CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Characterization of Glomerular Sox9⁺ Cells in Anti-Glomerular Basement Membrane Nephritis in the Rat

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Mechanisms of glomerular crescent formation and podocyte repair processes are still unclear. Therefore, we investigated the expression of the transcription factor Sox9 as a potential marker of a subpopulation of parietal epithelial cells (PECs) with potential regenerative properties. Glomerular Sox9 expression was characterized in detail in a rat anti-glomerular basement membrane (GBM) nephritis model using immunofluorescence and confocal laser scanning microscopy. In healthy kidneys Sox9 is expressed in a subpopulation of PECs restricted to approximately 20% to 50% of PEC nuclei and was highly conserved in all investigated species. During rat anti-GBM nephritis the number of glomerular Sox9⁺ cells increased and was associated with proliferation activity. In nephritic glomeruli Sox9 expression was not restricted to Bowman's capsule lining but was also found on cells of the glomerular tuft. Nearly all Sox9⁺ cells also expressed the PEC marker Pax8, whereas endothelial cells, mesangial cells, macrophages, and T lymphocytes lacked Sox9 expression. At the margins of crescents Sox9⁺/Pax8⁺ cells additionally expressed podocyte markers. In contrast, in sclerotic lesions a minority of Sox9⁺/Pax8⁺ cells expressed the myofibroblast marker α -smooth muscle actin. In glomerular Sox9⁺ cells Jagged 1 was up-regulated. During anti-GBM nephritis Sox9⁺ PECs proliferate and migrate onto the glomerular tuft. Future studies are needed to confirm the origin of Sox9⁺ cells from PECs and differentiation in both podocytes and/or myofibroblasts. (*Am J Pathol* 2018; 188: 2529–2541; <https://doi.org/10.1016/j.ajpath.2018.07.023>)

Crescent formation is a severe histologic hallmark that can occur in almost all glomerular diseases, being mainly composed of parietal epithelial cells (PECs), podocytes,^{1–4} and inflammatory cells such as macrophages.⁵ Podocytes are essential to maintain the glomerular filtration barrier. However, during crescent formation podocytes get lost or lose function, leading to impaired renal function.⁶ Because podocytes are terminally differentiated cells, they cannot divide and are unable to regenerate themselves.^{7–9} Several observations argue in favor of the existence of regenerative mechanisms, and it has been postulated that PEC migration on the glomerular tuft and differentiation into podocytes might play a key role in podocyte regeneration.^{10–18} However, there are also some indications of PEC participation in glomerulosclerosis after injury.¹¹ Although, CD133⁺/CD24⁺ PECs were shown to have the

potential to regenerate podocytes in humans,¹⁹ such markers are lacking in rodents, and similar markers occurring in different species are currently unknown. This has so far prevented a detailed analysis of the mechanisms of podocyte regeneration by a subpopulation of PECs. Emphasis should be placed on the identification of an adequate marker of PECs showing a regenerative potential. Therefore, in this study, our

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aim was to identify a marker of PECs with regenerative potential that is highly conserved over different species, including rodents and humans. As a promising candidate, Sox9, a member of the Sry-related high mobility group box of transcription factors²⁰ that is known as a marker for stem cell or progenitor populations during development and in several adult organs, was chosen.^{21,22} Accordingly, Sox9 activity plays a pivotal role in development of various tissues, including the skeleton,²³ and regeneration of adult organs such as the intestine, liver, lung, and pancreas.^{24–28} Moreover, Sox9 is critical for normal kidney development. It is expressed within nephron precursors and controls epithelial branching during the formation of the urinary tract system.²⁵ In addition, lineage tracing experiments in mice demonstrated that Sox9 expression in tubules constitutes an early transcriptional response to injury and that surviving Sox9⁺ tubule cells re-entered mitosis and were required for a successful regeneration of a functional tubular epithelium.^{29,30} Nevertheless, little is known about glomerular Sox9 expression.

Here, we present evidence for a Sox9 expression in a subpopulation of PECs of healthy glomeruli that is conserved among different species. Our data indicate that after injury Sox9 might be activated by Notch signaling and enables PECs to proliferate and migrate onto the glomerular tuft. Moreover, our study supports a potential role of Sox9-expressing cells in both podocyte regeneration and fibrosis.

Materials and Methods

Evaluation of Glomerular Sox9 Expression in Different Healthy Species and during Renal Development in the Rat

Expression of Sox9 was investigated in kidneys from healthy mice ($n = 6$), rats ($n = 11$), and pigs ($n = 3$). Furthermore, Sox9 expression was analyzed in human kidney tissue without evidence of renal disease ($n = 9$) obtained from distant portions of kidneys surgically excised because of the presence of a localized neoplasm. The use of tissue specimens was approved by the ethical committee of the Medical Faculty of the University of Erlangen.

In addition, glomerular Sox9 expression was investigated during renal development with the use of kidneys from three newborn rats collected 2 days after birth. In contrast to glomerulogenesis in humans, renal development in rats is still ongoing during the first 2 weeks after birth. In these biopsies S-shaped bodies can be found in the outer cortex and nearly fully developed glomeruli in the inner cortex.

Anti-GBM Nephritis Model in the Rat

The experimental protocol for the animal studies was approved by the German regional committee for animal care and use, which is equivalent to the US Institutional Animal Care and Use Committee, and authorized by the governmental department (Regierung von Unterfranken permit

55.2-2532-2-324). Prior animal studies were performed in strict accordance with the German Animal Welfare Act. Anti-glomerular basement membrane (GBM) nephritis was induced in 11 male Wistar Kyoto rats (WKY/NlcoCrI; Charles River, Sulzfeld, Germany) with a body weight of 200 to 220 g by a single i.v. tail injection of 30 μ g of mouse monoclonal anti- α 4(IV)NC1 of rat GBM antibody clone a84 (Chondrex Inc., Redmond, WA). Age- and weight-matched male Wistar Kyoto rats injected with phosphate-buffered saline served as controls ($n = 11$). For monitoring of kidney function rats were housed in metabolic cages before the start of the experiment and on days 6 and 13 to collect urine for 24 hours. Proteinuria was measured by using Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany). Survival biopsies were taken on day 7, and rats were sacrificed on day 14 after induction of anti-GBM nephritis.

