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Genetic Podocyte Lineage Reveals Progressive Podocytopenia with Parietal Cell Hyperplasia in a Murine Model of Cellular/Collapsing Focal Segmental Glomerulosclerosis

Taisei Suzuki,* Taiji Matsusaka,^{†‡} Makiko Nakayama,*
Takako Asano,[§] Teruo Watanabe,*
Iekuni Ichikawa,^{‡¶} and Michio Nagata*

From the Department of Pathology,* Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan; the Department of Pediatrics,[†] Vanderbilt University School of Medicine, Nashville, Tennessee; the Institute of Medical Science and Department of Medicine[‡] and the Department of Bioethics,[¶] Tokai University Medical School, Isehara, Kanagawa, Japan; and the Department of Pediatrics,[§] National Defense Medical College, Saitama, Japan

Focal segmental glomerulosclerosis (FSGS) is a progressive renal disease, and the glomerular visceral cell hyperplasia typically observed in cellular/collapsing FSGS is an important pathological factor in disease progression. However, the cellular features that promote FSGS currently remain obscure. To determine both the origin and phenotypic alterations in hyperplastic cells in cellular/collapsing FSGS, the present study used a previously described FSGS model in p21-deficient mice with visceral cell hyperplasia and identified the podocyte lineage by genetic tagging. The p21-deficient mice with nephropathy showed significantly higher urinary protein levels, extracapillary hyperplastic indices on day 5, and glomerular sclerosis indices on day 14 than wild-type controls. X-gal staining and immunohistochemistry for podocyte and parietal epithelial cell (PEC) markers revealed progressive podocytopenia with capillary collapse accompanied by PEC hyperplasia leading to FSGS. In our investigation, non-tagged cells expressed neither WT1 nor nestin. Ki-67, a proliferation marker, was rarely associated with podocytes but was expressed at high levels in PECs. Both terminal deoxynucleotidyl transferase dUTP nick-end labeling staining and electron microscopy failed to show evidence of significant podocyte apoptosis on days 5 and 14. These findings suggest that extensive podocyte

loss and simultaneous PEC hyperplasia is an actual pathology that may contribute to the progression of cellular/collapsing FSGS in this mouse model. Additionally, this is the first study to demonstrate the regulatory role of p21 in the PEC cell cycle. (Am J Pathol 2009, 174:1675–1682; DOI: 10.2353/ajpath.2009.080789)

Glomerulosclerosis is a common pathological process that promotes functional deterioration of the kidney irrespective of the cause of the disease. In addition to mesangial proliferation and matrix accumulation pathways that underlie glomerulosclerosis,^{1,2} recent studies suggest that alterations in resident epithelial cells are pivotal to the progression of glomerulosclerosis, and such alterations are typical in focal segmental glomerulosclerosis (FSGS).^{3,4}

The glomerulus consists of two resident epithelial cell types, ie, podocytes and parietal cells (PECs). Both cell types share the proliferative phenotype during glomerulogenesis. At the capillary loop stage, podocytes express specific markers of differentiation accompanied with cell cycle inactivation.^{5–7} Likewise, loss of podocytes has been shown to cause segmental glomerulosclerosis in postadaptive FSGS, and the amount of urinary podocytes is correlated with disease progression in human FSGS and experimental renal diseases.^{8–10} In contrast, epithelial cell hyperplasia is involved in cellular/collapsing FSGS.^{4,11–13}

Lesions in cellular/collapsing FSGS reveal unique features, and the proliferating epithelial cells typically overlie the collapsed tuft showing visceral cell hyper-

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Address reprint requests to Dr. Michio Nagata, Department of Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, Japan. E-mail: nagatam@md.tsukuba.ac.jp.

plasia.^{11,13,14} The origin of proliferating epithelial cells in cellular/collapsing FSGS has been investigated by immunostaining with specific cell markers in conjunction with proliferation markers. From the loss of podocyte markers, Barisoni et al concluded that hyperplastic epithelial cells in cellular/collapsing FSGS are de-differentiated podocytes.¹⁵ By contrast, we and another group have observed the expression of the PEC markers cytokeratin and Pax-2 in these lesions.^{16,17} Because immunostaining studies to identify cell markers are insufficient to determine cellular origin, whether visceral cell hyperplasia is the result of podocytes that have lost their podocyte markers and are expressing PEC markers *de novo*, or proliferating PECs that persistently express their own markers remains unclear. The interactions between two resident epithelial cells that mimic glomerulogenesis (ie, cell proliferation and phenotypic alterations) may play a key role in the progression of FSGS. Nevertheless, the molecular mechanisms orchestrating these epithelial interactions have not been elucidated.

