urinary albumin excretion (albumin/creatinine ratio) in the different groups (n = 5); H and I: Co-localization images and quantification of the epithelial-to-mesenchymal transition, indicated by altered E-cadherin and α-SMA expression, in the different groups (magnification × 630; scale bar: 20 μm); J and K: Immunohistochemical staining and quantification of glomerular expression of Rab5-GTP in the different groups (magnification × 400; scale bar: 20 μm); L and M: Immunofluorescence co-localization and quantification of nephrin and active Rab5 (Rab5-GTP) in the different groups (magnification × 630; scale bar: 20 μm). Data are expressed as mean ± SEM. $^{a}P < 0.05$; $^{b}P < 0.001$. DN: Diabetic nephropathy; NMN: Nicotinamide mononucleotide; AOC: Area of coverage.

which mainly act through metabolic and hemodynamic mechanisms. Accordingly, NMN could serve as a complementary therapy to current DN treatments, with the potential for additive or synergistic renoprotective effects. Future experimental and clinical studies should evaluate the efficacy, safety, and combinatorial benefits of NMN with established agents such as SGLT2 inhibitors or GLP-1 RAs. Taken together, our findings position NMN as a promising and potentially safer alternative or adjunct for dampening IL-6-driven inflammation and its downstream consequences in DN.

However, our study has several limitations. First, the use of cell lines (HK-2 PTECs and immortalized podocytes) might not fully recapitulate DN in humans. Validation using primary human cells or organoid models could provide additional translational relevance. Another notable limitation of our study is the use of the STZ-induced diabetic model, which closely mimics type 1 DM but differs in several important respects from the pathophysiology observed in DN that in patients with type 2 DM (T2DM), the predominant clinical form. Indeed, type 2 DN is typically associated with obesity, insulin resistance, and a distinct inflammatory and metabolic profile. Previous studies have demonstrated renoprotective effects of NMN administration in T2DM models, including db/db mice, which are characterized by obesity, insulin resistance, and marked kidney damage[23,24]. Thus, our findings, alongside these prior results, strongly support the potential broader applicability and therapeutic relevance of NMN for treatment of type 1 and type 2 DN. Future studies