Immunohistochemistry and Immunofluorescence Staining

For all single staining and colocalization studies kidneys were fixed in formalin, embedded in paraffin, and cut into sections of 2 μ m. After deparaffinization endogenous peroxidase was blocked by using 3% H₂O₂ for immunohistochemistry. Antigen was retrieved by using target retrieval solution (Dako Deutschland GmbH, Hamburg, Germany) and cooking in a pressure cooker for 2.5 minutes. After blocking for 10 minutes with 1% bovine serum albumin in 50 mmol/L Tris pH 7.4 sections were incubated overnight at 4°C for immunofluorescence staining and at room temperature for immunohistochemistry with the use of the following antibodies diluted in 1% bovine serum albumin in 50 mmol/L Tris pH 7.4. Sox9, a rabbit monoclonal antibody against Sry-related HMG box 9 (Abcam, Cambridge, UK) dilution 1:100; Sox9, a guinea pig antibody against mouse Sry-related HMG box 9 (a gift from Elisabeth Sock; Friedrich-Alexander-University Erlangen Nürnberg, Erlangen, Germany) dilution 1:200; PCNA, a mouse monoclonal antibody against proliferating cell nuclear antigen (Dako Deutschland GmbH) dilution 1:100; Thy-1 (clone Ox-7), WT1, a rabbit polyclonal antibody against Wilm's tumor antigen 1 (WT1) (Santa Cruz Biotechnology, Santa Cruz, CA) dilution 1:200; Ox7, a mouse monoclonal antibody against rat Thy-1 (Bio-Rad GmbH, München, Germany) dilution 1:50; Jagged 1, a rabbit polyclonal antibody against Jagged 1 (Acris Antibodies GmbH, Herford, Germany); CD31, a mouse monoclonal antibody against rat CD31 (Bio-Rad GmbH) dilution 1:50; CD68, a mouse monoclonal antibody against rat CD68 (clone ED-1; Bio-Rad GmbH) dilution 1:50; CD3, a monoclonal rabbit antibody against human CD3 (Zytomed Systems GmbH, Berlin, Germany) dilution 1:50; Pax8, a rabbit polyclonal antibody against Paired box gene 8 (Cell Marque, Rocklin, CA) dilution 1:50; Synaptopodin, a mouse monoclonal antibody against rat Synaptopodin (clone G1D4; Progen Biotechnik GmbH, Heidelberg, Germany) dilution 1:100; Podocalyxin, a goat

polyclonal antibody against human Podocalyxin (R&D Systems, Inc., Minneapolis, MN) dilution 1:100; Nephryn, a guinea pig polyclonal antibody against human Nephryn (Acris Antibodies GmbH) dilution 1:50; α -SMA, a mouse monoclonal antibody against α -smooth muscle actin (Dako, Glostrup, Denmark) dilution 1:50.

For single staining of Sox9 primary antibody was detected with biotinylated goat anti-rabbit IgG (BA-1000) or biotinylated goat anti-guinea pig (BA-7000), ABC-kit (PK-6100), and ImmPACT-DAB (SK-4105) as substrate (all from Vector Laboratories, Burlingame, CA), followed by periodic acid-Schiff staining.

Double and triple staining of Sox9 with glomerular cell markers was performed with the use of fluorescence-labeled secondary antibodies diluted 1:200 in 50 mmol/L Tris pH 7.4 and incubated for 30 minutes at room temperature. The following secondary antibodies were used: donkey anti-mouse IgG Alexa Fluor 568 (A21202), donkey anti-mouse IgG Alexa Fluor 647 (A31571), donkey anti-rabbit IgG Alexa Fluor 488 (A21206), donkey anti-rabbit IgG Alexa Fluor 568 (A10042), donkey anti-rabbit IgG Alexa Fluor 647 (A31573), donkey anti-goat IgG Alexa 633 (A21082), and goat anti-guinea pig IgG Alexa Fluor 488 (A32723) (all from Thermo Fisher Scientific, Waltham, MA).

Sections were also submitted to double-staining with two primary antibodies generated in rabbits (Sox9/Pax8). After application of the first anti-rabbit primary antibody sections were treated with Alexa Fluor 488 fluorescence-labeled donkey anti-rabbit Fab fragments (711-547-003; Jackson ImmunoResearch, Suffolk, UK) dilution 1:200 in 1% bovine serum albumin in 50 mmol/L Tris pH 7.4 for 30 minutes. After intensive washing in Tris buffer blocking was achieved by anti-rabbit Fab fragments (Jackson ImmunoResearch) dilution 1:50 in 50 mmol/L Tris pH 7.4 for 30 minutes. Thereafter, the second anti-rabbit primary antibody was applied. Because higher sensitivity was required for Jagged1 staining biotinylated goat anti-rabbit antibody followed by ABC-kit and DABImpact as substrate (all from Vector Laboratories) were used. After blocking with 1% H₂O₂ and Avidin–biotin blocking kit bound rabbit anti-Sox9 antibodies were detected with the use of alkaline phosphatase–conjugated secondary antibodies (Vector Laboratories) and Fast Red as chromogen.

Nuclei of cells were stained with DAPI in distilled water (0.2 μ g/mL) for 5 minutes, followed by rinsing in Tris buffer. Finally, sections were covered with Mowiol mounting medium (Calbiochem, La Jolla, CA) and analyzed with laser scanning confocal microscopy (LSM Zeiss 710 and Zen 2009 software version 5.5; Zeiss GmbH, Jena, Germany) and ImageJ software version 1.46r (NIH, Bethesda, MD).³¹

To confirm the specificity of the applied anti-Sox9 antibodies tissue was obtained from wild-type and central nervous system-specific Sox9-deficient mice generated in the laboratory of Michael Wegner and Elisabeth Sock at the Department of Biochemistry (Friedrich-Alexander-University Erlangen-Nürnberg).

Negative controls for immunostaining included either deletion or substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or preimmune rabbit IgG.

