Permanent Genetic Tagging of Podocytes: Fate of Injured Podocytes in a Mouse Model of Glomerular Sclerosis

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Injured podocytes lose differentiation markers. Therefore, the true identity of severely injured podocytes remains unverified. A transgenic mouse model equipped with a podocyte-selective injury induction system was established. After induction of podocyte injury, mice rapidly developed glomerulosclerosis, with downregulation of podocyte marker proteins. Proliferating epithelial cells accumulated within Bowman's space, as seen in collapsing glomerulosclerosis. In this study, the fate of injured podocytes was pursued. Utilizing Cre-loxP recombination, the podocyte lineage was genetically labeled with lacZ in an irreversible manner. After podocyte injury, the number of lacZ-labeled cells, which were often negative for synaptopodin, progressively declined, correlating with glomerular damage. Parietal epithelial cells, but not lacZ-labeled podocytes, avidly proliferated. The cells proliferating within Bowman's capsule and, occasionally, on the outer surface of the glomerular basement membrane were lacZ-negative. Thus, when podocytes are severely injured, proliferating parietal epithelial cells migrate onto the visceral site, thereby mimicking proliferating podocytes.

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Damage to podocytes has been implicated as a key step in the progression of glomerular diseases to glomerulosclerosis. Because podocytes lack the ability of postnatal proliferation, loss of podocytes is thought to be responsible for the irreversibility of glomerulosclerosis (1–4). Exceptionally, in collapsing focal segmental glomerulosclerosis (FSGS), podocytes are thought to proliferate (5–7). In both settings, injured podocytes lose all differentiation markers and their characteristic structure. Hence, the identity of injured podocytes has heretofore been determined solely on the basis of their anatomical location, while their true identity remains unverified.

Previously, we established a transgenic mouse line in which selective podocyte injury can be induced on demand (8). The transgenic mice (NEP25) express human (h)CD25 selectively on podocytes, and injection of a hCD25-targeted recombinant immunotoxin, anti-Tac(Fv)-PE38 or LMB2 (9), permits selective and inducible injury only to podocytes. After LMB2 injection, NEP25 mice developed severe nephrotic syndrome and subsequently renal failure. Podocytes and other glomerular cells were progressively damaged, finally leading to glomerulosclerosis. Shortly after the injection of LMB2, expression of podocyte-specific markers, including WT-1, synaptopodin and nephrin, disappeared. The disappearance of the markers may reflect either true loss of podocytes or downregulation of the markers.

Our study aimed to chase the fate of injured podocytes regardless of their status of differentiation by permanently labeling the podocyte lineage, utilizing the Cre-loxP system. This allowed us to identify podocyte-derived cells and to assert true loss of podocytes.

Materials and Methods

Animal Experiments

The Animal Experimentation Committee of Tokai University approved the protocol, in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

A Cre cDNA fragment obtained from pCre-Pac (Kurabo, Japan) (10) was combined with a 5.5kb *Nphs1* promoter fragment (8) and intron of the rabbit β-globin gene. The resultant transgene was injected into fertilized eggs obtained from BDF1 × C57BL/6N mating. The *Nphs1*-Cre mice (line 33) were used after backcrossing with C57BL/6N more than six times.

Nphs1-Cre/ROSA26-loxP mice (n = 3), *Nphs1*-Cre/ROSA26-loxP/NEP25 mice (n = 17), ROSA26/NEP25 mice (n = 12) were identified by PCR on tail DNA as described previously (8,11,12,13) and analyzed. *Nphs1*-Cre/ROSA26-loxP/NEP25 mice (n = 7, 6 and 4) were analyzed 10, 14 and 21 d after LMB2 injection (50 ng/g body weight [BW]), respectively.

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Histologic Analyses

Frozen renal sections (4 μm) were stained for lacZ as previously reported (14). Two percent glutaraldehyde-fixed frozen sections were microwaved for 15 min and stained with monoclonal anti–Ki-67 antibody (1:100; Novocastra, UK). Other histologic analyses were performed as described previously (8,14).

