The direction and role of phenotypic transition between podocytes and parietal epithelial cells in focal segmental glomerulosclerosis

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1Kidney and Vascular Pathology, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan; 2Department of Nephrology, Iizuka Hospital, Fukuoka, Japan; 3Department of Internal Medicine, Institute of Medical Science, Tokai University School of Medicine, Kanagawa, Japan; and 4Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Submitted 25 April 2013; accepted in final form 11 October 2013

Sakamoto K, Ueno T, Kobayashi N, Hara S, Takashima Y, Pastan I, Matsusaka T, Nagata M. The direction and role of phenotypic transition between podocytes and parietal epithelial cells in focal segmental glomerulosclerosis. Am J Physiol Renal Physiol 306: F98–F104, 2014. First published October 23, 2013; doi:10.1152/ajprenal.00228.2013.—Focal segmental glomerulosclerosis (FSGS) is a podocyte disease. Among the various histologies of FSGS, active epithelial changes, hyperplasia, as typically seen in the collapsing variant, indicates disease progression. Using a podocyte-specific injury model of FSGS carrying a genetic podocyte tag combined with double immunostaining by different sets of podocytes and parietal epithelial cell (PEC) markers [nestin/Pax8, Wilms’ tumor-1 (WT1)/claudin1, and podocalyxin/Pax2], we investigated the direction of epithelial phenotypic transition and its role in FSGS. FSGS mice showed progressive proteinuria and renal dysfunction often accompanied by epithelial hyperplasia, wherein 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal)-positive podocyte-tagged cells were markedly decreased. The average numbers of double-positive cells in all sets of markers were significantly increased in the FSGS mice compared with the controls. In addition, the average numbers of double-positive cells for X-gal/Pax8, nestin/Pax8 and podocalyxin/Pax2 staining in the FSGS mice were comparable, whereas those of WT1/claudin1 were significantly increased. When we divided glomeruli from FSGS mice into those with FSGS lesions and those without, double-positive cells tended to be more closely associated with glomeruli without FSGS lesions compared with those with FSGS lesions. Moreover, the majority of double-positive cells appeared to be isolated and very rarely associated with FSGS lesions (1/1,997 glomeruli). This study is the first to show the incidence and localization of epithelial cells with phenotypical changes in FSGS using a genetic tag. The results suggest that the major direction of epithelial phenotypic transition in cellular FSGS is from podocytes to PECs and that these cells were less represented in the active lesions of FSGS.

Podocytes are highly differentiated cells with a minimum capacity to regenerate (6, 8, 14), which suggests that podocytes maintain the highest differentiation status among glomerular cells and that PECs seem to hardly differentiate to podocytes after maturation. Since podocytes outgrown from isolated glomeruli aberrantly expressed the macrophage phenotype in vitro (13), it may be that podocytes undergo phenotypic transition and express PECs or macrophage markers, rather than PECs differentiating to podocytes under tissue injury.

By contrast, recent observations using immunohistochemistry for stem cell markers showed that PECs acquired the podocyte phenotype, suggesting the PEC as a podocyte precursor (15). In fact, a protein expression study is unable to determine the direction of differentiation even if the cell is shown to express different cell phenotypes simultaneously, because protein expression is a transient event and does not unequivocally guarantee cellular origin as well as gain of new functions.

The present study aimed to investigate the direction of epithelial phenotypic transition and discussed its relevance to the pathogenesis of FSGS, using triple transgenic mice (Nphs1-Cre/ROSA26-loxP/NEP25) as a FSGS model caused by podocyte-specific injury (7). With this system, we were able to trace the podocyte lineage, and double staining with podocytes and a PEC marker was able to reveal the incidence and localization of cells with phenotypic transition during FSGS progression (19).

Our results suggest that the major direction of epithelial phenotypic transition in FSGS is from podocyte to PEC. In addition, the low incidence and unassociated localization of
such cells in segmental lesions suggested that podocyte phenotypic transition is an isolated cellular response associated with glomerular injury.

