Renal Progenitor Cells Contribute to Hyperplastic Lesions of Podocytopathies and Crescentic Glomerulonephritis

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ABSTRACT

Glomerular injury can involve excessive proliferation of glomerular epithelial cells, resulting in crescent formation and obliteration of Bowman’s space. The origin of these hyperplastic epithelial cells in different glomerular disorders is controversial. Renal progenitors localized to the inner surface of Bowman’s capsule can regenerate podocytes, but whether dysregulated proliferation of these progenitors contributes to crescent formation is unknown. In this study, we used confocal microscopy, laser capture microdissection, and real-time quantitative reverse transcriptase–PCR to demonstrate that hypercellular lesions of different podocytopathies and crescentic glomerulonephritis consist of three distinct populations: CD133−CD24−podocalyxin (PDX)−nestin−renal progenitors, CD133−CD24+PDX−nestin−transitional cells, and CD133+CD24−PDX+nestin−differentiated podocytes. In addition, TGF-β induced CD133+CD24+ progenitors to produce extracellular matrix, and these were the only cells to express the proliferation marker Ki67. Taken together, these results suggest that glomerular hyperplastic lesions derive from the proliferation of renal progenitors at different stages of their differentiation toward mature podocytes, providing an explanation for the pathogenesis of hyperplastic lesions in podocytopathies and crescentic glomerulonephritis.


The traditional glomerular disease classification encompasses a bewildering array of descriptive pathologic entities and their clinical counterparts.1–10 Evidence suggests that depending on the cause and associated environmental factors, podocyte dysfunction or injury is a common factor underlying a broad spectrum of histopathologic patterns in which glomerular hyperplastic and/or sclerotic lesions are variably combined.3–10 In adult humans, either delayed or excessive wound healing can manifest in every injured epithelial tissue.11 Delayed healing is the consequence of an insufficient response of resident epithelial progenitor cells that are thus replaced by extracellular matrix (ECM), leading to fibrosis. Conversely, excessive healing is characterized by aberrant progenitor cell prolifera-
tion, referred to as hypertrophic scarring. In the glomerulus, the response to podocyte injury can involve aberrant ECM production and replacement of resident cells by areas of fibrosis, as described for the lesions found in patients with and experimental models of focal segmental glomerulosclerosis (FSGS). Conversely, excessive proliferation of resident glomerular epithelium may cause (pseudo)crescent formation and Bowman’s space obliteration, as seen in the hypercellular lesions of collapsing glomerulopathy and in crescentic glomerulonephritis.

Recent studies have provided the first evidence that podocytes can be regenerated from a resident population of renal progenitors localized along the inner surface of the Bowman’s capsule. In both humans and mice, these progenitors reach the glomerular tuft via the vascular pole, where complete differentiation into podocytes occurs. In humans, these cells are characterized by the presence of surface CD133 and CD24 and the expression of transcription factors Oct-4 and Bmi-1. More recently, we demonstrated that renal progenitors are a heterogeneous population of cells that express the stem cell markers CD133 and CD24 in the presence or absence of podocyte-specific markers such as PDX or nestin.

In this study, we hypothesized that, as a consequence of glomerular injury, resident renal progenitors of the Bowman’s capsule proliferate in attempt to replace injured podocytes and that if regeneration occurs in a dysregulated manner, then it can generate hyperplastic glomerular lesions, scarring, and eventually nephron loss. Hyperplastic lesions that are frequently observed in glomerular disorders, particularly in collapsing glomerulopathy and crescentic glomerulonephritis, originate from CD133+CD24+ renal progenitors at different stages of their differentiation toward mature podocytes. These results might reconcile seemingly contradictory results of the literature and suggest a novel intriguing explanation for the pathogenesis of these glomerular disorders.

