Changes in glomerular parietal epithelial cells in mouse kidneys with advanced age

Sebastian S. Roeder,1,4 Ania Stefanska,1 Diana G. Eng,1 Natalya Kaverina,1 Maria W. Sunseri,1 Bairbre A. McNicholas,1 Peter Rabinovitch,2 Felix B. Engel,3 Christoph Daniel,3 Kerstin Amann,3 Julia Lichtnekert,3 Jeffrey W. Pippin,3 and Stuart J. Shankland1

1Division of Nephrology, University of Washington, Seattle, Washington; 2Department of Pathology, University of Washington, Seattle, Washington; 3Department of Nephropathology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany; and 4Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany

Submitted 8 April 2015; accepted in final form 26 May 2015

The general population is living longer, highlighting the need to better understand the effects of aging on organ structure and function. Population-based studies have shown that impaired kidney function is common in the elderly, with a 15% prevalence in persons older than 70 yr of age (14, 15). The incidence of end-stage kidney disease is increasingly disproportionate higher in people over the age of 65 yr compared with younger individuals, and the need for dialysis in the octogenarian population has increased over 50% in the past decade (31). Functional kidney changes in aging are well documented in rats and humans (8, 73). There is a gradual decline in the glomerular filtration rate of between 0.63 and 0.75 ml·min⁻¹·yr⁻¹ (34, 55). There is also a decrease in renal blood flow, which is mainly confined to the renal cortex, whereas renal medullary blood flow remains relatively well preserved (67). Direct shunts exist between afferent and efferent arterioles bypassing the glomeruli in the juxtaglomerular (JG) zone (38). The effective renal blood flow progressively decreases to a greater extent than the glomerular filtration rate in the outer cortex (OC) because of increasing postglomerular vascular resistance, resulting in an increased filtration fraction (20). In aged rats, afferent arteriole resistance falls, allowing for a rise in glomerular capillary hydraulic pressure independent of changes in systemic pressure (3), leading to glomerulosclerosis (69).

Several well-documented structural glomerular changes accompany aging in rats (76) and humans (24), including progressive glomerulosclerosis from the accumulation of extracellular matrix proteins, mesangial expansion, and thickening of the glomerular basement membrane. The size of glomeruli increases with age due to compensatory hypertrophy, especially in JG glomeruli, which is accompanied by glomerular hyperfiltration and increased intraglomerular pressure (35). However, other studies have shown that glomerular volume either decreases (43) or does not change (27) with aging. Several studies in recent years have shown that podocytes are injured in aged kidneys (75), leading Floege et al. (21) to call kidney aging a “disorder of podocytes.” Podocyte number decreases with age, and a subset undergo hypertrophy (77). Nephrin levels are also reduced (77), and desmin (a marker of injury) is increased in aged podocytes (21). Wanner et al. (72) showed that podocytes are not replaced in 1-yr-old mice, which are considered middle aged. However, a subset of cells of renin lineage migrate in to the glomerular tuft in aged mice, of which several begin to express podocyte markers (47). In contrast, glomerular endothelial and mesangial cells increase in number to accommodate the increase in glomerular size (76).

Despite increasing knowledge of several glomerular changes in the aging kidney, our understanding of parietal epithelial cells (PECs) in aging is limited, especially at a time when the biology and roles of PECs are being better defined in health and disease (52, 57). Although rodents serve as a reliable model for studying the aged kidney as they do exhibit many of...
the functional and structural characteristics seen in humans (2, 37), little has been published regarding the impact of age on mouse kidneys. The purpose of the present study was to better describe the changes that occur in PECs in aged mice.

METHODS

Study animals and tissue processing. Twenty-seven-month-old C57BL/6 female mice (n = 8) were obtained from the National Institute on Aging (Charles River). These mice are considered advanced age, equivalent to humans aged 78 yr old (39, 70). Three-month-old C57BL/6 female mice (n = 7) were obtained from Jackson Laboratories (Bar Harbor, ME) and used as young control mice. Only female animals were studied to exclude sex differences. Mice were housed under standard conditions and handled according to guidelines of the Institutional Animal Care Committee of the University of Washington. At death, mice were perfused with 10 ml of ice-cold PBS to remove excess blood cells. After kidney bissection, one half of the kidney was fixed in 10% neutral buffered formalin (Globe Scientific, Paramus, NJ) at 4°C overnight, rinsed in 70% ethanol, processed, and then embedded in paraffin. The other half of the kidney was fixed for 45 min in 4% paraformaldehyde solution (Affymetrix, Santa Clara, CA) in PBS, washed in 30% sucrose at 4°C overnight, patted dry, rinsed briefly in OCT compound (Sakura Finetek, Torrance, CA), embedded in OCT, and frozen in a dry ice 100% ethanol bath.

Immunostaining. Immunoperoxidase staining was performed on 4-μm tissue sections from mouse kidney biopsies fixed in formalin and embedded in paraffin. Sections were deparaffinized using Histoclear (National Diagnostics, Atlanta, GA) and rehydrated in a graded series of ethanol. Antigen retrieval was performed by boiling in 10 mM citric acid buffer (pH 6.0 or 7.0) or in 10 mM EDTA buffer (pH 6.0). Non-specific antibody binding was blocked using background Buster (Accurate Chemical & Scientific, Westbury, NY) for 20 min at room temperature. When biotinylated secondary antibodies were used, endogenous biotin activity was suppressed with an avidin/biotin blocking kit (Vector Labs, Burlingame, CA). Antibodies were diluted in 1% IgG-free BSA (Sigma-Aldrich, St. Louis, MO) in PBS and incubated overnight at 4°C. Secondary antibodies and streptavidin conjugates were incubated for 1 h at room temperature.