**MATERIALS AND METHODS**

*Animal study.* The FSGS model is a previously established mouse model genetically expressing human CD25 limited to podocytes (NEP25 mouse) (7). The injection of human CD25-specific immunotoxin (LMB2) can induce selective injury in podocytes and cause severe proteinuria and histological characteristics of FSGS. For genetic podocyte tagging, we mated Nphs1-Cre/ROSA26-loxP mice with NEP25 mice to obtain Nphs1-Cre/ROSA26-loxP/NEP25 triple transgenic mice, in which we were able to monitor the podocyte lineage by means of β-galactosidase expression.

Mice were maintained at the animal facility of the Institutional Animal Use and Care Committee at the University of Tsukuba (registration no. 11–305) in accordance with our institutional guidelines. The mice were monitored and had free access to water and standard mice chow. Eight- to 12-wk-old mice were used for the experiments. A previous study revealed the appropriate amount of standard mice chow. Eight- to 12-wk-old mice were used for the experiments. A previous study revealed the appropriate amount of standard mice chow.

**RESULTS**

NEP25 FSGS mice revealed progressive podocyte depletion with epithelial hyperplasia. The podocyte-selective injury in NEP25 FSGS mice resulted in proteinuria with increases of serum creatinine and BUN levels on day 12 after immunotoxin injection. The significance of differences was accepted when the *P* value was <0.01.

**DISCUSSION**

The podocyte or alkaline phosphatase activity was visualized using dianımobenzidine (DAB substrate-chromogen system, Dako) or nitro blue tetrazolium (NBT/BCIP, Roche Diagnostics, Mannheim, Germany) according to the manufacturers’ instructions. For three sets of double staining, WT-1/claudin, nestin/Pax8, and podocalyxin/Pax2 antibodies for podocyte protein were incubated first and then the same staining procedure was done with PEC protein. Double-positive cells were identified as cells whose nuclei were stained by specific protein surrounded by cell cytoplasm staining with a specific protein.

Antigen detection with a mouse primary antibody, podocalyxin, or nestin on mouse tissues is complicated by high levels of background staining. The main cause of background staining is the binding of the secondary anti-mouse antibody to endogenous mouse tissue immunoglobulins and other components. To block this binding, we used preincubation with a primary antibody, a Fab fragment of unconjugated anti-mouse IgG, in combination with a secondary antibody, a biotin-conjugated Fab fragment anti-mouse IgG, for 1 h. Next, the antibodies including primary and secondary antibody were preincubated with mouse serum for 1 h. This method led to the most complete elimination of background staining and achieved satisfactory results.

The genetic podocyte tag was visualized by X-gal staining using β-galactosidase expression. Frozen sections (5 μm thick) were used for X-gal staining. Briefly, frozen sections were fixed in acetone at 4°C followed by incubation with X-gal staining solution [1 mM MgCl₂, 2 mM K3Fe(CN)6, 2 mM K4Fe(CN)6, and 1 mg/ml X-gal (GIBCO BRL, Gaithersburg, MD) in PBS] overnight at 37°C. To identify podocytes and PECs, immunohistochemistry was performed on paraffin sections (3 μm thick). In the double staining with X-gal and Pax8, the staining for X-gal was performed first. After the X-gal staining, the anti-Pax8 antibody for PEC protein was incubated as described earlier.

All of the glomeruli stained were individually captured by Lumina Vision Software and saved in a file. This system is able to allow an observer to determine whether the same cell profiles are double-positive cells or not in a blind manner. We analyzed over 60 glomeruli (range, 62–108 glomeruli) in each mouse. Double-positive cells were identified when two or three of the authors all observed as double positive.

**Statistics.** The results are expressed as means ± SE. Statistical significance was determined with a two-tailed Student’s *t*-test. In all calculations, the significance of differences was accepted when the *P* value was <0.01.
dase (X-gal) and Pax8, the PEC protein, clearly identified the cells under phenotypic transition from podocyte to PEC. Isolated X-gal/Pax8 double-positive cells were clearly detected in both controls and FSGS mice. The localization of X-gal/Pax8 double-positive cells was on the glomerular surface as well as Bowman’s capsule in both groups (Fig. 2, C, D, and G). It is notable that the hyperplastic epithelial lesions in the FSGS mice were mostly positive for Pax8, although X-gal/Pax8 double-positive cells were not associated with this lesion (Fig. 2H).