Glomeruli were graded using scores of 0 to 4 for 0%, <25%, <50%, <75%, and 100%, respectively, of the tuft area that was positive for lacZ or synaptopodin.

Results

Establishment of Nphs1-*Cre Transgenic Mouse Lines*

We obtained eight transgenic founder mice carrying the *Nphs1*-Cre transgene. Six founder mice produced transgenic offspring. In five lines, Northern analysis detected Cre mRNA in the kidney. Immunostaining revealed Cre protein in podocytes in four lines.

For Cre-mediated recombination, we mated these four *Nphs1*-Cre lines with two reporter lines, the CAG-CAT-Z (11) and ROSA26-loxP (12) (Figure 1). Because the lacZ gene of these reporter lines is separated from a ubiquitous promoter by a loxP-flanked DNA fragment, lacZ is expressed only when and where Cre recombinase excised the intervening fragment. Line 33 was found to be the best Cre line because all podocytes expressed lacZ intensely, while nonpodocyte renal cells were negative for lacZ. Transgenic mice of line 33 showed functionally and morphologically normal renal phenotype.

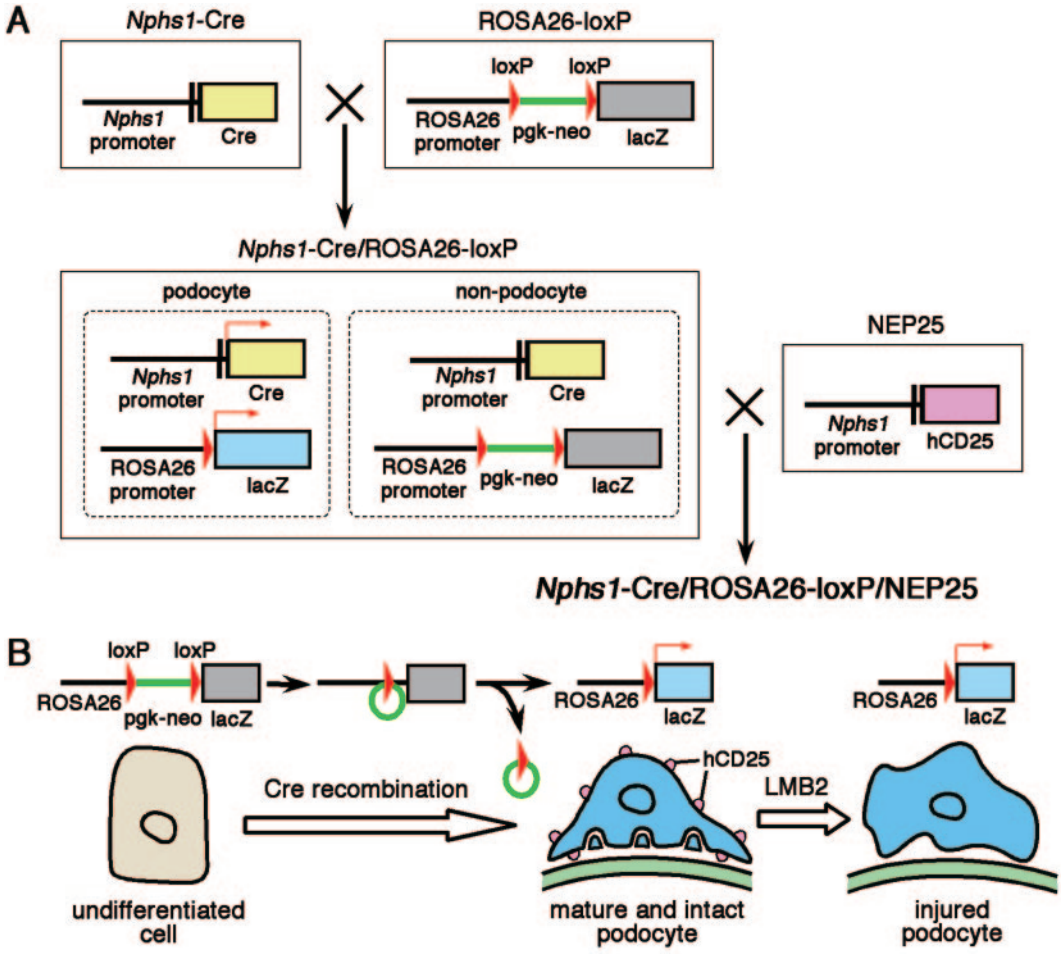

Figure 1. Genetic tagging of the podocyte lineage. (A) Generation of *Nphs1*-Cre/ROSA26-loxP/NEP25 mice. *Nphs1*-Cre mouse carries *Nphs1* promoter and the Cre recombinase gene (upper left). ROSA26-loxP mouse carries a ubiquitous promoter (ROSA26 promoter) with the lacZ gene (upper right). Without Cre recombinase, the lacZ gene is not expressed due to an interruption by a loxP-flanked DNA fragment (pgk-neo). In podocytes of *Nphs1*-Cre/ROSA26-loxP mouse, Cre-mediated recombination excises the loxP-flanked DNA fragment, thereby irreversibly activating the lacZ gene (lower left). The lacZ gene expression is driven by the ROSA26 promoter (not by *Nphs1* promoter) so that it is not downregulated in injured podocytes. To induce selective podocyte injury in this double transgenic mouse, we mated this mouse with NEP25 mouse (lower right), obtaining *Nphs1*-Cre/ROSA26-loxP/NEP25 mouse. (B) Permanent lacZ tagging of the podocyte lineage in *Nphs1*-Cre/ROSA26-loxP/NEP25 mouse. In