For immunoperoxidase staining, endogenous peroxidase activity was blocked by incubation in 3% H2O2 for 15 min. After primary antibody incubation and labeling with horseradish peroxidase, immunostaining was visualized by precipitation of diaminobenzidine (Sigma-Aldrich). Periodic acid-Schiff (PAS) staining was performed as a negative staining. A mean of 69 ± 4 PECs per glomerulus was assessed for each animal. The number of podocytes per glomerular cross section was biased by changes in glomerular size, the tuft area for each glomerulus was also measured with ImageJ (version 1.48, National Institutes of Health), and scale bars were applied to all pictures using this software. Using an unbiased stereology approach, we then calculated podocyte density by dividing the number of p57-stained cells in one glomerulus by the corresponding glomerular tuft area.

The number of PECs was assessed by PAX2/PAS staining as previously described (80, 81). PECs were identified as cells lining Bowman’s capsule that showed strong nuclear staining. An average of 98 ± 11 glomeruli were assessed for each animal. As the number of PECs was biased by changes in glomerular size, we also measured the Bowman’s capsule length of each glomerulus. Using an unbiased stereology approach, we then calculated PEC density by dividing the number of PAX2-stained cells in one glomerulus by the corresponding Bowman’s capsule length.

Quantification of CD44, vimentin, and α-SMA was performed on immunofluorescence-stained sections, with PAS counterstaining. A mean of 69 ± 3 glomeruli were assessed for the quantification of each antibody. A glomerulus was considered positive if there was at least one cell lining the Böving’s capsule that stained with the respective antibody.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 (La Jolla, CA). A two-tailed unpaired Student’s t-test was applied to compare means of groups, and P < 0.05 was considered statistically significant. Data are presented as means ± SD.
RESULTS

Glomerulosclerosis increased in aged mouse kidneys. The percentage of glomeruli with sclerosis was determined on PAS-stained sections and was categorized as either segmental or global. Because dramatic differences were noted between the glomeruli of the outer cortical (OC) and juxtamedullary (JM) regions, glomeruli from each location were evaluated separately to delineate these differences. Segmental sclerosis was increased in 27-mo-old mice with advanced age in both OC glomeruli (3 mo: 0.0 ± 0% vs. 27 mo: 2.6 ± 0.65%, P < 0.01) and JM glomeruli (3 mo: 0.0 ± 0% vs. 27 mo: 6.8 ± 1.8%, P < 0.01; Fig. 1). Although global sclerosis was not

Fig. 1. Glomerulosclerosis increases in mice with advanced age. Periodic acid-Schiff (PAS) staining was used to identify and quantitate glomerular scarring in young and old mice. Scale bars are provided. A: low-power magnification (×100) of PAS staining of a 27-mo-old (27m) mouse. The dashed line demarcates the juxtamedullary (JM) region on the left from the outer cortical (OC) region on the right. Arrows indicate the kidney capsule. *An obsolescent glomerulus. B: example of an aged glomerulus with segmental glomerulosclerosis. C: example of an aged glomerulus with global glomerulosclerosis. D: the percentage of glomeruli with segmental glomerulosclerosis increased in aged mice in both the OC and JM compared with young mice. Within the group of mice with advanced age, segmental glomerulosclerosis was more marked in JM glomeruli. E: the percentage of glomeruli with global glomerulosclerosis in JM glomeruli increased in aged mice compared with young mice. n.s., not significant; n/a, statistical analysis was not applicable as means equalled zero.
increased in the OC (3 mo: 0.0 ± 0% vs. 27 mo: 0.2 ± 0.2%, 
\( P = 0.37 \)), global glomerulosclerosis was increased in aged JM 
glomeruli (3 mo: 0.0 ± 0% vs. 27 mo: 2.34 ± 0.57%, \( P < 0.01 \)).

Glomerular tuft area increased and podocyte density 
decreased in aged mouse kidneys. Dramatic regional 
differences were noted between glomeruli of the OC and JM regions, as 
follows. First, glomerular tuft area was larger in JM glomeruli 
compared with OC glomeruli in 3-mo-old mice (JM: 4,477 ± 77.4 \( \mu \text{m}^2 \) vs. OC: 3,416 ± 121 \( \mu \text{m}^2 \), \( P < 0.0001 \); Fig. 2). 
Second, glomerular tuft area increased in the OC in 27-mo-old 
mice compared with 3-mo-old mice (3 mo: 4,477 ± 121 \( \mu \text{m}^2 \) vs. 
27 mo: 4,012 ± 117.6 \( \mu \text{m}^2 \), \( P < 0.01 \)). Third, glomerular 
tuft area also increased in JM glomeruli in 27-mo-old mice 
compared with 3-mo-old mice (3 mo: 4,477 ± 77.4 \( \mu \text{m}^2 \) vs. 27 
mo: 5,094 ± 79.7 \( \mu \text{m}^2 \), \( P < 0.0001 \)). Finally, in 27-mo-old 
mice, glomerular tuft area was larger in JM glomeruli compared 
with OC glomeruli (JM: 5,094 ± 79.7 \( \mu \text{m}^2 \) vs. OC: 
4,012 ± 117.6 \( \mu \text{m}^2 \), \( P < 0.0001 \)).