Quantitative Evaluation of Glomerular Sox9⁺ Cells

After immunohistochemistry, glomerular Sox9 staining was analyzed by light microscopy at $\times 40$ magnification. Quantitative evaluation was performed by counting double- and triple-positive cells per glomerular cross section (GCS) by analysis of at least 60 glomeruli per group with the use of confocal laser scanning microscopy. Double- and triple-positive cells were quantified for glomerular tuft and Bowman's capsule separately.

Statistical Analysis

After testing for normal distribution of values by using Kolmogorov-Smirnov test and finding that many data sets are not normally distributed, data were analyzed with Kruskal-Wallis test, followed by Dunn's multiple comparison test. $P < 0.05$ was accepted as statistically significant. Statistical analysis was performed with GraphPad Prism 5

Figure 1 Sry-related high mobility group box (Sox9) was expressed in a subpopulation of parietal epithelial cells (PECs) and was highly conserved over different species. **A–D:** Glomerular Sox9 expression was analyzed by using immunohistochemistry in renal tissue from healthy mouse (**A**), rat (**B**), pig (**C**), and humans (**D**). **Arrowheads** highlight Sox9⁺ cells. **E** and **F:** Rabbit (rb) anti-Sox9 antibody (ab) specificity was confirmed by immunohistochemical staining of a wild-type (WT) (**E**) and central nervous system (CNS)-Sox9 knockout (ko) mouse embryo (**F**). See also [Supplemental Figure S2](#). Scale bars = 50 μ m.

Figure 2 Glomerular Sry-related high mobility group box (Sox9)⁺ cells expressed parietal epithelial cell (PEC) markers and were localized on the glomerular tuft after induction of anti-glomerular basement membrane (GBM) nephritis. **A** and **B**: Immunohistologic staining for Sox9 with additional periodic acid-Schiff (PAS) staining in healthy (**A**) and GBM-nephritic (**B**) rats. **Arrowheads** highlight Sox9⁺ cells on the glomerular tuft (**B**). **C**: An overview of Sox9⁺/Paired box gene 8 (Pax8)⁺ cells on the glomerular tuft of anti-GBM-nephritic rat with the use of confocal laser scanning microscopy (above). **Boxed areas** are shown at higher magnification in the **bottom row**. **D–H**: Bar diagrams demonstrate the evaluation of the total number of glomerular Sox9⁺ cells (**D**), the number of Sox9⁺ cells lining the Bowman's capsule (**E**), the number of Sox9⁺/Pax8⁺ cells lining Bowman's capsule (**F**), the number of Sox9⁺ cells on the glomerular tuft (**G**), and the number of Sox9⁺/Pax8⁺ cells on the glomerular tuft (**H**). Data are expressed as means ± SEM. ***P* < 0.01, ****P* < 0.001. DAPI (blue staining) demarcates nuclei. Scale bars: 50 μm (**A** and **B**); 10 μm (**C**). gcs, glomerular cross section.

for Windows software version 5.02 (GraphPad Software Inc., San Diego, CA).

Results

Sox9 Is Expressed in a Subpopulation of PECs and Is Highly Conserved across Different Species

A rabbit antibody raised against human Sox9 was used. The human peptide sequences used for generation of this antibody is ≥98% identical to homologous Sox9 sequences in mouse, rat, and pig, as assessed by Basic Local Alignment Search Tool (NIH; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn) analysis. Kidney biopsies from different healthy species, including mouse (Figure 1A), rat (Figure 1B), pig (Figure 1C), and human

(Figure 1D), showed nuclear Sox9 expression in PECs lining Bowman's capsule, indicating highly conserved localization of Sox9⁺ cells over different mammalian species. However, in healthy glomeruli only a subpopulation of PECs expressed Sox9 (Figure 1, A–D). Specificity of the anti-Sox9 antibodies was confirmed by using embryos from wild-type and central nervous system-specific Sox9-deficient mice (Figure 1, E and F, and Supplemental Figure S1, A and B). In addition, the signals generated with both the guinea pig (Supplemental Figure S1C) and the commercial rabbit antibody (Supplemental Figure S1D) completely overlap (Supplemental Figure S1E), also indicating Sox9-specific detection with both antibodies. Controls that used unspecific rabbit IgG were completely negative in human, rat, and pig kidney tissue (Supplemental Figure S1, F–H). However, because the rabbit anti-Sox9 antibody showed less background staining, the rabbit anti-Sox9 antibody was preferentially used.

Figure 3 Glomerular Sry-related high mobility group box (Sox9)⁺ cells increased by proliferation. **A** and **B**: Immunofluorescence double staining for Sox9 and proliferating cell nuclear antigen (PCNA) in healthy (**A**) and anti-glomerular basement membrane (GBM) nephritic (**B**) rats. **Arrowheads** highlight Sox9⁺ cells in **A** and Sox9⁺/PCNA⁺ cells in **B**. Confocal laser scanning microscopy was used to evaluate the number of Sox9⁺/PCNA⁺ cells lining Bowman capsule (**C**), on glomerular tuft (**D**), and the total number of PCNA⁺ cells (**E**) in healthy and anti-GBM nephritic rats on days 7 and 14 after model induction. Data are expressed as means $\pm$ SEM. *** P < 0.001. DAPI (blue staining) demarcates nuclei. Scale bars = 30 μ m. gcs, glomerular cross section.

After Induction of Anti-GBM Nephritis the Number of Glomerular Sox9⁺ Cells Is Increased and Sox9⁺ Cells Additionally Appear on the Glomerular Tuft

