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Research paper

Knockdown of *NUP160* inhibits cell proliferation, induces apoptosis, autophagy and cell migration, and alters the expression and localization of podocyte associated molecules in mouse podocytes

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ABSTRACT

Genetic mutations in dozens of monogenic genes can lead to serious podocyte dysfunction, which is a major cause of steroid-resistant nephrotic syndrome (SRNS). The *NUP160* gene is expressed in both human kidney and mouse kidney. However, whether knockdown of *NUP160* impairs podocytes has not yet been established. Therefore, we knocked down *NUP160* by targeted short hairpin RNA (shRNA) in conditionally immortalized mouse podocytes and observed the effect of *NUP160* knockdown on the proliferation, apoptosis, autophagy and cell migration of podocytes. We also investigated the effect of *NUP160* knockdown on the expression and localization of podocyte associated molecules, such as nephrin, podocin, CD2AP and α-actinin-4. The knockdown of *NUP160* significantly inhibited the proliferation of podocytes by decreasing the expression of both cyclin D1 and CDK4, increasing the expression of p27, and inducing S phase arrest. The knockdown of *NUP160* promoted the apoptosis and autophagy of podocytes, and enhanced cell migration. The knockdown of *NUP160* decreased the expression of nephrin, podocin and CD2AP in podocytes, and increased the expression of α-actinin-4. The knockdown of *NUP160* also altered the subcellular localization of nephrin, podocin and CD2AP in podocytes. These results suggest that the knockdown of *NUP160* impairs mouse podocytes, i.e. inhibiting cell proliferation, inducing apoptosis, autophagy and cell migration of mouse podocytes, and altering the expression and localization of podocyte associated molecules, including nephrin, podocin, CD2AP and α-actinin-4.

1. Introduction

Nephrotic syndrome is a renal disease caused by disruption of the glomerular filtration barrier, resulting in massive proteinuria, hypoalbuminemia, hyperlipidemia and edema (Wiggins, 2007). According to its response to steroid therapy, nephrotic syndrome is classified into steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS) categories. SRNS has an immunological or genetic etiology (Trautmann et al., 2015). With the development of genetics and molecular biology, the contribution of genetic factors is increasingly emphasized in the pathogenesis of SRNS. To date, > 50 monogenic genes have been discovered to cause SRNS when mutated (Lovric et al., 2016). These include genes encoding slit diaphragm constituting molecules, such as *NPHS1*, *NPHS2*, *CD2AP*, genes encoding the actin cytoskeleton, such as *ACTN4*, *INF2*, *MYO1E*, genes encoding actin-regulating small GTPases of the Rho/Rac/Cdc42 family, such as *ARHGDIA*, *ARHGAP24*, *KANK*, and genes encoding nucleoporins, such as *NUP93*, *NUP107* and *NUP205*, among others. Mutations in *NPHS1*, *NPHS2* and *CD2AP* disrupt the integrity of the slit diaphragm and lead to congenital nephrotic syndrome, early onset autosomal recessive SRNS and early onset focal segmental glomerulosclerosis (FSGS), respectively (Kestilä et al., 1998; Boute et al., 2000; Löwik et al., 2007). Mutations in *ACTN4* change the cytoskeletal dynamics of podocytes and lead to adult onset autosomal dominant FSGS (Kaplan et al., 2000). Mutations in *ARHGDIA* change the migration ability of podocytes and lead to early onset SRNS (Gee et al., 2013). Mutations in *NUP93* inhibit podocyte proliferation and promote the apoptosis of podocytes and lead to early-childhood-onset SRNS (Braun et al., 2016). Mutations in

Abbreviations: CCK-8, Cell Counting Kit-8; FBS, fetal bovine serum; FSGS, focal segmental glomerulosclerosis; HRP, horseradish peroxidase; IFN-γ, interferon-γ; LSD, least significant difference; Nup160, nucleoporin 160 kDa; OD, optical density; PARP, poly (ADP-ribosyl) transferase; PI, Propidium iodide; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome

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NUP107 cause hypoplastic glomerulus structures and abnormal podocyte foot processes and lead to early-childhood-onset SRNS (Miyake et al., 2015).

The identification of monogenic causes of SRNS revealed that podocytes are a critical site in the pathogenesis of SRNS. Notably, all the SRNS related genes are localized to podocytes, and loss of their functions inescapably leads to podocyte dysfunction (Lovric et al., 2016). The *NUP160* gene maps to chromosome 11p, encoding nucleoporin 160 kDa (Nup160). The expression of Nup160 in both human kidney and mouse kidney was reported (https://proteomescout.wustl.edu/proteins/). However, whether the knockdown of *NUP160* impairs podocytes has not been established.

In this study, we knocked down *NUP160* by targeted short hairpin RNA (shRNA) in conditionally immortalized mouse podocytes, and observed the effect on the proliferation, apoptosis, autophagy and cell migration of podocytes via in vitro functional assays. We also investigated the effect of *NUP160* knockdown on the expression and localization of podocyte associated molecules including nephrin, podocin, CD2AP and α-actinin-4 by quantitative real-time polymerase chain reaction (qRT-PCR), western blotting and immunofluorescence assay.

2. Methods

2.1. Cell culture

Conditionally immortalized mouse podocytes were a gift from Prof. Peter Mundel (Department of Medicine, Mount Sinai School of Medicine, New York, USA) and were cultured as previously described (Mundel et al., 1997). Briefly, the conditionally immortalized mouse podocyte clone was cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin/streptomycin and 10 U/ml mouse interferon-γ (IFN-γ) at 33 °C (the permissive condition). To induce differentiation, podocytes were reseeded and cultured at 37 °C in six-well plates and 25 cm^2-flasks coated with 10 mg/ml of collagen type I after removal of IFN-γ, for at least 14 days (non-permissive condition).