Podocyte number was determined by nuclear staining for 
p57, as we have previously reported (66). In young mice, the 
absolute number of podocytes was higher in JM glomeruli 
compared with OC glomeruli (JM: 11.34 ± 0.3 p57-stained 
cells/glomerular cross section vs. OC: 9.42 ± 0.3 p57-stained 
cells/glomerular cross section, \( P < 0.0001 \); Fig. 2). However, 
compared with 3-mo-old mice, absolute podocyte number 
was significantly lower in 27-mo-old mice for both 
JM glomeruli (3-mo-old mice: 11.34 ± 0.30 p57-stained 
cells/glomerular cross section vs. 27-mo-old mice: 5.71 ± 0.2 
p57-stained cells/glomerular cross section, a 49.65% decrease, 
\( P < 0.0001 \)) and OC glomeruli (3-mo-old mice: 9.41 ± 0.03 
p57-stained cells/glomerular cross section vs. 27-mo-old 
mice: 5.33 ± 0.20 p57-stained cells/glomerular cross section, a 
43.43% decrease, \( P < 0.0001 \); Fig. 2). There was no significant 
difference in absolute podocyte number in older mice between 
JM and OC glomeruli (\( P > 0.05 \)).

Because of the changes in glomerular tuft area, unbiased 
stereology was performed to assess podocyte density. This 
revealed that in younger mice, podocyte density was lower in 
JM glomeruli compared with OC glomeruli (JM: 2,535 ± 58.74 
p57-stained cells/mm\(^2\) tuft area vs. OC: 2,759 ± 17.09 
p57-stained cells/mm\(^2\) tuft area, \( P < 0.01 \); Fig. 2). Likewise, 
podocyte density was significantly lower in older mice in both 
JM glomeruli (3-mo-old mice: 2,535 ± 58.74 p57-stained 
cells/mm\(^2\) tuft area vs. 27-mo-old mice: 1,126 ± 53.68 p57-
stained cells/mm\(^2\) tuft area, a 55.58% decrease, \( P < 0.0001 \)) 
and OC glomeruli (3-mo-old mice: 2,759 ± 17.09 p57-stained 
cells/mm\(^2\) tuft area vs. 27-mo-old mice: 1,341 ± 81.67 p57-
stained cells/mm\(^2\) tuft area, a 51.40% decrease, \( P < 0.0001 \)). 
Within the group of older mice, podocyte density was markedly 
lower in JM glomeruli (\( P < 0.05 \) vs. OC glomeruli).

These results show that glomerular tuft area was larger in JM 
glomeruli of young mice and in both OC and JM glomeruli of 
older mice. Podocyte number and density decrease markedly in

---

**Fig. 2. Podocyte number and density decrease in mice with advanced age.** Podocytes were identified by p57 staining (brown nuclear stain; examples indicated by arrowheads) in tissue counterstained with PAS from young and old mice at 3 mo (3m) and 27 mo of age, respectively. Representative images were taken under the same magnification (×400). Scale bars are provided. A–D: examples of p57 staining from the young OC (A), young JM (B), old OC (C), and old JM (D). E: in young and old mice, the glomerular tuft area was higher in JM glomeruli compared with OC glomeruli for their given age. The glomerular tuft area was higher in older mice for OC and JM glomeruli compared with young mice. F: in young mice, the absolute number of podocytes, as measured by p57-stained cells per glomerular cross section, was higher in JM glomeruli. In aged mice, p57 staining was significantly reduced in both OC and JM glomeruli. G: unbiased stereology showing that compared with young mice aged 3 mo, podocyte density (number of podocytes/tuft area) was lower in mice with advanced age at 27 mo in both OC and JM glomeruli. Within the group of old mice, podocyte density was lower in JM glomeruli compared with OC glomeruli.
aged mice, and podocyte density was further lower in aged JM glomeruli compared with OC glomeruli, where the majority of glomerulosclerosis occurred in 27-mo-old mice.

**Bowman’s capsule length increased and PEC density decreased in aged mouse kidneys.** Because of the increase in glomerular size with age, we also measured Bowman’s capsule length. As expected, the data mirrored the glomerular tuft area measurements, as follows. Bowman’s capsule length was higher in JM glomeruli compared with OC glomeruli in 3-mo-old mice (JM: 654.2 ± 12.6 μm vs. OC: 448.5 ± 9.8 μm, P < 0.0001; Fig. 3). Second, Bowman’s capsule length increased in the OC in 27-mo-old mice compared with 3-mo-old mice (3-mo-old mice: 448.5 ± 9.8 μm vs. 27-mo-old mice: 692.7 ± 21.1 μm, P < 0.5). Third, Bowman’s capsule length increased in JM glomeruli in 27-mo-old mice compared with 3-mo-old mice (3-mo-old mice: 654.2 ± 12.6 μm vs. 27-mo-old mice: 869.8 ± 25.8 μm, P < 0.0001). Finally, Bowman’s capsule length increased in JM glomeruli in 27-mo-old mice compared with 3-mo-old mice (3-mo-old mice: 654.2 ± 12.6 μm vs. 27-mo-old mice: 869.8 ± 25.8 μm, P < 0.0001).