Successful induction of anti-GBM nephritis was confirmed by measurement of significant proteinuria ([Supplemental Figure S2A](#)) and crescent formation in >50% of analyzed GCSs ([Supplemental Figure S2B](#)). In addition, the number of podocytes, as assessed by WT1 staining, was significantly reduced by $40.3\% \pm 1.2\%$ on day 7 and by $34.0\% \pm 1.4\%$ on day 14 ([Supplemental Figure S2C](#)). Interestingly, both proteinuria and glomerular crescent formation showed a tendency to decreased levels by 16% from day 7 to day 14 ([Supplemental Figure S2, A and B](#)). Although Sox9⁺ cells in healthy rats were restricted to PECs lining Bowman's capsule ([Figure 2A](#)), the number was significantly increased after induction of anti-GBM nephritis ([Figure 2B](#)). Moreover, in nephritic kidneys Sox9 expression was additionally detected on glomerular tuft cells ([Figure 2, B and C](#)). Quantitative evaluation of Sox9⁺ cells revealed an increase of 2.9-fold on day 7 and 4.3-fold on day 14 ([Figure 2D](#)). Separate analysis of Bowman's capsule and the glomerular tuft showed that the number of Sox9⁺ cells along Bowman's capsule increased 1.9-fold on day 7 (7.5 ± 0.4 cells/gcs) and 2.4-fold on day 14 (9.3 ± 0.5 cells/GCS) compared with healthy glomeruli (3.9 ± 0.3 cells/GCS) ([Figure 2E](#)). Almost all Sox9⁺ cells along Bowman's capsule also expressed the PEC marker Pax8, as assessed by immunofluorescence double staining ([Figure 2, E and F](#)). Of

note, along the Bowman's capsule of healthy glomeruli, 50.0% of the PECs expressed Sox9, after anti-GBM model induction on day 7, 67.2% and, on day 14, 77.9% of Pax8⁺ cells express Sox9 (data not shown). Although healthy kidneys lacked Sox9⁺ cells on the glomerular tuft, on day 7 after anti-GBM model induction, 4.2 ± 0.4 cells and on day 14 5.7 ± 0.5 cells/GCS were localized on the glomerular tuft ([Figure 2G](#)). Moreover, quantitative analysis showed that almost all Sox9⁺ cells expressed Pax8 on the glomerular tuft (94.5% on day 7 and 89.7% on day 14) ([Figure 2, G and H](#)). Interestingly, almost all Pax8⁺ cells on the glomerular tuft also expressed Sox9 (97.6%) (data not shown). Although on day 7 after induction of anti-GBM nephritis the number of tubular Sox9⁺ cells was comparable with healthy controls, a sixfold increase in Sox9⁺ tubular cells was also observed on day 14 ([Supplemental Figure S3](#)).

Sox9⁺ Cells Start to Proliferate after Induction of Anti-GBM Nephritis

To answer the question whether the increased number of Sox9⁺ cells in nephritic glomeruli may be due to proliferative activity, Sox9/PCNA double staining was performed by using immunofluorescence labeling. In the anti-GBM model glomerular Sox9⁺ cells showed significant proliferative activity along Bowman's capsule and on the glomerular tuft; however, proliferating Sox9⁺ cells in healthy rats were rarely found ([Figure 3, A–D](#)). Of interest, even on the

Figure 4 Endothelial cells, mesangial cells, macrophages, and T cells did not express Sry-related high mobility group box (Sox9). **A–D:** Double immunofluorescence staining was used to colocalize Sox9 and endothelial cell marker CD31 (**A**), mesangial cell marker Ox-7 (**B**), macrophage marker CD68 (**C**), and T-cell marker CD3 (**D**). **Arrowheads** highlight CD3⁺ T cells. DAPI (blue staining) demarcates nuclei. Scale bars = 30 μ m. GBM, glomerular basement membrane.

glomerular tuft 50.0% of the Sox9⁺ cells on day 7 and 24.9% on day 14 showed proliferative activity (data not shown). However, on day 7 and 14 the total number of proliferating glomerular cells was about four times higher than the corresponding total amount of Sox9⁺/PCNA⁺ cells after induction of anti-GBM nephritis (Figure 3, C–E), indicating additional high proliferative activity of Sox[−] cells presumably of mesangial cell origin.

After Induction of Anti-GBM Nephritis Sox9 Is Not Expressed by Endothelial Cells, Mesangial Cells, Macrophages, and T Cells

Most Sox9⁺ cells on the glomerular tuft expressed Pax8; therefore, they might originate from PECs. Of interest, most Sox9⁺ cells on the glomerular tuft expressed Pax8 at a lower level than Sox9⁺ cells lining Bowman's capsule.

Figure 5 On the glomerular tuft of anti-GBM nephritic rats, Sry-related high mobility group box (Sox9)⁺ cells expressed both parietal epithelial cells (PECs) and podocyte markers. Confocal laser scanning microscopy was used for colocalization of Sox9, the PEC marker Paired box gene 8 (Pax8), and the podocyte marker Synaptopodin (A and B) and the podocyte marker Podocalyxin (E–H). Arrowheads in the overview microphotographs above and respective details from Sox9⁺ podocytes with single channels (A and B) highlight Sox9⁺/Pax8⁺/Synaptopodin⁺ cells. The frequency of Sox9⁺/Pax8⁺/Synaptopodin⁺ (C) and Sox9⁺/Podocalyxin⁺ (D) cells. See also Supplemental Figure S4. Data are expressed as means $\pm$ SEM. ** P < 0.01, *** P < 0.001. DAPI (blue staining) demarcates nuclei. Scale bars = 20 μ m. GBM, glomerular basement membrane; gcs, glomerular cross section.

However, to exclude Sox9 *de novo* expression by other glomerular cells, colocalization studies with markers for different glomerular cell populations were performed. CD31⁺ endothelial cells (Figure 4A), Ox7⁺ mesangial cells (Figure 4B), ED1⁺ macrophages (Figure 4C), or CD3⁺ T cells (Figure 4D) were not positive for Sox9.

On the Glomerular Tuft, a Subpopulation of Sox9⁺ Cells Express Podocyte Markers

Of interest, immunofluorescence triple staining showed that a subpopulation of Sox9⁺/Pax8⁺ cells on the glomerular tuft were additionally positive for the podocyte marker Synaptopodin in nephritic kidneys (Figure 5, A–C). This observation was confirmed by analyzing Sox9⁺/Synaptopodin⁺/WT1⁺ cells (Figure 6). Furthermore, Sox9 costaining with other podocyte markers such as Podocalyxin (Figure 5, D–H) and Nephlin (Supplemental Figure S4) were performed in addition. All four markers yielded comparable numbers of Sox9⁺/podocyte marker⁺ cells (Figure 5, C and D). In most nephritic glomeruli, Sox9⁺/Pax8⁺/Synaptopodin⁺ cells were localized at the margins of crescentic lesions in close vicinity to regions that lacked podocyte markers (Figure 5, A and B). Although Sox9⁺/Pax8⁺/Synaptopodin⁺ cells were detected in about every second GCS on day 7 (0.7 ± 0.1 triple-positive cells/GCS) on day 14 on average every GCS showed one triple-

positive cell (1.5 ± 0.2 triple-positive cells/GCS) (Figure 5C). Comparable numbers of Sox9⁺ cells expressing podocyte markers were counted by using the Sox9/Synaptopodin/WT1 triple staining (Figure 6). Note, that, on day 7, 17.8% and, on day 14, 28.8% of all Sox9⁺ cells on the glomerular tuft were positive for the podocyte marker Synaptopodin (data not shown). Most of these cells were additionally positive for the PEC marker Pax8, which was, however, often expressed at a lower level than the Pax8 expression in Sox9⁺ cells lining Bowman's capsule (Figure 5, A and B).