undifferentiated cells, the lacZ gene is not expressed (left). During development, *Nphs1* promoter turns active, expressing Cre recombinase in podocytes. Cre deletes the pgk-neo and recombines ROSA26 promoter and the lacZ gene. Thus, lacZ is expressed in mature and intact podocytes under the regulation of ROSA26 promoter (middle). After LMB2 injection, lacZ expression remains in injured podocytes (right).

Stable Labeling of Podocytes

In *Nphs1*-Cre/CAG-CAT-Z and *Nphs1*-Cre/ROSA26-loxP mice, the lacZ gene is driven by β-actin–based CAG promoter and ROSA26 promoter, respectively, not by *Nphs1* promoter. In a pilot study, we injected LMB2 into *Nphs1*-Cre/CAG-CAT-Z/NEP25 triple transgenic mice and found that lacZ rapidly disappeared, before podocytes showed any discernible morphologic changes (data not shown). This indicated that CAG-lacZ is downregulated in podocytes affected by LMB2, and not suitable for our experimental purpose.

To test the stability of ROSA26 promoter activity, we mated NEP25 mice with the ROSA26 line. Unlike ROSA26-loxP reporter mice, the new double transgenic mice do not carry the loxP-flanked DNA insertion, and thus ubiquitously express lacZ without Cre recombination. In ROSA26/NEP25 mice, tissues were examined 3 to 21 d after LMB2 injection in widely ranging doses (5 to 50 ng/g BW). No lacZ-negative cells were found in any of the 962 glomeruli studied, which carry various degrees of epithelial injury or mesangial changes at any condition (Figure 2, A and B). These indicated that ROSA26-lacZ is not downregulated after LMB2 injection, thus is suitable for stable labeling of podocytes.

Loss of LacZ-Labeled Podocytes in Nphs1-*Cre/ROSA26-loxP/NEP25 Mice*

We mated *Nphs1*-Cre/ROSA26-loxP mice with NEP25 mice, obtaining *Nphs1*-Cre/ROSA26-loxP/NEP25 triple transgenic mice (Figure 1). Without LMB2, the kidneys of the triple transgenic mice showed normal morphology and lacZ staining similar to *Nphs1*-Cre/ROSA26-loxP mice. The lacZ staining pattern coincided with that of synaptopodin in the adjacent sections (Figure 2, C and D).