Double staining for WT1/claudin1, nestin/Pax8, and podocalyxin/Pax2. WT1-positive cells from control mice were clearly located at the podocyte position, whereas their numbers were decreased in the tufts of the FSGS mice. We found WT1-positive cells among hyperplastic epithelial lesions (Fig. 3, A and E). WT1/claudin1 staining clearly discriminated

Fig. 1. A: proteinuria in NEP25 focal segmental glomerulosclerosis (FSGS) mice and control mice. In the FSGS mice, no overt proteinuria was observed until day 3, and the urinary protein/creatinine ratio showed a significant increase on day 5 and peaked on day 12, compared with controls. B: serum creatinine and blood urea nitrogen (BUN) in FSGS mice and controls. Serum creatinine in FSGS mice was significantly increased on day 12 compared with controls. C: Wilms’ tumor (WT1)-positive cells in control mice and FSGS mice. The numbers of WT1-positive cells per glomerulus were 6.94 ± 0.44 in the control mice and 1.32 ± 0.17 in the FSGS mice (*P < 0.001).

Fig. 2. Representative glomerular profiles in control (A–D) and FSGS (E–H) mice. Periodic acid-methenamine silver (PAM) staining showed marked glomerular collapse with extracapillary hypercellularity resembling collapsing FSGS pathology in FSGS mice (E). Genetic tags visualized by 5-bromo-4-chloro-3-indoly1 β-D-galactoside (X-gal) staining (blue) showed limited localization on podocytes in controls and parietal epithelial cells (PECs) were entirely negative for X-gal staining (B). In the FSGS mice, blue tagging was almost absent in sclerotic lesions (F, arrow). Glomerular profiles are shown for double staining for X-gal and Pax8 (C, D, G, and H). In the controls, Pax8 was restricted to the PEC nuclei, and X-gal was restricted in the cell cytoplasm of podocytes. Double-positive cells occasionally existed along Bowman’s basement membrane (C and D, arrow). In the FSGS mice, isolated X-gal- and Pax8-coexpressing cells were found (G, arrow), and hypercellular lesions were composed almost entirely of Pax8-positive cells. No X-gal-coexpressing cells were found (H).
podocytes and parietal cells separately in the controls, but occasional double-positive cells were identified in the FSGS mice (Fig. 3, B and F). Nestin/Pax8 double-positive cells were identified in control mice as well as FSGS mice. In the controls, nestin/Pax8 staining also clearly discriminated each cell type (Fig. 3C), and double-positive cells were very rarely found on the glomerular basement membrane (GBM) and Bowman’s capsule. In the FSGS mice, they were localized not in the hyperplastic lesions but around them in an isolated manner (Fig. 3G). Podocalyxin/Pax2 staining also discriminated each cell type in the controls, and a few isolated double-positive cells were found in the FSGS mice (Fig. 3, D and H).

Frequency of glomeruli with double-positive cells in the different sets of markers. Since PEC-lineage cells were not genetically traceable in this study, we compared the prevalence of each set of marker double-positive cells. If the prevalence of double-positive cells in each set of marker was at similar levels as that in X-gal/Pax8 staining, it may be likely to conclude that the major direction of phenotypic transition is from podocyte to PEC. In all four sets of markers, the average numbers of double-positive cells per glomerulus in the FSGS mice were significantly increased compared with controls [X-gal/Pax8 (×10⁻²); 1.59 ± 0.39 vs. 5.82 ± 0.5, WT1/claudin1 (×10⁻²); 1.12 ± 0.33 vs. 8.4 ± 0.79, nestin/Pax8 (×10⁻²); 1.47 ± 0.46 vs. 5.09 ± 0.66, podocalyxin/Pax2 (×10⁻²); 1.62 ± 0.8 vs. 5.87 ± 0.77] as shown in Fig. 4. In addition, the numbers of nestin/Pax8 and podocalyxin/Pax2 double-positive cells in the FSGS mice were comparable to those of X-gal/Pax8. Since genetic tagging is the most reliable marker of podocyte-lineage cells, the comparable incidence of double-positive cells in podocalyxin/Pax2 and nestin/Pax8 suggested that these double-positive cells were mostly podocytes expressing PEC markers. By contrast, the numbers of WT1/claudin1 double-positive cells in FSGS mice were significantly higher than those of the other three sets of markers, including X-gal/Pax8.

Localization of double-positive cells. The double staining of X-gal/Pax8 was done with frozen sections. This method enables the visualization of double-positive cells, but it was occasionally difficult to detect the exact locations of these cells in the present study. In this context, we used paraffin-embedded sections with double immunostaining for three sets of markers for podocytes and PECs, and we estimated the numbers and localization of double-positive cells. Table 1 summarizes the results.

When all of the double-positive cells in each group were pooled, the incidences of double-positive cells in controls were comparable among the markers (nestin/Pax8, 8/554; WT1/claudin1, 6/536; and podocalyxin/Pax2, 9/564). The localization of double-positive cells was seen in the tuft as well as the vascular pole. To test whether double-positive cells were associated with FSGS lesions, we divided the glomeruli of FSGS mice into those with or without FSGS lesions. Interestingly, double-positive cells in all three sets of markers appeared predominantly in glomeruli without FSGS lesions compared with those with FSGS lesions. WT1/claudin1 double-positive cells in glomeruli without FSGS lesions were predominantly found on the vascular pole and Bowman’s capsule compared with the other markers. Generally, double-positive cells were present in an isolated manner in both the controls and FSGS mice. Importantly, we found only single double-positive cells within the FSGS lesions, detected by WT1/claudin1 staining, among 1,997 glomeruli from FSGS mice. These findings indicated that...
epithelial transdifferentiation was not predominantly associated with FSGS lesions.

**DISCUSSION**

In the present study, we attempted to examine the direction of phenotypic transition between podocyte and PEC and its association with the FSGS lesions. The great advantage of this study was the use of podocyte reporter mice to identify podocyte-lineage cells even in the damaged glomeruli, since cell marker studies with immunostaining techniques occasionally show unstable expression patterns. To estimate the incidence and precise localization of cells with phenotypic transition, we prepared multiple sets of markers for podocytes and PECs, i.e., nestin/Pax8, WT1/claudin1, and podocalyxin/Pax2, for double immunostaining.

Our first observation is that podocyte-specific injury provoked significantly increased epithelial phenotypic transition in FSGS. The previous observations of podocyte phenotypic changes in FSGS in humans were based on the double-immunostaining of podocyte markers and PECs or macrophage markers and demonstrated the presence of each protein in double-positive cells in collapsing FSGS or recurrent FSGS (3, 15, 17, 23). Although these findings suggested PECs as a resource for podocytes in physiological and pathological conditions, it has been still undetermined whether PEC transdifferentiate to podocyte functionally. As immunostaining alone is not able to detect the direction of cell differentiation, a genetic tagging system monitoring PECs is needed. Unfortunately, the present study was unable to monitor the PEC lineage by genetic tagging, but the comparable incidence of nestin/Pax8 and podocalyxin/Pax2 double staining with that of X-gal/Pax8 in FSGS mice suggested that podocyte-expressed PEC protein was much more predominant than PEC-expressed podocyte protein. Of note, we found a significantly increased incidence of WT1/claudin1 double-positive cells in FSGS compared with other sets of markers. Since we observed a similar incidence of double-positive cells among X-gal/Pax8, nestin/Pax8, and podocalyxin/Pax2 staining, significantly increased double-positive cells for WT1/claudin1 are likely to be PECs expressing WT1. Ohse et al. (11) have shown increases in WT1/claudin1 double-positive cells in different models of glomerular diseases. We also found WT1/claudin1 double-positive cells predominantly on Bowman’s capsule in glomeruli without FSGS lesions. Since not only mature podocytes but also a precursor for podocytes and PECs in the S-shaped body, or even undifferentiated nephrogenic mesenchyme, equally expressed WT1 (22), aberrant WT1 expression in PECs in disease may represent either PECs that differentiated toward podocytes or PECs that dedifferentiated to a PEC progenitor, including undifferentiated mesenchyme. Further studies are needed to determine the function of aberrant WT1 protein in PECs in disease.

<table>
<thead>
<tr>
<th>Number and localization of double-positive cells</th>
<th>WT1/Claudin1</th>
<th>Nestin/Pax8</th>
<th>Podocalyxin/Pax2</th>
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<tbody>
<tr>
<td>Controls (n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double-positive cells/total glomerular number</td>
<td>6/536</td>
<td>8/554</td>
<td>9/564</td>
</tr>
<tr>
<td>Tuft</td>
<td>4</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Vascular pole</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Bowman’s capsule</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FSGS (n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double-positive cells/total glomerular number</td>
<td>55/651</td>
<td>34/672</td>
<td>40/674</td>
</tr>
<tr>
<td>FSGS lesion (−)</td>
<td>39/55</td>
<td>29/34</td>
<td>31/40</td>
</tr>
<tr>
<td>Tuft</td>
<td>16</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>Vascular pole</td>
<td>11</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Bowman’s capsule</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FSGS lesion (+)</td>
<td>16/55</td>
<td>5/34</td>
<td>9/40</td>
</tr>
<tr>
<td>Tuft</td>
<td>7</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Vascular pole</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bowman’s capsule</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>FSGS lesion</td>
<td>1</td>
<td>0</td>
<td>0</td>
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</table>

FSGS, focal segmental glomerulosclerosis.
Our next interest was to investigate the role of epithelial phenotypic transition in FSGS progression. In the FSGS mice, all sets of podocyte and PEC marker double-positive cells were indeed significantly increased compared with the controls. Of note, these cells were predominant in the glomeruli without FSGS lesions compared with those with such lesions. In addition, most such cells in the glomerular profile were single and isolated. Of note, we identified the localization of double-positive cells in control and FSGS mice. In the control mice, double-positive cells were found in similar numbers by nestin/Pax8, WT1/claudin1, and podocalyxin/Pax2 staining. Notably, some of these cells were located at the vascular pole. Since PECs transit to podocytes at the vascular pole, and since previous studies suggested the presence of epithelial cells with a phenotype that is intermediate between those of podocytes and PECs at the vascular pole (1, 15), it is likely that podocyte and PEC marker double-positive cells were present at the vascular pole in the normal glomeruli.

In the FSGS mice, double-positive cells for nestin/Pax8 and podocalyxin/Pax2 were predominantly located on the tuft in both glomeruli with and those without FSGS lesions. In addition, double-positive cells were mainly associated with glomeruli without FSGS lesions and were not associated with segmental lesions, typically having epithelial hyperplasia in the glomeruli with FSGS lesions. In fact, we found only one double-positive cell in segmental sclerosis among 1,997 glomeruli from the FSGS mice. This confirmed our previous observation of a lack of accumulation of genetic podocyte-tagged cells in epithelial hyperplasia in this model and another model of FSGS (19, 21). Taking all of these findings together, we surmise that podocytes expressing PEC proteins are rarely admixed or incidentally stray into active epithelial changes in FSGS. Because this model of FSGS progressed acutely and rapidly showing active cellular lesions and the present study focused on these particular lesions, actual involvement of podocytes with the PEC phenotype for FSGS pathogenesis remains to be elucidated.

In conclusion, this study is the first to show that epithelial phenotypic transition was increased and that its direction was predominantly from podocyte to PEC in a mouse model of FSGS. We suggest that podocytes with phenotypic transition expressing PEC markers participate less in active FSGS lesions and a type of cellular response caused by progressive podocyte loss.

GRANTS

This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center of Cancer Research, and was also supported by the basic research support program of the Japanese Society of Nephrology and by Grants-in-Aid for Scientific Research of the Japan Society for the Promotion of Science (KAKEN; research project no. 22590877) and Progressive Renal Disease Research of the Ministry of Health, Labor, and Welfare of Japan.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