RESULTS

Distribution of Distinct Subsets of CD133+CD24+ Renal Progenitors in the Bowman’s Capsule of Adult Healthy Human Kidneys

We first evaluated the expression of the renal progenitor markers CD133 and CD24 in 15 healthy human kidneys by using laser confocal microscopy. In adult human glomeruli, coexpression of CD24 and CD133 was restricted to renal progenitors (Figure 1A), as described previously. CD133+CD24+ renal progenitors also expressed claudin-1 (Figure 1B), caveolin-1, cytokeratin, and vascular cellular adhesion molecule 1 (VCAM-1; data not shown), markers that within the glomerulus are expressed by the parietal epithelial cells (PECs) but not by podocytes. On the basis of the expression of renal progenitor markers CD133 and CD24 and of podocyte markers such as nestin or PDX, we confirmed that the cells lining Bowman’s capsule are arranged in a precise sequence within the Bowman’s capsule and consist of three distinct populations of cells, as already reported. A subset of more undifferentiated cells expressing CD133 and CD24 in the absence of nestin and PDX localized to the urinary pole of the Bowman’s capsule (Figure 1). A transitional population (progenitor podocytes) expressing CD133 and CD24, as well as nestin and PDX, usually localized between the urinary and the vascular pole (Figure 1). Finally, more differentiated cells expressing neither CD133 nor CD24 but exhibiting the podocyte markers localized at the vascular pole of Bowman’s capsule and were contiguous with differentiated podocytes (Figure 1).

Distinct Subsets of CD133+CD24+ Cells Are Major Constituents of Hyperplastic Lesions in Podocytopathies

We then evaluated the possible contribution of different progenitor cell subsets as well as tuft podocytes to the generation of intraglomerular hyperplastic lesions of patients affected by two types of podocytopathies, collapsing glomerulopathy, and FSGS.

Collapsing Glomerulopathy.

In collapsing glomerulopathy, hyperplastic intraglomerular lesions consisted of high numbers of cells (range 12 to 59; mean 32.9 ± 12.6) (Figure 2A). Triple-label immunofluorescence demonstrated that 50 to 75% of total cells represented immature progenitors, which expressed the renal progenitor markers CD133 and CD24 but not nestin or PDX (Figure 2). In addition, tuft podocytes, expressing nestin or PDX in the absence of CD24 and CD133, represented 10 to 40% of total cells. Finally, a population representing 2 to 15% of the cells were CD133+CD24+ nestin+, thus exhibiting a transitional phenotype and suggesting their nature of progenitor podocytes (Figure 2B). Similar results were obtained by using PDX instead of nestin as a podocyte marker (Figure 2C).

Focal Segmental Glomerulosclerosis.

In FSGS, glomeruli showed mild signs of hyperplasia and intraglomerular lesions consisted of lower numbers of cells (range 4 to 23; mean 11.1 ± 6.6). Again, even the FSGS lesions were mostly represented by CD133+CD24+nestin− cells (55 to 82% of the cells; Figure 2, D through F). In addition, tuft podocytes, expressing nestin or PDX in the absence of CD24 and CD133, represented 6 to 30% of total cells. Finally, 5 to 25% of the cells were CD133+CD24−nestin−, thus representing progenitor podocytes (Figure 2E). Similar results were obtained when PDX was used as a podocyte marker instead of nestin (data not shown).

In agreement with previous results, the CD133+CD24+ renal progenitors within the lesion of both collapsing glomerulopathy and FSGS were also characterized by coexpression with the PECs markers claudin-1 (Figure 2F), cytokeratin, caveolin-1, and VCAM-1 (data not shown). Only rare cells of the lesions expressed α-smooth muscle actin (α-SMA; a
marker for activated mesangium and myofibroblasts) or CD68 (a marker for monocytes/macrophages), but these cells did not coexpress CD133 or CD24 (data not shown).

To provide additional evidence in favor of these observations, we performed quantitative real-time reverse transcriptase–PCR (RT-PCR) on hyperplastic lesions obtained through laser capture microdissection of affected glomeruli. As a comparison, we used identical areas obtained from healthy glomerular tufts, because in healthy adult human kidneys, the glomerular tuft does not contain renal progenitors, whereas it contains a high number of mature podocytes. The hyperplastic lesions expressed significantly higher levels of the renal progenitor markers CD133 and CD24 and of the transcription factors Oct-4 and Bmi-1, which are specific for renal progenitors, in comparison with healthy glomerular tufts (Figure 2G). By contrast, the podocyte markers nestin, podocin, and nephrin were consistently more expressed in the healthy glomerular tuft in comparison with hyperplastic lesions (Figure 2H).