After LMB2 injection into *Nphs1*-Cre/ROSA26-loxP/NEP25 mice, the pathologic changes of the kidney were essentially the same as NEP25 single transgenic mice, including vacuolar degeneration of podocytes and parietal epithelial cells (PEC), PEC proliferation, mesangial expansion, mesangiolysis, hyalinosis, and glomerular sclerosis. In some glomeruli, even detached podocytes were clearly stained for lacZ (Figure 2E).

We compared lacZ and synaptopodin staining in adjacent sections in *Nphs1*-Cre/ROSA26-loxP/NEP25 mice 10 d after LMB2 injection (Figure 2, F and G). Synaptopodin disappeared more extensively than lacZ. We semiquantified lacZ- and synaptopodin-stained areas in 112 glomeruli using scores from 0 to 4; 61% of glomeruli had lower scores for synaptopodin than

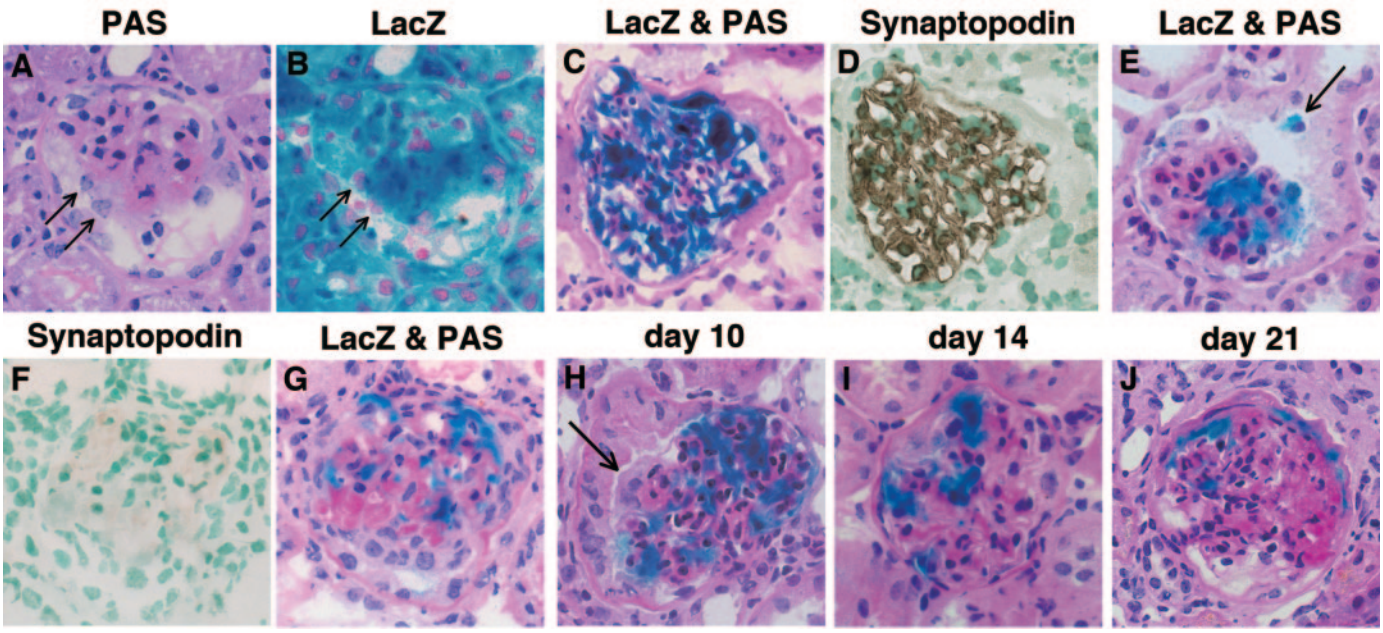

Figure 2. Stable lacZ labeling of podocytes. (A and B) Stability of the ROSA26 promoter. Selective podocyte injury was induced by LMB2 injection in ROSA26/NEP25 mice. Periodic acid-Schiff (PAS) staining (A) showed an injured glomerulus with degenerated podocytes (arrows). The adjacent section (B) showed that all cells including injured podocytes (arrows) were intensely stained for lacZ (blue). (C and D) Specificity of the lacZ labeling of podocytes in *Nphs1*-Cre/ROSA26-loxP/NEP25 mice. The stain pattern of lacZ (C) and synaptopodin (D, brown) in the adjacent sections of a normal glomerulus coincided with each other, demonstrating that all podocytes are specifically stained for lacZ. (E through J) Glomerular morphology of *Nphs1*-Cre/ROSA26-lox P/NEP25 mice after LMB2 injection. In damaged glomeruli, a detached podocyte (arrow) was clearly stained for lacZ. Note that lacZ-positive cells disappeared in the left upper area of the glomerulus, indicating that podocytes are lost in this area. In a severely damaged glomerulus, synaptopodin staining (F) was diminished globally, whereas lacZ (G) in the adjacent section was retained in a segmental pattern. (H, I, J) A representative glomerulus stained for lacZ is presented for each time point. LacZ-labeled podocytes were progressively lost. (J) Sclerosis is evident. Note that a part of the glomerular basement membrane (GBM) is covered by lacZ-negative cells (arrow). Magnification, ×400.

those of lacZ. This confirmed that synaptopodin is downregulated before podocytes are lost.

