Expression of a Novel Stress-inducible Protein, Sestrin 2, in Rat Glomerular Parietal Epithelial Cells

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ABSTRACT

Sestrin 2, initially identified as a p53 target protein, accumulates in cells exposed to stress and inhibits mammalian target of rapamycin (mTOR) signaling. In normal rat kidneys, sestrin 2 was selectively expressed in the PECs, identified by the marker PGP9.5. In adriamycin nephropathy, sestrin 2 expression decreased in PECs on day 14, together with increased expression of phosphorylated S6 ribosomal protein (P-S6RP), a downstream target of mTOR. Sestrin 2 expression was markedly decreased on day 42, coinciding with glomerulosclerosis and severe periglomerular fibrosis. In puromycin aminonucleoside nephropathy, decreased sestrin 2 expression, increased P-S6RP expression, and periglomerular fibrosis were observed on day 9, when massive proteinuria developed. These changes were transient and nearly normalized by day 28. In crescentic glomerulonephritis, sestrin 2 expression was not detected in cellular crescents, whereas P-S6RP increased. In conditionally immortalized cultured PECs, the forced downregulation of sestrin 2 by shRNA resulted in increased expression of P-S6RP, and increased apoptosis. These data suggest that sestrin 2 is involved in PEC homeostasis by regulating the activity of mTOR. In addition, sestrin 2 could be a novel marker of PECs, and decreased expression of sestrin 2 might be a marker of PEC injury.

KEY WORDS: sestrin-2, glomerular parietal epithelial cells, mTOR
Introduction

Sestrin 2 is a stress-inducible protein, initially identified as a hypoxia-responsive gene product (7). Sestrin 2 accumulates within cells exposed to stress, and plays an important role in suppressing the production of reactive oxygen species (ROS) and protecting cells from oxidative damage (6). In addition, sestrin 2 inhibits mammalian target of rapamycin (mTOR) signaling through a redox-independent mechanism, by activating 5′-adenosine monophosphate-activated protein kinase (AMPK) and phosphorylating tuberous sclerosis protein 2 (TSC2) (5). Although sestrin 2-knockout mice are reported to be fully viable and to not display any gross developmental abnormalities (28), a recent study showed that deletion of sestrin 2 exacerbates obesity-induced mTOR activation, glucose intolerance, insulin resistance, and hepatosteatosis (18).

Target of rapamycin (TOR) is a Ser/Thr kinase that was originally identified in yeast mutants resistant to the effect of rapamycin (29). Subsequently, mammalian TOR (mTOR) was cloned in mammalian cells (29). mTOR constitutes a part of two distinct multiprotein complexes, TOR complex 1 (TORC1), which is sensitive to rapamycin, and TORC2, which is not sensitive to rapamycin (4, 29). Activated TORC1 directly phosphorylates two proteins, p70 ribosomal protein S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1), which stimulate ribosome biogenesis and translation to increase the cell mass (13). In turn, phosphorylated p70S6K phosphorylates S6 ribosomal protein (S6RP), which also stimulates translation (29).

Persistent mTOR activation is associated with diverse diseases including cancer, allograft rejection, autoimmune disorders, cardiovascular diseases and metabolic disorders (29). Studies in animals are underway to identify the roles of mTOR signaling in the pathogenesis of kidney diseases such as glomerular diseases, polycystic kidney, and renal cancer (13). Genetic deletion of TORC1 in mouse podocytes induces proteinuria and progressive glomerulosclerosis, whereas
genetic reduction of podocyte-specific TORC1 in diabetic animals suppresses the development of diabetic nephropathy (10, 14).

Among resident cells in the glomeruli, glomerular parietal epithelial cells (PECs) remain poorly understood (22). Recent studies have shown that PECs are dynamic and constantly responsive to cues within the glomeruli (22). In juvenile mice, PECs have been shown to migrate to become podocytes (1). PECs also contribute to the development of the sclerotic lesions in focal segmental glomerulosclerosis. (26). In addition, PECs are also reported to function as a second barrier of the glomerular filtrate, with their tight junctions to prevent filtered protein from escaping into the extraglomerular space (21).

In this study, we first demonstrated that sestrin 2 was predominantly expressed in normal rat PECs. We further evaluated the expression of sestrin 2 and mTOR signaling in normal and diseased kidneys and attempted to determine the role of sestrin 2 and the association between sestrin 2 and mTOR signaling by using conditionally immortalized cultured PECs.

Methods

Animals and experimental protocol

All rats were purchased from Charles River Japan (Kanagawa, Japan) and fed a standard diet and given water ad libitum. Six-week-old male Wistar rats were used to investigate the normal kidneys. For induction of adriamycin (ADR) nephropathy, 6-week-old male Wistar rats were administered a single injection of 7.5 mg/kg of ADR (doxorubicin hydrochloride; Sigma-Aldrich St. Louis, MO) via the tail vein; on days 0, 8, 14 and 42 after the injection, the rats were euthanized by injection of pentobarbital sodium (Kyoritsu Pharmaceutical, Tokyo, Japan) and kidney samples were harvested. For induction of puromycin aminonucleoside (PAN)
nephropathy, 6-week-old male Wistar rats were administered a single injection of 100 mg/kg of PAN (Sigma-Aldrich, St. Louis, MO) via the tail vein, as described previously (27); on days 0, 9, and 28 after injection, the rats were sacrificed. For induction of crescentic glomerulonephritis, 7-week-old male Wistar Kyoto (WKY/NCrlCrj) rats were administered a single injection of a nephritogenic monoclonal antibody at 80 μg/body (clone a84; Iwai Chemicals Company, Tokyo, Japan) via the tail vein; On day 10 after the induction, kidney samples were harvested.

All animal experiments were carried out in accordance with the institute of Experimental Animal Research of Gunma University and were handled using protocols approved by the Animal Care Committee of Gunma University.

**Measurement of urinary protein excretion**

Twenty-four-hour urine was collected under normal conditions and each week after the induction of nephritis using a metabolic cage. The urinary protein concentration was determined using a Bio-Rad protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan).

**Primary antibodies**

The primary antibodies used in this study were as follows; rabbit polyclonal anti-sestrin 2 antibody (ProteinTech Group, Chicago, IL), mouse monoclonal anti-PGP9.5 antibody (clone 13C4; Gene Tex, Irvine, CA), mouse monoclonal anti-β actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit monoclonal anti-phospho-S6 ribosomal protein (P-S6RP) antibody (Ser 235/236; Cell signaling Technology, Beverly, MA), mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (clone 1A4; Sigma-Aldrich), rabbit anti-PAX2 antibody (Invitrogen Corporation, Camarillo, CA), rabbit monoclonal anti-phospho-p70 S6 kinase antibody (Thr 389; Cell signaling), and rabbit monoclonal anti-phospho-4E-BP1 antibody (Thr 37/46; Cell signaling).
Immunohistochemistry

Kidneys were fixed in formalin and embedded in paraffin. Four-micrometer sections were stained with periodic acid-Schiff (PAS). For the immunohistochemical analysis, 4-µm sections were deparaffinized and rehydrated. Antigens were retrieved by microwaving at 500 W for 10 min in 10 mmol/l citric acid. Endogenous peroxidase activity was blocked with periodic acid (Nichirei, Tokyo, Japan) for 45 sec. The sections were incubated with the primary antibodies at 4°C overnight, followed by incubation with the biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at room temperature and with horseradish peroxidase-avidin (Vector Laboratories) for 30 min at room temperature. Color development was performed using diaminobenzidine tetrahydrochloride solution (Nichirei). Sections were counterstained with methyl green or PAS.

Quantification by immunohistochemistry

The glomerular damage was quantified by grading the severity of the glomerulosclerosis and mesangial expansion on PAS-stained sections, on a scale of 0 to 4 (0, no lesion; 1, 0 to 25%; 2, 25 to 50%; 3, 50 to 75%; 4, 75 to 100%) as described previously (11). Fifty glomeruli from each rat were evaluated, and the average score was calculated. Sestrin 2 expression in the PECs and periglomerular α-SMA expression were quantified on a scale of 0 to 4 (0, no lesion; 1, 0 to 25%; 2, 25 to 50%; 3, 50 to 75%; 4, 75 to 100% around the Bowman’s capsule) in each glomerulus. The average score of 50 glomeruli per section was obtained. The number of PAX2-positive cells attached to the Bowman’s capsule was counted in 50 glomeruli per section. The percent area of P-S6RP-positive PECs per glomerulus was assessed using Photoshop CS6 (Adobe, San Jose, CA), as follows. First, the total number of pixels corresponding to the
glomerulus was counted in the captured image of each glomerulus. After removing the
glomerular tuft, the number of pixels showing P-S6RP positivity (brown area) within the
Bowman’s capsule was counted. The percent P-S6RP-positive area was calculated using the
formula; number of pixels showing P-S6RP positivity / total number of pixels corresponding to the
glomerulus x 100 (%). The average percentage of 50 glomeruli per section was calculated.