PECs were identified by immunostaining for PAX2 (45). Absolute PEC number was quantified by counting positively stained nuclei. In young mice, absolute PEC number was significantly higher in JM glomeruli compared with OC glomeruli (JM: 4.8 ± 0.15 PAX2-stained nuclei/glomerular cross section vs. OC: 2.8 ± 0.14 PAX2-stained nuclei/glomerular cross section, P < 0.0001; Fig. 3). Compared with 3-mo-old mice, PEC number was higher in 27-mo-old animals in OC glomeruli (3-mo-old mice: 2.8 ± 0.14 PAX2-stained nuclei/glomerular cross section vs. 27-mo-old mice: 3.9 ± 0.18 PAX2-stained nuclei/glomerular cross section, P < 0.001) but was unchanged in JM glomeruli (3-mo-old mice: 4.8 ± 0.15 PAX2-stained nuclei/glomerular cross section vs. 27-mo-old mice: 4.8 ± 0.27 PAX2-stained nuclei/glomerular cross section, P = 0.97; Fig. 3). As previously noted, there was no significant difference in absolute podocyte number in older mice between JM and OC glomeruli (P > 0.05). Absolute PEC number, however, was higher in JM glomeruli compared with OC glomeruli in 27-mo-old mice (JM: 4.8 ± 0.27 PAX2-stained nuclei/glomerular cross section vs. OC: 3.9 ± 0.18 PAX2-stained nuclei/glomerular cross section, P < 0.05).

Next, unbiased stereology was used to determine PEC density by dividing the number of PAX2-stained nuclei by Bowman’s capsule length (Fig. 3). In young mice (3 mo), PEC density was higher in JM glomeruli compared with OC glomeruli (JM: 7.32 ± 0.20 PAX2-stained cells/mm Bowman’s capsule length vs. OC: 6.32 ± 0.24 PAX2-stained cells/mm Bowman’s capsule length, P < 0.001). Compared with young mice, PEC density decreased significantly in aged kidneys (age: 27 mo) in both OC glomeruli (5.59 ± 0.24 PAX2-stained
cells/mm Bowman’s capsule length, a 11.60% decrease vs. 3 mo, \( P < 0.05 \) and JM glomeruli (5.48 ± 0.22 PAX2-stained cells/mm Bowman’s capsule length, a 25.16% decrease vs. 3 mo, \( P < 0.0001 \)). In older mice, PEC density was similar in OC and JM glomeruli (\( P > 0.05 \)).

These data show that in young mice, absolute PEC number and PEC density are higher in JM glomeruli. Overall PEC number significantly increased in 27-mo-old OC glomeruli, whereas PEC density is reduced in elderly mouse kidneys, more so in JM glomeruli, similar to the decrease in podocyte density in this region.

**Extracellular matrix proteins increased in Bowman’s capsule in aged PECs.** PECs normally adhere to Bowman’s capsule, which is constitutively composed of several specific extracellular matrix proteins (for a review, see Ref. 44). To determine if extracellular matrix proteins of Bowman’s capsule changed with advanced age, immunohistochemistry was performed for HSPG (Perlecan), the laminin \( \alpha_1/\beta_1 \)-chain, and conformational determinants on collagen type IV, particularly \( \alpha_1, \alpha_2, \) and \( \alpha_6 \), which are all normally present in Bowman’s capsule but are expressed in low levels or absent in the glomerular basement membrane (36, 41).

As expected, staining for HSPG was detected along Bowman’s capsule in young mice (Fig. 4). HSPG staining increased substantially along Bowman’s capsule in older mice (Fig. 4). The laminin \( \alpha_1/\beta_1 \)-chain stained Bowman’s capsule in young mice but was decreased in older mice (Fig. 4). As expected in young mice, collagen type IV stained Bowman’s capsule, the tubular basement membrane, the mesangial matrix, and the basement membrane of the vasculature. However, in mice with advanced age, collagen type IV staining intensity increased markedly along Bowman’s capsule and, to a lesser extent, in glomerular basement membranes as well (Fig. 4). Staining for collagen type I was also performed, which increased in a periglomerular location in 27-mo-old mice but not within glomeruli (not shown).

**CD44 and ERK phosphorylation increase in aged kidneys.** Because two extracellular matrix proteins were increased in Bowman’s capsule in aged mice, we next examined CD44, a marker of activated PECs, a state in which PECs produce extracellular matrix proteins in experimental and human focal segmental glomerulosclerosis (FSGS) (19, 59, 60). In the present study, CD44 expression was assessed by immunohistochemistry. In young mice, CD44 staining in cells lining Bowman’s capsule was present in only 1.3 ± 0.9% of OC glomeruli and 12.2 ± 2.7% of JM glomeruli (\( P < 0.01 \); Fig. 5).

As expected from previous reports (19, 59, 60), only very occasional CD44-stained circulating cells were detected within the glomerular tuft. However, the percentage of glomeruli staining positive for CD44 along Bowman’s capsule increased significantly in mice with advanced age in both the OC (16.88 ± 1.9%, \( P < 0.0001 \) vs. 3 mo) and JM (51.33 ± 4.4%, \( P < 0.0001 \) vs. 3 mo, \( P < 0.0001 \) vs. OC in 27-mo-old mice; Fig. 5).

