Parietal epithelial cells maintain the epithelial cell continuum forming Bowman’s space in focal segmental glomerulosclerosis

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Summary statement
A novel role of parietal epithelial cells (PECs) in glomerulosclerosis is described. PECs seem to restore the glomerular epithelial continuum, which may avert further loss in glomerular function.

Abstract
In the glomerulus, Bowman’s space is formed by a continuum of glomerular epithelial cells. In focal segmental glomerulosclerosis (FSGS), glomeruli show segmental scarring, a result of activated PECs invading the glomerular tuft. The segmental scars interrupt the epithelial continuum. However, non-sclerotic segments seem to be preserved even in glomeruli with advanced lesions. We studied the histology of the segmental pattern in Munich Wistar Frömter (MWF) rats, a model for secondary FSGS. Our results showed that matrix layers lined with PECs cover the sclerotic lesions. These PECs formed contacts with podocytes of the uninvolved tuft segments, restoring the epithelial continuum. Formed Bowman’s spaces were still connected to the tubular system. Furthermore, in biopsies of patients with secondary FSGS we also detected matrix layers formed by PECs, separating the uninvolved from the sclerotic glomerular segments.

While PECs have a major role in the formation of glomerulosclerosis, we showed that in FSGS, PECs also restore the glomerular epithelial cell continuum that surrounds Bowman’s space. This process may be beneficial and indispensable for glomerular filtration in the uninvolved segments of sclerotic glomeruli.

Introduction
The kidneys are, amongst others, responsible for the removal of waste from our blood, reabsorption of nutrients, regulation of the body’s fluid balance and blood pressure. The functional units of the kidneys are the nephrons, consisting of a glomerulus, the filtration unit, and a tubular system. In the glomerulus four main cell types can be identified: endothelial cells, podocytes (visceral epithelial cells), mesangial cells and parietal epithelial cells (PECs). While mesangial cells have a more structural supportive role, endothelial cells and podocytes form together with the glomerular basement membrane the filtration barrier that is responsible for blood filtration and the formation of pro-urine (Arif and Nihalani, 2013). The glomerular capillary tuft is surrounded by Bowman’s capsule creating Bowman’s space, into which the filtrate enters after passing through the glomerular filtration barrier.
PECs line Bowman’s capsule facing the urinary space. They are connected to the proximal tubular cells at the urinary pole and podocytes at the vascular pole, thereby an epithelial cell continuum is created, ensuring that the pro-urine which is formed is directed to the tubular system.

The glomerulus is vulnerable to injury from inflammatory, metabolic, and other disease processes. Injury to the glomerulus can lead to proteinuria (Shankland et al., 2014). Progressive glomerular injury is characterized by the development of focal segmental glomerulosclerosis (FSGS). FSGS may be found without an undefined etiology, in this case it is called “idiopathic” or “primary” FSGS. When FSGS develops due to an underlying cause e.g., hypertension, viral infections, obesity or medications, it is called secondary FSGS (Kuppe et al., 2015, Fogo, 2015). The diagnosis of FSGS indicates that some of the glomeruli appear morphologically normal (focal) and that the affected glomeruli are only partially (segmental) sclerotic (Jennette et al.). The focal and segmental pattern of sclerosis in FSGS distinguishes scarring related to specific diseases from nonspecific global sclerosis, affecting the entire tuft, occurring at any age and increasing with ageing (Fogo, 2015). In FSGS the affected segments of the glomeruli show variable amounts of sclerosis, cellularity, hyaline accumulations and adhesions between the glomerular tuft and Bowman’s capsule.

It is very likely that the functional segments are still perfused and filter, provided that the glomerulus is still connected to a functional proximal tubule. Only the presence of foot process effacement in the unaffected glomeruli and in the uninvolved (non-sclerotic) segments of sclerotic glomeruli suggest that the structural damage is more widespread than indicated by the histologic lesions (Silva and Pirani, 1988). The segmental development of FSGS depends on local activation of PECs induced by podocyte injury (Smeets et al., 2011, Kriz, 2003, LeHir and Kriz, 2007, Nagata, 2017, Nagata and Kriz, 1992). Activated PECs migrate onto the capillary tuft and deposit extracellular matrix (ECM), leading to the development of segmental sclerotic lesions (Kuppe et al., 2015, Smeets et al., 2011). It is intriguing that also in kidneys with more advanced disease and older FSGS lesions, the lesions still can appear in a segmental pattern. This suggest that the uninvolved segments of the glomerulus are somehow relatively stable without marked progression of the glomerular lesion.

Glomerular function is tightly regulated and is highly dependent on a correct anatomical configuration, which consists of perfused capillaries accompanied by intact glomerular filtration barrier that is delimited by Bowman’s capsule with a glomerulotubular connection. Also, the ability to develop the necessary pressure equilibrium within the glomerulus, is only possible when the glomerular anatomical configuration is intact.

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We hypothesize that in glomeruli with segmental lesions the anatomical configuration of the uninvolved segments is preserved or restored and functional, which prevents complete loss of the affected glomeruli and explains the segmental appearance of FSGS.

In the present study, we investigate the anatomical configuration of glomeruli with FSGS and question how uninvolved (non-sclerotic) segments are functionally preserved or restored. In particular, we focus on the location and interplay between podocytes and PECs in segmentally sclerotic glomeruli in Munich Wistar Frömter rat model for secondary FSGS, and in human kidney biopsies of patients with secondary FSGS.