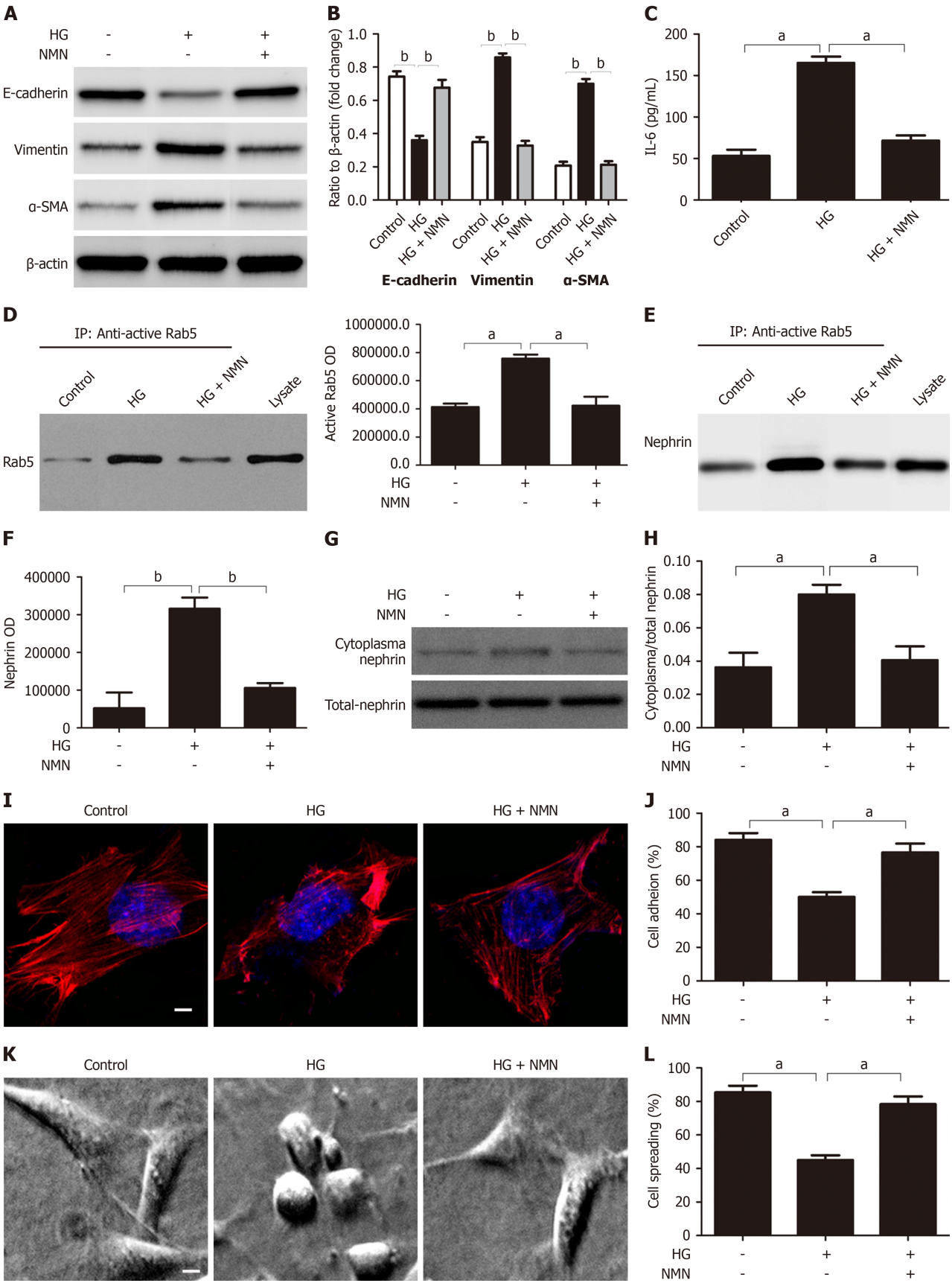

Figure 7 Nicotinamide mononucleotide protection depends on crosstalk between proximal tubular epithelial cell and podocytes. A and B: Western blot analysis of epithelial-to-mesenchymal transition markers in HK-2 cells that received different treatments and quantification of these results (n = 3); C: IL-6 secretion by HK-2 cells that received different treatments (n = 3); D: Co-immunoprecipitation of active Rab5 in podocytes cultured in different conditioned media and quantification of these results; E and F: Co-immunoprecipitation of nephrin and active Rab5 in podocytes cultured in different conditioned media and quantification of the results (n = 3); G and H: Western blot analysis of cytoplasmic nephrin (C-nephrin) and total nephrin (T-nephrin) from podocytes cultured in different conditioned

media and quantification of C-nephrin/T-nephrin ratio (n = 3); I: Representative confocal microscopy images of podocytes cultured in different conditioned media (magnification: × 1000; scale bar: 50 μm; red: Cytoskeleton; blue: Nuclei); J: Quantification of podocyte adhesion after culture in different conditioned media (n = 3); K: Representative electron microscopy images of podocyte spreading after culture in different conditioned media (magnification: × 400; scale bar: 20 μm); L: Quantitative analysis of podocyte spreading after culture in different conditioned media (n = 3). $^{a}P < 0.05$; $^{b}P < 0.001$. HG: High-dose glucose; NMN: Nicotinamide mononucleotide.

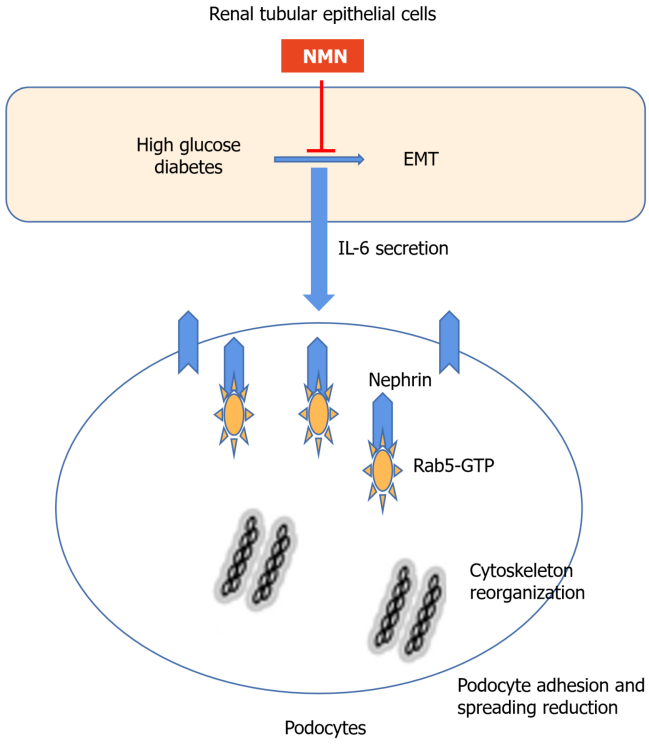

Figure 8 Possible mechanism of crosstalk between proximal tubular epithelial cells and podocytes and effect of nicotinamide mononucleotide during diabetic nephropathy. High-dose glucose induces the epithelial-mesenchymal transition and secretion of IL-6 by proximal tubular epithelial cells (PTECs). Podocytes sense this IL-6, and this leads to the binding of internalized nephrin with active Rab5, followed by disruptions of the cytoskeleton, podocyte adhesion, and podocyte spreading. nicotinamide mononucleotide blocks signaling from the PTECs, decreases the binding of nephrin with active Rab5, and ameliorates cellular damage. EMT: Epithelial-mesenchymal transition; NMN: Nicotinamide mononucleotide.

employing T2DM models are warranted to more precisely define the clinical applicability of NMN. Moreover, although our study specifically highlights IL-6, other cytokines such as TNF-α and TGF-β also play significant roles in the inflammatory milieu that contributes to DN. In fact, TNF-α and TGF-β signaling can modulate renal inflammation and fibrosis [57-59], suggesting that a broader inflammatory network may interact with IL-6/Rab5 signaling in the pathogenesis of DN. Future studies that examine these other cytokines in conjunction with IL-6 will help clarify their possible interactions.

CONCLUSION

In summary, our results identified a novel mechanism that underlies the injury of podocytes during DN, in which IL-6 released by PTECs activates Rab5 in podocytes, which then induces nephrin endocytosis. We also found that NMN can effectively protect against DN. These findings highlight the IL-6/Rab5 axis as an additional therapeutic target and suggest that metabolic supplementation (NMN) could be a simple and viable strategy to halt or slow the progression of DN when used with existing treatments.

FOOTNOTES

Author contributions: Gao P and Wu XY contribute equally to this study as co-corresponding authors; Zha DQ performed experimental work, data collection, and statistical analyses, and drafted the manuscript; Gao P designed and supervised the study, interpreted data, and critically revised the manuscript; Wu XY contributed to study conception, provided technical guidance, analyzed the experimental results, and critically revised the manuscript; all authors read and approved of the final manuscript.

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Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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L-Editor: A
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