We have recently found that staining for p-ERK activation loop residues Thr202/Tyr204 and Thr185/Tyr187 increased in PECs in experimental FSGS (unpublished observations). Accordingly, double staining was performed to determine if activated PECs in aged glomeruli coexpressed p-ERK. As we have previously reported (12), p-ERK staining was not detected in young glomeruli in the present study. In contrast, as shown in Fig. 5, p-ERK staining was indeed increased in aged glomeruli and was restricted to cells lining Bowman’s capsule. Nearly 100% of PECs that stained for CD44 in aged kidneys coexpressed p-ERK. Taken together, a subset of PECs in aged kidneys de novo express CD44 and activated ERK.

**A subset of epithelial-to-mesenchymal transition markers increased in aged PECs.** Because of the strong correlation between scarring and certain epithelial cells undergoing epithelial-to-mesenchymal transition (EMT) (29), we next sought to determine if PECs began to express EMT markers in aged kidneys. Double staining for collagen type IV, a constitutive matrix protein of Bowman’s capsule, was used to easily dis-
27-mo-old mice: 60.63 \pm 0.01) and JM glomeruli (3-mo-old mice: 37.14 \pm 0.001). In 27-mo-old mice, compared with 3-mo-old young mice; the percentage was higher in JM glomeruli. C: In contrast, in mice aged 27 mo, considered as advanced age, CD44 staining was detected in cells lining Bowman’s capsule in the OC (arrowheads). An occasional CD44-positive cell was detected in the capillary loops (arrows). D: CD44 staining was readily detected in cells lining Bowman’s capsule in the JM of 27-mo-old mice (arrowheads). CD44-positive cells were detected in the capillary loops (arrows). E: the percentage of glomeruli with CD44-stained cells on Bowman’s capsule increased in 27-mo-old aged mice compared with 3-mo-old young mice; the percentage was higher in JM glomeruli. F–H: confocal microscopy images of immunofluorescent staining in aged JM glomeruli (magnification: \times 400). F and G: staining for CD44 (F: red; arrowheads show examples) and p-ERK (G: green; arrowheads show examples) increased in cells lining Bowman’s capsule. H: CD44 and p-ERK colocalized (yellow).

Glomerular immunostaining for the EMT markers E-cadherin (5, 79) and \(\beta\)-catenin (5, 79) were not significantly different in aged kidneys compared with young kidneys (\(P > 0.05\); data not shown). Because we have recently reported that smooth muscle (SM)22 was increased in PECs in experimental FSGS (40), immunostaining for SM22 was also performed on young and aged kidneys. SM22 was barely detected in young mice, with no changes in older mouse kidneys (\(P > 0.05\) vs. young mice; data not shown).

Using collagen type IV to demarcate Bowman’s capsule, these results are consistent with the de novo expression of two EMT markers in aged PECs that were barely detected in young mice. Expression of these EMT markers in 27-mo-old mice was more pronounced in glomeruli of the JM region, matching the more severely injured phenotype of glomeruli in that area.

De novo expression of pericyte but not senescent markers in aged PECs. Studies have shown that a subset of myofibroblasts derive from pericytes, and, in this state, they are typically profibrotic (18). Given that \(\alpha\)-SMA increases in activated pericytes (30), and given that our data in the present study showed that PECs were activated (de novo expression for CD44) in aging, we aimed to determine if PECs begin to express pericyte markers (NG2, PDGF receptor-\(\beta\), and CD146) with advanced age (Figs. 7–9).

NG2 staining was barely detected in PECs in young mice but was increased substantially in PECs in aged mice (Fig. 7). As expected, staining for NG2 was absent when the IgG isotype...
control was substituted for the primary antibody and when primary or secondary antibodies were omitted (not shown). To ensure that NG2 staining in PECs was indeed positive, triple staining for pericyte markers in the kidney interstitium was used as a positive control. As expected, pericyte staining for NG2, α-SMA, and PDGF receptor-β were all detected in individual images, and NG2 and PDGF receptor-β staining merged when images were overlapped. All three makers overlapped in the stroma surrounding larger vessels.

The results of the immunofluorescent staining for the PDGF receptor-β are shown in Fig. 8. As expected, PDGF receptor-β staining was detected in a mesangial distribution in 3-mo-old mice, but not in PECs. However, in 27-mo-old mice, PDGF receptor-β staining was detected in PECs as well as in mesangial cells. Staining for PDGF receptor-β was not detected when the IgG isotype control was substituted for the primary antibody. The percentage of glomeruli with positive α-SMA staining in cells lining Bowman’s capsule was significantly higher in 27-mo-old OC and JM glomeruli compared with young mice.
in PECs in young mice, similar to that in normal human PECs (9). In 3-mo-old mice, CD146 staining was detected in mesangial cells and tubular epithelial cells, as previously reported in humans (16). However, CD146 staining was increased in PECs of aged mice at 27 mo. Staining for CD146 was not detected when the IgG isotype control was substituted for the primary antibody. Stromal cells of the interstitium were used as a positive control, because interstitial pericytes normally stain for CD146. Our results showed that in the kidney interstitium, staining was positive for CD146, which overlapped with PDGF receptor-β (PDGFRβ; D3, green) to create a white color when merged (D4).