Figure 1. Assessment of CD24 and CD133 expression in normal human kidney is shown. (A) Both CD24 and CD133 are, within the glomerular structure, exclusively expressed by the epithelial cells lining the Bowman’s capsule in adult human glomeruli. NHK, normal human kidney. (B) CD24+CD133+ renal progenitors also expressed claudin-1 (blue). (C) Double-label immunofluorescence for CD133 (green) and nestin (red) demonstrating the existence of cells expressing CD133 in absence of the podocyte marker nestin, which localize to the urinary pole (UP) of the Bowman’s capsule, and cells expressing CD133 and nestin, which localize between the UP and the vascular pole (VP) of the Bowman’s capsule (arrow). Glomerular podocytes also appear as CD133−nestin+. To-pro-3 counterstains nuclei. (D) Double-label immunofluorescence for CD133 (green) and PDX (red) demonstrating the existence of cells expressing CD133, which localize to the UP of the Bowman’s capsule, and cells expressing CD133 and PDX (arrows), which localize between the UP and the VP of the Bowman’s capsule. Glomerular podocytes also appear as CD133−PDX+. To-pro-3 counterstains nuclei. (D') High magnification of the UP. Arrow points to cells showing double labeling for CD133 and PDX. To-pro-3 counterstains nuclei. Magnifications: ×400 in A through C; ×200 in D; and ×560 in D' and D''.

CD133+CD24+ Cells Are Major Constituents of the Tip Lesion

Glomerular tip lesion has been associated with FSGS, minimal-change nephropathy, membranous nephropathy, diabetic nephropathy, and postinfectious glomerulonephritis, which suggests that it does not represent a specific disease; however, this lesion consists of a collection of cells localized at the urinary pole of the Bowman’s capsule, suggesting a possible involvement of CD133−CD24+ renal progenitors in its generation. In our study, a tip lesion consisted of 16.9 ± 8.4 (range 6 to 23) cells. Most of these cells were positive for CD24 and CD133 (Figure 3, A and B). In particular, 60 to 85% of total cells were CD133+CD24+nestin−, whereas 8 to 16% of the cells were CD133+CD24+nestin+ (progenitor podocytes). Tuft podocytes represented 5 to 20% of total cells. Similar results were obtained when PDX was used as a podocyte marker instead of nestin (Figure 3C). Finally, quantitative real-time RT-PCR performed on hyperplastic lesions obtained through laser cap-
ture microdissection of affected glomeruli confirmed that also in the tip lesions CD133, CD24, Oct-4, and BmI-1 are consistently more expressed in comparison with healthy glomerular tufts (Figure 3D). By contrast, nestin, podocin, and nephrin were more expressed in the healthy glomerular tuft in comparison with the tip lesions (Figure 3E).

**Distinct Subsets of CD133⁺CD24⁺ Cells Are Major Constituents of Hyperplastic Lesions in Crescentic Glomerulonephritis**

In crescentic glomerulonephritis, hyperplastic lesions consisted of 35.9 ± 11.3 (range 9 to 53) cells. In the lesions analyzed, 45 to 80% of total cells were CD133⁺CD24⁺nestin⁺. In addition, CD133⁻CD24⁻nestin⁺ cells (tuft podocytes) were 5 to 50% of total cells in hyperplastic lesions, whereas a population representing 3 to 18% of the cells were CD133⁺CD24⁺nestin⁺ (progenitor podocytes), thus exhibiting a transitional phenotype (Figure 4, A and C). Similar results were obtained when PDX was used as a podocyte marker instead of nestin (Figure 4B). CD133⁺CD24⁺ cells within the crescents and along Bowman's capsule were also characterized by coexpression of claudin-1 (Figure 4D), caveolin-1 (Figure 4E), cytokeratin, and VCAM-1 (data not shown). Only rare cells of the lesions expressed α-SMA (data not shown). Using CD68 as a macrophage marker, we found that the majority of cases of crescentic glomerulonephritis have CD68⁺ cells in crescents, although these cells were rare in the “early” cellular crescents analyzed (Figure 4F). Double labeling for CD133 or CD24 and CD68 or α-SMA showed no cells coexpressing these two antigens (data not shown). To provide further support to these results, we analyzed the mRNA levels of CD133 and CD24 of the renal progenitor–specific transcription factors Oct-4 and BmI-1 and of the podocyte markers nestin, podocin, and nephrin. Quantitative real-time RT-PCR performed on hyperplastic lesions obtained through laser capture microdissection from the kidneys of patients with crescentic glomerulonephritis demonstrated that these latter expressed significantly higher levels
of CD133 and CD24, Oct-4, and Bmi-1 in comparison with healthy glomerular tufts (Figure 4G). By contrast, the podocyte markers nestin, podocin, and nephrin were consistently more expressed in the healthy glomerular tuft in comparison with the crescentic lesion (Figure 4H).

