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Original Article

Detection of podocyte proliferation in rat nephropathy models by retrovirus-EGFP tracing technology

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Abstract: Podocyte depletion is a pivotal pathogenesis for many types of glomerulonephritis. There is expanding literatures suggests that the podocyte injury and loss may be replenished by podocyte regeneration or by transferring from the parietal progenitor cell proliferation. But the mechanism underlying podocyte responding to injury and repair in diverse glomerulonephritis is still not fully understood. The lack of an effective method to find direct evidence of podocyte division *in vivo* is a limitation for the research. In this study, we used retrovirus-enhanced green fluorescent protein tracing technology to capture the proliferated podocytes immediately after mitosis in rat adriamycin nephropathy and passive Heymann nephritis models. The proliferated podocytes in glomeruli were captured by detecting GFP as well as BrdU via immunohistochemical staining. GFP and BrdU positive cells were detected by immunohistochemical staining, which overlaps the WT1 positive, in glomeruli of rat adriamycin nephropathy and passive Heymann nephritis models. These results indicate damaged podocyte is able to proliferate in rat glomerulonephritis models, and pMSCV-EGFP tracing technology is a significant method for further studying of podocytes injury and repair in glomerulonephritis.

Keywords: Podocyte injury, podocyte proliferation, rat nephrosis, retrovirus vector

Introduction

Podocyte, a component of the glomerular filtration membrane, is susceptible to injury. Podocyte depletion, which is a pivotal factor in the pathogenesis of a majority of glomerular diseases, results in the destruction of the glomerular filtration barrier leading to proteinuria [1-3]. The study of podocyte injury is a hotspot recently in the field of kidney disease. There are many literatures about the podocyte injury and depletion in glomerulonephritis which may be replenished by podocyte regeneration in HIV-associated nephropathy and idiopathic collapsing glomerulopathy [4, 5], or be compensated by transferring from the parietal progenitor cell of the glomerulus capsule wall [6, 7]. However, because the experimental method to observe podocyte growth in vivo is limited, lack of direct evidence of podocyte division in most common glomerulonephritis, the mechanism underlying podocyte injury and repair in diverse glomerulonephritis is still not fully understood. It is significant, therefore, to develop a method in vivo experiment to provide more convincing evidence of the proliferation of podocytes in common glomerulonephritis.

In this study, a novel retrovirus-enhanced green fluorescent protein (pMSCV-EGFP) tracing technology was used to detect the proliferative podocytes in two rat nephropathy models, including cytotoxic nephrosis induced by adriamycin (ADR) and immune-mediated models of passive Heymann nephritis (PHN).

pMSCV-EGFP is constructed from an oncovirus subfamily of retroviruses characterized by the dependence on host cell proliferation for completion of the viral life cycle. The viral DNA does

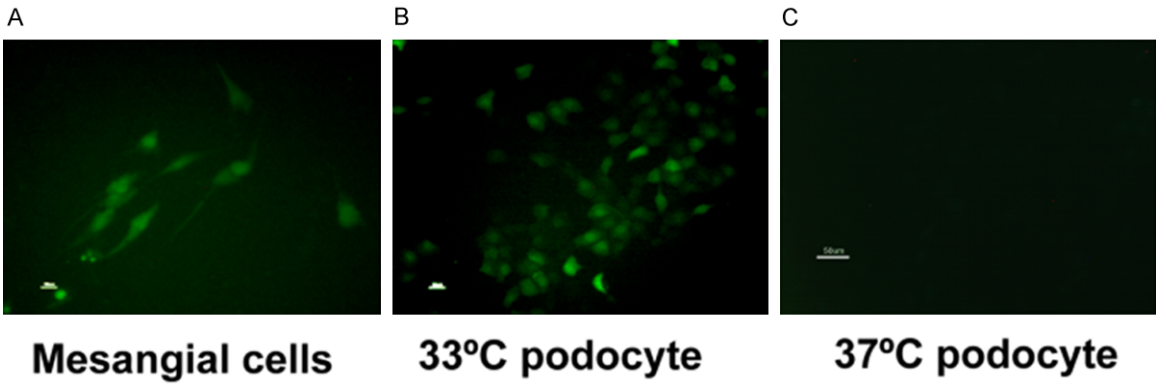

Figure 1. The effect of retrovirus vector infection on proliferated cells *in vitro*. A. Mesangial cells cultured by the pMSCV-EGFP conditioned medium for 48 h, GFP positive cells were observed by fluorescence microscope; B. Dedifferentiated podocytes under 33°C cultured by the pMSCV-EGFP conditioned medium for 48 h, GFP positive cells were observed by fluorescence microscope; C. Differentiated podocytes under 37°C cultured by the pMSCV-EGFP conditioned medium for 48 h, GFP positive cells were observed by fluorescence microscope. Scale bars represent 50 μm.