Notch 3 staining is increased in PECs in aged mouse kidneys. We next stained for Notch 3 because Notch has been implicated in PEC activation and EMT in glomerular disease (64, 71) and because Notch 3 has been reported to be profibrotic in kidneys (17). Notch 3 staining was faintly detected in PECs in young mice (Fig. 10). In contrast, staining for Notch 3 was markedly increased in PECs in aged kidneys but not in other glomerular cells (Fig. 10).

DISCUSSION

The majority of advanced aging studies in the kidney have been limited to humans and rats, with only few reports in mice. Moreover, despite the role of PECs in health and proteinuric glomerular diseases being better understood in recent years, the effects of advanced age on PECs are still poorly defined beyond showing that ceruloplasmin is increased in PECs in aged rats (78). In the present study, we describe that mice with advanced age (27 mo of age, which is equivalent to a human age of 78 yr) (22, 25, 39, 70) develop age-related glomerular changes. The effects of age on PECs include reduced PEC density, de novo activation, and changes in marker expression consistent with EMT. These phenotypic changes are more pronounced in glomeruli in the JM region compared with the OC and likely account for the increase in HSPG and collagen type IV in Bowman’s capsule of aged mice.

The results of the present study in mice with advanced age show several features considered characteristic of aging (24, 76), as follows. First, aged mice developed segmental and global glomerulosclerosis. Sataranatatajan et al. (56) showed that transforming growth factor-β and a subset of micro-RNAs were associated with increased matrix production in very old mice. Noteworthy was that glomerular scarring was substantially more prominent in JM glomeruli. The overall percentage of glomeruli with sclerosis in male mice (the sex used in the present study) was 30%, while in humans, the percentage of glomeruli with sclerosis is typically lower (24, 51, 54). This might reflect the well-characterized lower incidence of scarring in female mice (the sex used in the present study), species...
differences, and a lower incidence of kidney risk factors in older mice, such as hypertension. Second, glomerular hypertrophy, defined as an increase in glomerular size, was present in aged mice similar to other species. Third, like in humans and rats (77), podocyte number was markedly reduced in aged mice. Podocyte changes and damage have been well characterized in aged rat and human kidneys, including depletion, reduced nephrin staining, and increased desmin expression (21, 48, 72, 75, 77).

However, to our knowledge, this is the first study to use unbiased stereology to show that age-related depletion in podocyte density is more pronounced in JM glomeruli compared with OC glomeruli. Our results show that absolute podocyte number per glomerular cross section decreased in aged kidneys similarly in JM and OC glomeruli. However, because glomerular size increased more in JM glomeruli of aged mouse kidneys, podocyte density was lower in this region of the kidney. Given the well-established links between reduced podocyte number and glomerulosclerosis (74), it is noteworthy that JM glomeruli also had more age-associated scarring in mice. Together, these features validate the use of mice as a model to define the effects of aging on glomeruli, which is critical for future studies that can use genetically altered mice for mechanistic studies. As discussed below, it is worth highlighting that there are substantial changes in JM glomeruli with aging beyond the well-described size alterations.

A first major finding in the present study was an age-related decrease in the density of PECs, which was more pronounced in JM glomeruli. Our results showed that the length of Bowman’s capsule increased in both JM and OC glomeruli, coincident with overall glomerular hypertrophy. Differences in glomerular size have been previously shown based on kidney location in humans (63). Using Bowman’s capsule length as the denominator for unbiased stereology, our results in younger mice showed that PEC density was higher in JM glomeruli compared with OC glomeruli. However, in aged kidneys, PEC density decreased significantly in both JM and OC glomeruli, being more pronounced in JM glomeruli. The absolute number of PECs increased in OC glomeruli but did not change in JM glomeruli with age. These data in very old mice confirm our previous report (80) in middle-aged rats, where overall PEC number also increased. However, this is the first report, to our knowledge, measuring PEC density in very old mice and separately for the OC and JM regions. Taken together, both podocyte and PEC density decrease with advanced age, and both changes are more pronounced in JM glomeruli. The impact of location on glomerular epithelial cell number and density is highlighted in the present study, as combining the data from both glomerular locations might lead to misinterpretations.

Wiggins et al. (77) showed that in aged kidneys, glomerular mesangial and endothelial cells increase in number, appropriately when glomerular volume increases. Thus, they showed no changes in the density of these cells. In contrast, therefore, the present study shows that age negatively impacts the density of both glomerular epithelial cell types (podocytes and PECs). A report (77) by others has suggested candidate mechanisms for the age-related decline in podocyte number, such as ROS. The mechanisms underlying the age-related decrease in PEC num-

![Immunofluorescent staining for PDGFRβ increases in PECs in mice with advanced age.](http://ajprenal.physiology.org/)

