Urinary Podocyte Loss Is a More Specific Marker of Ongoing Glomerular Damage than Proteinuria

Donghai Yu,*† Arndt Petermann,* Uta Kunter,* Song Rong,*‡ Stuart J. Shankland,‡ Juergen Floege*

*Department of Medicine, Division of Nephrology and Clinical Immunology, University of Aachen, Germany; †Tongji Medical College, Huazhong University of Science and Technology; Wuhan; China; ‡Department of Medicine, Division of Nephrology, University of Washington School of Medicine, Seattle, Washington

Podocyte loss contributes to the development of glomerulosclerosis. Although podocyte detachment has been recognized as a new mechanism of podocyte loss in glomerular diseases, its time course and relationship to disease activity are not known. Urinary excretion of viable podocytes was quantified in two models of transient glomerular injury, i.e., rats with puromycin aminonucleoside-induced nephrosis (PAN) and mesangiproliferative nephropathy (anti-Thy 1.1 nephritis model), as well as in a model of continuous glomerular injury, i.e., hypertensive nephropathy (5/6-nephrectomy model), and in aging rats. The number of glomerular Wilms’s tumor (WT)-1–positive podocytes and the glomerular expression of cell-cycle proteins in vivo were assessed. Urinary podocyte loss occurred in both primary (PAN) and secondary (anti-Thy 1.1 nephritis) in parallel to the onset of proteinuria. However, subsequently proteinuria persisted despite remission of podocyturia. In continuous glomerular injury, i.e., after 5/6-nephrectomy, podocyturia paralleled the course of proteinuria and of systemic hypertension, whereas no podocyturia became detectable during normal aging (up to 12 mo). Despite podocyte detachment of varying degrees, no decrease in glomerular podocyte counts (i.e., WT-1 positive nuclei) was noted in either disease model. Podocyturia in the PAN and anti-Thy 1.1 nephritis model was preceded by entry of glomerular podocytes into the cell cycle, i.e., cyclin D1, cdc2, and/or proliferating cell nuclear antigen (PCNA) expression. Podocyturia is a widespread phenomenon in glomerular disease and not simply a reflection of proteinuria because it is limited to phases of ongoing glomerular injury. The data suggest that podocyturia may become a more sensitive means to assess the activity of glomerular damage than proteinuria.


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Address correspondence to: Dr. Arndt Petermann, Division of Nephrology and Clinical Immunology, RWTH Aachen, Pauwelstrasse 30, Aachen NRW 52057, Germany. Phone: +49-0-241-8089-531; Fax: +49-0-241-8082-446; E-mail: apetermann@ukaachen.de

D.Y. and A.P. contributed equally to the study.

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turia may better reflect ongoing glomerular damage than proteinuria. Finally, we investigated whether viable podocytes were lost into the urine at a specific point within the cell cycle.

Materials and Methods

Experimental Design

All animal experiments were approved by the local review boards.

In Vivo Studies

Puromycin Aminonucleoside-Induced Nephrosis. Puromycin is a podocyte toxin inducing loss and fusion of podocyte foot processes as observed, for example, in human minimal change nephropathy (17,18). Puromycin (150 mg/kg body weight) (Sigma-Aldrich Co, St. Louis, MO) was injected intravenously into male Sprague-Dawley rats (Charles River Co., Sulzfeld, Germany) weighing 120 to 140 g (n = 6 per time point). The 24-h urine samples were collected at days 0, 2, 3, 5, 10, and 15, and urine protein excretion was determined by the sulfosalicylic acid method as previously reported (19). After the 24-h urine collection, bladder urine was obtained from all rats, after which rats were euthanized and kidneys processed for immunostaining and quantitation of cell cycle activity.

Anti-Thy 1.1 Nephritis Model. The anti-Thy 1.1 nephritis model of mesangiproliferative nephritis was induced in male Wistar rats (Charles River Co) weighing 180 to 200 g by intravenous injection of mouse monoclonal antibody to the Thy 1.1 antigen (clone OX-7; 1 mg/kg body weight; Medizagnost; Reutlingen, Germany). Animals were euthanized at days 0, 1, 4, 5, 20, and 30 (n = 6 per group at each time point), and urine and kidney samples were obtained as described.