Jagged1 Is Up-Regulated in Sox9⁺ Cells

To investigate potential activators of Sox9 the expression of the Notch-signaling molecule Jagged1 was tested in healthy and anti-GBM nephritic rats by using immunohistochemical double staining. Of interest, Sox9⁺ PECs expressed Jagged1; however, glomerular Jagged1-staining was weak (Figure 7A). After induction of anti-GBM nephritis glomerular Jagged1 was increased. Almost all glomerular Sox9⁺ cells expressed Jagged1, indicating Jagged1 was a potential activator of Sox9 during anti-GBM nephritis as reported in recent studies that investigated a remnant kidney injury model.³² In agreement, Sox9⁺/Jagged1⁺ cells were detected on day 14 in crescents (Figure 7B).

Figure 6 A subpopulation of Sry-related high mobility group box (Sox9)⁺ cells on the glomerular tuft expresses podocyte markers Wilm's tumor antigen 1 (WT1) and synaptopodin. Confocal laser scanning microscopy was used for colocalization of Sox9, nuclear podocyte marker WT1, and the membrane podocyte marker Synaptopodin. **A:** In healthy kidneys Sox9⁺ cells were restricted to Bowman's capsule; **arrowheads** mark Sox9⁺ cells on Bowman's capsule in single and merged channels. **B and C:** On day 14 after nephritis induction Sox9⁺/WT1⁺/Synaptopodin⁺ cells could be detected on glomerular tuft marked by **arrowheads**. **D:** The frequency of Sox9⁺/WT1⁺/Synaptopodin⁺ cells. Data are expressed as means $\pm$ SEM. $**P < 0.01$, $***P < 0.001$. DAPI (blue staining) demarcates nuclei. Scale bars = 30 μ m. GBM, glomerular basement membrane; gcs, glomerular cross section.

In Glomerular Lesions a Minority of Sox9⁺ Cells Express the Myofibroblast Marker α -Smooth Muscle Actin

To investigate the potential involvement of Sox9⁺ cells in fibrosis, immune double staining was performed for Sox9 and the myofibroblast marker α -SMA. After induction of anti-GBM nephritis a small number of Sox9⁺/ α -SMA⁺ cells were found along Bowman's capsule (Figure 7, C and E) and within glomerular lesions (Figure 7, D and F), indicating that Sox9⁺ cells might also be involved in the development of fibrosis.

During Renal Development Sox9 Is Expressed in Podocyte Precursors

During renal development Sox9 has been identified to be essential for branching morphogenesis,²⁵ but characterization of Sox9-expressing cells especially in developing glomeruli was lacking. In newborn rats, the early stages of glomerular

development could still be found in the outer cortex. Sox9 was highly expressed in developing tubular cells in S-shaped bodies (Figure 8, A and D) and premature glomeruli (Figure 8, B and E) but almost lacking in Podocalyxin⁺ podocytes (Figure 8, D and E). However, pronounced Sox9 expression was detected in podocytes (Figure 8, C and F) and PECs (Figure 8C) in early mature glomeruli, indicating a potential role of Sox9 in later differentiation of podocytes.

Discussion

PECs are cells lining Bowman's capsule, but they might be important as a reservoir to regenerate podocyte and tubular cells.³³ However, PECs consist of different subpopulations with different potential. Here, we describe for the first time the expression of the transcription factor Sox9 in healthy and nephritic rats as a marker of a PEC subpopulation, which is highly conserved among different species,

Figure 7 Almost all glomerular Sry-related high mobility group box (Sox9)⁺ cells expressed Jagged 1 and a minority expressed the myofibroblast marker α -smooth muscle actin (α -SMA). **A** and **B**: Jagged 1 (brown staining) was detected predominantly in Sox9⁺ (pink staining) cells in healthy (**A**) and anti-glomerular basement membrane (GBM) nephritic glomeruli (**B**). **C** and **D**: Immunofluorescence staining for Sox9/Pair box gene 8 (Pax8)/ α -SMA in healthy (**C**) and anti-GBM nephritic rats (**D**). **Arrowheads** highlight Sox9⁺/Pax8⁺/ α -SMA⁺ cells along Bowman's capsule and on the glomerular tuft. **E** and **F**: The numbers of Sox9⁺/Pax8⁺/ α -SMA⁺ cells lining the Bowman capsule (**E**) and on the glomerular tuft (**F**) in healthy and anti-GBM nephritic rats were evaluated on days 7 and 14 after model induction. Data are expressed as means $\pm$ SEM. ****** P < 0.01, ******* P < 0.001. Nuclei were stained with DAPI (blue staining). Scale bars: 50 μ m (**A** and **B**); 30 μ m (**C** and **D**). gcs, glomerular cross section.