Fig. 8. Immunofluorescent staining for PDGFRβ increases in PECs in mice with advanced age. Confocal microscopy images (magnification: ×400) are shown of JM glomeruli from young mice aged 3 mo and old mice aged 27 mo. Glomeruli are indicated by dashed circles. Scale bars are provided. Nuclei were stained blue with DAPI. A: PDGFRβ staining (green) was detected in cells in the glomerular tuft in young mice in a mesangial distribution, but not along Bowman’s capsule. B: in old mice, PDGFRβ was detected in cells lining Bowman’s capsule (arrowheads). C: PDGFRβ was not detected when the primary antibody was substituted with an IgG isotype control. D: double staining for PDGFRβ (D1, green) and the mesangial cell marker α5-integrin (D2, red) and nuclei stain with DAPI (D3, blue); colocalization of PDGFRβ and α5-integrin was observed when the images were merged (D4, yellow, arrows). These results show that PDGFRβ staining increased in PECs in aged kidneys.
ber were not studied. Apart from the increase in Bowman’s capsule length, used as the denominator for unbiased stereology, likely biological mechanisms include cell death, which has been reported in PECs (12), and PEC detachment (1). Altered autophagy must be considered as it has been in aged podocytes (28), although we found no changes in active mTOR staining in aged PECs. The absence of p21 staining (58) suggests that PEC senescence is less likely. Although not studied, we speculate that, like in other cell types (62), self-renewal by proliferation is likely also reduced with advanced age. Finally, age-related changes to extracellular matrix proteins of Bowman’s capsule to which PECs normally adhere might alter their survival and/or attachment.

Data from the present study showed that collagen type IV and HSPG, but not laminin or collagen type I, are increased in Bowman’s capsule of aged mice. The cellular source for the increase in matrix proteins is likely PECs. CD44 is considered a marker of PEC activation, a state in which PECs are typically profibrotic (19, 46, 59, 60). A second major finding in the present study was the de novo expression of CD44 in aged PECs. Similar to these reports, the present study showed that CD44 staining was barely detected in glomeruli of young mice. However, in aged mice, half of all JM glomeruli and 16% of OC glomeruli contained PECs that stained for CD44. These data suggest that activated PECs in aging produce increased matrix proteins, similar to that recently described in diabetic

Fig. 9. CD146 immunofluorescent staining increases in aged PECs. Confocal microscopy images (magnification: ×400) are shown of JM glomeruli from young mice aged 3 mo and old mice aged 27 mo. Glomeruli are indicated by dashed circles. Scale bars are provided. Nuclei were stained blue with DAPI. A: staining for CD146 (magenta) was detected in mesangial cells, a subset of cortical tubular cells, and pericytes (dashed arrow), but not in PECs. B: in 27-mo-old kidneys, CD146 staining was detected in PECs (arrowheads). C: CD146 staining was not detected when the primary antibody was substituted with an IgG isotype control. D: positive controls for CD146 in the kidney interstitium of the medullary region. CD146 stained perivascular cells (D1, arrows), which also stained for α-SMA (D2) and PDGFRβ (D3) and overlapped when merged (D4).

Fig. 10. Notch 3 immunofluorescent staining is increased in PECs of aged mice. Confocal microscopy images (magnification: ×400) are shown of JM glomeruli from young mice aged 3 mo and old mice aged 27 mo. Glomeruli are indicated by dashed circles. Scale bars are provided. Nuclei were stained blue with DAPI. A: Notch 3 staining was faintly detected in cells lining Bowman’s capsule in young animals (red, arrowhead). Dashed arrows show internal positive controls for Notch 3 in vessels. B: in old mice, Notch 3 staining was markedly increased in cells along Bowman’s capsule (arrowheads). C: when Notch 3 antibody was substituted with the IgG isotype control, staining was not detected.
states in vitro and in vivo (26). In aged glomeruli, CD44-stained cells were typically restricted to Bowman’s capsule and only rarely seen on the glomerular tuft in a podocyte distribution. As expected, occasional CD44-stained circulating cells were detected in the capillary loops. This differs from experimental and human studies in FSGS, where a subpopulation of CD44-expressing PECs have migrated to the tuft and are in part responsible for the segmental or global scarring (19, 46, 59, 60). One can only speculate on such differences, including a reduced migratory capacity for PECs with advanced age, or that the window when this occurred was earlier (or later) than the time course studied.

Renal epithelial cells oftentimes undergo EMT in disease (29). Biological functions are regularly altered after EMT, including an increased production of extracellular matrix proteins (29). Because certain matrix proteins were increased along Bowman’s capsule with aging, we examined several markers reported to indicate EMT, recognizing that these might vary according to cell type. A third major finding in the present study was the increased expression of the EMT markers α-SMA and vimentin in PECs in aged mice. Staining for the EMT markers SM22, E-cadherin, and β-catenin were not altered in PECs with age. EMT has been previously reported in PECs (4, 65). However, this is the first time showing EMT in aged PECs. We can only speculate on the biological implications of the increased α-SMA and vimentin staining by aged PECs. In the context of increased matrix proteins in Bowman’s capsule with aging, it is important to note that these EMT markers have been associated with increased matrix protein accumulation. For example, α-SMA-expressing cells in the liver are profibrogenic (42). In many epithelial cells, EMT reprogramming has been associated with the development of fibrosis (33). Vimentin stabilizes collagen mRNAs (11), and, more recently, urinary vimentin has been used as a biomarker for kidney fibrosis in patients (10). Taken together, one might consider that the increase in EMT markers is associated with PEC matrix production and accumulation in aging.