5/6-Nephrectomy Rat Model. Rats with 5/6 nephrectomy served as a model of hypertensive progressive renal damage. In brief, unilateral right-sided nephrectomy was followed 1 wk later by ligation of two of three renal artery branches in male Sprague-Dawley rats (Charles River Co) weighing 180 to 200 g. Healthy age-matched rats were used as controls. Urine samples were obtained and animals were euthanized at weeks 1, 2, 4, 8, and 12 after the second operation (n = 5 per group at each time point). Mean arterial BP was measured at the different time points by the tail-cuff method (Softron Indirect Blood Pressure Meter; Bp-98A; Softron Co. Ltd, Tokyo, Japan).

Aging Rat Model. To study if podocyte loss is induced in healthy animals solely by aging, Sprague-Dawley rats (Charles River Co) were studied at the ages of 1, 2, 3, 6, and 12 mo (n = 6 per group at each time point).

Bladder Urine Collection and Cell Culture

To collect sterile bladder urine, animals were anesthetized by ether inhalation and an intramuscular injection of a mixture containing 80% of 2% Rompun (Bayer Vital GmbH, Leverkusen, Germany) and 20% of 10% Ketamine (CEVA Tiergesundheit GmbH, Duesseldorf, Germany). A butterfly needle was then inserted into the tail vein and a bolus of 5 to 6 ml/200 g body weight of normal saline was injected over 2 to 5 min to induce a forced diuresis as described previously (10). Next, the abdomen was opened through a longitudinal incision. The urinary bladder was punctured with a 1-ml syringe, and urine (0.8 to 1 ml) was removed and rapidly transferred into a sterile 15-ml tube. Urine pH was partially neutralized by adding 11 ml of prewarmed PBS (Life Technologies, Paisley, UK). After centrifugation at 1200 × g at room temperature for 5 min, the supernatant was removed and discarded, and the cell pellet resuspended in 12 ml of culture medium for rat podocytes (20). The medium was made up of 338 ml DMEM medium (Biochrom AG; Berlin, Germany), 113 ml Ham’s F-12 (Biochrom), 25 ml 10% FBS, 5 ml penicillin/streptomycin, 5 ml glutamine (2 mmol/L; all from Life Technologies), 5 ml HEPES (Sigma, Taufkirchen, Germany), 5 ml sodium bicarbonate (Sigma), and 5 ml trace element mix (BioSource, Solingen, Germany).

The resuspended pellet was divided into 2-ml aliquots and seeded into six-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) coated with collagen I (Bovine Collagen I; Collaborative Biomedical Products, Bedford, MA). Tissue culture plates were incubated at 37°C in 95% air/5% CO₂. Twenty-four h after seeding, red blood cells and nonattached cells were removed by washing with PBS. Adherent cells were subsequently grown at 37°C in DMEM medium containing 5% FBS, which was replaced every 3 d.

After 24 h of cell culture, the number of individual adherent cells was counted and morphologic changes were documented. Cultivated cells were further characterized by immunofluorescent staining with podocyte-specific antibodies as described previously (10).

Immunostaining

To assess podocyte cell-cycle activity in the different disease models, kidney samples from rats with PAN, anti-Thy 1.1 nephritis, 5/6-nephrectomy, and aging rats were fixed in formalin, embedded in paraffin, and cut into 2-μm thick sections. Indirect immunoperoxidase staining was performed as reported previously (7) using the following primary antibodies and overnight incubation at 4°C: cyclin D1 (mouse monoclonal; Neomarkers, Fremont, CA), cdc2 (mouse monoclonal; Neomarkers), Wilm’s tumor (WT-1) (mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA), and proliferating cell nuclear antigen (PCNA) (mouse monoclonal; Oncogene, Schwalbach, Germany). Controls included omitting the primary antibody and substituting the primary antibody with irrelevant mouse IgG.

Tissue sections were boiled in citric acid (10 mmol; pH 6.0) for 10 min to unmask antigens. Nonspecific background staining was reduced with the avidin/biotin blocking kit (Vector Laboratories Inc, Burlingame, CA) and Background Buster (Innovex Biosciences, Fichmond, CA). A biotinylated horse anti-mouse secondary antibody (Vector) was then added and detected using the ABC-Kit (Vector). Antigen was finally visualized using diaminobenzidine (Sigma) with nickel chloride enhancement. To recognize binucleated or multinucleated podocytes, WT-1 staining was followed with PAS-staining.

To detect cell-cycle active podocytes in situ, double immunostaining was performed. Tissue sections were boiled in citric acid and nonspecific background staining was reduced with the avidin/biotin blocking kit as described. Staining was performed with a polyclonal rabbit anti-podocin antibody (a kind gift of Peter Mundel, Department of Medicine and Anatomy, Albert Einstein College, New York, NY) overnight at 4°C, followed by a biotinylated horse anti-rabbit secondary antibody (Vector) and the ABC-Kit (Vector). Antigen was finally visualized using diaminobenzidine (Sigma) with nickel chloride enhancement for approximately 6 min. Nonspecific background was then reduced by Background Buster followed by an overnight staining with an antibody either against cyclin D1 or PCNA at 4°C. A biotinylated horse anti-mouse secondary antibody was then added and detected using the AEC staining kit (Sigma) as described (21).

Quantitation of Immunostaining

The glomerular expression of each cell-cycle protein was evaluated in a blinded fashion in control and diseased animals at each time point. For this, 30 consecutive glomerular cross-sections were evaluated in each study and control animal by counting the number of cells staining positively for each antigen. Because all glomerular cells can express cdc2 and cyclin D1, and because this study was specifically targeted to podocytes, we counted only those PCNA and cyclin D1 cells that also


1734 Journal of the American Society of Nephrology
expressed podocin as a podocyte-specific cell marker. Because we were not able to establish convincing double immunostaining for cdc2 and different podocyte-specific proteins, we counted those cdc2 cells that located to typical podocyte positions, i.e., to the edge of the glomerular tuft. In the case of WT-1, all positive glomerular cells were counted, because in the renal glomerulus WT-1 is specifically expressed by podocytes (22).

The number of WT-1–positive cells per glomerular cell volume was calculated using the thin and thick section method for estimation of podocyte number, glomerular volume, and glomerular volume per podocyte as described (23). In brief, mean glomerular volume in a given tissue was calculated by measuring 50 glomerular diameters and calculating the mean glomerular diameter. From these data, the maximal glomerular diameter can be derived using the Weibel formula (maximum diameter = 4/π × mean measured diameter). Because the glomerulus is seen as a sphere, mean glomerular volume can be derived from these data. To calculate the podocyte number per glomerulus, sections of different thickness (3 and 9 μm) were cut from the same tissue and WT-1–positive nuclei counted in each. The difference in podocyte counts between thin and thick sections is directly proportional to the difference in slice thickness. For both thin and thick sections, the mean podocyte number per glomerular tuft area is calculated. Differences in podocyte number are related to the difference in section thickness (6 μm). By dividing the known difference in section thickness by the podocyte number per glomerular area, one can derive the glomerular volume per podocyte. The mean podocyte number per tuft is equal to the mean glomerular volume divided by glomerular volume per podocyte.

Mean values per time point were calculated and the results were expressed as the number of cells staining positively per 30 glomerular cross-sections in the case of cdc2, cyclin D1, and PCNA or as the number of cells per 50 glomerular volumes in the case of WT-1.

Statistical Analysis
All data are expressed as mean ± SD. Statistical significance (defined as P < 0.05) was evaluated using the Bonferroni t test and Spearman test.

Results
Viable Podocytes Can Be Cultivated from the Urine of Study Rats in Different Disease Models
Similar to recent data obtained in the rat model of passive Heymann nephritis (10), the induction of other glomerular diseases in rats was paralleled by the appearance of podocytes in the urine. The cells were viable and attached to the culture dishes. After 24 h of culture, colonies of cells with similar morphologic features were visible (Figure 1A). During the following days, they showed an increase in cell size and other characteristic changes in cell morphology (Figure 1B) as described recently in urinary cells cultivated from rats with passive Heymann nephritis (10). Approximately one-third of the podocytes cultured ex vivo exhibited polyoidy, again confirming earlier observations (10). By immunofluorescent staining with antibodies against nephrin and podocin, all cells in culture stained positively for these specific podocyte proteins from day 1 of cell culture onwards (data not shown). However, in this study we failed to note any increase in cell numbers over time, which is in contrast to our recent data obtained in passive Heymann nephritis (10). Rather, despite changing the culture medium every third day, cells rounded up and finally detached over a time course of approximately 15 d.

Although an intravenous fluid bolus was used before harvesting the cells from the bladder to standardize experimental conditions, we noted that a similar amount of cells also appeared in the urine without fluid loading (data not shown). More importantly, loss of these cells into the urine was diseasespecific, because with or without fluid loading, such cells were noted in normal control rats.

Urinary Podocyte Loss Exhibits a Characteristic Time Course in Each Disease Model and Can Be Dissociated from Proteinuria
In healthy control rats as well as in aging rats of up to 1 yr of age, we consistently failed to detect any podocytes in the urine (data not shown) despite the slow development of age-dependent proteinuria (1-mo-old rats: 26 ± 2 mg/24 h versus 12-mo-old rats: 84 ± 11 mg/24 h).

After the induction of PAN in rats, low numbers of urinary podocytes first appeared in some animals on days 3 and 5 (Figure 2A). On day 10 after disease induction, podocyturia increased massively in all animals and reached 8000 or more cells after 24 h of culture (Figure 2A). These changes closely paralleled the onset of marked proteinuria (Figure 2A). However, whereas proteinuria remained high on day 15, podocyturia declined sharply to near baseline (Figure 2A).

In the anti-Thy 1.1 model of mesangioproliferative nephritis, low-grade podocyturia (approximately 30-fold lower than that observed in PAN) was transiently detected on days 4 and 5 but at no other time points (Figure 2B). Days 4 and 5 of the disease are characterized by the onset of massive mesangial cell proliferation and matrix expansion, which subsides at later time points such as days 21 and 30 (24,25). As in rats with PAN, proteinuria increased in parallel with the occurrence of podocyturia but persisted on day 21 when podocyturia had already subsided (Figure 2B).

Podocyturia in rats with 5/6-nephrectomy, in which glomerular injury in contrast to PAN and anti-Thy 1.1 nephritis is continuous, followed a completely different time course. It was first detected in some animals at week 1 and 2 after disease induction and markedly increased toward the end of the observation period, i.e., week 12 (Figure 2C). Also, in 5/6-nephrectomized rats, podocyturia did not dissociate from protein-
uria and rather exhibited a significant correlation with proteinuria (Figure 3A).

In contrast to PAN and anti-Thy 1.1 nephritis, the 5/6-nephrectomy model is characterized by systemic hypertension (Table 1). We therefore investigated a potential relationship between podocyturia and mean arterial BP. As shown in Figure 3B, BP and the extent of podocyturia were positively correlated. Urinary Podocyte Loss Is Not Associated with a Significant Decrease in Glomerular WT-1–Positive Cells

WT-1 is a nuclear protein specific for podocytes and parietal glomerular epithelial cells in the adult kidney (22). WT-1–positive cells within the glomerular tuft excluding the parietal epithelium were counted at each time point in aging rats as well as in rats with PAN, anti-Thy 1.1 nephritis, and 5/6-nephrectomy, and the number per glomerular cell volume was calculated as described (23) to determine whether podocyte loss into the urine led to a reduction of glomerular podocyte numbers.

Despite a trend toward lower podocytes per glomerulus in both the PAN and anti-Thy 1.1 nephritis model (Figure 4, A and B), none of the changes reached statistical significance.

Assessment of glomerular WT-1 expression was notable for the appearance of WT-1–positive binucleated or multinucleated cells in all disease models examined (Figure 4D) but not in normal controls or aging rats.

Increase in Cell-Cycle Markers Precedes Podocyturia

In our experiments, PCNA was used as a marker of cell-cycle activity, cyclin D1 as a marker of the G1 phase, and cdc2 as a marker of the G2/M-phase (26,27). In the case of PCNA and cyclin D1, double immunostaining with podocin was performed to prove the podocyte character of cell-cycle active cells. In healthy control animals, rare glomerular cells expressed either of these cell-cycle markers (Figure 5, C and D). Rarely, expression of PCNA or cyclin D1 was noted in normal podocytes (Figure 5, C and D). Also, no increase in cell-cycle activity above this low baseline level occurred during normal aging (data not shown). Immunohistological control stains, in which
The primary antibody was replaced by irrelevant mouse IgG, resulted in the complete loss of immunoreactivity (data not shown).

In rats with PAN, PCNA cyclin D1 and cdc2 expression was noted in cells within the glomerular tuft (Figure 5, A and B) and in podocytes. When podocytes were assessed separately, the number of PCNA or cyclin D1-positive podocytes increased significantly on day 2 after disease induction and thereby precede the increase of the G2/M-phase marker cdc2 by 1 d (Figure 5E). All three markers showed maximum expression on day 10 and decreased thereafter (Figure 6A), thus mirroring the course of podocyturia (Figure 2A).

In the anti-Thy 1.1 nephritis model, expression of all three cell-cycle markers in podocytes increased on day 2 and reached a maximum on day 4 to 5 (Figure 6B), i.e., paralleling the podocyte loss in urine. Whereas cell-cycle markers subsequently remained elevated (Figure 6B), podocyturia returned to baseline (Figure 2B).

After 5/6-nephrectomy, we failed to detect any significant changes in cell-cycle activity, i.e., PCNA, cyclin D1, or cdc2 expression, over the whole period of observation (Figure 6C).

**Discussion**

In this study we asked whether podocyturia might provide clinically relevant information on disease activity. We also have attempted to gain more insight into consequences and potential mechanisms of podocyturia.

We therefore first re-established the method of harvesting and cultivating urinary podocytes. We confirmed our previous data (10) that under the culture conditions chosen a virtually pure podocyte population was cultured. At variance with our previous studies in the rat model of passive Heymann nephritis, however, no in vitro proliferation of podocytes ex vivo could be detected in any of the models investigated. Whether this discrepancy related to the disease models or to subtle differences in culture conditions in two different laboratories is currently unknown. However, the nonproliferative state of the urinary podocytes cultured in this study may more closely reflect the podocyte phenotype in vivo.

Although we have attempted to standardize our experimental conditions by intravenous fluid loading before obtaining bladder urine, we have probably detected only a subset of excreted podocytes. First, we have quantified viable podocytes only, and of these only the cells that were able to attach to the cell culture dish. In the case of damaged, dying, or even apoptotic urinary podocytes, it is likely that their phenotypic characteristics, such as the expression of marker proteins, are altered, which may preclude an unequivocal identification of such cells as podocytes by protein or RNA quantitation methods such as Western blot, immunofluorescence, or reverse-transcription PCR. Finally, fluid intake and voiding habits of the experimental animals immediately before the study period might affect podocyte excretion and may be difficult to control. However, because the central aim of our work was to develop a method to assess ongoing glomerular damage, many of these limitations will have little effect as long as the method is practical and reproducible.

The first major result of our study was that podocyturia and proteinuria can be separated experimentally. Whereas the onset of proteinuria was paralleled by the onset of podocyturia in both the PAN and anti-Thy 1.1 nephritis models, proteinuria persisted in late disease stages, whereas podocyturia disappeared. These observations suggest that the detection of proteinuria cannot distinguish between a persistent defect of the glomerular barrier and ongoing injury, whereas podocyturia may be more specific for “active” injury. Both models are notable for their “single-shot” nature of the injurious condition, i.e., glomerular damage was transient, which may explain the complete and rapid disappearance of podocyturia at 2 to 3 wk after disease induction. Importantly, this course of events was observed not only in PAN, in which the toxin puromycin aminonucleoside directly induces oxidative podocyte damage (28,29), but also in anti-Thy 1.1 nephritis. In the latter model, the primary injury, i.e., injection of a complement-activating anti-Thy 1.1 antibody, is highly selective for mesangial cells. However, in the course of mesangial cell destruction, intraglomerular capillaries are destabilized, leading to capillary dilatation and intraglomerular microaneurysms at 3 to 4 d after disease induction (5). All of this will contribute to secondary podocyte damage via altered physical forces, e.g., stretching (30,31), and/or potential biochemical alterations of the normal GBM in the course of the disease. Subsequently, massive mesangial cell proliferation and angiogenic repair re-establish intraglomerular forces between day 5 and 10 (25), which should terminate podocyte stress and can explain the disappearance of podocyturia at late stages of anti-Thy 1.1 nephritis.

This interpretation, namely podocyturia reflecting “active” ongoing glomerular damage better than proteinuria, is also supported by our observations in the 5/6-nephrectomy model. Here, in the face of continuing glomerular hyperfiltration and systemic hypertension, podocyturia never subsided and rather mirrored proteinuria. The causal role of hypertension in driving podocyte loss is also supported by our observation of a significant correlation between BP and the extent of podocyturia.

We next assessed whether podocyturia in the various models is mirrored by a decrease in glomerular podocytes numbers. Similar to a recent report (23), we used the expression of WT-1, a transcription factor of major importance for podocyte differentiation (22), to quantify podocyte numbers. Because of the complex architecture of the podocyte, counting of a nuclear
podocyte antigen appears preferable to cytoplasmic proteins such as synaptopodin or membrane proteins such as podocalyxin and nephrin. However, assessing podocyte numbers by WT-1 quantification would be of limited value if a major regulation of WT-1 expression occurs in disease. In fact, dedifferentiation of podocytes in vivo, as occurs in collapsing or HIV nephropathy, does result in complete loss of WT-1 expression (32,33). However, apart from such extreme cases, in diseases more relevant for our study, i.e., hypertension, IgA-nephropathy, minimal change, and membranous nephropathy, the podocytic WT-1 expression is well preserved (32,34). Furthermore, WT-1 expression is also maintained in immortalized podocytes in vitro during proliferation and differentiation (20,35), as well as in embryonal podocytes in vivo (22).

The second major finding of our study was that urinary podocyte loss did not reduce the number of glomerular podocytes in the three disease models to a significant extent. Especially in the PAN model, this observation was unexpected because the toxin puromycin aminonucleoside induces early podocyte apoptosis in vivo (36), followed by pronounced excretion of viable podocytes in urine (Figure 2A). However, this initial podocyte death and the loss of cell–cell contact inhibition may provide a stimulus for repair in surviving podocytes to cover the resulting gaps by cell division. Consistent with this hypothesis, we detected entry of podocytes in situ into the G1 phase in early PAN (Figure 6A). Because only WT-1–positive nuclei were assessed, our data can not differentiate between true podocyte cell division and the appearance of binucleated or multinucleated podocytes. In agreement with observations of this study (Figures 1 and 4D), the latter have been detected in glomerulosclerosis in situ (9,37), as well as in the urine (10,12). Because multinucleated cells can only be identified if the cell is sectioned at the level of both or more nuclei, the real percentage of binucleated or multinucleated podocytes likely is underestimated. As a side note, both in this and in our former study, approximately one third of the podocytes cultured from urine were binucleated or multinucleated (10) and it is therefore possible that such cells are particularly prone to detachment from the GBM. Taken together, the observation that glomerular podocyte numbers did not decrease to a significant degree in either model can probably be explained by the compensatory increase of binucleated or multinucleated podocytes in situ, which was noted in all models and, more importantly, likely reflects a relatively low absolute podocyte shedding against the huge total number of adherent glomerular podocytes.

The third major finding of our study was that entry of podocytes into the G1 phase of the cell cycle, as indicated by cyclin D1 expression, at least in the acute disease models preceded detectable podocyte loss. Furthermore, the peak of podocytic cell cycle activity coincided with the peak of podocyturia, which extends and confirms our previous data in passive Hey-
mann nephritis (10). Because of the chronic and less synchronized character of damage, these changes will be less obvious in the 5/6-nephrectomy model. Taken together, these observations argue against the hypothesis that cell-cycle entry is a mere response to detachment and urinary loss of podocytes. Rather, they suggest that the attempt of podocytes to enter mitosis and cell division is associated with altered attachment to the GBM, e.g., via transient retraction of primary and/or secondary foot processes. Given the glomerular filtration forces and flow characteristics, this altered attachment may in some podocytes result in complete detachment from the GBM with subsequent loss into the urinary space.

In summary, using three different models of podocyte damage in vivo with transient or continuing injury as well as primary or secondary injury, we present evidence that urinary excretion of viable podocytes appears to be confined to the phase of “active” ongoing glomerular damage, whereas the detection of proteinuria can not distinguish between ongoing damage and persistent glomerular defects of the barrier function. We hypothesize that podocyturia may be derived from an attempt of podocytes in situ to cover gaps by entering the cell cycle, which at least in some podocytes may result in rounding off and detachment from the GBM. We conclude that podocyturia may bear the clinical potential to become a unique means of assessing ongoing, i.e., “active,” glomerular damage.

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