Proliferating Cells in Podocytopathies and Crescentic Glomerulonephritis Are CD133+CD24+

To evaluate which cells contributed to the generation of hyperplastic lesions through their proliferation, we evaluated the expression of the proliferation marker Ki67 in combination with CD133, CD24, PDX, and nestin. Triple-label immunofluorescence demonstrated that proliferating epithelial cells in collapsing glomerulopathy and crescentic glomerulonephritis mostly expressed CD133 and CD24 in the absence of podocyte markers, such as PDX and even nestin (Figure 5). Interestingly, however, a relevant subset of proliferating cells consisted of a transitional population of cells (progenitor podocytes) coexpressing not only the progenitor markers CD133 and CD24 but also the podocyte markers nestin and PDX (Figure 5). Finally, Ki67 was virtually never observed on cells (tuft podocytes) that expressed nestin and PDX in the absence of CD133 and CD24 (Figure 5). Although the number of proliferating cells was consistently lower in FSGS, CD133+CD24−nestin− (renal progenitors) and CD133+CD24−nestin+ (progenitor podocytes) were the only cells to show proliferative potential. Results for podocytopathies and crescentic glomerulonephritis are quantified in Figure 5, A and C. Similar results were observed also in the tip lesions (data not shown).

Cultured CD133+CD24+ Renal Progenitors Produce ECM Components in Response to TGF-β Treatment.

Progression of glomerular injury toward glomerulosclerosis is characterized by the evolution from an early cellular glomerular lesion to a fibrous lesion, which is related to progressive accumulation of ECM.24,25 TGF-β is the major determinant of the matrix accumulation26–30; therefore, to evaluate whether renal progenitors can also produce and deposit the ECM in response to TGF-β, we analyzed the effects of TGF-β treatment on the production of α1 and α3 chain of collagen IV, the α1 chain of collagen I, fibronectin, and laminin by renal progenitors in comparison with podocytes. To obtain podocytes (CD133−CD24−PDX+), in the absence of cells with a transitional phenotype (CD133+CD24−PDX+), we recovered CD133−CD24+PDX+ podocytes by immunomagnetic separation or directly from cultured glomeruli after complete digestion of the Bowman’s capsule, as reported in the Concise Methods section. Treatment of renal progenitors with TGF-β resulted in a markedly increased expression of all ECM molecules tested at different time points, as as-
sessed by real-time quantitative RT-PCR (Figure 6). By contrast, podocytes showed only a slight increase of the \( \alpha_3 \) chain of collagen IV and of fibronectin after TGF-\( \beta \) treatment.

**DISCUSSION**

Until now, theories explaining the origin of aberrant epithelial cells in collapsing glomerulopathy and crescentic glomerulonephritis have been controversial.5,31–43 One possibility is that these cells are exclusively of parietal epithelial origin,34,38,40,42 whereas others suggested that some dedifferentiated podocytes acquire markers of PECs5,31,32,35,36,39,41; however, more recent studies demonstrated that the intermediate filament protein nestin, which is specifically expressed during any stage of podocyte development and in the mature cell, may allow the investigation of podocyte involvement in glomerular disorders.23,43

In this study, we hypothesized that hyperplastic epithelial cells of human glomerulopathies might derive from the two distinct populations of CD133\(^+\)CD24\(^-\)CD68\(^-\) renal progenitors resident in the Bowman’s capsule of the adult human kidney: More undifferentiated progenitors lacking podocyte markers (CD133\(^+\)CD24\(^+\)PDX\(^+\)nestin\(^-\)) and transitional cells (progenitor podocytes) that additionally express podocyte markers (CD133\(^+\)CD24\(^-\)PDX\(^+\)nestin\(^+\)).16 Studies in mice have documented that these progenitors are responsible for podocyte replacement,17 consistent with the human findings reported here showing the presence of progenitor cells in disease conditions characterized by podocyte loss.

In all glomerular diseases under consideration here, combined findings by confocal microscopy, laser capture microdissection, and real-time quantitative RT-PCR showed that the most represented cells of hyperplastic lesions are CD133\(^+\)CD24\(^-\)CD68\(^-\) cells within the lesion. CD133, CD24, Oct-4, Bmi-1, nestin, podocin, and nephrin mRNA quantification in identical areas of hyperplastic lesions of crescentic glomerulonephritis or healthy glomerular tuft as obtained with laser capture microdissection. Data are means ± SEM of four independent experiments. Magnifications: 400 in A, A', B, C, and F; 200 in C', D, D', E, and E'; and 600 in B'.

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**Figure 4.** Phenotypic analysis of the cells involved in hyperplastic lesions of crescentic glomerulonephritis is shown. (A) Assessment of the co-localization among CD24, CD133, and nestin. In crescents, the involved epithelial cells were positive for both CD24 (red) and CD133 (green). Merged images are in yellow. Rare hyperplastic cells exhibited a triple labeling for CD133, CD24, and nestin (blue). Merged images are in white. (A') High magnification of the hyperplastic lesion. (B) Assessment of the co-localization among CD24, CD133, and PDX. In crescents, the involved epithelial cells were positive for both CD24 (red) and CD133 (green). Merged images are in yellow. Rare hyperplastic cells exhibited a triple labeling for CD133, CD24, and PDX (blue). Merged images are in white. (B') High magnification of the hyperplastic lesion. (C) Assessment of the co-localization among CD24 (red), PDX (blue), and nestin (green). In crescents, the involved epithelial cells were mostly positive for CD24 but negative for PDX or nestin. Rare hyperplastic cells exhibited a triple labeling for CD24, PDX, and nestin. Merged images are in white. (C') High magnification of the hyperplastic lesion. (D and E) Assessment of the co-localization between the progenitor cell markers CD24 (green) and CD133 (red) and the PEC markers claudin-1 (D, blue) and caveolin-1 (E, blue). All four markers were expressed by the epithelial cells lining the Bowman’s capsule and the proliferative cells involved in the lesions. A great overlap was observed in the staining pattern of the three markers (white). (D' and E') High magnification of the hyperplastic lesion. (F) Absence of co-localization between CD24 (red) and CD68 (green) in hyperplastic lesions. Arrow points to CD68\(^+\) cells within the lesion. (G and H) CD133, CD24, Oct-4, Bmi-1, nestin, podocin, and nephrin mRNA quantification in identical areas of hyperplastic lesions of crescentic glomerulonephritis or healthy glomerular tuft as obtained with laser capture microdissection. Data are means ± SEM of four independent experiments. Magnifications: ×400 in A, A', B, C, and F; ×200 in C', D, D', E, and E'; and ×600 in B'.
data, immature progenitors (nestin–) or podocyte progenitors (transitional cells nestin+) all express markers of proliferation in contrast with differentiated podocytes that do not express proliferation markers (Ki67–). Whether these differentiated podocytes are native to the tuft or derive from progenitors cannot be established. These findings also provide a possible explanation for the results observed in some transgenic mouse models in which tagged podocytes actively proliferate within hyperplastic lesions. Indeed, because both podocytes of the tuft and podocyte progenitors of the Bowman’s capsule express podocyte markers, albeit at different levels, they might be tagged using a podocyte-specific promoter. Alternatively, different results might also be explained by the distinct experimental systems used. Indeed, it is likely that more than one mechanism leads to the same morphologic damage. Thus, glomerular abnormalities reflect patterns of injury rather than diseases.

One major observation in this article is that renal progenitors of hyperplastic lesions display diverse proliferative response in different diseases. This greatly helps in identifying relevant pathways that initiate or influence intraglomerular cell proliferation/differentiation. Interestingly, Le Hir et al. demonstrated that crescents in proliferative glomerulonephritis develop at cell bridges between podocytes and PECs that disrupt epithelial cell polarity. Our data suggest that in crescentic glomerulonephritis, attachment of podocytes to PECs and/or disruption of progenitor cell polarity initiates an abnormal proliferation of progenitors, resulting in crescentic disease. Given the strict similarity in numbers of proliferating progenitors, this concept may be applied to collapsing glomerulopathy. Indeed, in mice that express the Thy-1.1 antigen on podocytes, injection of anti-Thy-1.1 mAb induces a massive podocyte injury, with acute albuminuria and collapse of glomerular tuft, prominent extracapillary proliferation, and frequent attachments of hypertrophic podocytes to Bowman’s capsule as seen in collapsing glomerulopathy.

A related process would explain the generation of the tip lesion, that, according to some authors, has been categorized as a transcategorical entity, observed at the urinary pole. Here, CD133+CD24+PDX–nestin– progenitors were again the main constituent of hyperplastic lesions. As previously reported, the replacement of podocytes in the tuft follows a gradient, with novel podocytes being progressively added from the vascular stalk. Because the tip podocytes represent the “oldest” podocytes of the tuft, they might be the first to die and thus to be replaced by renal progenitors at sites of attachment at the urinary pole. Interestingly, the tip lesion occurs in several proteinuric conditions, including podocytopathies, membranous nephropathy (where it is present in 64% of cases), postinfectious glomerulonephritis, and diabetic nephropathy. On this basis, Haas et al. argued that the tip lesion is a response to prolonged heavy proteinuria. Consistently, experimental evidence in vitro and in models of disease documented that the exposure of podocytes to excessive amounts of plasma proteins promotes podocyte dysfunction and injury followed by tuft adhesion and sclerosis.

The key finding in FSGS was a less prominent proliferation of CD133+CD24+ progenitors despite their comparable per-
percentage in respect to those found in crescentic disease or other podocytopathies, suggesting that FSGS would be at least markedly accelerated by an insufficient proliferation of resident progenitors to replace injured podocytes, resulting in release of ECM and generation of scar. That this could be the case is indicated by our in vitro finding of production and deposition of high amounts of ECM by CD133<sup>+</sup>CD24<sup>+</sup>PDX<sup>+</sup>progenitors exposed to TGF-β, which is also highly secreted by podocytes in response to proteinuria.<sup>50</sup> In contrast, an abnormally high regenerative activity by resident renal progenitors would result in hyperplasia, similar to the hypertrophic scarring observed in skin keloids. Reasons underlying this different response might be related to the type of injury or to the different genetic background of patients, as suggested by a recent study<sup>52</sup> demonstrating that induction of a podocyte damage leads to glomerular injury with segmental lesions and mild intraglomerular proliferation of PECs in wild-type mice but to a complete histologic pattern of collapsing glomerulopathy related to high PECs proliferation in mice knockout for the cell cycle inhibitor p21.<sup>31</sup>

Finally, the evolution of the hyperplastic lesion is likely to be dependent also on the severity of podocyte injury. Data previously reported by Wiggins et al.<sup>3</sup> suggested that remission can occur as long as <20% of podocytes are lost. Accordingly, results in transgenic mice support the ability of PECs to replace approximately 10% of adult podocytes during adolescence.<sup>17</sup> By contrast, 20 to 40% podocyte depletion results in scarring, and when podocyte depletion is >60%, the glomerulus dies and the nephron is lost.<sup>3</sup> On the basis of the results of this and previous studies, we suggest that podocytopathies can undergo remission because of replacement of injured podocytes by the Bowman’s capsule progenitors, suggesting the potential use of renal progenitors for the treatment of patients with FSGS.<sup>16,53</sup>

Taken together, these results provide a new point of view for the pathogenesis of different types of podocytopathies and crescentic glomerulonephritis and allow us to suggest renal progenitors of the Bowman’s capsule as a novel potential target for the setup of therapies for glomerular disorders.<sup>53</sup>

**CONCISE METHODS**

**Patients**

We studied a total of 43 tissue specimens from patients with various types of glomerular disorders. To address the possible participation of renal progenitors to the response to podocyte injury, we analyzed biopsies with mild to severe degree of intraglomerular proliferation. In particular, on the basis of the taxonomy by Barisoni et al.<sup>9,10</sup> we
analyzed kidney specimens of 13 patients with two different types of podocytopathies: collapsing glomerulopathy (n = 6) and FSGS (n = 7). Among podocytopathies, collapsing glomerulopathy and FSGS were selected because they represent the extreme possible responses to podocyte injury. Indeed, collapsing glomerulopathy is characterized by high intraglomerular epithelial hyperplasia, whereas FSGS is characterized by podocyte detachment/death leading to podocytopenia, often associated with a mild degree of intraglomerular hyperplasia and segmental sclerosis.\textsuperscript{8,10} In addition, we analyzed biopsies from patients with a tip lesion (n = 5). Whereas collapsing glomerulopathy and FSGS are included in the proposed taxonomy,\textsuperscript{9,10} the tip lesion, according to some authors,\textsuperscript{8,46-49} has been categorized as a transcategorical entity. The tip lesion was included in our study because it consists of a collection of cells localized at the urinary pole of the Bowman’s capsule, thereby suggesting a possible involvement of CD133\textsuperscript{+}CD24\textsuperscript{+} renal progenitors in its generation. In addition, we studied kidney biopsy specimens of 25 patients who had crescentic glomerulonephritis. The histologic diagnosis was IgA nephropathy (n = 8), ANCA glomerulonephritis (n = 9), lupus nephritis (n = 4), and Schönlein-Henoch disease (n = 4). The patients were selected on the existence of extracapillary proliferation in sections of the cryo-specimens. In addition, macroscopically normal portions of renal tissue were obtained from 15 patients undergoing nephrectomy because of renal cell carcinoma, in accordance with the recommendations of the ethical committee of the Azienda Ospedaliero-Universitaria Careggi, Florence, on human experimentation.

**Immunofluorescence and Confocal Microscopy**

We performed double labelings for CD24 and CD133. In addition, we stained for both markers separately on serial sections. We assessed the localization of the hyperplastic lesions in periodic acid Schiff–stained sections and made a comparison with the localization of CD24\textsuperscript{+} and CD133\textsuperscript{+} cells in immunostained consecutive sections. Two-micro-meter acetone-fixed cryosections were incubated with antibodies against CD24 (clone SN3; Santa Cruz Biotechnology, Santa Cruz, CA) or against CD133 (CD133/1 clone AC133; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The primary antibodies were detected with goat anti-mouse IgG1-Alexa 488 (Molecular Probes, Leiden, Netherlands). For double labeling, the sections were incubated with anti-CD24 and subsequently with anti-CD133 (CD133/2 clone 293C3; Miltenyi Biotec GmbH). The primary antibodies were detected with goat anti-mouse IgG1-Alexa 488 and goat anti-mouse IgG2b-Alexa 546 (Molecular Probes). In control sections, primary antibodies were omitted. The sections were examined using a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Digital images were made at exactly the same positions within the consecutive section and automatically overlaid using the Leica CW4000 CytoFISH software (Leica Microsystems, GmbH) to obtain the merged image.

Confocal microscopy was performed on 5-μm sections of renal frozen tissues or on cell cultures by using a LSM 510 META laser confocal microscope (Carl Zeiss, Jena, Germany), as described previously.\textsuperscript{12-14}