The cell cycle inhibitor p21 has been suggested to play unique roles in glomerular injury. Pippin et al showed that up-regulation of p21 is associated with sublytic C5b-9-induced podocyte injury, suggesting that p21 limits podocyte re-entry into the cell cycle in the setting of cell injury.¹⁸ Conversely, *de novo* expression of p21 was detected in hyperplastic epithelial cells in human glomerular diseases.¹⁹ These studies suggest a role for p21 in glomerular diseases; however, its function remains unclear. Interestingly, p21-deficient mice with glomerulonephritis present a pathology that is characteristic of visceral epithelial hyperplasia leading to FSGS. Furthermore, these cells express ezrin, a podocyte marker; the authors concluded that the lack of p21 promoted podocyte mitogenicity, resulting in collapsing FSGS.²⁰ This model may provide an opportunity to study *in situ* interactions of epithelial cells that participate in cellular/collapsing FSGS.

Recently, genetic tagging has enabled us to trace the lineage of individual cells, even after the cells have become phenotypically transformed by proliferation or injury. Specifically, we and another group have established podocyte-specific Cre-expressing mice crossed with ROSA26 mice.^{21,22}

Here, we investigated podocyte lineage in a previously established cellular/collapsing FSGS model in p21-deficient mice to characterize the cellular origin and phenotypic alterations by genetic tagging. Our results suggest that progressive podocytopenia with PEC hyperplasia forms the basis of FSGS in this model. In addition, a novel function of p21 as a cell cycle regulator for PECs was identified.

Materials and Methods

Transgenic Mice and Experimental FSGS

Nphs1-Cre/ROSA-loxP mice and p21^{-/-} mice were mated, and *Nphs1*-Cre/ROSA-loxP/p21^{-/-} mice were generated.²² *Nphs1*-Cre/ROSA-loxP/p21^{+/+} mice were used as

controls. To induce FSGS, duck anti-rabbit kidney antibody was injected intraperitoneally into mice.²³ The antibody was injected into 10- to 12-week-old male mice on 2 consecutive days, and they were sacrificed on day 5 or 14.²⁰ Urine was collected before sacrifice. The kidneys were perfused with phosphate-buffered saline. Tissue blocks were fixed either in 10% formalin or 2% glutaraldehyde for histological examination. In addition, blocks were embedded in OCT compound for cryosectioning. For X-gal staining, the kidneys were perfused with phosphate-buffered saline, followed by perfusion with fixative solution consisted of 10% formaldehyde (Wako, Osaka, Japan), 0.2% glutaraldehyde, and 10% polyoxyethylene (9) octylphenyl (Wako) in phosphate-buffered saline. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tsukuba (Ibaraki, Japan), and the study was approved by the Institutional Review Board of the University of Tsukuba.

Staining and Morphological Analysis

Paraffin sections were processed for periodic acid-Schiff staining, a periodic acid-silver methenamine staining, and Masson's trichrome staining. LacZ expression was visualized by X-gal staining of frozen sections 3 μ m thick.

Figure 1. Urinary protein and glomerular lesions. **A:** The ratio of urinary protein to creatinine was significantly greater in p21^{-/-} mice with nephropathy than both in p21^{-/-} mice without nephropathy and p21^{+/+} mice with nephropathy on day 5 ($P < 0.01$, $n = 5$ per group). On day 14, no significant difference was observed between p21^{-/-} and p21^{+/+} mice, both with nephropathy. **B:** Extracapillary hyperplastic index on day 5. The index was significantly greater in p21^{-/-} mice than in p21^{+/+} mice, both with nephropathy ($P < 0.05$, $n = 5$ per group). **C:** Glomerular sclerosis index. The index of p21^{-/-} mice was significantly higher than that of p21^{+/+} mice, both with nephropathy, on day 14 ($P < 0.05$, $n = 5$ per group).