Western blot analysis
Western blot analysis was performed as described elsewhere, with some modifications (27).
In brief, protein was extracted from the cultured PECs using RIPA lysis buffer (1 × TBS, 1%
Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, Santa Cruz)
containing PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz). The
protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) in
accordance with the manufacturer’s directions. Ten μg of the protein extract was separated on
4-20% precast polyacrylamide gels (Nippon Bio-Rad) and transferred to a polyvinyl difluoride
membrane (Immobilon-P; Millipore, Bedford, MA). After blocking with 2% bovine serum albumin
to reduce nonspecific antibody binding, the membrane was incubated with primary antibodies
overnight at 4ºC, washed with Tris-buffered saline (20mM Tris-HCl, 150mM NaCl, and 0.1%
Tween 20), and incubated with an alkaline phosphatase-conjugated anti-rabbit IgG or
anti-mouse IgG antibody (Promega, Madison, WI) at room temperature for 2 hours. After further
washing, detection of the bound antibody was performed using chromogen
5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich).

Cell culture
Conditionally immortalized mouse PECs were generated as previously described (23). Cells
were cultured on a 100-mm type-I collagen-coated culture dish (Iwaki, Tokyo, Japan) in RPMI 1640 containing penicillin, streptomycin, amphotericin B, and 2% fetal bovine serum. Cells were propagated at 33°C with 5 U/ml recombinant mouse interferon-γ (Millipore, Billerica, MA) and differentiated at 37°C in the absence of interferon-γ for 10 days.

**Sestrin 2 knockdown with small hairpin RNA (shRNA)**

Sestrin 2 silencing was performed using shRNA by a previously described method (24). In brief, pLKO.1-puro lentiviral plasmids encoding shRNA for mouse sestrin 2 or nontarget control (NTC), together with a puromycin resistance gene, were purchased from Thermo Scientific Open Biosystems (Huntsville, AL). HIV-1-based lentiviral particles were generated in HEK293FT cells (American Type Culture Collection, Manassas, VA) by co-transfection of shRNA or NTC shRNA with pCMV R8.91 and pCMV-VSV-G using FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN). Cultured PECs were infected with the lentiviral particles in the presence of 8 μg/ml polybrene for 5h at 33°C. The effective viral titer was confirmed by observation of the cell survival in the presence of 3 μg/ml puromycin (Invivogen, San Diego, CA). Five shRNA clones were tested for their ability to silence sestrin 2 mRNA, and the best-performing clone was selected.

**Measurements of apoptosis**

Apoptosis was measured by Hoechst 33342 (Dojindo Molecular Technologies, Tokyo, Japan) staining and by careful morphological analysis, as previously reported (12). The apoptotic cells were counted in six randomly selected fields, and expressed as a percentage in triplicate. In addition, the degree of apoptosis was also measured by measurement of the caspase-3 activity using the APOPCYTO caspase colorimetric assay kit (Medical & Biological Laboratories, Nagoya, Japan) in accordance with the manufacturer’s instructions.
Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). Differences between two groups were compared using the two-tailed t test. Comparisons of multiple groups were performed by ANOVA and if the ANOVA revealed significance, Tukey’s test was applied, using IBM SPSS statistics 21 (IBM SPSS, Tokyo, Japan). P values < 0.05 were considered as indicative of statistically significance.

Results

Expression of sestrin 2 in the normal rat kidney

Immunohistochemical staining of the normal rat kidney revealed selective expression of sestrin 2 along the Bowman’s capsule (Fig. 1, A and B). The staining pattern of sestrin 2 was similar to that of PGP9.5 (Fig. 1C), a well-known marker of PECs (25), indicating that sestrin 2 was predominantly expressed in the PECs. Staining was absent elsewhere in the kidney, and not detected when the primary antibody was removed as a negative control (data not shown).

Sestrin 2 staining decreases in ADR nephropathy

To determine the sestrin 2 expression in PECs in proteinuric disease conditions, the ADR model of focal segmental glomerulosclerosis was induced in rats. As shown in Figure 2A, urinary protein excretion increased progressively after ADR injection. Severe glomerulosclerosis was observed in PAS-stained sections of the kidney on day 42 (Fig. 2B and Fig. 3A-D). Quantitation shows that sestrin 2 staining in PECs was unchanged until day 8, but decreased on day 14, with markedly decreased staining on day 42 (Fig. 2C and Fig. 3E-H). In contrast, α-SMA staining,
which was observed only in the afferent or efferent arterioles on day 8 after ADR injection, was
detected along Bowman’s capsule on day 14, with marked increase of its expression on day 42
(Fig. 2D and Fig. 3I-L). However, the α-SMA-positive cells were not PECs, because they were
located outside the Bowman’s capsule, as shown in Figure 4A and 4B.

**S6RP staining increases in ADR nephropathy**

Because sestrin 2 is considered a negative regulator of the mTOR pathway (5), the
phosphorylation levels of S6RP, a direct phosphorylation target of mTOR, was examined using
anti-phospho S6RP antibody. P-S6RP staining in PECs was barely detected in control rats. In
contrast, P-S6RP staining increased significantly by day 14 (Fig. 2E, Fig. 3M-P). As shown in
Figure 4C and 4D, the P-S6RP-positive cells were found along Bowman’s capsule, in contrast to
the α-SMA-positive cells (Fig. 4A and 4B). In addition, examination of serial sections of the
kidney in ADR nephropathy revealed a reciprocal relationship between the expression of sestrin
2 and that of P-S6RP; i.e., PECs which showed increased P-S6RP expression showed
decreased sestrin 2 expression, whereas PECs with sustained sestrin 2 expression did not show
P-S6RP expression (Fig. 4F and 4G). The number of PECs in ADR nephropathy was examined
by PAX2 staining, because PAX2 was expressed in the nucleus of PECs (2), allowing to count
the number of PECs. The number of PAX2-positive cells decreased slightly with the progression
of ADR nephropathy (Fig. 2F and Fig. 3Q-T).

**Sestrin 2 expression in PAN nephropathy**

The expression of sestrin 2 was examined in PAN nephropathy, a model of minimal-change
nephrotic syndrome. As shown in Figure 5A, urinary protein excretion was markedly increased
on day 9 of PAN nephropathy, but was almost normal by day 28. Although glomerulosclerosis in
PAN nephropathy, this was much less severe as compared to ADR nephropathy (Fig. 5B and Fig. 6A-C). Sestrin 2 staining was moderately decreased on day 9 but increased again on day 28 (Fig. 5C and Fig. 6D-F). In contrast to sestrin 2 expression, the number of α-SMA-positive cells outside the Bowman’s capsules was increased on day 9, but decreased again by day 28 (Fig. 5D and Fig. 6G-I). The reciprocal relationship between sestrin 2 and P-S6RP was also observed in PAN nephropathy (Fig. 5C, E and Fig. 6J-L), where P-S6RP increased on day 9, when sestrin 2 was reduced. The number of PAX2-positive cells was slightly increased on day 28 (Fig. 5F and 6M-Q).

**Sestrin 2 expression in crescentic glomerulonephritis**

PECs proliferate to form crescents in crescentic glomerulonephritis (22). To determine the sestrin 2 expression in crescents, WKY rats were injected with anti-GBM antibody. Massive proteinuria was observed at day 10 (4.0 ± 3.8 vs. 229.3 ± 27.5 mg/day, day 0 vs. day 10, P < 0.01). As shown in Figure 7, sestrin 2 staining was not detected in the cellular crescents, but was detected in cells lining Bowman’s capsule not participating in the crescent. In contrast, P-S6RP staining increased in a subpopulation of cells within the crescents.

**Downregulation of sestrin 2 by shRNA increased the activity of mTOR**

To examine the role of sestrin 2 in PECs, conditionally immortalized cultured mouse PECs were silenced for sestrin 2 using specific shRNA. Reduced expression of sestrin 2 protein was confirmed by western blot analysis compared to PECs transfected with NTC (Fig. 8A and 8B). Sestrin 2-silenced PECs cultured under growth-restrictive conditions for 10 days had increased levels of phosphorylated 4E-BP1, p70S6K and S6RP, which are direct downstream targets of mTOR (Fig. 8A and 8C).
To determine the biological role of sestrin 2 in cultured PECs, apoptosis was measured. In transfected PECs with reduced levels of sestrin 2- apoptosis was increased measured by Hoechst 33342 staining and caspase-3 activity (Fig.9).

Discussion

Recent studies have shown that many renal cell types express genes unique to that cell, which may serve not only as specific ‘markers’ for identification, but are also likely serve cell-type-specific functions (22). Examples in podocytes include nephrin, podocin, NEPH1, GLEPP-1, podocalyxin and synaptopodin (20). In PECs, unique proteins include PAX2, PAX8, the tight junction proteins claudin-1 or occludin, ubiquitin-related protein PGP9.5, and the intermediate filament protein cytokeratin (2, 21, 23, 25). The first major finding of this study was that sestrin 2 was predominantly expressed in the glomerular PECs in normal rats. Sestrin 2 is a novel p53 target protein that is known to accumulate in cells exposed to stress (17). Just recently, increased expression of sestrin 2 was reported in renal proximal tubules in a model of renal ischemia-reperfusion injury (15). In addition, upregulation of sestrin 2 expression was shown in cultured renal tubular cells (NRK-52E) exposed to oxidative stress (15). Taken together, the results of the current study show that in contrast to other cell types where sestrin 2 expression is related to stressors, sestrin 2 is constitutively expressed in normal PECs.

The expression of sestrin 2 in PECs was examined in three experimental models of nephrotic syndrome. The second major finding was that sestrin 2 staining in PECs was closely linked with proteinuria. In both the ADR and PAN models of podocyte injury characterized by proteinuria, sestrin 2-staining PECs was decreased, and this coincided with the presence of proteinuria. This was further highlighted by transient proteinuria in PAN being accompanied by a
transient decline in sestrin 2 staining. Finally, in the anti-GBM model, reduced sestrin staining accompanied the proteinuria, but the decrease was selectively in the proliferating cells of the glomerular crescents. Taken together, although the models used where diseases of podocytes or the GBM, changes to sestrin 2 occurred in PECs, where it is normally expressed.

Because sestrin 2 is known to inhibit mTOR signaling by activating AMPK and phosphorylation of TSC2 (5), we also examined the downstream phosphorylation targets of mTOR, with an emphasis on P-S6RP. The third major finding of our study was the paradoxical expression of decreased expression of sestrin 2 with increased expression of P-S6RP during the periods of heavy proteinuria in both the ADR nephropathy and PAN nephropathy models. This data suggests that sestrin 2 regulates the activity of mTOR in the PECs in both health and disease conditions.

Interestingly, increased periglomerular fibrosis, which was demonstrated by the accumulation of α-SMA-positive cells around the Bowman’s capsule was observed when the PECs showed decreased sestrin 2 and increased P-S6RP expression. In addition, periglomerular fibrosis was reversible in the PAN nephropathy model when the proteinuria resolved and expression of sestrin 2 was restored in the PECs. PECs have tight junctions and are considered to serve as a permeability barrier (21). Therefore, we hypothesize that heavy proteinuria induces PEC injury, which in turn results in the disruption of their tight junctions that allows leakage of protein into the extraglomerular space, followed by the development of periglomerular fibrosis.

In contrast to the reversible changes of sestrin 2 expression and periglomerular fibrosis in the PAN nephropathy model, which is a model of transient nephrotic syndrome, progressive decrease of sestrin 2 expression was observed in the ADR nephropathy model, which is a model of progressive nephrotic syndrome. In the ADR nephropathy model, the severe decrease of
sestrin 2 expression in the PECs was also associated with a decrease in the number of PECs, severe periglomerular fibrosis and increased glomerular sclerosis, suggesting that prolonged PEC injury was closely linked to the glomerular and periglomerular injury. Although we could not detect any apoptotic cells within the Bowman's space by TUNEL staining (data not shown) on day 42 of ADR nephropathy, we suppose that the reduction in the number of PEC was due to apoptosis or detachment of the PECs under the condition of reduced sestrin 2 expression. Because apoptotic cells are likely detached and washed away in the urinary ultrafiltrate, there may only be a small window for the detection of apoptotic PECs in vivo (8). Previous in vitro studies have shown that silencing of sestrin 2 increased apoptosis, including of the renal tubular cells (3, 6, 15, 19). The fourth major finding of our study was that silencing of sestrin 2 in cultured PECs increased phosphorylation of the downstream targets of TORC1 (4E-BP1, p70S6K and S6RP) and induced apoptosis. Because immature PECs cultured under growth-permissive conditions stop proliferating and differentiate under growth-restrictive conditions, we supposed that the dysregulation of mTOR activity was associated with the apoptosis of PECs in vitro. However, the precise mechanisms underlying the role of sestrin 2 in PEC survival need to be determined in the future.

The above data show that decreased expression of sestrin 2 was associated with a decrease in the number of PECs in the ADR nephropathy model and cultured PECs. Meanwhile, we observed that, in the crescentic glomerulonephritis model, in which PECs are considered to proliferate to form crescents, sestrin 2 expression was decreased in the cells within the crescent, whereas P-S6RP expression was highly increased in these cells. Considering the role of mTOR in cell growth and proliferation (9, 29), the increased activity of mTOR in these cells seems explicable. Recently, Kurayama et al. reported increased expression of P-S6RP in the PECs before apparent crescent formation and further increase in the expression in the crescentic
lesions in rat anti-GBM nephritis (16). In addition, they studied the effects of the mTOR inhibitor, everolimus, in this crescent model. Interestingly, early treatment with the mTOR inhibitor led to increased cellular necrosis of the PECs, whereas later treatment reduced glomerular crescent formation. These findings demonstrate the complex role of the mTOR pathway in the PECs.

Taken together, Figure 10 shows our working hypothesis about sestrin 2 expression and mTOR activity in the PECs in normal and diseased kidneys based on the present data. In the normal rat kidney, sestrin 2 is predominantly expressed in the PECs and minimal mTOR activity is detected. These conditions may be required for maintenance of the homeostasis of PECs. In injured PECs, decreased expression of sestrin 2 and increased activity of mTOR are observed. In the PAN nephropathy or ADR nephropathy model, injured PECs lose their barrier function and the leakage of protein around the Bowman's space causes periglomerular fibrosis. In addition, sustained injury of the PECs in the ADR nephropathy model leads to apoptosis and/or detachment of PECs, which in turn causes glomerulosclerosis. In contrast, in anti-GBM nephritis, decreased expression of sestrin 2 and increased activity of mTOR are associated with proliferation of the PECs to form crescents. Although the reason why decreased sestrin 2 expression and increased mTOR activity are associated with different outcomes in the PECs still remains unclear after this study, our data show that sestrin 2 could be a novel marker of PECs and that decreased expression of sestrin 2 might be a marker of PEC injury.

GRANTS
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DISCLOSURES
None of the authors have any competing interests to declare.
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FIGURE LEGENDS

Fig. 1. Immunohistochemical staining of sestrin 2 in the normal rat kidney. A and B: Sestrin 2 was selectively expressed in the PECs. C: PGP9.5, a known marker of PECs was also principally expressed in the PECs. Original magnification: A, x100; B and C, x400.

Fig.2. Changes of the urinary protein excretion, glomerulosclerosis and expression of each of the proteins in the PECs in the rat model of ADR nephropathy.

Rats were injected with ADR and the urinary protein excretion and kidney sections were examined on days 0, 8, 14 and 42 (n = 6, at each time-point). A: Urinary protein excretion (UP). B: Glomerulosclerosis (GS). C: Sestrin 2. D: α-SMA. E: P-S6RP. F: PAX2. The degree of glomerulosclerosis (B) was estimated on the PAS-stained kidney sections as described in the methods section. The degrees of sestrin 2 (C), α-SMA (D), P-S6RP (E), PAX2 (F) expression were estimated on the immunohistochemically stained kidney sections using each specific antibody as described in the methods section. * p < 0.01 vs. day 0. † p < 0.01 vs. day 8. ‡ p < 0.05 vs. day 8. § p < 0.01 vs. day 14.

Fig.3. Immunohistochemical staining for each protein in the PECs during ADR nephropathy.

Rats were injected with ADR and the kidney sections were examined on day 0 (A, E, I, M, Q), day 8 (B, F, J, N, R), day 14 (C, G, K, O, S), and day 42 (D, H, L, P, T). A-D: PAS staining. Severe glomerulosclerosis was observed on day 42 (D). E-H: Sestrin 2. Sestrin 2 expression decreased on day 14 (G and G') with an even more marked decrease observed on day 42 (H and H'). I-L: α-SMA. α-SMA expression was not detected on day 0 (I and I') or day 8 (J and J'), except in the small arteries (J: arrow). Expression of α-SMA was detected around the basement
membrane of the Bowman’s capsule on day 14 ($K$ and $K'$: arrows) and markedly increased on day 42 ($P$ and $P'$: arrows). $M$-$P$: P-S6RP. Along the Bowman’s capsule, weak P-S6RP expression was detected in some cells ($M$: arrows), but most cells were negative for P-S6RP expression on day 0 ($M$ and $M'$: arrow heads). Within the glomerular tufts, some cells showed strong staining for P-S6RP ($M$ and $M'$: asterisks). These cells were identified as podocytes based on their localization. Strong expression of P-S6RP was observed in the cells along the Bowman’s capsule on day 14 ($O$ and $O'$: arrows) and day 42 ($P$ and $P'$). $Q$-$T$: PAX2. PAX2 was detected in the cells along the Bowman’s capsule, in a nuclear localization. The number of PAX2-positive cells was mildly decreased on day 42 ($T$ and $T'$). Original magnification: x400.

Fig.4. Immunohistochemical stainings for α-SMA, P-S6RP and sestrin 2 in a rat model of ADR nephropathy.

A and B: α-SMA immunostaining counterstained with PAS staining on day 42 in the model of ADR nephropathy. α-SMA-positive cells localized outside the Bowman’s capsule (B: arrow). $C$-$E$: P-S6RP immunostaining counterstained with PAS staining on day 14 of ADR nephropathy. P-S6RP-positive cells mainly localized within the Bowman’s capsule ($D$ and $E$: arrow). A few P-R6RP-positive cells were also detected outside the Bowman’s capsule ($E$: arrowheads). $F$: P-S6RP. $G$: Sestrin 2. Serial sections of P-S6RP ($F$) and sestrin 2 ($G$) on day 14 of ADR nephropathy showed a reciprocal relationship between sestrin 2 and P-S6RP expressions. PECs which showed strong P-S6RP expression ($F$: arrows) showed faint sestrin 2 expression ($G$: arrows). In contrast, PECs which showed no P-S6RP expression ($F$: arrowheads) showed sustained sestrin 2 expression ($G$: arrowheads). Original magnification: x400.

Fig.5. Changes of the urinary protein excretion, glomerulosclerosis and expression of
each of the proteins in the PECs of the rat model of PAN nephropathy.

Rats were injected with PAN and the urinary protein excretion and kidney sections were examined on days 0, 9 and 28 (n = 6, at each time-point). **A**: Urinary protein excretion (UP). **B**: Glomerulosclerosis (GS) **C**: Sestrin 2. **D**: α-smooth muscle actin (α-SMA). **E**: P-S6RP. **F**: PAX2.

The degree of glomerulosclerosis (**B**) was estimated on the PAS-stained kidney sections as described in the methods section. The degrees of sestrin 2 (**C**), α-SMA (**D**), P-S6RP (**E**), PAX2 (**F**) expressions were estimated on the immunohistochemically stained kidney sections using each specific antibody as described in the methods section. * p < 0.01 vs. day 0. ** p < 0.05 vs. day 0. # p < 0.01 vs. day 9.

**Fig.6. Immunohistochemical staining for each protein in the PECs in a rat model of PAN nephropathy.**

Rats were injected with PAN and the kidney sections were examined on day 0 (**A**, **D**, **G**, **J**, **M**), day 9 (**B**, **E**, **H**, **K**, **N**), and day 28 (**C**, **F**, **I**, **L**, **O**). **A-C**: PAS staining. **D-F**: Sestrin-2. Sestrin 2 expression decreased on day 9 (**E** and **E'**), but was restored on day 28 (**F** and **F'**). **G-I**: α-SMA. Strong expression of α-SMA was detected around the basement membrane of the Bowman’s capsule on day 9 (**H** and **H’**: arrows), but disappeared on day 28 (**I** and **I’**). **J-L**: P-S6RP. P-S6RP was detected weakly in some cells along the Bowman’s capsule, (**J** and **J’**: arrows), but most cells were negative for P-S6RP expression on day 0 (**J** and **J’”**: arrowheads). Strong expression of P-S6RP was observed in the cells along the Bowman’s capsule on day 9 (**K** and **K’**: arrows), but the expression almost disappeared on day 28. Within the glomerular tufts, some cells showed strong staining for P-S6RP (**J** and **J’**: asterisks). **M-O**: PAX2. PAX2 was detected in the cells along the Bowman’s capsule in a nuclear localization. Original magnification: x400.
Fig. 7. Immunohistochemical stainings for sestrin-2 and P-S6RP in anti-GBM antibody-induced glomerulonephritis. WKY rats were injected with anti-GBM antibody and the kidney sections were examined on day 0 (A, C, E; serial sections) and day 10 (B, D, F; serial sections). A and B: Periodic acid-Schiff (PAS) staining. Cellular crescents were observed on day 10 (B: arrows). C and D: Sestrin-2. Sestrin-2 expression was not detected in the area of the crescent formation (D: arrows). E and F: P-S6RP. Strong P-S6RP expression was detected within the crescents (F: arrows). Original magnification: x400.

Fig. 8. Effect of sestrin 2 downregulation on the expression of mammalian target of rapamycin (mTOR) in conditionally immortalized cultured PECs. Conditionally immortalized cultured PECs were transfected with shRNA targeting sestrin 2 or non-target control (NTC). Transfected PECs were then cultured under growth-restrictive conditions for 10 days and the proteins were extracted for western-blot analysis. A: Western-blot analysis for sestrin 2 and the downstream targets of mTOR. Sestrin 2 expression was reduced in the PECs transfected with shRNA targeting sestrin 2 as compared with that in the PECs transfected with NTC. Phosphorylation of the downstream targets of mTOR: P-4E-BP1, P-p70S6K and P-S6RP expressions were also increased in the PECs transfected with shRNA targeting sestrin 2 as compared with that in the PECs transfected with NTC shRNA. B: Relative expression of sestrin 2 protein detected by western-blot analysis. As compared with that in the PECs transfected with NTC, a significant reduction of sestrin 2 protein expression was observed in the PECs transfected with sestrin 2 shRNA. * p < 0.01. B: Relative expression of the proteins downstream of phosphorylated mTOR. The expressions of P-4E-BP-1, P-p70S6K and P-S6RP were
significantly increased in the PECs transfected with sestrin 2 shRNA as compared with that in the PECs transfected with NTC. ** p < 0.05.

**Fig.9. Effect of sestrin 2 downregulation on cellular apoptosis in conditionally immortalized cultured PECs.** Conditionally immortalized cultured PECs were transfected with shRNA targeting sestrin 2 or non-target control (NTC). Transfected PECs were then cultured under growth-restrictive conditions for 10 days and the degree of apoptosis was determined. 

A-C: Hoechst 33342 staining in cultured PECs. A: PECs transfected with NTC showed faint apoptosis. B: Increased apoptosis was observed in the PECs transfected with sestrin 2 shRNA. C: Apoptosis as determined by Hoechst 33342 staining was significantly increased in the PECs transfected with sestrin 2 shRNA than in the PECs transfected with NTC. * p< 0.01. D: The degree of apoptosis as determined by caspase-3 staining was also significantly increased in the PECs transfected with sestrin 2 shRNA as compared with that in the cells transfected with NTC. * p< 0.01.

**Fig.10. Schema. Sestrin 2 and activity of mTOR in normal and diseased PECs.** The details are described in the text.
Figure 1
Figure 2
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**Figure 3**
Figure 4
Figure 5
Figure 6
Figure 8
Figure 9

(A) NTC

(B) Sestrin 2 shRNA

(C) Apoptotic cells

(D) Caspase-3 activity

Figure 9
Sestrin 2
mTOR

Normal PECs

Maintenance of homeostasis

Injured PECs

Sestrin 2
mTOR

Loss of barrier function
Apoptosis and/or detachment
Proliferation

Periglomerular fibrosis
Glomerulosclerosis
Crescent formation

Figure 10