Ten, 14 and 21 d after LMB2 injection, more than 50% of the glomerular tuft area was lacZ-negative in 27, 81, and 95% of glomeruli, respectively. Concurrently, segmental or global sclerosis was observed in 18, 23, and 60% of glomeruli at each time point (Figure 2, H, I, J). These data demonstrate that podocytes were indeed progressively lost temporally correlating with progression of sclerosis.

Origin of Proliferating Visceral Epithelial Cells

After LMB2 injection, NEP25 mice showed avid cell proliferation within Bowman's capsule, forming up to two layers, but not a crescent. In some glomeruli, the glomerular tufts were surrounded by a monolayer of cuboidal epithelial cells, which morphologically resembled fetal immature podocytes and had no apparent connection to PEC with quiescent appearance (Figure 3A). These cuboidal cells were positive for cytokeratin, a marker for PEC, and negative for synaptopodin (Figure 3, B and C). The cuboidal epithelial cells were found to incorporate bromodeoxyuridine, demonstrating active DNA synthesis in these cells (Figure 3, D and E).

We identified the origin of these proliferating cells by lacZ staining. Both the cells proliferating within Bowman's capsule and the visceral epithelial cells on the outer surface of the glomerular basement membrane (GBM) were negative for lacZ (Figure 3, F and G). In addition, none of the lacZ-positive cells were stained for Ki-67 in the double-stained sections (Figure 3H).

Discussion

In this study, we chased the fate of injured podocytes in a genetic model of FSGS (NEP25 mice) by permanently tagging the podocyte lineage with the lacZ gene. This allowed us to identify podocytes regardless of their status of differentiation, and thus to assert true loss of podocytes.

Previous studies showed that the number of podocytes decreases with the development of glomerulosclerosis (15–18). In a classic method engineered by Weibel (19), the number of podocytes is counted under the electron microscope. Because injured podocytes lose characteristic structures, identification of podocytes is based on anatomical location. Alternatively, podocytes are identified by podocyte-specific markers, WT-1 or GLEPP1 (20–22). However, these markers are downregulated in certain circumstances, particularly when podocytes are se-

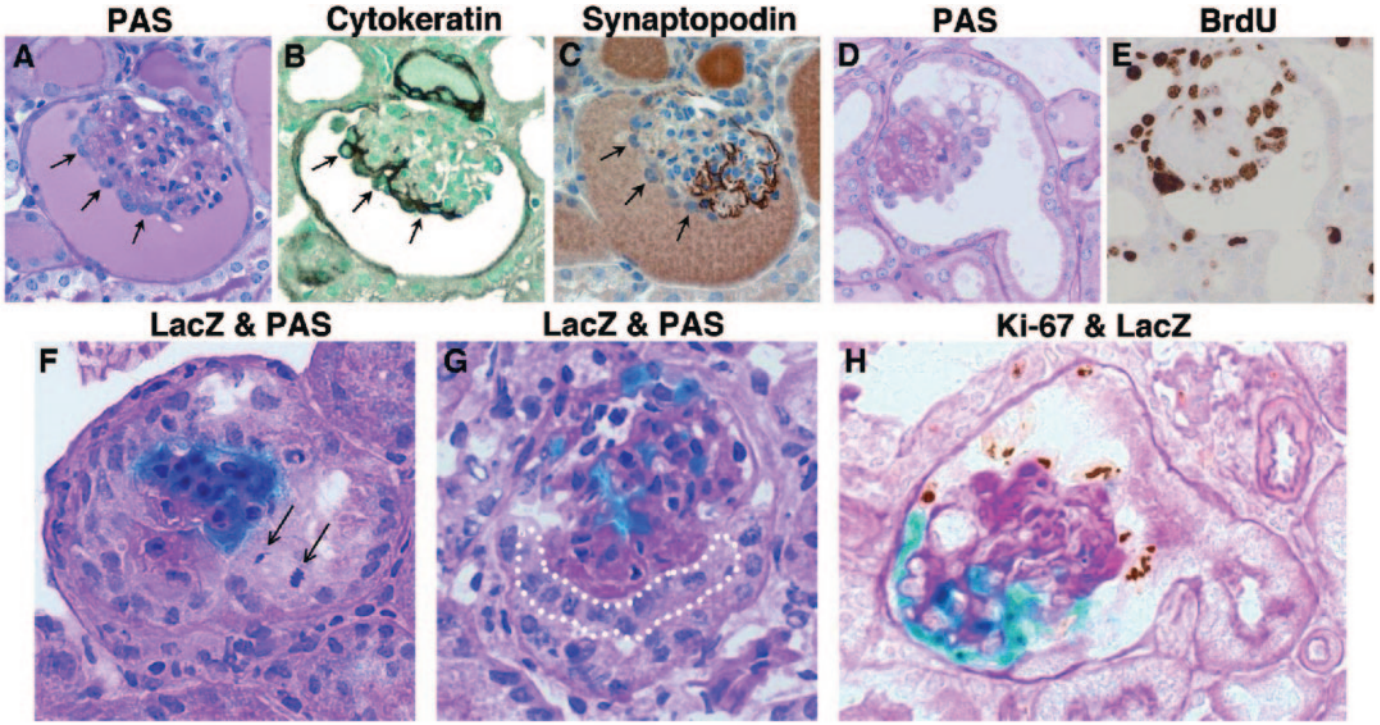

Figure 3. Origin of proliferating epithelial cells. (A through E) Cuboidal visceral epithelial cells in NEP25 mice. (A) Sclerotic glomerulus is surrounded by a monolayer of cuboidal epithelial cells (arrows). Immunostaining of the adjacent sections showed that these cells are positive for cytokeratin (B) and negative for synaptopodin (C). Proliferating cells were labeled by a continuous infusion of bromodeoxyuridine (BrdU) for 14 d after LMB2 injection. PAS staining (D) showed that cuboidal epithelial cells had piled up around a sclerotic glomerulus. These cells were positive for BrdU in the adjacent section (E). (F through H) Origin of proliferating epithelial cells in *Nphs1*-Cre/ROSA26-loxP/NEP25 mice. (F) The proliferating cells within Bowman's capsule with a mitotic figure (arrows) were negative for lacZ. (G) The cuboidal visceral epithelial cells on the outer surface of the GBM (enclosed by dotted line) were negative for lacZ. (H) Epithelial cells proliferating within Bowman's capsule were positive for Ki-67 (brown), a proliferation marker, and negative for lacZ. Magnification, ×400.

verely injured. Therefore, these methods can count only intact or mildly injured podocytes, and are not suitable for quantifying the podocytes in severely injured glomeruli undergoing sclerosis.