Briefly, the sections were incubated at 37°C overnight in X-gal solution. Paraffin sections 2 μ m thick were stained with anti-synaptopodin antibody (mouse monoclonal antibody, 1:1 dilution; PROGEN Biotechnik, Heidelberg, Germany) as a podocyte-specific marker using a Vector M.O.M. Peroxidase Kit (Vector Laboratories, Burlingame, CA) and an Avidin/Biotin Blocking Kit (Vector Laboratories) in accordance with the manufacturers' instructions. Anti-WT1 antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-nestin antibody (mouse monoclonal antibody, 1:50 dilution; Chemicon, Temecula, CA) were also used as podocyte-specific markers. Anti-laminin antibody (mouse monoclonal antibody, 1:60 dilution; Sigma-Aldrich, St. Louis, MO) was used to outline the glomerular basement membrane (GBM). Anti-cytokeratin antibody (rabbit polyclonal antibody, 1:50 dilution; Dako, Glostrup, Denmark), anti-Pax-2 antibody (rabbit polyclonal antibody, 1:50 dilution; Zymed Laboratories Inc., San Francisco, CA) and anti-claudin-1 antibody (rabbit polyclonal antibody, 1:300 dilution; Invitrogen, Carlsbad, CA) were used as PEC markers. Incubation of all rabbit polyclonal antibodies was followed by EnVision+ Single Reagent (Dako) reaction. To avoid mouse-on-mouse nonspecific reactions, a modification of the ARK (Dako) detection procedure was used. Mac-3 antibody (rat monoclonal, 1:50 dilution; BD Biosciences, San Jose, CA) was used to stain macrophages. Anti-Ki-67 antibody (rat monoclonal, 1:100 dilution; Dako) was used to detect cell cycle activity. Biotinylated rabbit anti-rat IgG (1:100 dilution; Chemicon) was used as a secondary antibody, followed by reaction with peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan). Peroxidase activity was visualized using Liquid

DAB+ Substrate Chromogen System (Dako). Glomerular binding of duck IgG (1:20 dilution; Nordic Immunology, Tilberg, The Netherlands) or mouse IgG (1:80 dilution; MP Biomedical, Aurora, OH) was quantified via immunofluorescence. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (Apoptosis in Situ Detection Kit; Wako) was performed to detect apoptotic cells.

Urinary Protein and Creatinine

The urinary concentrations of protein and creatinine were measured using a Hitachi 7170 autoanalyzer (Hitachi High-Technologies, Tokyo, Japan).

Histological Analysis

Glomerular pathology was assessed by semiquantitative grading using extracapillary hyperplastic and sclerosis indices. The severity of hyperplasia and sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, extracapillary hyperplasia or sclerosis of <25% of the glomerulus; 2+, 3+, and 4+, extracapillary hyperplasia or sclerosis of 25–50%, 50–75%, and >75% of the glomerulus, respectively. The whole-kidney average sclerosis index was obtained by averaging scores from all glomeruli on one section.²⁴ TUNEL-positive cells in glomeruli (over 50 per mouse), including Bowman's space and capsule, were counted, and the means were calculated.

Figure 2. Representative micrographs showing cellular/collapsing FSGS in *Nephr1-Cre/ROSA26-loxP/p21^{-/-}* mice. Low-magnification view of the renal cortex in *p21^{+/+}* mice (A) and *p21^{-/-}* mice (B) both with nephropathy on day 5. *p21^{-/-}* mice with nephropathy showed microcystic tubules with protein casts. Glomerular profiles in *p21^{-/-}* mice with nephropathy on day 5 (C and D) and day 14 (E and F). C: The markedly collapsed glomerular tuft is surrounded by hyperplastic epithelial cells with cytoplasmic vacuolization and occasional protein droplets. D: The collapsed glomerular tuft is surrounded by hyperplastic visceral epithelial cells. Note the presence of a visceral-parietal epithelial bridge. E: A segmentally collapsed tuft formed a synechia to the Bowman's capsule in the tip domain. F: Segmental glomerulosclerosis and hyalinosis were noted. Epithelial hyperplasia was rarely observed in *p21^{-/-}* mice with nephropathy on day 14. A and B: Masson's trichrome stain; C–F: periodic acid silver methenamine. Scale bar = 200 μ m (A, B) and 20 μ m (C–F).

Statistical Analysis

Student's *t*-test was used for urinary protein analysis, and the two-sided Mann-Whitney *U*-test was used for statistical analyses of differences in extracapillary hyperplastic indices, glomerular sclerosis indices, and TUNEL analyses.