Results

Matrix layers enclose healthy glomerular segments

The histopathology of segmental glomerulosclerotic lesions was studied in kidneys of the Munich Wistar Frömter (MWF) rat, a model for secondary FSGS. Most mouse and rat models are very acute, with extensive PEC proliferation in the initial phase and sclerosis development within a few days or weeks, and thus do not mimic the chronic disease development, generally seen in many patients. MWF rats have been widely used to study histopathological changes in FSGS, because the sclerotic pattern observed in the glomeruli closely resembles the ones seen in human FSGS. We studied the morphology of the sclerotic and the uninvolved glomerular segments and in particular the boundaries between both segments in PAS stained kidney sections from MWF rats with early and advanced FSGS lesions. The FSGS lesions were mostly seen in the perihilar area of the glomerulus. The vast majority of the lesions were segmental and only in the oldest rats, with the most advanced disease state, globally sclerotic glomeruli were observed. The uninvolved segments of the glomeruli with FSGS showed a normal morphology with open capillaries and some scattered erythrocytes that may imply capillary blood flow at the time of kidney sampling (Fig. S1). These normal appearing segments seemed to be delimited by Bowman’s capsule despite the presence of segmental sclerotic lesions. Closer examination of Bowman’s capsule surrounding the uninvolved segment of the glomerular tufts revealed that the original Bowman’s capsule bifurcated into other matrix layers lined by epithelial cells (Fig. 1, Fig. S2). These layers form a connection with the unaffected tuft area, separating the non-sclerotic from the diseased glomerular segments (Fig. 1, Fig. S2).
Parietal epithelial cells form a boundary between sclerotic and uninvolved segments

The cells that are present on the matrix layer branching from the original Bowman’s capsule have a PEC-like phenotype as they have a flat cell body with cytoplasm that is hardly visible (Fig. 1, Fig. S2). We performed immunofluorescent stainings to investigate the cellular composition at this particular region. Double immunofluorescence staining for either LKIV69, a marker specific for matrix produced by PECs (Smeets et al., 2014) (Fig. S3), or SSeCKS or claudin-1, markers for PECs (Burnworth et al., 2012, Colvin and Chang, 2019), and synaptopodin, a marker for podocytes, revealed that the matrix layers covering the sclerotic areas were indeed formed and lined by PECs (Figs 2, 3 A-D). In addition, we observed that these matrix layers were connected to the glomerular tuft that is lined with synaptopodin-positive podocytes. Notably, these connections resemble the connections between PECs and podocytes at the hilum of the glomerulus (Figs 2, 3 A-D).

Analysis of the ultrastructure by electron microscopy showed thin epithelial cells layers consisting of PECs extending from Bowman’s capsule. These cell layers covered the sclerotic segments of the glomerulus and formed a connection between Bowman’s capsule and podocytes of the healthy segments of the glomerular tuft (Fig. 3E). LKIV69 immunostaining was examined by indirect immunoelectron microscopy. Bifurcation of Bowman’s capsule could be detected. PECs located on a LKIV69-positive matrix layer, branching from the original capsule were identified (Fig. 3F).

The parietal epithelial cells that form the connection with the podocytes express CD44

In previous studies it has been shown by us and others that PECs are involved in the development of FSGS lesions (Dijkman et al., 2005, Kuppe et al., 2015, Smeets et al., 2011, Shankland et al., 2014). These PECs are hypertrophic with enlarged cell bodies and nuclei. In addition, these activated PECs show de novo expression of the glycoprotein CD44 (Eymael et al., 2018, Fatima et al., 2012). This phenotype is different from that of the PECs lining the matrix layers covering the sclerotic lesions of affected glomeruli, as these PECs resemble normal quiescent PECs of Bowman’s capsule. Nevertheless, immunofluorescence staining for CD44, SSeCKS and synaptopodin revealed that the flat PECs that form the new matrix layer between the sclerotic and uninvolved glomerular segments are CD44-positive (Fig. 3 G-J). This was true for all sclerotic glomeruli we scored in the tissue slices of the rats (n=4). In addition, in some glomeruli also flat PECs of the original Bowman’s capsule were CD44-positive.
Bowman’s spaces of the uninvolved segments of sclerotic glomeruli are still connected to the proximal tubules

The glomerulus can only filter the blood if it is connected to a tubular system. Although, we observed that Bowman’s spaces surrounding the uninvolved segments of sclerotic glomeruli were created by epithelial connections between PECs and podocytes, we also questioned whether these spaces were connected to the proximal tubules. This means that all spaces in a single glomerulus should be connected to each other and to the proximal tubule. To test this hypothesis, serial sections of the rat kidneys were immunohistochemically stained for the PEC markers claudin-1 and SSeCKS and the podocyte marker nephrin (Fig. 4A, B). All serial sections were scanned and registered. Registration of the sections allowed to stack the sections (Movie1-4) and to create 3D reconstructions. To study the Bowman’s spaces created by the restored epithelial continuum, we annotated all spaces within the glomerulus that were surrounded by PECs and podocytes. 3D images of the Bowman’s spaces were created using binary masks (Fig. 4C, D). Analysis of the 3D images enabled us to determine whether the spaces in a glomerulus were connected to each other and to the proximal tubule. In three MWF rats, we annotated at least 35 glomeruli per rat. Glomeruli which were about at least 90% visible in the serial sections were scored. Two rats (both 41 weeks old) showed that 82% and 79% of the scored sclerotic glomeruli had restored Bowman’s spaces and were still connected to the proximal tubule. The third rat was 54 weeks old and showed in only 58% of the scored sclerotic glomeruli that Bowman’s spaces were connected to the proximal tubule. The other glomeruli were often atubular. These results indicate that PECs possibly maintain a functional Bowman’s space for the uninvolved glomerular segments, which is connected to the proximal tubule.