Figure 8 During renal development Sry-related high mobility group box (Sox9) was expressed in podocyte precursors. **A–C:** Sox9⁺ cells were marked in different glomerular developmental stages, including S-shaped bodies (**A**), premature glomeruli (**B**), and early mature glomeruli (**C**) with the use of kidneys from newborn rats and immunohistochemistry. **Black arrowheads** mark Sox9⁺ parietal epithelial cells (PECs) in early mature glomeruli. **D–F:** Colocalization studies were performed with Sox9 and the podocyte marker Podocalyxin. **White arrowheads** mark tubular Sox9⁺ cells (**D** and **E**) and podocyte precursors (**F**). Nuclei were stained with DAPI (blue staining). Scale bars = 20 μm. gcs, glomerular cross section.

including mouse, rat, pig, and humans and exerts specific properties. In humans CD133⁺/CD24⁺ PECs were identified to have the capacity to regenerate podocytes.¹⁹ However, these markers are not expressed in rodents; therefore, they are not suitable for the characterization of PECs in animal models. In this study, Sox9 was studied as a potential progenitor marker for podocytes because previous studies in different organs, including salivary gland, intestine, pancreas, and liver, established Sox9 as a marker for progenitor cells.^{26,34–37} In addition, in the kidney Sox9⁺ tubular cells were already identified as tubular progenitor cells.^{29,30} In healthy glomeruli, the subpopulation of Sox9⁺ PECs represented approximately one-half of all Pax8⁺ PECs. In contrast, in the anti-GBM nephritis model Sox9⁺ PECs started to proliferate, resulting in an increase of Sox9⁺/Pax8⁺ cells up to 77.9% of all PECs. High proliferative activity of Sox9⁺ cells were similarly reported in the kidney after tubular injury, resulting in expansion of Sox9⁺ cells that contribute to regeneration of proximal tubule, loop of Henle, and distal tubule segments.^{29,30} Furthermore, in other organs it has been shown that Sox9⁺ cells can generate daughter cells by proliferation.^{24,26–28} Although increased proliferative activity was clearly observed in Sox9⁺ glomerular cells, it cannot be excluded that PECs *de novo* express Sox9 after injury. Thus, it remains unclear whether the Sox9⁺ cells on the glomerular tuft descend from a distinct Sox9⁺ progenitor population along Bowman's capsule or whether they also descend from PECs that *de novo* express Sox9 on injury. Accordingly, although two studies identified the up-regulation of Sox9 as an early event after tubule injury, there is an ongoing debate whether the reparative capacity is elicited by tubular epithelial cells²⁹ or because of the response of renal progenitor cells.³⁰

Podocytes are essential for maintaining the glomerular filtration barrier, and loss of a critical number of intact podocytes leads to impaired glomerular function and progressive glomerulosclerosis.⁶ Accordingly, glomerular

crescent formation occurs in almost all glomerular diseases as a consequence of podocyte loss. In this study, an anti-GBM nephritis model that resulted in podocyte loss and crescent formation was used. Because podocytes are terminally differentiated cells, they are unable to regenerate by proliferation.³⁸ Nevertheless, several observations argue in favor of the existence of podocyte regenerative mechanism. Under normal physiological circumstances a small amount of living podocytes are steadily discharged into the urine.^{39–41} In the absence of a regenerative mechanism, their number is too high to guarantee lifelong sufficient glomerular podocytes. In addition, in humans and animal models early glomerular disease stages can completely regress.^{7–9} Therefore, alternative mechanisms for podocyte regeneration have been postulated. Nevertheless, until now it has remained controversial whether PECs, cells of the renin lineage, and/or other cell types contribute to podocyte regeneration.⁴²

Experimental studies confirmed that most crescent-forming cells originate from PECs.⁴³ However, PECs might also have the capability to replace lost podocytes by migration onto the glomerular tuft. In this study almost all PECs located on the glomerular tuft expressed Sox9, indicating a potential role for Sox9 in the migratory activity of PECs. This assumption was supported by the observation that knockdown of Sox9 in thyroid cancer cells inhibits invasiveness of these cells.⁴⁴ Furthermore, tamoxifen-CRE-induced Sox9 inhibition impairs renal recovery after ischemia/reperfusion,²⁹ indicating an important role of Sox9 in repair processes of tubular epithelial cells. Moreover, after induction of anti-GBM nephritis Sox9⁺ cells that express podocyte markers were frequently detected. This observation was confirmed by using the four different podocyte markers, Podocalyxin, Synaptopodin, WT1, and Nephlin. Together with their morphologic appearance, this makes us believe that these Sox9⁺ cells are indeed podocytes. Furthermore, we suggest that Sox9⁺ podocytes

originate from PECs because these cells still co-express the PEC marker Pax8 on a low level. So far, marker expression analysis and cell lineage—tracing experiments in different animal models of aging, glomerular hypertrophy, and podocyte injury have been conducted to investigate whether PECs are able to participate in podocyte regeneration. Accordingly, several studies clearly support the hypothesis of PEC dedifferentiation, migration on the glomerular tuft by cellular bridges formed by activated, matrix-producing PECs,⁴⁵ and finally differentiation into podocytes.^{11,13–16} Moreover, Lasagni et al¹⁸ reported that after disease remission after injury induction, a genetic PEC reporter signal has been traced to cells that express the podocyte marker WT1. Our observation of low Pax8 expression in Sox9⁺ podocytes suggest that Sox9 may allow dedifferentiation of PECs, followed by differentiation into podocytes. Similar observations have been reported for other organs, including liver, pancreas, lung, and hair follicle, showing differentiation of Sox9⁺ cells into functional cells.^{24,26–28} Our experimental setting strongly supports a role of Sox9⁺ PECs in podocyte regeneration. However, lineage tracing experiments that are currently not available in the rat are necessary for confirmation.

Sox9⁺ PECs not only had the potential to regenerate podocytes but also co-expressed α -SMA in some cells after injury, indicating differentiation of Sox9⁺ cells into a myofibroblast-like phenotype. Although co-expression of Sox9 with mesangial, endothelial, or inflammatory cell markers could not be identified, the ability of Sox9⁺ cells to differentiate into two different phenotypes underlines the progenitor/stem cell character of Sox9⁺ cells. However, both Sox9⁺ PEC phenotypes show different localization: Sox9⁺ podocytes were restricted to the edge or outside the glomerular crescent, myofibroblast-like Sox9⁺ cells were always located within crescents. The formation of these two phenotypes might be influenced by differences in microenvironments.