2.2. Designing shRNA oligos

We employed the replication-incompetent lentiviral vector pLKO.1 (Sigma, St. Louis, MO, USA) chosen by the RNAi Consortium (TRC) for cloning and expressing shRNA sequences. The coding sequences of *Mus musculus NUP160* mRNA (NM_021521) were searched for the interference target sites. The potential targets were oligonucleotide sequences starting with A or G as the transcription initiation point of the U6 promoter followed by 20 nucleotides, with a stretch of more than four Ts as the terminal signal for RNA pol III. By BLAST search, we chose three potential interference targets. Two cohesive cloning sites of *Age*I and *Eco*RI were designed at the ends of the forward and reverse insert template with a loop sequence (CTCGAG). Table 1 shows the list of target sequences.

Table 1
Sequences of *NUP160*-shRNAs.

	Sense	Anti-sense
NUP160 shRNA-1	5′-CCGGCCCTATGTGAATCTG CATAATCTCGAGATTATGC AGATTCACATAGGGTTTTTG-3′	5′-AATTCAAAAACCCTATGTG AATCTGCATAATCTCGAGA TTATGCAGATTCACATAGGG-3′
NUP160 shRNA-2	5′-CCGGTGATCTTGCAGCAGC TATTAACTCGAGTTAATAG CTGCTGCAAGATCATTTTTG-3′	5′-AATTCAAAAATGATCTTGC AGCAGCTATTAACTCGAGT TAATAGCTGCTGCAAGATCA-3′
NUP160 shRNA-3	5′-CCGGCGGCAAATTGAAATT CTGGAACTCGAGTTCCAGA ATTTCAATTTGCCGTTTTTG-3′	5′-AATTCAAAAACGGCAAATT GAAATTCTGGAACTCGAGT TCCAGAATTTCAATTTGCCG-3′

2.3. Construction of recombinant RNA interference plasmids

The pLKO.1-TRC cloning vector contains a 1.9 kb stuffer that is released upon digestion with *Eco*RI and *Age*I. The forward and reverse oligonucleotides were annealed, and the resulting double stranded DNA was ligated into pLKO.1 digested with *Eco*RI and *Age*I. Then, *Escherichia coli* Stlb3 competent cells were transformed with the above ligation products, and spread on LB agar plates containing 100 μg/ml ampicillin. After confirming the presence of the shRNA insert by digestion with *Eco*RI and *Nco*I, and further verified by sequencing with pLKO.1 sequencing primers, the recombinant plasmids were prepared with E.Z.N.A.® Plasmid Mini Kit II (Omega Bio-Tek, Norcross, GA, USA).

2.4. Production of lentivirus particles

To produce lentivirus, pLKO.1 shRNA plasmid, packaging plasmids psPAX2 and envelope plasmid pMD2.G were co-transfected into HEK293T cells using PEI in accordance with the standard procedure. After 12 h, the culture media were replaced with 5 ml fresh DMEM/F12 containing 10% FBS and penicillin/streptomycin. After 24 and 48 h, the supernatant was harvested, centrifuged to remove cellular debris, and filtered. The pLKO.1-shC002 vector (Sigma) was used as the control plasmid.

2.5. NUP160 knockdown in mouse podocytes

To achieve stable knockdown of *NUP160* in mouse podocytes, the lentivirus containing media was added to mouse podocytes. After 24 h, the virus-infected cells were selected with puromycin (2 μg/ml) for an additional 10 days to establish sublines of mouse podocytes with stable knockdown of *NUP160*.

2.6. qRT-PCR

Total RNA was extracted from cells using the total RNA kit (Omega Bio-Tek) following the manufacturer's protocol. Then, cDNA was obtained by reverse transcription (HiScript® Q RT SuperMix for qPCR; Vazyme, Nanjing, China). qRT-PCR was performed with an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Roche, Mannheim, Germany). The primer sequences used for qRT-PCR analysis are listed in Table 2. The relative amount of mRNA of each gene in each sample was calculated using the $2^{-\Delta\Delta CT}$ method. The expression of GAPDH was used as an internal control.

2.7. Western blotting

Cells were lysed with RIPA lysis buffer with protease and phosphatase inhibitors. Total proteins were quantified using the bicinchoninic acid (Pierce, Rockford, AL, USA) method. After denaturation, samples were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with TBST containing 4% BSA at room temperature for 1 h. Then, the membranes were incubated with primary antibodies at 4 °C overnight. Subsequently, the membranes were incubated with secondary antibodies for 1 h at room temperature. The membranes were then developed with WesternBright ECL (Advansta, Menlo Park, CA, USA) for imaging. Image lab software was used to acquire and analyze imaging signals. Both primary and secondary antibodies for western blotting were as follow: rabbit anti-NUP160 mAb (1:1000, ab74147, Abcam, Cambridge, MA, USA), rabbit anti-cyclin D1 mAb (1:200, SP4, ab16663, Abcam), rabbit anti-CDK4 mAb (1:2000, EPR17525, ab199728, Abcam), mouse anti-p27 mAb (1 μg/ml, SX53G8, ab193379, Abcam), rabbit anti-Caspase3 mAb (1:1000, 8G10, 9665, Cell Signaling Technology, Boston, MA, USA), rabbit anti-cleaved PARP mAb (Asp214) (1:1000, D6X6X, 94885, Cell