Podocytes in uninvolved segments show normal foot-process morphology

Next we studied the morphology of the podocytes on a ultrastructural level. Although we observed a normal immunostaining for synaptopodin, we cannot exclude severe foot-process effacement, hampering glomerular filtration. To study the foot process morphology in detail, we performed super resolution microscopy to visualize the foot process morphology. We focussed on podocytes in the uninvolved areas of sclerosed glomeruli that were enclosed by SSeCKS-positive parietal epithelial cells. We compared the podocyte foot-process morphology observed in the uninvolved areas with the morphology observed in non-sclerosed glomeruli of the same kidneys. Large areas in the uninvolved segments showed a normal foot-process morphology (Fig. 5B, C, arrows, B’, C’), that resembled the morphology observed in non-sclerosed glomeruli glomeruli (Fig. 5A, A’). However, we also observed some areas showing a disturbed staining pattern (Fig 5C, arrowhead). Such areas were
also observed in the non-sclerotic glomeruli (Fig 5A, arrowhead). Quantitative assessment of the images using Podocyte Exact Morphology Measurement Procedure (PEMP) allowed the quantification of the foot process morphology by measuring the filtration slit density (FSD). We observed a reduced FSD in the involved segments compared to the non-sclerotic glomeruli, indicating some degree of foot process effacement compared to the non-sclerotic glomeruli.

**Parietal epithelial cells form matrix layers between sclerotic and uninvolved segments in human FSGS lesions**

To translate our findings from the MWF rats to the human situation we examined kidney biopsies from nine patients with FSGS lesions. In these biopsies we found glomeruli that resemble the histopathological pattern seen in the MWF rat model (Fig. 6 and 7). LKIV69 in combination with ANXA3 and synaptopodin staining of the biopsies revealed that connections are formed between podocytes and PECs at the site of sclerosis (Fig. 6). PAS-, synaptopodin-, claudin-1, and LKIV69 stainings also showed that newly formed matrix layers had been formed by PECs that lay close to podocytes from the uninvolved segments (Fig. 7 A, C, E, F, H, J). Of note, the PECs forming the connection to the podocytes were in most lesions CD44-negative, although in some lesions CD44-positive PECs were observed (Fig. 7 B, D, G, I).

**Discussion**

In this study, we investigated segmental sclerotic lesions in MWF rats, an experimental model for secondary focal segmental glomerulosclerosis. We identified that PECs, next to their involvement in the formation of glomerulosclerosis, also participate in the maintenance of the epithelial continuum forming Bowman’s space, which is indispensable for glomerular filtration.

In our study, we discovered matrix layers that branch off the original Bowman’s capsule, covering sclerotic lesions, and that formed a connection with the remnant glomerular tuft segments. These matrix layers were produced and lined by PECs. The findings are summarized in Figure 8.

Also, in human biopsies showing secondary FSGS we detected PECs that form a matrix layer that separates uninvolved from sclerotic glomerular areas and formed connections with the uninvolved tuft segments, indicating that the phenomenon seen in the sclerotic glomeruli of MWF rats occurs in humans as well. In our study, we based our conclusions on findings in secondary FSGS, whether this process can also occurs in primary FSGS has to be elucidated.
The role of PECs in FSGS lesions or crescents in rapidly progressive glomerulonephritis have been reported earlier (Kuppe et al., 2015, Smeets et al., 2014, Kuppe et al., 2019, Dijkman et al., 2005, Smeets et al., 2011, Gaffney, 1982, Smeets et al., 2009). In these studies, activated PECs are described to be responsible for the formation and progression of sclerotic lesions. Activated PECs proliferate and migrate to the glomerular tuft, while excreting extracellular matrix, which leads to the formation of sclerotic lesions. In this study, we could confirm the involvement of CD44-positive, activated PECs in sclerosis formation in secondary FSGS. However, we also observed that a subpopulation of PECs is present on matrix layers that branched off the original Bowman’s capsule, separating the sclerotic from the uninvolved glomerular segments. An interesting finding was that the morphology of these PECs was similar to that of normal PECs of Bowman’s capsule (flat squamous epithelial cells). This phenotype is different from that of activated PECs that have been described to cause glomerulosclerosis, as activated PECs show marked hypertrophy with and enlarged cuboidal cell bodies and enlarged nuclei (Gaffney, 1982). Although PECs that were located on the matrix layers that surround the uninvolved segments of sclerotic glomeruli resemble quiescent PECs, they did express CD44. In a previous study we showed that CD44 is involved in proliferation and migration of PECs (Eymael et al., 2018), processes that may also be important for the PECs that cover the matrix layers lining the sclerotic lesions, observed in this study.

The PECs described in our study with the MWF rats appeared not to be a direct part of the glomerular scar but lined the sclerotic lesions and formed connections between the original Bowman’s capsule and the uninvolved segments of the glomerular tuft. Similar observations have been described in a study by Kriz et al. (Kriz et al., 1998). In this particular study, the sequence of histopathologic events leading from an initial glomerular injury to segmental sclerosis was studied in Fawn-hooded hypertensive rats (FHH). Similar to the MWF rats used in our study, the lesions were consistently associated with the glomerular vascular pole. In the FHH rats, this was attributed to expansion of primary branches of the afferent arteriole and subsequent podocyte injury and detachment. At the sides of podocyte detachment, PECs form tuft adhesions to Bowman’s capsule. Also in these rats, it was described that PECs demarcated the boundaries between the affected and still intact tuft remnants (Kriz et al., 1998). However, in the current study, we also focused on the connection between the PECs and the podocytes on the uninvolved tuft segments. The transition between PECs to podocytes at these connections seemed to be similar to the normal transition from PECs to podocytes at the hilum of the glomerulus. Consequently, an epithelial cell continuum and thus an enclosed Bowman’s space was created. Restoration of Bowman’s space is important for glomerular function since an enclosed Bowman’s space is essential for the formation of normal capsular hydrostatic pressures and thus for glomerular filtration. In fact, we observed that the
connection between the newly formed matrix layer and the glomerular tuft showed a similar curvature as the original Bowman’s capsule, suggesting the presence of capsular hydrostatic pressure. We suppose that due to the restoration of the Bowman’s space the non-scarred segments can function even when a large part of the glomerulus is sclerotic. We also observed erythrocytes in capillaries in the remaining uninvolved tuft segments, indicating that these segments could still be perfused.