Jagged1 was stained as a potential signal transduction molecule of the Notch pathway involved in activation and action of Sox9 during repair processes. Earlier studies described that Notch signaling is essential in renal development and plays a key role in cell-fate decisions and differentiation.⁴⁶ However, in mature glomeruli the Notch pathway becomes silent.⁴⁷ In contrast, in glomerular disease (eg, during collapsing focal segmental glomerulosclerosis) Notch-signaling is activated in PECs.⁴⁸ Notch activation differentially regulates renal progenitors and differentiation, as shown for isolated renal progenitors.⁴⁹ Almost all glomerular Sox9⁺ cells co-expressed the Notch signaling molecule Jagged1, indicating that Sox9 was activated by the Notch pathway. In this model it could not be proven that Sox9 activation was Jagged1 dependent. However, Sox9 activation by the Notch pathway was recently reported in a model of chronic kidney disease.³²

Some reports in mice have highlighted a pivotal role of Sox9 activity in development of various tissues, including

the kidney.²⁵ Accordingly, lineage tracing experiments revealed that during kidney development Sox9-expressing cells contributed to most nephron segments, except for the glomerulus.^{25,30} By investigation of newborn rat kidneys it was confirmed that Sox9 is lacking in S-shaped bodies during glomerular development. However, Sox9 expression was detectable in early podocytes from premature glomerular developmental stages and absent in all adult glomerular tuft cells, indicating a potential role of Sox9 during the podocyte differentiation processes. Thus, the re-expression of Sox9 during kidney repair after injury might involve the recapitulation of developmental processes.

Conclusion

These data provide evidence for species-conserved Sox9 expression in a subpopulation of PECs that can expand after glomerular injury. Sox9 might act as a marker of a subpopulation of PECs, allowing migration and differentiation into selected cell types, including podocytes and myofibroblasts. As a result, these data support a role of Sox9-expressing PECs in both podocyte regeneration and fibrosis.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.07.023>.

References

1. Moeller MJ, Soofi A, Hartmann I, Le Hir M, Wiggins R, Kriz W, Holzman LB: Podocytes populate cellular crescents in a murine model of inflammatory glomerulonephritis. *J Am Soc Nephrol* 2004, 15: 61–67
2. Morita T, Suzuki Y, Churg J: Structure and development of the glomerular crescent. *Am J Pathol* 1973, 72:349–368
3. Min KW, Gyorkey F, Gyorkey P, Yium JJ, Eknayan G: The morphogenesis of glomerular crescents in rapidly progressive glomerulonephritis. *Kidney Int* 1974, 5:47–56
4. Boucher A, Droz D, Adafer E, Noel LH: Relationship between the integrity of Bowman's capsule and the composition of cellular crescents in human crescentic glomerulonephritis. *Lab Invest* 1987, 56: 526–533
5. Jennette JC, Hippi CG: The epithelial antigen phenotype of glomerular crescent cells. *Am J Clin Pathol* 1986, 86:274–280
6. Kriz W, Gretz N, Lemley KV: Progression of glomerular diseases: is the podocyte the culprit? *Kidney Int* 1998, 54:687–697
7. Fries JW, Sandstrom DJ, Meyer TW, Rennke HG: Glomerular hypertrophy and epithelial cell injury modulate progressive glomerulosclerosis in the rat. *Lab Invest* 1989, 60:205–218