Results

Proteinuria and Glomerular Pathology

Urinalysis indicated that the protein to creatinine ratio was significantly greater in $p21^{-/-}$ mice than in $p21^{+/+}$ mice, both of which exhibited nephropathy, on day 5 ($P < 0.01$), whereas the protein to creatinine ratio was similar between groups on day 14 (Figure 1A). Figure 2 shows the sequential glomerular lesions in $p21^{-/-}$ mice with nephropathy. In $p21^{-/-}$ mice on day 5, the glomerular lesions showed focal or global tuft collapse with extracapillary hyperplasia, which was frequently observed in both mono-layered and multilayered visceral cells. In addition, we observed prominent tubular microcystic dilatation with proteinaceous casts in $p21^{-/-}$ mice, while no such changes were apparent in $p21^{+/+}$ mice. Tuft necrosis with fibrin exudate, mesangial proliferation, inflammatory infiltrates, and Bowman's capsular destruction were not observed. Hyperplastic epithelial cells occasionally had cytoplasmic vacuoles or reabsorption droplets. Careful inspection of serial sections occasionally revealed bridges connecting visceral hyperplastic cells and parietal epithelial cells. Glomeruli with segmental sclerosis on day 14 included "tip" lesions and tuft collapse with matrix accumulation and hyalinosis. The extracapillary hyperplastic index on day 5 was significantly higher in $p21^{-/-}$ than in $p21^{+/+}$ mice ($P < 0.05$, Figure 1B). Extracapillary cellular lesions included hypercellular and/or monolayer lesions; both lesion types were localized to the visceral side of the collapsed tuft segment. The glomerular sclerosis index was higher in $p21^{-/-}$ than in $p21^{+/+}$ mice (Figure 1C). Immunohistochemistry revealed that hypercellular and monolayer extracapillary lesions frequently expressed cytokeratin and Pax-2, but not synaptopodin or macrophage markers (Figure 3, A and B; data not shown in Pax-2 and Mac-3). Immunofluorescence analysis revealed glomerular binding of anti-mouse, but not anti-duck, IgG on the GBM (data not shown).

Genetic Identification of Podocytes by X-gal Staining

In all cells of the podocyte lineage in *Nphs1-Cre/ROSA26* mice, the lacZ marker gene is irreversibly activated after Cre-mediated excision by nephrin promoter activation. Thus, podocyte lineage cells are theoretically stably labeled with lacZ, even when they are injured and lose podocyte markers. The result reveals that blue X-gal staining was exclusively limited to podocytes under normal conditions (Figure 4). All surface cells of normal

Figure 3. Immunohistochemistry and X-gal staining of glomeruli in $p21^{-/-}$ mice with nephropathy. **A:** Synaptopodin staining was absent in visceral epithelial cells on the collapsed tuft (arrows), whereas preserved tufts were covered with synaptopodin-positive cells (arrowhead). **B:** Bowman's capsule and a collapsed glomerular tuft were entirely covered with keratin-positive epithelial cells. Visceral-parietal epithelial bridges expressed keratin. **C:** Double staining for laminin and X-gal showed a few X-gal-negative cells on the glomerular basement membrane, which was delineated by laminin staining (arrowheads). **D:** WT1 and LacZ were co-expressed in the right half of this glomerular surface, whereas hyperplastic epithelial cells in the left portion were negative for both WT1 and X-gal (arrowheads). **E:** Ki-67 and X-gal double-positive cells were rarely observed in glomeruli. Ki-67 staining was sometimes observed among parietal epithelial cells. **F:** Ki-67-positive cells in the hyperplastic lesion were virtually white. Scale bar = 20 μ m.

glomeruli were X-gal-positive, whereas those of segmentally sclerotic glomeruli were composed of both X-gal-positive and -negative cells. Visceral cell hyperplasia ultimately consisted of purely unstained cells, with no admixture of blue-stained cells. In addition, no blue cells are presented beneath the non-tagged hyperplastic epithelial cells. Monolayer lesions on areas of tuft collapse/sclerosis were also unstained. We occasionally found an unstained bridge connecting parietal and visceral aspects of the epithelium but rarely isolated blue cells on the parietal side (parietal podocytes). With advancing sclerosis, glomeruli showed a reduction in the number of blue cells, indicating progressive podocytopenia with sclerosis.