Observing the described phenomenon, the question remains which signalling pathways and cell-cell interactions drive migration of PECs towards the remaining podocytes on the non-sclerotic tuft remnants. In general, local podocyte loss seems to be the basis of segmental sclerosis formation. A local response is initiated to cover the naked glomerular basement membrane (GBM). Podocyte hypertrophy and PECs covering the GBM are possible mechanisms to prevent protein leakage, functioning as wound healing process (Nagata et al., 2017).

Although the molecular pathways resulting in PEC activation are largely unknown, several signalling routes have been identified to have a possible role in PEC activation and migration of PECs onto the glomerular tuft (e.g. CD44, pERK1/2, mTOR, Wnt-β-catenin, angiotensin II, MIF, CD9 and CXCL12/CXCR4 signalling) (Ito et al., 2020, Lazareth et al., 2019, Miesen et al., 2017, Nagata, 2017, Romoli et al., 2018). At this moment one can only speculate on the sequence of events resulting in the restoration of the epithelial continuum. It is likely that PECs become activated and migrate to the tuft due to chemotactic signals, as described by Ito et al. In this study it was shown that injured podocytes increase migration inhibitory factor (MIF) and stromal cell-derived factor 1 (SDF-1, CXCL2) expression that stimulates CD44 expression and CD44-mediated migration in PECs (Ito et al., 2020). PECs migrate along the sclerosed denuded GBM. Next, contact inhibition at the side of podocyte-PEC contacts may result in a stable epithelial cell continuum formed by the PECs and podocytes.

In general, the described process may be important to keep the glomeruli functional and one may speculate that in case of remission or successful treatment the glomeruli remain (partly) functional, without further progression of FSGS. A recent case-study by Hamroun et al. may support this theory. In this particular study, a kidney with recurrence of primary FSGS was retransplanted 8 months after transplantation and reimplanted in another patient. At time of reimplantation the kidney showed marked FSGS and diffuse podocyte foot process effacement. In the 4- and 12-months biopsies after reimplantation the kidney still showed FSGS lesions in over two-thirds of the glomeruli. However, foot process effacement was reduced and kidney function was restored. Indicating that the lesions were inactive and did not affect kidney function (Hamroun et al., 2021).
In conclusion, this study shows that PECs are not only involved in (secondary) FSGS formation but also in the maintenance of the glomerular epithelial cell continuum so that the function of Bowman’s space is preserved and the pro-urine created by the uninvolved segments can still be directed to the proximal tubule. To get more insight into the filtration capacity of the uninvolved segments, perfusion studies using fluorescently-labelled tracers should be performed. In this case, the integrity of the restored Bowman’s space could be investigated. Another question that remains is whether the PECs that form the epithelial continuum as described in the current paper are differentially regulated compared to PECs actively involved in FSGS lesions. This is of importance in respect to the idea to target the processes driving PEC activation and to prevent or attenuate sclerosis. If PECs also fulfil a beneficial role in sclerotic glomeruli, inhibition of for instance PEC migration may have adverse effects.

**Material and methods**

**Animal experiments**

In the present study we used Munich Wistar Frömter (MWF) rats as a model for secondary focal segmental glomerulosclerosis. Initiated by an autosomal gene modification on chromosome 6, MWF rats show a reduced number of nephrons. Spontaneous development of hypertension contributes to injury to intermediate/small vessels and podocytes, which results in progressive glomerular scarring with age and finally renal dysfunction (Hackbarth et al., 1991). After a time period of 13-14 weeks MWF rats develop proteinuria and FSGS development starts at about 20 weeks of age. For our experiments only male MWF rats (n=8) with an age of 41-54 weeks were biopsied. The rat tissue was intravenous perfused using 3% (w/v) paraformaldehyde, before the kidneys were sampled. The isolated renal tissue was fixed in 4% (w/v) formalin and embedded in paraffin. The rats were obtained from Professor Kreutz, Department of Clinical Pharmacology and Toxicology, Charité Centrum für Therapieforschung, Charité - Universitätsmedizin Berlin, Germany (Schulte et al., 2012). All animals were housed under standard conditions. Animal procedures were approved by the German government officials (LANUV NRW 50.203.2 – AC 10/06) and performed in accordance with the European Communities Council Directive (86/609/EEC).
Patient biopsies

Archived kidney biopsies were selected with consent from the local ethics board of the Radboud university medical center (file number 2018-4563). Available patient data is depicted in table 1. Patients 6 to 9 were analysed for renal pathology consultation, but were not treated in the Radboudumc. Clinical data of these 4 patients is not available.

Staining methods

Stainings were performed on formalin fixed and paraffin embedded 4 µm tissue sections. The histopathology was analysed in Periodic acid–Schiff (PAS) stained kidney sections of the MWF rats. PAS staining was performed automatically with the Tissue-Tek® Prima™ (Sakura, version 20) histochemical staining machine. In short, slices were deparaffinised, washer with demi water and treated with periodic acid for 10 min. Next, slices were again washed in demi water, 30 min incubated in Schiff's reagent and rinsed with lukewarm water. After washing in streaming water, slices were incubated with haematin for 10 min and finally washed with demi.