In addition to acquiring EMT markers, aging in PECs was accompanied by the enhanced expression of several additional markers, including NG2, PDGF receptor-β, and CD146, together considered markers of pericytes (50). We did not detect any changes in the senescent marker p21 in aged PECs, nor the major autophagy protein mTOR. Whether the acquisition of these markers by PECs with age is pathological in the sense that normal biological functions are altered and/or that cells in these states are profibrotic and/or have reduced progenitor capacity could not be determined. One might speculate what the biological significance is of enhanced pericyte markers in aged PECs. Numerous studies have suggested that pericytes are myofibroblast precursors in kidney and nonkidney cells (50). Thus, one consideration is that the increase in expression for NG2, PDGF receptor-β, and CD146 in aged PECs suggests a reprogramming of PECs from an epithelial cell to a pericyte-myofibroblast-like cell, leading to an increase in matrix accumulation. Noteworthy is that blockade of PDGF receptor signaling reduces renal fibrosis (13, 33). Because glomerular epithelial cells do not typically express PDGF receptor-β, it was critical for us to use negative and positive controls in the present study.

Several studies have demonstrated that a subset of PECs serve as adult podocyte progenitors (6, 32). In contrast to humans, where a subset of PECs normally express the progenitor markers CD133/CD24 (53), mice do not express such antigens, nor other well-defined PEC progenitor markers. NCAM has been used in rats as a PEC progenitor marker (7), and, although PECs normally stain for NCAM in young mice, levels did not change in 27-mo-old mice in the present study. An important question is if aging impacts any regenerative capacity of PECs to serve as podocyte progenitors. Wanner et al. (72) showed that in middle-aged mice (1 yr old), PECs did not regenerate podocytes. Our group has reported that in middle-aged rats, a subset of cells lining Bowman’s capsule coexpress both podocyte and PEC protein (80), although the biological significance of such “transition cells” is not known. If PECs do indeed serve as adult podocyte progenitors, the data in the present study would strongly suggest that this biological role would likely be substantially reduced in advanced age because of several of the age-related changes in PECs, including a decrease in PEC density, their increased activation (defined by CD44 expression), and additional alterations in markers, including EMT proteins.

We acknowledge several limitations of the present study. Given the descriptive nature of this study, we cannot accurately determine the pathogenic role of the observed changes in PEC number, PEC EMT, or of the other markers described. Nor can we determine the underlying mechanisms of the observed changes. Two potential mechanisms are considered, ERK and Notch 3, albeit with descriptive associations. In the present

![Fig. 11. Schematic proposal for changes in glomerular PECs in mice with advanced age. In young mice, confluent PECs (blue color with green nuclei) attach to Bowman’s capsule. With advanced age, there is a decrease in PEC density (most pronounced in JM glomeruli). Of the remaining PECs, a subset become activated and express CD44 (red) and are likely profibrotic. Another PEC subset undergoes EMT (purple), whereas another increases expression for markers typical of pericytes (orange). These events are temporally associated with, and may even be secondary to, enhanced ERK activation and Notch 3 expression in aged PECs. We speculate that with advanced age, the combination of PEC activation, possible EMT, and pericyte-myofibroblast acquisition leads to increased matrix production by PECs and contributes to glomerulosclerosis. These events, together with reduced PEC density, likely limit any potential progenitor capacity for PECs to function as podocyte progenitors in aging.](http://ajprenal.physiology.org/)

AJP-Renal Physiol • doi:10.1152/ajprenal.00144.2015 • www.ajprenal.org
study, p-ERK staining was significantly increased in aged glomeruli, and double staining showed that p-ERK colocalized to PECs coexpressing CD44. We have recently reported that the active form of ERK, p-ERK, was de novo expressed in activated PECs in experimental FSGS (unpublished observations and Ref. 12). Taken together, the de novo coexpression of p-ERK in CD44-activated PECs correlates strongly with glomerulosclerosis of aging. Notch 3 was significantly increased in PECs with aging and may play a mechanistic role for two reasons. First, Notch has been implicated in PEC activation and EMT in glomerular disease (64, 71), and, second, Notch 3 has been reported to be profibrotic in kidneys (17). Unfortunately, we did not obtain renal function data from these mice with advanced age. Clearly, followup functional and mechanistic studies are required.

In summary, very old mice exhibit several of the characteristic glomerular features of aging previously identified in humans and rats. However, the data from the present study are the first to show that PECs do undergo several age-related changes (Fig. 11), including a decrease in PEC density that is more pronounced in JM glomeruli, enhanced activation, and increased expression of markers considered to represent EMT. The consequences of these changes are beyond the scope of this study, but we speculate that the major ones are glomerulosclerosis, a thickened Bowman’s capsule, and possibly a reduced reservoir of progenitors for podocyte regeneration. Our data suggest that ERK and Notch are candidate pathways for future study.

ACKNOWLEDGMENTS

The present work was performed in (partial) fulfillment of the requirements for obtaining the “Dr. Med.” degree from the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU).

GRANTS

S. Roeder was supported by a scholarship of the Interdisciplinary Center for Clinical Research (IZKF) of the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU). This work was supported by National Institutes of Health Grants P30-AI-013280, RO1-HL-101186, RO1-AG-038550, R24-DK-094768-01, R01-DK-093493-02, 5-R01-DK-056799-10, 5-R01-DK-056799-12, 1-R01-DK-097598-01A1, P30-AG-013280, RO1-HL-101186, and RO1-AG-038550. This work was also supported by an Emerging Fields Initiative for Cell Cycle in Disease and Regeneration from the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