Double staining with X-gal and laminin revealed that the few cells attached to the GBM were X-gal-negative and were not connected to the parietal cell layer in this section. Hyperplastic epithelial cells were ultimately WT1-negative, whereas X-gal-positive cells in the same glomeruli strongly expressed WT1. Ki-67-positive blue cells were rarely observed; they were isolated and not found within the hyperplastic epithelial lesions. Ki-67-positive cells were sometimes present on Bowman's capsule but were X-gal-negative. Visceral epithelial hyperplasia occasionally ($<10\%$) contain Ki-68-positive cells and they are virtually X-gal-negative (Figure 3, C–F). Histological serial section analysis for X-gal, nestin, and claudin-1

Figure 4. X-gal staining under differential interface contrast microscopy. **A:** A $p21^{+/+}$ mouse (control, without nephropathy). Tagged cells covered the outermost portion of the glomerular tuft; however, they were not observed in any other portion of the nephron. **B–E:** $p21^{-/-}$ mice with nephropathy on day 5. **B:** An isolated monolayer of non-tagged cells covered a portion of the glomerular tuft (arrowhead). **C:** A monolayer of white cells (arrowheads) covered a portion of the collapsed tuft with continuity to parietal cells via a visceral-parietal bridge (arrow). Note that the bridging cells were white. **D:** Most of the collapsed tuft surface was covered with a monolayer of non-tagged cells. **E:** Marked hyperplastic epithelial cells (arrowhead) were white. **F:** Very few blue cells remained in glomeruli with advanced sclerosis on day 14. Scale bars = 10 μm .

staining revealed that non-tagged cells involved in visceral cell hyperplasia were negative for nestin but positive for claudin-1 (Figure 5).

Ultrastructure of Epithelial Hyperplasia

Transmission electron microscopy revealed that hyperplastic visceral cells had tight cell junctions and were composed solely of epithelial cells. Epithelial lesions were composed of either clusters of cells or single extended cell buds showing clear continuity with cuboidal parietal cells lining Bowman's capsule (Figure 6). Collapsed tuft segments often had subendothelial hyaline deposition. Notably, hyperplastic cell clusters were not necessarily clustered opposite the naked GBM. Podocytes frequently contained cytoplasmic vacuoles and absorption droplets and showed foot process effacement or detachment. In some cases, extended single cells with brush borders migrated to the GBM. The GBM facing the epithelial lesions lacked podocytes, and cells lining the GBM did not form foot processes.

Apoptosis

TUNEL-positive cells were occasionally observed in glomeruli, but these were relatively rare. The number of TUNEL-positive cells per glomerulus showed no statistically significant difference between $p21^{+/+}$ and $p21^{-/-}$

Figure 5. Histological serial section analysis for X-gal (**A**) and nestin (**B**) staining, and X-gal (**C**) and claudin-1 (**D**) staining. Non-tagged visceral hyperplastic cells were negative for nestin and positive for claudin-1, whereas blue cells within the collapsed tuft were positive for nestin. Scale bars = 10 μm .

mice at both times they were examined (Figure 7). TUNEL-positive cells were most frequent within hypercellular lesions or parietal cells. Electron microscopy revealed that apoptotic bodies were not present in podocytes in any tissue sample examined, but apoptotic bodies were observed within the hyperplastic epithelium (Figure 6a).

Discussion

The present study, which examined a unique murine model of FSGS exhibiting visceral epithelial hyperplasia,²⁰ provided two important insights. First, genetically tagging the podocyte lineage revealed progressive podocytopenia combined with PEC hyperplasia in cellular/collapsing FSGS; second, $p21$ appears to play a regulatory role in the PEC cell cycle. Although the lesions were induced by intraperitoneal injection of duck anti-rabbit kidney antibody into mice, glomerular lesions revealed reproducible visceral cell hyperplasia with tuft collapse leading to FSGS, as originally described.²⁰ As noted previously,²⁰ we also failed to find distinct tuft necrosis, fibrin exudates, inflammatory infiltrates, or rupture of Bowman's capsule, which are characteristic pathology of necrotizing crescentic glomerulonephritis observed in anti-GBM antibody-induced nephritis provoked by intravenous antibody injection. However, we did observe tubular microcystic dilatation, one of the histological features of collapsing FSGS in humans.²⁵ Thus, the pathological changes observed in this model are more consistent with cellular/collapsing FSGS than with crescentic glomerulonephritis. Although glomerular binding