Regarding immunofluorescent (IF) stainings, all antibodies were diluted in PBS-BSA 1% (v/v). Primary antibodies were incubated either for 1 h at room temperature or overnight at 4°C. Secondary antibodies were incubated 1 h at room temperature and diluted 1:200. Used primary antibodies can be found in table 2. The following secondary antibodies were used: Alexa Fluor 488 donkey anti-goat IgG (H+L) (A11055, ThermoFisher), Alexa Fluor 568 donkey anti-goat IgG (H+L) (A11057, ThermoFisher), Alexa Fluor 647 donkey anti-mouse IgG (H+L) (ab150107, Abcam), Alexa Fluor 647 donkey anti-rabbit IgG (H+L) (A31573, ThermoFisher), Alexa Fluor 568 donkey anti-rabbit IgG (H+L) (A10042, ThermoFisher).

For the detection of the PEC matrix, LKIV69 (1:50, kindly provided by Dr. T. van Kuppevelt) and anti-VSV Glycoprotein−Cy3 (1:400; monoclonal mouse antibody, clone P5D4, C7706, Merck) were used. IF stained slices were mounted with DAPI Fluoromount-G® (0100-20, SouthernBiotech). IF Images were captured using the automated high-content microscope (DMI6000B, Leica microsystems) or Keyence BZ-9000 Microscope (Keyence Deutschland GmbH, Neu-Issenburg, Germany).

A double immunohistochemically (IHC) staining was performed to detect podocytes and PECs on 4 µm consecutive sections of the embedded rat kidney tissue (3 series of 3 different rats). In the double IHC staining a horseradish peroxidase (HRP) and alkaline phosphatase (AP) detection were combined. To avoid nonspecific binding of the secondary antibody, slices were blocked with 20% (v/v) normal horse serum. Endogenous biotin, biotin receptors, and avidin were blocked with the
Avidin/Biotin blocking Kit (SP-2001, Vector laboratories). To detect podocytes, slices were incubated with an anti-nephrin antibody (table 2). As secondary antibody a horse anti-goat biotinylated antibody was used (1:200; BA-9500, Vector laboratories). Endogenous peroxidases were blocked with 0.3% (v/v) hydrogen peroxide in PBS. VECTASTAIN®ABC-AP staining Kit (PK-5000, Vector laboratories) was applied followed by incubation of the StayBlue/AP solution (ab178453, Abcam), resulting in a blue nephrin staining in the podocytes. For the follow-up HRP-reaction, slices were blocked with 20% (v/v) goat serum. To ensure an optimal staining of PECs, we used two different antibodies directed against the cytoplasmic protein SSeCKS and against the membrane PEC marker protein claudin-1. To detect both PEC makers we used a Brightvision poly HRP anti-rabbit antibody (VWRKDPVR110HRP, Immunologic). PECs were stained red after incubation with the AEC substrate system (ab64252, Abcam). IHC stained slices were covered with Fluoromount-G® (0100-01, SouthernBiotech).

Human biopsies were stained with a 3,3′-Diaminobenzidine (DAB) followed by a PAS stain. In short, endogenous peroxidases were blocked with 3% (v/v) hydrogen peroxide in PBS. Endogenous biotin, biotin receptors, and avidin were blocked with the Avidin/Biotin blocking Kit (Vector laboratories SP-2001). Tissue slices were blocked with 20% (v/v) serum. Primary antibodies were used as described in table 2. HRP-labelled secondary antibodies against rabbit, mouse or goat were used (Vector laboratories). Slices were incubated with VECTASTAIN® ABC-HRP Kit, Peroxidase (Standard) (Vector laboraties, PK-6100) followed by DAB. After dehydration, a PAS stain was applied and slices were mounted.

**Slide registration and 3D reconstruction of Bowman’s space**

To establish the architecture of the Bowman’s capsule and consequently Bowman’s space in affected glomeruli, 3D images of the sclerotic glomeruli were made. Using the Pannoramic P250 Flash II tissue scanner (3DHistech, camera: CIS VCC-FC60FR19CL, 0.24 µm pixel⁻¹) and corresponding computer software (Pannoramic Scanner, version 1.22.0.67865), light microscopic images of the histological slides were digitalized. Each stack of IHC slides was reconstructed by a pairwise registration of neighbouring slides. A stack consisted out of as a series of images made of the consecutive cuts in every 4 µm from the same paraffin embedded tissue block. The slide image in the middle of each stack was selected as a starting point and the three-dimensional reconstruction was obtained by concatenating all pairwise results. The pairwise registration is a three-step registration pipeline consisting of a robust pre-alignment, a parametric registration computed on coarse resolution images, and a high-resolution nonlinear registration (Lotz et al., 2019, preprint). In all three steps the Normalized Gradient Fields distance that measures the alignment of image gradients is
minimized (Haber and Modersitzki, 2007). In the nonlinear registration curvature regularization is added to the distance term to favour smooth deformations without foldings (Fischer and Modersitzki, 2003). After registration, glomeruli of interest were selected using the Automated Slide Analysis Platform (ASAP) and serial images of these selected glomeruli were created. Using ImageJ, Bowman’s spaces of the single images of one glomerulus were annotated and binary masks were created to create 3D reconstructions with the ImageJ 3D viewer plugin.

*Super resolution imaging and filtration slit density measurements*

Sample processing and subsequent imaging was performed as described before (Artelt et al., 2018). In short, 4 µm paraffin sections were directly mounted on coverslips (VWR). To correct for PFA-induced autofluorescence, samples were incubated with 100 mM glycine in PBS for 10 minutes. Samples were blocked with 1% (v/v) fetal bovine serum, 1% (v/v) goat serum, 1% (v/v) bovine albumin and 0.1% (v/v) cold fish gelatin in PBS at RT for 1 hour. Primary antibodies against nephrin (guinea pig, Progen GmbH, 1:100) and SSeCKS (Table 2, 1:200) were diluted in blocking solution and detected by a secondary anti-guinea pig antibody (1:800) and anti Cy3-labeled polyclonal goat anti-rabbit IgG (H+L) (1:600) (both from Jackson Immuno Research, Hamburg, Germany) diluted in blocking solution. 3D-Structured Illumination Microscopy (SIM) images were acquired using a Zeiss Elyra PS.1 system. Using Zeiss ZEN black software, 3D-SIM images were reconstructed. Podocyte PEMP was performed using FIJI and a custom-build macro (Siegerist et al., 2017). Analysis was performed in two different MWF Rat glomeruli. FSD was measured in 8 (4 sclerotic and 4 non-sclerotic) glomeruli.