not enter the nucleus until mitosis [8] and productive infection by the retrovirus can be prevented in the absence of host cell proliferation [9, 10]. It has been used to study neurogenesis in the olfactory bulb of adult mice after selective impairment of dopaminergic neurons [11] and in the hippocampus of adult Mongolian gerbils with transient global ischemia [12]. In slices of ischemic rat brain, the GFP positive neurons were co-positive for markers of gamma-aminobutyric acid (GABA) and cholinergic neurons, proving proliferative neurons can differentiate into functional cells [13]. Therefore, a retroviral vector carrying GFP has been showed to be an ideal marker for cell division in experiments *in vivo* [11-16].

We injected pMSCV-EGFP via tail veins into rat models of ADR-induced nephrosis and PHN to capture the proliferated podocytes via the detection of GFP by immunohistochemical staining. In addition, the BrdU was also detected by immunohistochemical staining. The results indicated that certain damaged podocytes have a capacity for regeneration with GFP positive in rat glomerulonephritis. Our work demonstrated that retrovirus-enhanced green fluorescent protein (pMSCV-EGFP) tracing technology is a significant method for further studying podocytes injury and repair in glomerulonephritis *in vivo*.

Materials and methods

Retrovirus vector transduction

The retrovirus production protocol was as described [14]. Briefly, Platinum-E (Plat-E; Cell Biolabs Inc., San Diego, CA, USA) cells, a potent retrovirus packaging cell line [17], were prepared at 80% confluence then transfected with pMSCV-EGFP (a gift from Dr Huang, State Key Laboratory of Medical Neurobiology of Fudan University, Shanghai, China) using Lipofectamine®2000 (Invitrogen, Carlsbad, CA, USA). The supernatant containing the retrovirus was collected at 48 hour post transfection, centrifuged and filtered, and the final pellet was suspended and kept at -80°C as described [13].

Animal models

ADR-induced nephrosis: In total, 24 male Sprague Dawley rats received left unilateral nephrectomy under sodium pentobarbital (40 mg/kg intraperitoneally) anesthesia. The rats were divided randomly into two groups; 6 rats were assigned to the control group and the other 18 rats constituted the ADR nephrosis group. One week later, ADR (Haizheng Co. Zhejiang, China) dissolved in 0.9% (w/v) NaCl was injected i.v. (5 mg/kg body weight) into rats of the ADR nephrosis group (twice with an interval of 7 days), and the control rats received the same volume of 0.9% (w/v) NaCl. At weeks 2, 5 and 10 following the second injection, the rats were anesthetized and sacrificed. At 48 hours before sacrifice, pMSCV-EGFP conditioned medium was injected i.v. (5 ml/kg body weight) into rats of the ADR nephrosis group and the control group by single injection into the tail. 24 hours later, BrdU (Sigma, MO, USA) dissolved in 0.9% NaCl (50 mg/kg body weight) was in-