Our study visualized injured podocytes by permanent genetic tagging that persisted beyond synaptopodin downregulation, and demonstrated that the number of podocytes declined progressively with exacerbation of glomerular damage. In glomeruli with advanced global sclerosis, lacZ-labeled cells were scarce. We found no evidence for proliferation or transdifferentiation of podocyte-derived cells. Moreover, many lacZ-negative cells proliferated within Bowman's capsule, and some were located on the outer surface of the GBM. Thus, these results demonstrated that both the podocyte-specific proteins and the anatomical location of podocytes fail to reliably identify podocytes when they are injured.

Some studies suggest that podocytes can be lost without alteration in glomerular structure (16,17,20). In our model, before lacZ-labeled podocytes were lost, the glomeruli showed severe damage, including accumulation of periodic acid-Schiff (PAS)-positive materials, hyalinosis, and proliferation of PEC, indicating that true loss of podocytes does not necessarily precede glomerular damage, but can occur in parallel with the advancement of glomerular damage toward glomerular sclerosis.

In several experimental settings, podocytes are shown to proliferate (23–25). In collapsing FSGS, including HIV-associated nephropathy (HIVAN), despite lack of the characteristics of mature podocytes, the epithelial cells clustering on the outer surface of the GBM are regarded as dedifferentiated podocytes because they are proliferating in the area apparently separated from Bowman's capsule (5–7). On the other hand, in human idiopathic FSGS, monolayer cellular lesions of epithelial cells that covered the sclerotic glomeruli were reported to be of parietal origin because these cells have several characteristics unique to PEC and are connected to PEC (26,27). In this regard, we generated transgenic mice expressing HIV-1 genes in podocytes. These mice developed collapsing FSGS accompanied by avid cell proliferation within the glomerulus. Most proliferating cells were positive for cytokeratin, and we found no epithelial cells doubly positive for Ki-67 and desmin, a marker for injured podocytes (28).

The NEP25 model does not develop collapsing FSGS, but instead some globally sclerotic glomerular capillaries become surrounded by a monolayer of cuboidal epithelial cells. Similar to the monolayer lesions in idiopathic FSGS, they lack the characteristics of podocytes, but express cytokeratin, a marker for PEC. The monolayer lesions were sometimes observed in glomeruli with apparently quiescent PEC, suggesting the possibility of podocyte transdifferentiation. However, our study unequivocally demonstrates that these cells do not originate from podocytes. Avid proliferation of PEC suggests that they originated from PEC and have migrated onto the outer surface of the GBM, thereby mimicking proliferating podocytes. It will therefore be of interest to extend our approach to studying the origin of proliferating glomerular epithelial cells in HIVAN. The proliferating epithelial cells, heretofore regarded as podocytes, may, in part, include cells derived from PEC.

Using another podocyte injury model, Thy-1.1 transgenic mice, Smeets *et al.* (29) found that after induction of podocyte damage by an injection of anti–Thy-1.1 antibody, PEC proliferated and produced extracellular matrix, forming adhesions. Smeets *et al.* proposed that proliferation of PEC plays an important role in the formation of FSGS. In our model, similar PEC proliferation was also observed after podocyte injury. PEC proliferate and overlay the denuded GBM to replace lost podocytes.

Of note, using a similar genetic labeling method, Moeller *et al.* (25) followed the podocyte lineage in the anti-GBM glomerulonephritis model. They found that podocyte-derived cells without expression of podocyte differentiation markers transdifferentiated into crescentic cells, proliferated, and contributed to the formation of crescents. In our model, we observed no signs of redistribution, transdifferentiation, or proliferation of lacZ-positive cells, *i.e.*, podocytes. It should be noted that avid inflammatory reactions occur in the anti-GBM model, whereas in the NEP25 model glomerular injury is triggered by suppression of protein synthesis in podocytes, and no inflammatory cells are observed in injured glomeruli. Different injury processes may account for the different responses of podocytes.

In summary, by permanently labeling the podocyte lineage, we demonstrated that podocytes are progressively lost in parallel with the progression of glomerular injury, without transforming into other type of cells. In addition, our study indicates that when podocytes are severely injured, proliferating PEC migrate onto the visceral site, *i.e.*, the outer surface of the GBM, thereby mimicking proliferating podocytes.

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