*Immunoelectron microscopy*

LKIV69 immunostaining was examined by indirect immunoelectron microscopy (IEM), using immunoperoxidase labelling on 20 µm frozen sections. One-millimeter-thick kidney slices were immersion-fixed in a mixture of 10 mm periodate, 75 mm lysine, and 2% (w/v) paraformaldehyde, pH 6.2 (PLP), for 3 h. The slices were washed in PBS for 30 min and cryoprotected by immersion in 2.3 m sucrose solution for 1 h. Finally, tissues were snap-frozen in liquid nitrogen. Cryosections (20 µm) were rinsed in PBS for 1 h and then incubated with the LKIV69 antibody diluted in PBS-BSA 1% (v/v) for 18 h at 4°C, followed, after three washes with PBS, by incubation with a secondary antibody
anti-VSV diluted in PBS-BSA 1% (v/v). After three washes in PBS, the sections were incubated with a tertiaryperoxidase-labelled rabbit anti-mouse antibody diluted in PBS containing 1% BSA. After three washes in PBS, the sections were incubated in PBS, pH 7.4, containing diaminobenzidine (DAB) medium for 10 min, followed by DAB with the addition of 0.003% (v/v) hydrogen peroxide for 7 min. The sections were washed in distilled water, post-fixed in Palade buffer containing 1% (w/v) for 30 min at 4°C, dehydrated, and embedded in Epon812 (Merck). Ultrathin sections were examined in a JEOL 1200 EX2 electron microscope (JEOL).

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The authors wish to thank Lars Damen and Arie Oosterhof (Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, the Netherlands) for their contribution in the production and delivery of LKIV69 and Muradije Demirel-Andishmand (Department of Pathology, Radboud university medical center, Nijmegen, the Netherlands) for cutting consecutive sections of the embedded MWF rat tissue for us.

Competing interests

The authors declare that they do not have any conflicts of interest. The results presented in this article have not been published previously.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. Additional information and data is available from the corresponding author on reasonable request.
Author contributions statement

L.M. was involved in project administration, investigation, formal analysis, visualization and original draft preparation. P.B. was involved in software, methodology, formal analysis and original draft preparation. B.W., F.M., T.S., E.B., V.D., N.E., were involved in investigation and resources. J.E. and R.W. were involved in original draft review and editing. J.L., N.W., M.v.E., J.v.d L. were involved in software, resources and original draft review and editing. E.S. was involved in conceptualization. T.H. v. K. was involved in resources. M.J.M. was involved in resources, original draft writing and editing. J.F.M.W., J.J., B.S. were involved in conceptualization, investigation, original draft writing and editing, visualization, supervision and funding acquisition.

References


Figure 1 | Matrix layers branching from the original Bowman’s capsule line the sclerotic areas of glomeruli. Kidney tissue slices of MWF rats were PAS stained. (A, B, C) Glomeruli with segmental sclerosis. (A’, B’, C’) Zooms (3x) of the marked areas (rectangles) seen in images A, B, C receptively. Arrows indicate bifurcations of original Bowman’s capsule. Matrix layers branch off Bowman’s capsule and are lined with flat epithelial cells. The matrix layers separate the uninvolved from the sclerotic segments of the glomerular tuft. Asterisks indicate sclerosis.
Figure 2 | Connections between PEC matrix and the glomerular tuft at sclerotic areas are formed. Synaptopodin (green), LKIV69 (red) and DAPI (blue) expression is shown. (A) A non-sclerotic glomerulus of a MWF rat is shown. The glomerular tuft lined with podocytes is connected with the LKIV69-positive Bowman’s capsule at the vascular pole, reflecting the connection between PEC matrix and podocytes at this position. (A’) Zoom (4x) of the marked area (rectangle) seen in picture A. Dashed circles mark the area of the connection between PEC and podocytes at the vascular pole. (B, C, D) Sclerotic glomeruli of MWF rats containing connections between synaptopodin- and LKIV69-positive matrix at the side of sclerosis are shown. (B’, C’, D’) Zooms (4x) of the marked areas (rectangles) seen in picture B, C, D, respectively. The circles highlight connections between LKIV69-positive matrix and podocytes at the sclerotic areas. The dashed circle highlights the connection of the PECs and podocytes at the vascular pole. Single channels are shown in Fig. S4.
Figure 3 | Connections between CD44-positive PECs and the glomerular tuft at the sclerotic areas are formed. (A) A non-sclerotic glomerulus of a MWF rat is displayed. The glomerular tuft lined with podocytes (red) is connected with the SSeCKS-positive PECs (green) at the vascular pole (arrows), reflecting the connection between PECs and podocytes at this position. (B) A sclerotic glomerulus of a MWF rat is shown containing connections (arrows) between synaptopodin (red) and SSeCKS (green) expressing cells at the side of sclerosis. The sclerotic area is marked with asterisks. The arrowhead indicates bifurcation of Bowman’s capsule lined by PECs. IF images of the single channels are shown in Fig. S5. (C) An image of a healthy glomerulus of a MWF rat is depicted. Claudin-1 signal (red) and synaptopodin signal (green) are in contact at the vascular pole (arrows). (D) A sclerotic glomerulus of a MWF rat is depicted showing contact of claudin-1 (red) and synaptopodin (green) signal at the boundaries of healthy and sclerotic glomerular parts (arrows). Sclerosis is marked with asterisks. IF images of the single channels are shown in Fig. S6. (E) Electron Micrographs are depicted, showing a sclerotic (asterisks) and uninvolved segment of a glomerulus. Parietal epithelial cells (PECS, blue) form a border between the sclerosed capillaries (asterisks) and the healthy glomerular segment. The PECs line a thin matrix layers, which covers loose material within the sclerosed segment. The last PEC is positioned against a podocyte (yellow), identified by the presence of foot processes (arrows). (F) An electron Micrograph is depicted, showing a sclerotic glomerulus with bifurcation of Bowman’s capsule (arrowhead). LKIV69 immunostaining was examined by indirect immunoelectron microscopy (black granular staining, arrows). Red colour annotation depicts the original Bowman’s capsule. Violet colour annotation highlights the branching of the capsule and PECs located on LKIV69-positive matrix (inset, zoom). (G-J) PECs that make new connections with podocytes express CD44 (green). Synaptopodin expression is depicted in violet. SSeCKS expression is depicted in red. PECs that make new connections with the glomerular tuft are CD44-positive (arrows). Sclerosis is marked with an asterisk.
**Figure 4| Restored Bowman’s space is connected to the proximal tubule.** Example of the 3D construction of Bowman’s space. (A) Kidney slices are stained for PECs (claudin-1 and SSeCKS, red) and podocytes (nephrin, blue). A sclerotic glomerulus is depicted. The red dashed line represents the original position of the PECs. Due to sclerosis (asterisk), Bowman’s space (yellow, arrow) is altered and the PECs surround a non-sclerotic segment of the glomerular tuft (black dashed line). (B) Serial images of the sclerotic glomerulus seen in A. The images show different amount of sclerosis. The image in the lower right corner is the same as seen in A. (C) Binary masks of the annotated Bowman’s spaces seen in the images in B. (D) 3D reconstruction of Bowman’s space of the binary masks seen in C. The 3D reconstruction reveals that altered Bowman’s space depicted in the glomerulus in A (arrow) is still connected to the rest of the Bowman’s space and to the proximal tubule.
**Figure 5** | Uninvolved tuft areas contain podocytes with normal podocyte foot process morphology.