Figure 6. Transmission electron micrographs in $p21^{-/-}$ mice with nephropathy on day 5. **A:** A low magnification view of a glomerulus with segmental epithelial hypercellularity. Note the polyoid structure of visceral hyperplastic epithelial cells and the marked vacuolation in some epithelial cells. **Inset:** apoptotic body within a hyperplastic epithelial cell (rectangle). The polyoid showed a connection to parietal epithelial cells and directly covered the glomerular basement membrane; podocytes were absent. A tuft with marked subendothelial hyaline deposition (arrow) was covered with podocytes showing foot process effacement. **Inset magnification:** $\times 1500$. **B:** A cluster of epithelial cells and a single cell attached to the collapsed tuft surface. The glomerular basement membrane in this area lacks podocytes, and the epithelial processes without foot processes showed faint attachment to the glomerular basement membrane. **Inset:** Some cells showed brush borders on the surface (rectangle). Original magnification of both pictures, $\times 600$. **Inset magnification:** $\times 3000$.

of mouse IgG suggests the involvement of autoimmunity, the precise mechanism of FSGS in this mouse model remains unclear.

The cellular origin of hyperplastic lesions in cellular/collapsing FSGS is a controversial issue due to the lack of lineage studies. Barisoni et al reported immunohistochemical evidence of dysregulated podocytes in hyperplastic visceral epithelial cells that had lost their podocytic markers.¹⁵ This concept has been supported by an immunostaining study in transgenic mice carrying the HIV-1 gene.²⁶ However, because podocytes and PECs are phenotypically similar when they proliferate during glomerulogenesis, identification through immunostaining alone is insufficient, even though hypercellular lesion localize to the visceral side. In the same model of cellular FSGS used here, Kim et al reported that hyperplastic

Figure 7. Apoptosis in glomeruli. **A:** TUNEL staining in tissue collected from $p21^{-/-}$ mice with nephropathy on day 5 showed positive cells among PECs and inner glomerular cells. **B:** Although apoptotic cells appeared more frequent in $p21^{-/-}$ mice, the frequency of glomeruli with TUNEL-positive cells was not significantly different between $p21^{-/-}$ mice and $p21^{+/+}$ mice with nephropathy on day 5 or 14 ($P > 0.05$, $n = 5$ per group). Scale bar = $10 \mu\text{m}$.

cells were of podocyte origin, based on their visceral localization and the expression of ezrin, a podocyte marker.²⁰ Podocyte lineage tracing by genetic tagging in this study demonstrated that hyperplastic visceral epithelial cells were not podocyte-derived. Cellular lesions consisted only of X-gal-negative cells without the presence of blue cells (podocytes), and these cells were negative for podocyte markers but basically expressed PEC markers, including cytokeratin, Pax-2, and claudin-1.²⁷ In addition, Ki-67 positive cells were predominantly non-tagged cells, and the podocytes rarely expressed Ki-67, which indicates that podocytes can re-enter the cell cycle.^{28,29} Nonetheless, this is insufficient to guarantee cell division (proliferation) even in tagged cells expressing Ki-67. Taken together, we conclude that the great majority of hyperplastic epithelial cells in this model of FSGS are of PEC origin. Although this strongly suggests that hyperplasia in cellular/collapsing FSGS is derived from PECs, it is not yet known whether the phenomenon is limited to this model. Smeets et al investigated the epithelial phenotype in a collapsing FSGS model in podocyte-specific Thy-1.1 transgenic mice. By immunostaining, they showed that hyperplastic cells expressed PEC markers and that the extracellular matrices were synthesized by PECs.³⁰ The same epithelial features of PEC hyperplasia

were observed in HIV- and pamidronate-associated collapsing FSGS in humans.³¹ Moreover, three-dimensional analysis showed a clear connection between visceral hyperplasia and the parietal wall in graft recurrence of human idiopathic focal FSGS, even though a connection was not apparent in the initial section.³² Our observations strongly support these previous studies, leading us to conclude that hyperplastic epithelial cells are of PEC origin rather than derived from proliferating podocytes expressing the PEC phenotype *de novo* in cellular/collapsing FSGS. Further cell lineage studies are needed to test this hypothesis in various models of FSGS, including a transgenic mice model of HIV-related collapsing glomerulopathy.

Whether PEC hyperplasia is an independent event or a reaction to podocytopenia in this model is a matter of interest. Electron microscopy demonstrated that podocytes were not present beneath hyperplastic PECs, suggesting that podocyte loss is a prerequisite for PEC hyperplasia. However, our previous studies in NEP 25 mice, which show severe podocytopenia with collapsed glomeruli and subsequent sclerosis, did not show visceral cell hyperplasia.²² Thus, podocyte loss alone may be insufficient to induce PEC hyperplasia. Instead, the visceral PEC proliferation as observed in this model of FSGS may depend on additional intrinsic signaling factors abnormalities, such as p21 deficiency.

Roles of the p21 family (p21, p27, and p57) in FSGS have been previously investigated. We demonstrated the repression of p27 and p57 and the expression of cytokeratin in hyperplastic epithelia in human FSGS and in crescentic glomerulonephritis, suggesting that p27 and p57 repression may be involved in PEC proliferation.^{33,34} The role of p21 in FSGS is still controversial. Shankland et al reported that *de novo* expression of p21 is associated with cellular proliferation in FSGS and collapsing glomerulopathy.¹⁹ In contrast, Srivastava et al reported a decrease of p21 in collapsing FSGS.³⁵ As the actual cellular origin in each study is not known, it may be that each group observed different cell types. In this regard, our observations of increased PEC hyperplasia that resulted in glomerulosclerosis in p21-deficient mice suggest a novel function of p21 that limits PEC proliferation in glomerular disease. Since PECs in mice frequently have the features of tubular epithelia, our finding may be supported by the previous notion that down-regulation of p21 in tubular epithelial cells is involved in the cystogenesis via cell proliferation in polycystic kidney disease.³⁶ Note that we observed elongated, single PECs that stretched out and attached directly to the naked GBM in p21-deficient mice, suggesting a novel role for p21 in suppressing PEC migration. Taken together, our results suggest that p21 deficiency results in the migration and proliferation of PECs, ultimately resulting in visceral epithelial hyperplasia in this model of FSGS.

Podocyte proliferation has been suggested to occur in crescentic glomerulonephritis, and Moeller et al found a population of podocytes in the crescent by lineage studies.³⁷ In addition, Thorner et al used nestin as a marker of podocytes and found a considerable portion of nestin-expressing cells in the crescent.³⁸ These reports suggest

podocyte proliferation *in vivo*. In our study of FSGS, the tagged cells did not form the cell nests, which is a feature of proliferation. In addition, binucleated podocytes and double staining with X-gal and Ki-67 were scarcely observed. These results imply that podocytes are resistant to stimuli promoting cell cycle re-entry, even when lacking the strong cell cycle inhibitor, p21.

We were not able to show the mechanism of podocytopenia in this disease. On day 5, glomerular histology revealed various degrees of hyperplastic lesions, including early and advanced lesions, and we detected glomerular binding of mouse, but not duck, IgG; these results suggest that autoimmune mechanisms are involved in lesion formation. Therefore, glomerular changes on day 5 may be sufficient to reveal hyperplastic lesions at a relatively early stage. In this phase, however, we did not observe significant podocyte apoptosis via TUNEL staining and electron microscopy, as reported in previous studies. Because we observed apoptotic PECs in PEC hyperplasia, the apoptotic cells found in our study and that of Kim et al²⁰ may be derived from PECs during or after cell proliferation. Regardless, our study does not suggest a definitive role for apoptosis in progressive podocytopenia in this model. Since it has been reported that p21 mediates podocyte apoptosis *in vitro*,³⁹ the p21 deficiency in this model is likely to have no effect on podocyte apoptosis. Further study is clearly needed to determine the mechanism of the podocytopenia in cellular/collapsing FSGS in this model.

In conclusion, this study used genetic tagging in a mouse model of cellular/collapsing FSGS and provided the first direct evidence that epithelial interactions such as podocytopenia with reactive PEC hyperplasia promote FSGS. Although the precise mechanism of podocyte loss is not clear, the results do not support the recent concept of podocyte proliferation in cellular/collapsing FSGS. Our results also suggest a novel role of p21 in controlling the cell cycle of PECs.

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