8. Marshall CB, Shankland SJ: Cell cycle and glomerular disease: a minireview. *Nephron Exp Nephrol* 2006, 102:e39–e48
9. Pabst R, Sterzel RB: Cell renewal of glomerular cell types in normal rats. An autoradiographic analysis. *Kidney Int* 1983, 24:626–631
10. Appel D, Kershaw DB, Smeets B, Yuan G, Fuss A, Frye B, Elger M, Kriz W, Floege J, Moeller MJ: Recruitment of podocytes from glomerular parietal epithelial cells. *J Am Soc Nephrol* 2009, 20:333–343
11. Eng DG, Sunseri MW, Kaverina NV, Roeder SS, Pippin JW, Shankland SJ: Glomerular parietal epithelial cells contribute to adult podocyte regeneration in experimental focal segmental glomerulosclerosis. *Kidney Int* 2015, 88:999–1012
12. Grahammer F, Wanner N, Huber TB: Podocyte regeneration: who can become a podocyte? *Am J Pathol* 2013, 183:333–335
13. Shankland SJ, Smeets B, Pippin JW, Moeller MJ: The emergence of the glomerular parietal epithelial cell. *Nat Rev Nephrol* 2014, 10:158–173
14. Swetha G, Chandra V, Phadnis S, Bhonde R: Glomerular parietal epithelial cells of adult murine kidney undergo EMT to generate cells with traits of renal progenitors. *J Cell Mol Med* 2011, 15:396–413
15. Sagrinati C, Netti GS, Mazzinghi B, Lazzeri E, Liotta F, Frosali F, Ronconi E, Meini C, Gacci M, Squecco R, Carini M, Gesualdo L, Francini F, Maggi E, Annunziato F, Lasagni L, Serio M, Romagnani S, Romagnani P: Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. *J Am Soc Nephrol* 2006, 17:2443–2456
16. Bariety J, Mandet C, Hill GS, Bruneval P: Parietal podocytes in normal human glomeruli. *J Am Soc Nephrol* 2006, 17:2770–2780
17. Moeller MJ, Smeets B: Role of parietal epithelial cells in kidney injury: the case of rapidly progressing glomerulonephritis and focal and segmental glomerulosclerosis. *Nephron Exp Nephrol* 2014, 126:97
18. Lasagni L, Angelotti ML, Ronconi E, Lombardi D, Nardi S, Peired A, Becherucci F, Mazzinghi B, Sisti A, Romoli S, Burger A, Schaefer B, Buccoliero A, Lazzeri E, Romagnani P: Podocyte regeneration driven by renal progenitors determines glomerular disease remission and can be pharmacologically enhanced. *Stem Cell Reports* 2015, 5:248–263
19. Ronconi E, Sagrinati C, Angelotti ML, Lazzeri E, Mazzinghi B, Ballerini L, Parente E, Becherucci F, Gacci M, Carini M, Maggi E, Serio M, Vannelli GB, Lasagni L, Romagnani S, Romagnani P: Regeneration of glomerular podocytes by human renal progenitors. *J Am Soc Nephrol* 2009, 20:322–332
20. Wright EM, Snopek B, Koopman P: Seven new members of the Sox gene family expressed during mouse development. *Nucleic Acids Res* 1993, 21:744
21. Kamachi Y, Kondoh H: Sox proteins: regulators of cell fate specification and differentiation. *Development* 2013, 140:4129–4144
22. Sarkar A, Hochedlinger K: The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* 2013, 12:15–30
23. Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, Wolf U, Tommerup N, Schempp W, Scherer G: Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* 1994, 79:1111–1120
24. Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, Jacquemin P, Pierreux CE, Clotman F, Lemaigre FP: Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. *Gastroenterology* 2009, 136:2325–2333
25. Reginensi A, Clarkson M, Neirijnck Y, Lu B, Ohya T, Groves AK, Sock E, Wegner M, Costantini F, Chaboissier MC, Schedl A: SOX9 controls epithelial branching by activating RET effector genes during kidney development. *Hum Mol Genet* 2011, 20:1143–1153
26. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, Sander M: SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A* 2007, 104:1865–1870
27. Turcatel G, Rubin N, Menke DB, Martin G, Shi W, Warburton D: Lung mesenchymal expression of Sox9 plays a critical role in tracheal development. *BMC Biol* 2013, 11:117
28. Vidal VP, Chaboissier MC, Lutzkendorf S, Cotsarelis G, Mill P, Hui CC, Ortonne N, Ortonne JP, Schedl A: Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. *Curr Biol* 2005, 15:1340–1351
29. Kumar S, Liu J, Pang P, Krautzberger AM, Reginensi A, Akiyama H, Schedl A, Humphreys BD, McMahon AP: Sox9 activation highlights a cellular pathway of renal repair in the acutely injured mammalian kidney. *Cell Rep* 2015, 12:1325–1338
30. Kang HM, Huang S, Reidy K, Han SH, Chinga F, Susztak K: Sox9-positive progenitor cells play a key role in renal tubule epithelial regeneration in mice. *Cell Rep* 2016, 14:861–871
31. Abramoff MD, Magalhaes PJ, Ram SJ: Image processing with ImageJ. *Biophotonics Int* 2004, 11:36–42
32. Ma Q, Wang Y, Zhang T, Zuo W: Notch-mediated Sox9(+) cell activation contributes to kidney repair after partial nephrectomy. *Life Sci* 2018, 193:104–109
33. Romagnani P: Toward the identification of a “renopoietic system”? *Stem Cells* 2009, 27:2247–2253
34. Chatzeli L, Gaete M, Tucker AS: Fgf10 and Sox9 are essential for the establishment of distal progenitor cells during mouse salivary gland development. *Development* 2017, 144:2294–2305
35. Formeister EJ, Sionas AL, Lorraine DK, Barkley CL, Lee GH, Magness ST: Distinct SOX9 levels differentially mark stem/progenitor populations and enteroendocrine cells of the small intestine epithelium. *Am J Physiol Gastrointest Liver Physiol* 2009, 296:G1108–G1118
36. Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S: Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. *Nat Genet* 2011, 43:34–41
37. Paganelli M, Nyabi O, Sid B, Evraerts J, El Malmi I, Heremans Y, Dolle L, Benton C, Calderon PB, van Grunsven L, Heimberg H, Campard D, Sokal E, Najimi M: Downregulation of Sox9 expression associates with hepatogenic differentiation of human liver mesenchymal stem/progenitor cells. *Stem Cells Dev* 2014, 23:1377–1391
38. Pavenstadt H, Kriz W, Kretzler M: Cell biology of the glomerular podocyte. *Physiol Rev* 2003, 83:253–307
39. Hara M, Yamamoto T, Yanagihara T, Takada T, Itoh M, Adachi Y, Yoshizumi A, Kawasaki K, Kihara I: Urinary excretion of podocalyxin indicates glomerular epithelial cell injuries in glomerulonephritis. *Nephron* 1995, 69:397–403
40. Vogelmann SU, Nelson WJ, Myers BD, Lemley KV: Urinary excretion of viable podocytes in health and renal disease. *Am J Physiol Renal Physiol* 2003, 285:F40–F48
41. Yu D, Petermann A, Kunter U, Rong S, Shankland SJ, Floege J: Urinary podocyte loss is a more specific marker of ongoing glomerular damage than proteinuria. *J Am Soc Nephrol* 2005, 16:1733–1741
42. Shankland SJ, Pippin JW, Duffield JS: Progenitor cells and podocyte regeneration. *Semin Nephrol* 2014, 34:418–428
43. Smeets B, Uhlig S, Fuss A, Mooren F, Wetzels JF, Floege J, Moeller MJ: Tracing the origin of glomerular extracapillary lesions from parietal epithelial cells. *J Am Soc Nephrol* 2009, 20:2604–2615
44. Huang J, Guo L: Knockdown of SOX9 inhibits the proliferation, invasion, and EMT in thyroid cancer cells. *Oncol Res* 2017, 25:167–176

45. Dijkman H, Smeets B, van der Laak J, Steenberg E, Wetzels J: The parietal epithelial cell is crucially involved in human idiopathic focal segmental glomerulosclerosis. *Kidney Int* 2005, 68:1562–1572
46. Asanuma K, Oliva Trejo JA, Tanaka E: The role of Notch signaling in kidney podocytes. *Clin Exp Nephrol* 2017, 21:1–6
47. Chen L, Al-Awqati Q: Segmental expression of Notch and Hairy genes in nephrogenesis. *Am J Physiol Renal Physiol* 2005, 288:F939–F952
48. Ueno T, Kobayashi N, Nakayama M, Takashima Y, Ohse T, Pastan I, Pippin JW, Shankland SJ, Uesugi N, Matsusaka T, Nagata M: Aberrant Notch1-dependent effects on glomerular parietal epithelial cells promotes collapsing focal segmental glomerulosclerosis with progressive podocyte loss. *Kidney Int* 2013, 83:1065–1075
49. Lasagni L, Ballerini L, Angelotti ML, Parente E, Sagrinati C, Mazzinghi B, Peired A, Ronconi E, Becherucci F, Bani D, Gacci M, Carini M, Lazzeri E, Romagnani P: Notch activation differentially regulates renal progenitors proliferation and differentiation toward the podocyte lineage in glomerular disorders. *Stem Cells* 2010, 28:1674–1685