(A) Super resolution microscopy image of a non-sclerotic glomerulus in a MWF rat stained for podocin (green) and SSeCKS (red). Podocytes show a dense twisted podocin staining pattern. Some areas showed a disturbed pattern for podocin (arrowhead). (A’) Zoom of the selected area (inset) in A. (B, C) images of sclerotic glomeruli. The uninvolved (non-sclerotic) segments, surrounded by SSeCKS- positive PECs, show a comparable dense twisted podocin staining pattern as observed in the non-sclerotic glomeruli. Next to areas with normal appearing foot-processes we also observed some areas with a disturbed pattern (B, arrowhead). (B’, C’) Zooms of the selected areas (insets) in B and C, respectively. (D) Filtration slit density (FSD) measurement by Podocyte Exact Morphology Measurement Procedure (PEMP). The FSD in the uninvolved areas was lower and showed more variation compared to the FSD measured in non-sclerosed glomeruli (* P <0,05, ** P <0,01).
Figure 6 | In humans, PECs and podocytes form connections at the site of sclerosis. Glomeruli of patients with FSGS lesions are depicted, stained with PAS staining (A-G) or synaptopodin, LKIV69 and ANXA3 (A'-A''', B'-B''', C'-C''', D', E', F', G'). (A-A''') A glomerulus of a patient suffering from early membranous nephropathy (patient no. 1 of table 1) is depicted. (B-B''') A glomerulus of a patient suffering from chronic TMA (patient no. 2 of table 1) is shown. (C-C''', D-D') Two glomeruli of a
patient suffering from MGRS are shown (patient no. 3 of table 1). (E, E') A glomerulus of a patient suffering from membranous nephropathy is shown (patient no. 4 of table 1). (F, F') A glomerulus of a patient suffering from diabetic glomerulosclerosis is depicted (patient no. 5 of table 1). (G, G') A glomerulus of a patient suffering from diabetic glomerulosclerosis is shown (patient no. 6 of table 1). Glomeruli shown in PAS staining reflect the same glomeruli shown in immunofluorescent staining next to the PAS image. Squares with dashed lines (A', B', C', D, E, G) show areas of zoom. Zoom images are depicted next to the image containing the squares. White circles highlight connections between synaptopodin and LKIV69 and ANXA3 staining signal.
Figure 7 In humans, CD44-positive PECs form a matrix layer between uninvolved and sclerotic glomerular segments. (A-G) Glomeruli of two patients suffering from necrotizing glomerulonephritis are depicted (patients no. 7 and no. 8 of table 1). (H-J) A glomerulus of a patient suffering from nephrosclerosis is shown (patient no. 9 of table 1). (A, C, F) PAS and claudin-1 (brown) stain show
that PECs (arrows) separate the sclerotic (asterisks) from the uninvolved segments. (H) PAS and synaptopodin (brown) staining show that small and flat cells line the sclerotic area (arrow) and that these cells are adjacent to functional, synaptopodin-positive podocytes. (E, J) Claudin-1 (green) and LKIV69 (PEC matrix, red) IF stainings show that PECs form the matrix layer (arrows), separating the uninvolved and sclerotic (asterisks) glomerular segments. Single channels are presented in Fig. S7. (B, D, G, I) The PECs that form the new matrix layers show CD44 expression (brown, arrows). Asterisks depict sclerosis.
Figure 8 | Schematic illustration of the observation described in this study.