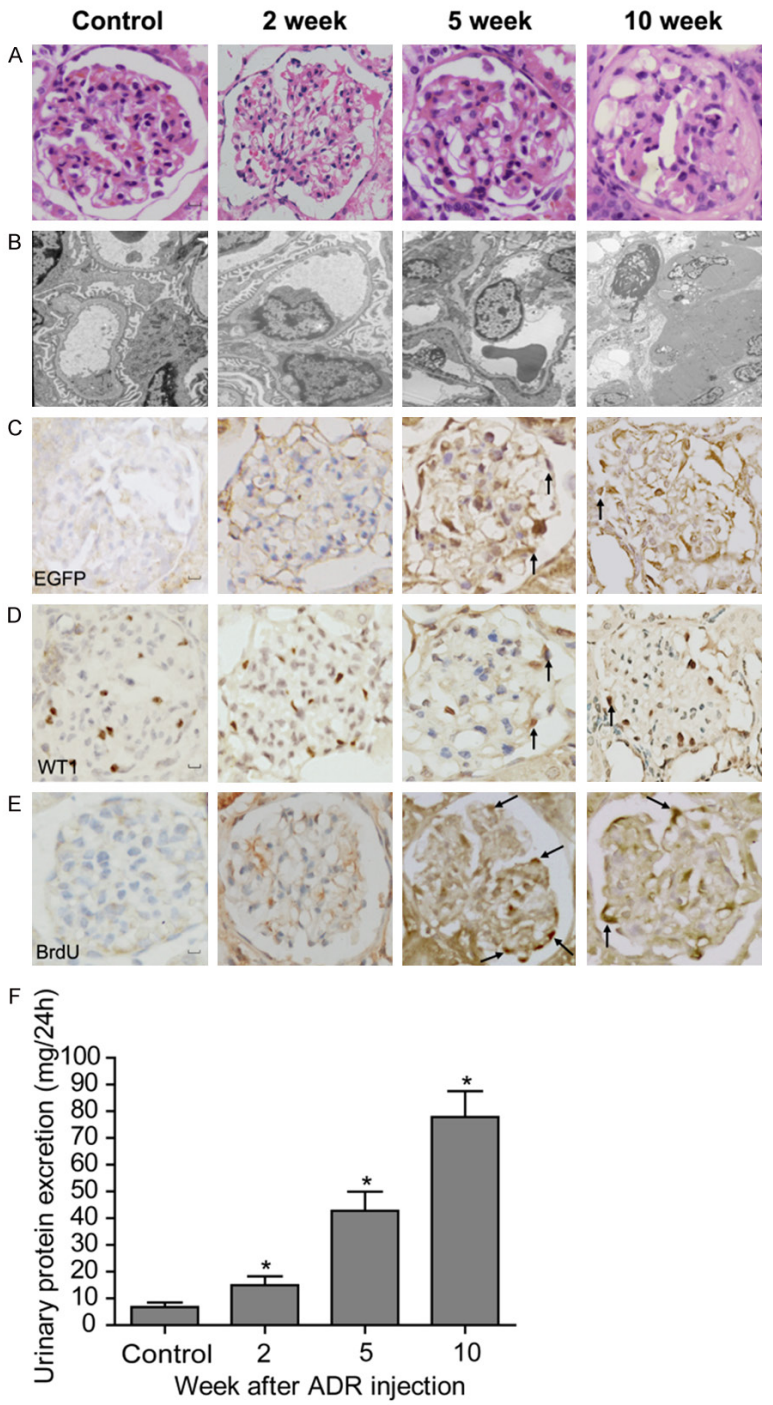

Figure 2. Proliferated podocytes detected in adriamycin (ADR)-induced nephrosis. A. H&E-staining of glomeruli from ADR nephrosis; B. Electron microscopy of glomeruli from ADR nephrosis; C. Immunostaining for enhanced green fluorescent protein (GFP) in ADR nephrosis; D. Immunostaining for the podocyte-restricted markers WT1 on successive sections for C. Black arrows (↑), both WT1-positive and GFP-positive cells. E. Immunostaining for 5-bromo-2-deoxyuridine(BrdU) in ADR nephrosis; F. The 24 hours proteinuria levels in different groups of ADR-induced rat models, $*P < 0.05$ 2 week, 5 week or 10 week versus control (n = 6), the values are mean ± SD and paired means were analyzed using Student's two-tailed *t*-test. Scale bars represent 50 μm.

traperitoneally injected into the ADR nephrosis group and the control group [15]. Urine samples were collected during the next 24 hours. Urine protein excretion was assayed by Labway Clinical Laboratory (Shanghai, China). The kidney tissues were prepared for the measurement of protein and for pathology and immunohistochemical studies analysis.

The PHN model: The PHN model of experimental membranous nephropathy was induced by single i.v. injection of rabbit anti-Fx1A antibody (5 ml/kg body weight) into the tail as described [18, 19]. Each of the 6 control rats was injected with normal rabbit serum. Six rats of the PHN group were sacrificed under anesthesia on days 5, 8 and 12. The pMSCV-EGFP injection, urine collection and kidney tissue procedures were as described above.

The study protocol was approved by the Animal Experiment Ethics Committee of Shanghai Medical College, Fudan University (reference number 20100223). All experimental procedures were in accord with the approved guidelines of Shanghai Medical College, Fudan University.

Immunohistochemical staining

The deparaffinized kidney sections (4 μm thick) from ADR-induced nephrosis and PHN rats were boiled in a microwave oven in 0.3% (v/v) H_2O_2 in 10 mM sodium citrate buffer (pH 6.1). The sections

were then probed with primary antibodies against green fluorescent protein (GFP; 1:100, Cell Signaling Technology, Danvers, MA, USA), BrdU (1:100, Santa Cruz); WT1 (1:50, Changdao Bio Co., Shanghai, China) for 1 hour at 37°C and overnight at 4°C, and biotinylated secondary antibodies (1 hour at 37°C). 3,3'-Diaminobenzidine was used as the chromogen and hematoxylin was used as the nuclear counterstain. The primary antibody was replaced by phosphate-buffered saline as the negative control.

The number of podocytes per sectional glomerulus was determined by counting the number of WT-1 expressing cells in each glomerulus from paraffin sections. Each slide (20 glomeruli per section and n = 6 per group) were observed independently by three investigators blinded to the identity of the paraffin section [20]. The mean rate of EGFP positive podocyte number to podocytes numbers per sectional glomerulus was determined for each group.

Cell culture and treatment

Plat-E (a gift from Dr Yu, Laboratory of Molecular Medicine, School of Life Sciences, Fudan University, Shanghai, China) was cultured as described [17] and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% (v/v) fetal bovine serum containing 10 mg/ml blasticidin (Sigma) and 1 mg/ml puromycin (Sigma) at 37°C.

Conditionally immortalized mouse podocyte 5 (MPC5) was cultured as described [21]. Briefly, MPC-5 cells were maintained under growth-permissive conditions at 33°C and 5% (v/v) CO_2 in RPMI-1640 medium (Gibco, Santa Ana, CA, USA) with 10% (v/v) fetal bovine serum, and 50-10 U/ml IFN-γ (Sigma) were used to maintain MPC-5 cells in proliferating condition. To induce differentiation, podocytes were shifted to non-permissive conditions at 37°C without IFN-γ for 14 days.

Mesangial cells were maintained in DMEM (Gibco) with 10% (v/v) fetal bovine serum containing 5% CO_2 at 37°C.

Podocytes and mesangial cells were infected by retrovirus vector via cultured by pMSCV-EGFP conditioned medium for 48 h, and then, GFP positive cells were observed by fluorescence microscope.

Statistical analysis

All of the statistical analyses used SPSS software and the values are expressed as mean ± SD and paired means were analyzed using Student's two-tailed *t*-test. Statistically significant difference was set at $P \leq 0.05$, and highly significant difference was set at $P \leq 0.01$.

Results

Effect of retrovirus vector infection on proliferated cells in vitro

To determine the effect of retrovirus vector infection, the pMSCV-EGFP conditioned medium was used to infect cultured mesangial cells and podocytes *in vitro*. The result showed that some mesangial cells and dedifferentiated podocytes (MPC5) under 33°C were positive after infection by retrovirus, but the mature podocytes under 37°C was negative (**Figure 1A-C**), which indicate that the pMSCV-EGFP conditioned medium is effective to infect proliferated podocytes.

Detection of GFP and BrdU positive podocytes in rat ADR-induced nephrosis

Under light microscopy, the pathologic changes of rat nephrotic models were observed after ADR treatment. Glomeruli were nearly normal in the ADR nephrosis group at 2 weeks, with only some tubular proteinaceous casts. At 5 weeks, the mesangial matrix increased slightly in glomeruli as a minimal change disease. At 10 weeks, the change of focal segmental glomerulosclerosis was observed, with a portion of global sclerosis, severe tubular damage and proteinaceous casts (**Figure 2A**). Electron microscopy showed a few effacements of foot processes in glomerular capillary loops at 2 weeks, diffuse effacements of processes at 5 weeks and focal hyalinization of glomeruli at 10 weeks (**Figure 2B**). Urine was collected for 24 hours, allowing calculation of urinary protein excretion/24 h. Proteinuria increased significantly with time in ADR-induced nephrosis rats (**Figure 2F**, $P < 0.05$).

The immunohistochemical results showed cells in glomeruli were GFP negative at 2 weeks, more cells were GFP positive at 5 weeks, and less at 10 weeks. Moreover, the GFP positive cells were distributed primarily at the periphery of the glomeruli capillary loop and overlapped

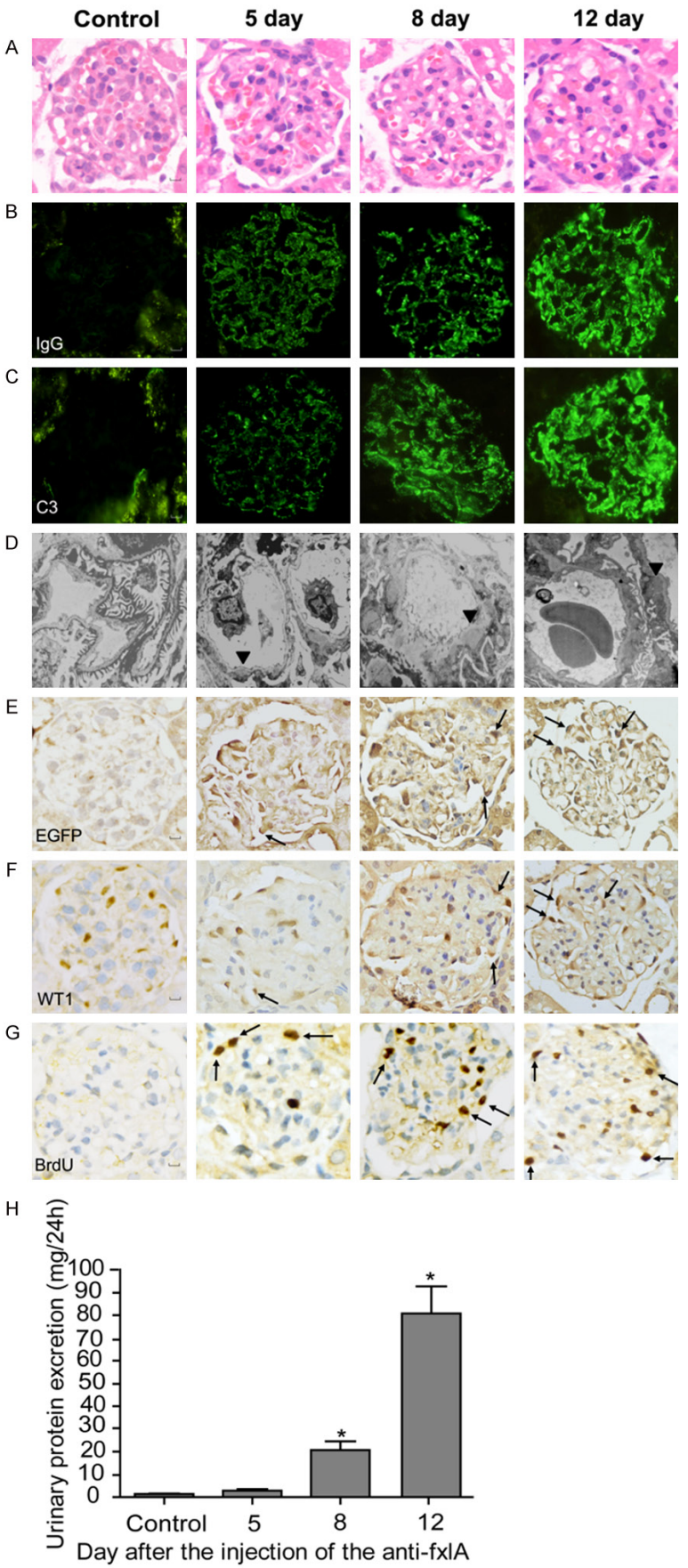

Figure 3. Proliferated podocytes detected in passive Heymann nephritis (PHN). A. H&E-staining of glomeruli from PHN groups; B. Immunofluorescence for IgG in PHN groups; C. Immunofluorescence for C3 in PHN groups; D. Electron microscopy of glomeruli from PHN groups. Filled triangle (▲), the electron dense deposits. E. Immunostaining for GFP in PHN groups; F. Immunostaining for WT1 on successive sections for E. Black arrows (↑), both WT1-positive and GFP-positive cells. G. Immunostaining for BrdU in PHN groups; H. The 24 hours proteinuria levels in different groups of PHN rat models, $*P < 0.05$ 8 day or 12 day versus control (n = 6), the values are mean ± SD and paired means were analyzed using Student's two-tailed *t*-test. Scale bars represent 50 μm.

the WT1-positive cells (**Figure 2C**, **2D**). The result of staining with BrdU was in accord with the result of EGFP-positive staining (**Figure 2E**).

Detection of GFP and BrdU positive podocytes in the PHN model

A podocyte is the target of immune-mediated injury in glomerular diseases such as membranous nephropathy [22].

In the PHN model, no significant change was observed under light microscopy, except mild thickening of the glomerular basement membrane (GBM) at 5, 8 and 12 days (**Figure 3A**). Immunofluorescence microscopy showed IgG and C3 were positive in a granular pattern along the GBM beginning at 5 days (**Figure 3B**, **3C**). Electron microscopy revealed granular electron-dense deposits located in the subepithelial region, effacement of the podocyte foot process and basement membrane thickening at

Table 1. The rate of GFP positive podocyte in rat nephropathy models (data showed as mean ± SD)

	ADR nephrosis				Passive Heymann nephritis			
	Control	2 week	5 week	10 week	Control	5 day	8 day	12 day
WT1+	13.52±1.86	12.31±1.86	10.11±1.85	6.64±1.44	13.81±1.73	12.48±1.82	10.33±1.50	8.46±1.61
GFP+	0	0	1.42±0.54	0.68±0.52	0	0.92±0.51	1.21±0.57	1.49±0.69
%	0	0	13.82±4.57	10.01±7.98	0	7.52±4.25	11.37±4.38	17.79±7.70

WT1+, the WT1 positive cells number/glomerulus; GFP+, both GFP and WT1 double positive cells number/glomerulus; %, the rate of the GFP positive podocyte.

5, 8 and 12 days (**Figure 3D**). The 24 h urinary protein excretion increased at 5 days ($P > 0.05$) and increased significantly at 8 and 12 days (**Figure 3H**, $P < 0.05$).

Immunohistochemistry staining showed more cells were GFP positive in glomeruli at 5, 8 and 12 days (**Figure 3E**) and distributed at the periphery of the glomerular capillary loop, which overlapped WT1-positive podocytes (**Figure 3F**). The result of BrdU-staining was consistent with that of EGFP-positive staining (**Figure 3G**).

Detection rate of GFP positive podocyte number in rat nephropathy models

WT1 is often used to quantify podocyte numbers under physiological and pathological conditions [4, 23-26]. In this study, the rate of GFP positive podocyte number to podocytes numbers per sectional glomerulus was determined in each glomerulus from paraffin sections of rat nephropathy models. In the ADR nephrosis model, the rate of GFP positive podocyte number was 13.82% and 10.01% at 5 and 10 weeks respectively. In PHN models, the rate was 7.52%, 11.37% and 17.79% at 5, 8 and 12 days respectively (**Table 1**).

These results indicate that podocyte proliferation does exist in ADR nephrosis and PHN.

Discussion

Owing to the complex anatomy of the glomerulus and the structural function served by podocytes on the basement membrane, the renewing podocyte might be rare and, therefore, hard to detect [27], making it difficult to investigate podocyte states *in vivo*. In this study, the retrovirus-EGFP tracing method was used to help the identification of proliferative podocytes in the glomeruli of rat nephropathy models. The integration of viral DNA and the production of viral proteins occur only after cells complete mitosis [8]. Therefore, cells stained GFP-positive in this study indicated they had undergone cell division recently. Within the rat models, immunohistochemical showed GFP-positive cells were overlapped the WT1-positive cells, suggesting rat podocytes possess proliferative potential after injury.

There are still different opinions about whether podocyte depletion could be compensated by proliferation from itself in most common types of glomerulonephritis. Some studies showed cyclin kinase was up regulated in these types of nephritis, however, the number of podocytes was still lower in glomerulonephritis compared to the normal control. No convincing direct evidence of podocyte division has been reported, which might be the reason to cause the problem complicated.

By injecting a retroviral vector into rat nephropathy models for the first time, our work has shown the proliferation of podocytes in diseased glomeruli. This encouraging finding signals the existence of podocyte division and proliferation in rat cytotoxic nephrosis and immune-mediated glomerulonephritis. Moreover, we have observed aggregation of three or four podocytes in local lesions in some occurrences of human nephritis during electron microscopic examination of renal biopsy and in PHN model (**Figure 4A**, **4B**). These phenomena also suggest the podocytes have undergone mitosis.

In the past decade, following the explosion of molecular and cellular research in podocyte biology, studies have suggested podocyte injury has a significant role in the pathogenesis of a majority of glomerular diseases. Reduction of the number of podocytes in a damaged glomerulus is crucial for the development of proteinuria and glomerulosclerosis. As long as the podocyte loss is limited, restitution or repair is possible, showing the glomerular architecture can be remodeled [28]. There is an

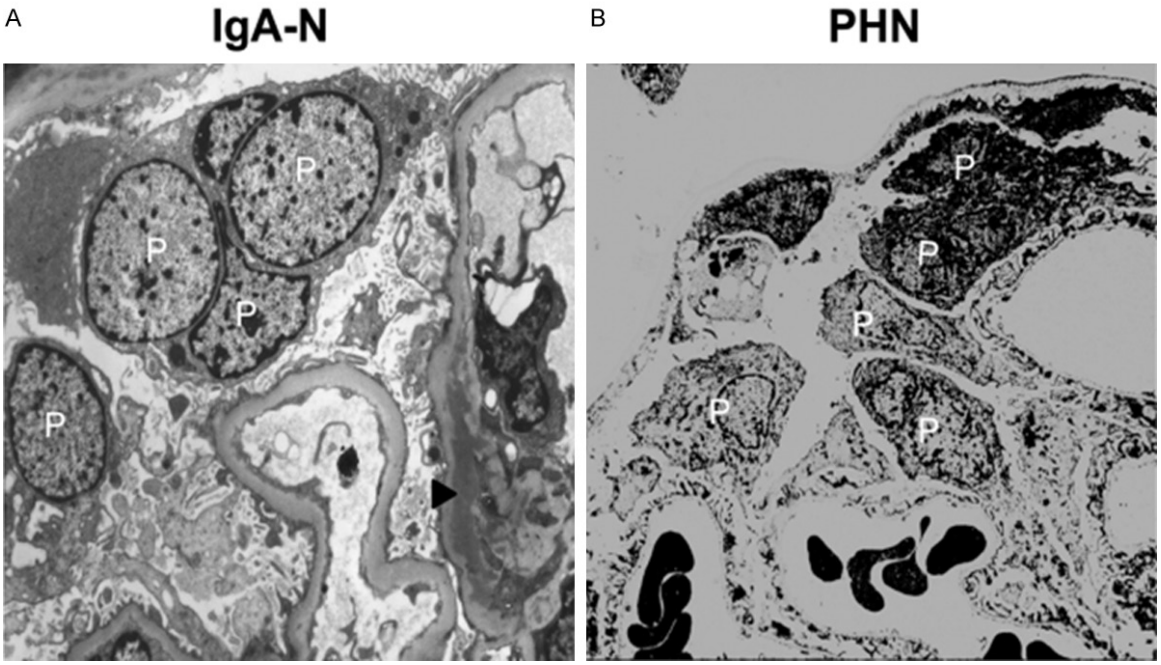

Figure 4. Aggregation of podocytes in local lesions in immune-mediated nephropathy. A. Electron microscopy of glomeruli from IgA-N patients; B. Electron microscopy of glomeruli from PHN rats. P, podocyte; filled triangle (▲), the electron-dense deposits.

expanding literature showing glomerular injury can be repaired by resident renal progenitor cells localized at the urinary pole of Bowman's capsule for replacement of depleted human and murine podocytes [6]. On the other hand, podocyte itself also shows the proliferative potential, for example, the proliferation of podocytes occurs in the rare collapsing variant of focal segmental glomerulosclerosis [4]. The proliferation and participation of podocytes in the formation of crescents has been shown in human anti-GBM disease and class IV diffuse proliferative lupus GN [29]. Shkrelis reported well differentiated (or mature) podocytes of rat could reversibly enter the cell cycle by conditional expression of the telomerase protein component (TERT) gene, showing telomerase and the Wnt/β-catenin pathway were involved in podocyte proliferation and in podocyte disease states [27]. All these data suggest depletion of podocytes in glomeruli diseases might be compensated by cell proliferation from parietal progenitor cell or from podocytes itself. Our experiments *in vivo* demonstrated that GFP positive podocytes overlapped WT1 positive in rat nephropathies could support this hypothesis for podocyte renewal, but its source still needs further investigation. Our data provide an effective method to observe the changes of podocyte injury directly, which might be beneficial for deeply approaching about podocyte injury and repair in glomerular diseases.

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Disclosure of conflict of interest

None.

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