The double immunolabeling was performed by using the following primary antibodies: CD133 (clone 293C3)/Nestin (Chemicon, Temecula, CA), CD133 (clone 293C3)/PDX (222328; R&D Systems, Minneapolis, MN), CD24/CD68 (EBM11; Dako, Glostrup, Denmark), and CD133 (clone 293C3)/WT-1 (F6; Santa Cruz Biotechnology). The secondary appropriate antibodies were the following: Goat anti-mouse IgG2b-Alexa 488, goat anti-rabbit IgG-546, goat antimouse IgG2a-Alexa 546, goat anti-mouse IgG1-546 and goat anti-rabbit IgG-488 (Molecular Probes). The triple immunolabeling was performed by using the following primary antibodies: CD133 (clone 293C3)/CD24/nestin, CD133 (clone 293C3)/CD24/PDX, CD24/PDX/nestin, CD24/CD133 (clone 293C3)/claudin-1 (ab15098; Abcam, Cambridge, UK), CD24/CD133 (clone 293C3)/caveolin-1 (clone N20; Santa Cruz Biotechnology), and CD24/PDX/Ki67 (pAb; Abcam, Cambridge, UK). The secondary appropriate antibodies were the following: Goat anti-mouse IgG2b-Alexa 488 or -546, goat antimouse IgG1-546 or -488, and goat anti-rabbit IgG-633 or -488 (Molecular Probes). For each adult normal renal sample, two independent observers assessed all of the glomerular sections for all of the markers. Twenty randomly selected glomeruli were assessed from at least three sections from each patient to quantify the percentage of CD133\textsuperscript{+}CD24\textsuperscript{+} nestin\textsuperscript{−}, CD133\textsuperscript{+}CD24\textsuperscript{−} nestin\textsuperscript{+}, and CD133\textsuperscript{−}CD24\textsuperscript{−} nestin\textsuperscript{+} cells over the total number of cells of the lesion or over the total number of Ki67\textsuperscript{+} cells in hyperplastic lesions of all of the glomerulopathies analyzed.

**Laser Capture Microdissection and Real-Time Quantitative RT-PCR**

Frozen renal specimens were subjected to laser capture microdissection as described previously.\textsuperscript{54} Areas with identical proportions (mean area 50,000 μm\textsuperscript{2}) of glomerular hyperplastic lesions or healthy glomerular tufts were dissected from sections of biopsies or normal tissue specimens, respectively. Microdissected tissue areas were collected for RNA extraction using the RNeasy Micro kit (Qiagen, Hilden, Germany). Samples were used for TaqMan real-time RT-PCR analysis according to previously described protocols.\textsuperscript{55} CD133, CD24, Bmi-1, nestin, podocin, and nephrin quantification was performed using primers and probes from Assay on Demand kits (Applied Biosystems, Warrington, UK). Primers and probes for Oct-4 were described previously.\textsuperscript{12} Collagen α3 (IV), collagen α1 (IV), collagen α1 (1), laminin, fibronectin, and glyceraldehyde-3-phosphate dehydrogenase quantification was performed using Assay on Demand kits (Applied Biosystems) after stimulation of cells with 5 ng/ml TGF-β. Messenger RNA levels were quantitatively analyzed by comparing experimental levels with standard curves generated with serial dilutions of the same positive sample. TaqMan RT-PCR was performed as described previously.\textsuperscript{55}

**Cell Cultures**

To recover CD133\textsuperscript{+}CD24\textsuperscript{+} cells and CD133\textsuperscript{−}CD24\textsuperscript{−} PDX cells (podocytes), we first depleted total renal cells for CD45 and CD31 and then labeled them with PE-conjugated anti-PDX mAb (R&D Systems), followed by magnetic labeling with anti-PE Multisort microbeads using the anti-PE Multisort Kit (Miltenyi), as described previously.\textsuperscript{16} To obtain podocytes, we removed magnetic beads by using Multisort release reagent and PDX cells treated with a second magnetic separation for CD133 (CD133 Cell Isolation Kit; Miltenyi). By
contrast, to obtain CD133<sup>+</sup>CD24<sup>+</sup> cells, we directly treated total renal cells depleted for CD45 and CD31 with a magnetic separation for CD133. The purified cell fractions consisted of >98% of CD133<sup>+</sup>CD24<sup>+</sup> or CD133<sup>−</sup>CD24<sup>−</sup> PDX<sup>+</sup> cells. Alternatively, podocytes were obtained from cultured glomeruli after complete digestion of the Bowman’s capsule, as described previously. Expression of WT1, nephrin, and CD133 was checked to evaluate the purity of podocyte cultures obtained through glomerular outgrowth. Cells were cultured as described previously.

Statistical Analysis
The results are expressed as means ± SEM. Comparison between groups was performed by the Mann-Whitney test or the Wilcoxon test, as appropriate.

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DISCLOSURES
None.

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