(A) A healthy glomerulus is depicted. PECs line Bowman’s capsule (orange) that surrounds the glomerular tuft and forms Bowman’s space. (B) In segmental sclerotic glomeruli the uninvolved glomerular segments are still surrounded by a Bowman’s capsule (orange) despite the presence of sclerotic lesions (grey). At the borders of the FSGS lesions the original Bowman’s capsule branches off (arrows) and matrix layers, lined by PECs, form a connection between the capsule and the podocytes of the uninvolved tuft segment. Within a single section of a glomerulus multiple spaces can be formed (spaces a and b), which in the whole glomerulus (3D) are still connected to each other and to the proximal tubule.

Table 1 | Pathological findings and parameters of kidney function

<table>
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<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>sCreat (umol/l)</th>
<th>sAlb (g/l)</th>
<th>UPCR g/10 mmol</th>
<th>Pathology findings/conclusions</th>
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<td>1</td>
<td>M</td>
<td>70</td>
<td>192</td>
<td>35</td>
<td>1.02</td>
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<td>2</td>
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<td>76</td>
<td>128</td>
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<td>24</td>
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<td>9</td>
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Table 2 | Antibodies used in immunostainings

<table>
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<tr>
<td>SSeCKS (pAb, made in rabbit, kindly provided by Prof E. Gelman)</td>
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<td>Synaptopodin (pAb, sc-21537, lot L0414, Santa Cruz)</td>
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<td>CD44 (clone 8E2, mAb, lot 2, 5640S, Cell signaling)</td>
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<tr>
<td>Claudin-1 (pAb, ab15098, lot GR282937-7, Abcam)</td>
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<tr>
<td>ANXA3 (pAb, HPA013398, lot A101914, Atlas antibodies)</td>
<td>1:200</td>
</tr>
<tr>
<td>Nephrin (pAb, AF4269-SP, lot ZMU02117071, R&amp;D systems)</td>
<td>1:300</td>
</tr>
</tbody>
</table>
**Fig. S1. Erythrocytes are present in non-sclerotic glomerular segments.** (A-C) Sclerotic glomeruli of MWF rats. Synaptopodin (blue) and SSeCKS and claudin-1 (red) expression is seen. In the non-sclerotic segments synaptopodin expression is preserved. Erythrocytes (arrows) are found in between the podocyte signal, reflecting the capillary lumen. Sclerosis is marked with asterisks.

**Fig. S2. Matrix layers of the original Bowman’s capsule line the sclerotic areas of glomeruli.** Kidney tissue slices of MWF rats were PAS stained. (A, B, C, D) 4 different glomeruli with segmental sclerosis are depicted. Arrows indicate the newly formed matrix layers lined with cells. Asterisks indicate sclerosis.
Fig. S3. LKIV69 expression represents the presence of PECs. (A-D) A healthy MWF rat glomerulus is shown. (E-H) A sclerotic MWF rat glomerulus is shown. Synaptopodin (violet), LKIV69 (red), SSeCKS (green) and DAPI (blue) signalling is depicted. The signal of SSeCKs seen in the cell body of the PECs overlap with the matrix signal LKIV69.
Fig. S4. Connections between PEC matrix and the glomerular tuft at sclerotic areas are formed—single channels. (A, D, G, J) The green colour reflects the synaptopodin signal seen in podocytes. (B, E, H, K) The red colour reflects the LKIV69 matrix deposited by the glomeruli. (C, F, I, L) Merge images of the LKIV69 and synaptopodin signal, including DAPI staining (blue). Fig. S5.
Fig. S5. Connections between PECs and the glomerular tuft at the sclerotic areas are formed, SSeCKS and synaptopodin single channels. (A-C) A non-affected glomerulus and (D-F) a sclerotic glomerulus of MWF rat tissues are depicted. Synaptopodin (red, A, D), SSeCKS (green, B, E) and merge images (C, F) are shown.

Fig. S6. Connections between PECs and the glomerular tuft at the sclerotic areas are formed, claudin-1 and synaptopodin single channels. (A-C) A healthy MWF rat glomerulus is shown. (D-F) A sclerotic MWF rat glomerulus is shown. Synaptopodin (green, A,D) and claudin-1 (red, B, E) expression is depicted. (C,F) Merge images are shown. Contact points between claudin-1 and synaptopodin signal can be seen.
Fig. S7. In humans PECs form matrix layers between uninvolved and sclerotic glomerular segments - single channels. (A-F) Sclerotic human glomeruli are shown. LKIV69 (red, A, D) and claudin-1 (green, B, E) expression is depicted. (C,F) Merge images are shown.
Movie 1. Stacked images of a sclerotic glomerulus. Registration of the images of the stained consecutive MWF tissue sections allowed the creation of serial images and stacking of selected glomeruli. Here, an example of a non-sclerotic glomerulus is given.

Movie 2. Stacked images of a sclerotic glomerulus. Registration of the images of the stained consecutive MWF tissue sections allowed the creation of serial images and stacking of selected glomeruli. Here, an example of a non-sclerotic glomerulus is given.
Movie 3. Stacked images of a non-sclerotic glomerulus. Registration of the images of the stained consecutive MWF tissue sections allowed the creation of serial images and stacking of selected glomeruli. Here, an example of a non-sclerotic glomerulus is given.

Movie 4. Stacked images of a non-sclerotic glomerulus. Registration of the images of the stained consecutive MWF tissue sections allowed the creation of serial images and stacking of selected glomeruli. Here, an example of a non-sclerotic glomerulus is given.
Supplementary information on antibody validation

For the following antibodies, validation was performed by the manufacturers: Synaptopodin (pAb, sc-21537, lot L0414, Santa Cruz), CD44 (clone 8E2, mAb, lot 2, 5640S, Cell signalling), Claudin-1 (pAb, ab15098, lot GR282937-7, Abcam), Nephrin (pAb, AF4269-SP, lot ZMU02117071, R&D systems).

Information on the anti-SSeCKS antibody can be found in the following paper:

Information on LKIV69 can be found in the following paper: