

Significance of Osteopontin Expression in the Progression of Human Focal Segmental Glomerulosclerosis

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Background : Osteopontin (OPN) is a cytokine related to cell-matrix adhesion and cell survival and is expressed in the distal convoluted tubules in normal adult kidneys. Only one *in vitro* study has investigated the role of OPN in mechanically stretched podocytes and their actin cytoskeleton rearrangement. **Methods :** Glomerular OPN expression was investigated in biopsies from patients with human idiopathic focal segmental glomerulosclerosis (FSGS) (n = 25) and in normal renal biopsies (n = 16) by immunohistochemistry. **Results :** OPN was expressed in the podocytes from patients with FSGS. OPN expression increased in podocytes from both non-sclerotic hypertrophic and sclerotic glomerular tufts in patients with FSGS compared to the podocytes in normal controls. **Conclusions :** The results suggest that OPN plays a role in the early adaptive response of podocytes to the increased mechanical load caused by glomerular hypertrophy preceding FSGS. OPN was involved in cell-matrix adhesion and influenced the detachment delay of podocytes from the glomerular basement membrane and apoptosis.

Key Words : Osteopontin; Podocyte; Glomerulosclerosis, focal segmental; Morphometric analysis

Osteopontin (OPN) is an arginine-glycine-aspartate (RGD) containing phosphoprotein known as an early T cell activator-1. OPN is a cytokine acting through $\alpha v \beta 3$ and stimulates an increase in bcl-2 activity and thereby opposes alternative signals that lead to an apoptotic response.^{1,2} As a cellular product, OPN is capable of forming cell-cell and cell-matrix bridges via its RGD sequence. The RGD sequence is an integrin binding motif common to many extracellular matrix (ECM) proteins; thus, OPN has also been classified as an ECM protein.

Endlich *et al.*³ reported that OPN pre-coating might contribute to F-actin reorganization and the formation of radial actin stress fibers in mechanically stretched podocytes, which are involved in podocyte foot process attachment to the stretched surface glomerular basement membrane that occurs in focal segmental glomerulosclerosis (FSGS).⁴ However, it is unclear which OPN, the soluble cytokine type or matrix type, plays a role in FSGS pathogenesis. Recent studies have provided evidence that glomerular hypertrophy precedes podocyte detachment from the glomerular basement membrane and that OPN mRNA expression increases in the glomeruli of experimental hyperten-

sive nephropathy models.^{5,6} In human FSGS, OPN mRNA expression increases in glomeruli. OPN is not expressed in the normal human adult glomeruli; however, it is expressed in the distal convoluted tubules and thick ascending limbs of the loop of Henle in the normal human adult kidney.⁷ The goal of this study was to investigate whether OPN is expressed in podocytes in both non-sclerotic hypertrophic and sclerotic glomerular tufts from patients with FSGS. Additionally, whether OPN is expressed in the podocytes of both glomeruli from patients with FSGS and glomeruli from normal controls was evaluated. Finally, OPN expression and the role it might play in the formation and progression of segmental glomerular sclerosis in human FSGS was studied.

MATERIALS AND METHODS

Patients

Twenty-five patients with FSGS presented with clinical fea-

tures of generalized edema and proteinuria from 2001 to 2006. All 25 patients were diagnosed with idiopathic FSGS based on the following criteria: absence of any other systemic diseases; presence of FSGS morphological characteristics including glomerular hypertrophy, mesangial matrix expansion, and segmental sclerosis in the peri-hilar area or glomerular tip or any glomerular tuft; and absence of glomerular tuft collapse. Tests for blood urea nitrogen (BUN) and serum creatinine concentrations, as well as a urinalysis were performed using standard laboratory procedures. Before the renal biopsy, each patient had a 24-hour urine collection for creatinine clearance (glomerular filtration rate, GFR) and urinary protein excretion. Nephrotic-range proteinuria was defined as proteinuria ≥ 3.5 g/day. Renal insufficiency was defined as a serum creatinine > 1.5 mg/dL or as a GFR < 80 mL/min/1.73 m². Normal controls were selected from patients presenting for military service or employment health screening. Sixteen biopsies had non-diagnostic abnormalities in patients who initially presented with an abnormal routine urinalysis. One patient had mild proteinuria and 15 had mild to moderate hematuria on the urinalysis; all had a 24-hour urine analysis.

Tissue processing and immunohistochemistry

A portion of the tissue was placed in 10% phosphate buffered formalin fixative for more than 12 hours, washed in tap water, and embedded in paraffin. Up to three sections were cut in sequence and two or more sections were placed on each slide. Immunohistochemistry was performed on 25 formalin-fixed paraffin-embedded renal biopsy specimens from patients with FSGS and 16 normal controls. The technical control used from the nephrectomy tissues was obtained, as described previously, using an anti-OPN antibody.

Briefly, each serial 3 μ m paraffin section underwent deparaffinization and was rehydrated in serial ethanol solutions. The tissue sections were immersed in methanol containing 0.3% H₂O₂ for 30 minutes to block endogenous peroxidase activity. Before the sections were exposed to the antibodies, they were incubated in 0.1 N citrate buffer to retrieve the unmasked antigens and placed in a microwave at 600 W for 3-5 minutes. An anti-OPN mouse anti-human monoclonal antibody was used (1 : 400, American Research Products, Inc., Belmont, MA, USA). The podocytes were identified with a mouse monoclonal antibody against synaptopodin (1 : 200, Progen Biotechnik GmbH, Heidelberg, Germany). Detection was conducted with Vectastatin ABC Elite kits, and/or with Vectastatin ABC AP

kit (Vector Laboratories, Burlingame, CA, USA) for double stain. Next, the sections were labeled with 3,3'-diaminobenzidine tetrahydrochloride (DAB; DakoCytomation, Glostrup, Denmark) for 1 minute, or with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP, Vector Laboratories) for 5 minutes and then counter stained with 1% Mayer's hematoxylin.

Evaluation of immunohistochemistry and morphometric analysis

To quantify glomerular OPN expression, the number of OPN-positive podocytes was counted per glomerulus, and the mean positive OPN podocyte number per glomerulus of each case was calculated [$N_{(OPN^+ GVEC)}$]. The glomerular density was determined using light microscopic sections at a magnification of $\times 400$ using the BA210 Digital Microscope Based Slide Scanning System with image analyzing software Digilab 2.0 (Shinhan Scientific Co., Ltd., Seoul, Korea; Motic Incorporation Ltd., Xiamen, China). The maximal planar area of each glomerulus [$A_{(glom)}$] could be measured by tracing each glomerulus on serial sections. The mean glomerular tuft volume (MGV) could be then calculated from $MGV = [A_{(glom)}]^{3/2} \times 1.38/1.01$, as described previously.⁸ The number of OPN-positive podocytes was calculated [$N_{(OPN^+ GVEC)}$], and the glomerular area [$A_{(glom)}$] was evaluated in each patient with FSGS and in the normal controls. The mean number of OPN-positive podocytes per each glomerular area [$N_{(OPN^+ GVEC)}/A_{(glom)}$] was calculated to quantify the density of OPN-positive podocytes per glomerulus.

Statistical analysis

The data were expressed as the mean \pm standard deviation. Continuous and non-continuous values were compared between the two groups with the student's t-test and χ^2 test. A p-value < 0.05 was considered significant.

RESULTS

Clinical features of patients with FSGS and normal controls

The ages of the patients with FSGS ranged from 19 to 79 years (44.4 ± 15.6), and the normal controls ages ranged from 19 to 59 years (38.3 ± 13.6) (Table 1). Thirteen men and ten women were included in the former group, and ten men and

Table 1. Clinical characteristics of patients with focal segmental glomerulosclerosis and normal controls

Variable	FSGS	NC	p-value
No. of cases	25	16	
Age (yr)	44.4 ± 15.6 (19-79)	38.3 ± 13.6 (19-59)	0.333
Gender			
Males	13	10	0.540*
Females	12	6	
Biochemistry			
BUN (mg/dL)	19.3 ± 12.9 (1.3-137.8)	13.3 ± 3.2 (8.5-17.0)	0.039
sCr (mg/dL)	1.4 ± 0.7 (0.5-8.49)	0.8 ± 0.2 (0.5-0.92)	0.005
Urinalysis			
Proteinuria positive	25	1	0.000*
negative	0	15	
Hematuria positive	11	12	0.063*
negative	14	4	
24-hour-urinalysis	16	13	
24 urine volume	2,009.7 ± 1,225.4 (890-510)	1,327.1 ± 721.2 (490-2,500)	0.156
GFR (mL/min/1.73 m ²)	88.7 ± 52.8 (5-298)	107.4 ± 29.2 (65-153)	0.755
Protein excretion (mg/day)	3,666.2 ± 3,403.9 (104.0-7148.3)	126.3 ± 264.9 (1.0-222.0)	0.000

p-values are evaluated by student's t-test or by χ^2 test.

FSGS, focal segmental glomerulosclerosis; NC, normal controls; BUN, blood urea nitrogen; sCr, serum creatinine; GFR, glomerular filtration rate.

six women were in the latter group. The serum BUN (19.3 ± 12.9 mg/dL vs 13.3 ± 3.2 mg/dL) and creatinine (1.4 ± 0.7 mg/dL vs 0.8 ± 0.2 mg/dL) levels were significantly higher in the patients with FSGS than in the normal controls ($p < 0.05$). Heavy proteinuria was present in all 25 patients with FSGS at the time of biopsy ($p < 0.01$). Among the normal controls, one patient with mild proteinuria had a serum BUN (20.0 mg/dL) and creatinine (1.3 mg/dL) that were slightly elevated; however, after several weeks of follow-up the serum levels of BUN and creatinine, including the proteinuria and hematuria, returned to normal following a urinalysis, and the renal biopsy findings were similar to the other controls. The mean amount of proteinuria in the FSGS group was larger than in the control group ($3,666.2 \pm 3,403.9$ mg/day vs 126.3 ± 264.9 mg/day, $p < 0.01$). The mean GFR tended to be less in the FSGS group than the control group (88.7 ± 52.8 mL/min/1.73 m² vs 107.4 ± 29.2 mL/min/1.73 m²); however, these differences were not significant.

Morphometric analysis data and OPN expression features in the patients with FSGS

The mean glomerular area [$A_{(glom)}$] increased in the patients with FSGS compared with the normal controls ($[17.30 \pm 4.57] \times 10^3 \mu\text{m}^2$ vs $[12.49 \pm 2.58] \times 10^3 \mu\text{m}^2$, $p < 0.01$). The mean volume of glomeruli [$V_{(glom)}$] increased in the patients with FSGS compared to the normal controls ($[3.19 \pm 1.24] \times 10^6 \mu\text{m}^3$ vs $[1.94 \pm 0.60] \times 10^6 \mu\text{m}^3$, $p < 0.01$) (Table 2). OPN expression

Table 2. Osteopontin expression in podocytes from patients with focal segmental glomerulosclerosis and normal controls

	FSGS	NC	p-value
Mean $N_{(glom)}$	7.08 ± 5.27	17.00 ± 9.31	0.001247
Mean $A_{(glom)}$ ($\times 10^3 \mu\text{m}^2$)	17.30 ± 4.57	12.49 ± 2.58	0.000165
Mean $V_{(glom)}$ ($\times 10^6 \mu\text{m}^3$)	3.19 ± 1.24	1.94 ± 0.60	0.000158
Mean $N_{(OPN^+ GVEC)}$	9.90 ± 4.99	0.45 ± 0.57	0.000000
Mean $N_{(OPN^+ GVEC)/A_{(glom)}}$ ($\times 10^6/\mu\text{m}^2$)	618.10 ± 370.38	37.10 ± 46.91	0.000000

p-values are evaluated by student's t-test.

FSGS, focal segmental glomerulosclerosis; NC, normal controls; $N_{(glom)}$, number of glomeruli; $N_{(OPN^+ GVEC)}$, number of osteopontin positive podocytes per glomerulus; $A_{(glom)}$, area of glomeruli; $V_{(glom)}$, volume of glomeruli; $N_{(OPN^+ GVEC)/A_{(glom)}}$, number of osteopontin positive podocytes per glomerular area.

was localized in the podocytes in patients with FSGS. OPN expression increased in the podocytes from both hypertrophic non-sclerotic and segmentally sclerotic glomerular tufts. OPN expression also increased in a few podocytes from globally sclerotic glomeruli in patients with FSGS compared to the podocytes in normal controls (Fig. 1A-D). OPN expression was scarcely observed in any other glomerular cells from patients with FSGS, except for a few monocytes trapped in the glomerular capillary lumen and attenuated glomerular parietal epithelial cells (data not shown). OPN expression was frequently noted in the distal convoluted tubules with luminal dilation adjacent to the age-related globally sclerotic glomeruli. Synaptopodin was expressed along the glomerular capillary tufts from the non-sclerotic hypertrophic glomeruli but not along the segmentally sclerotic

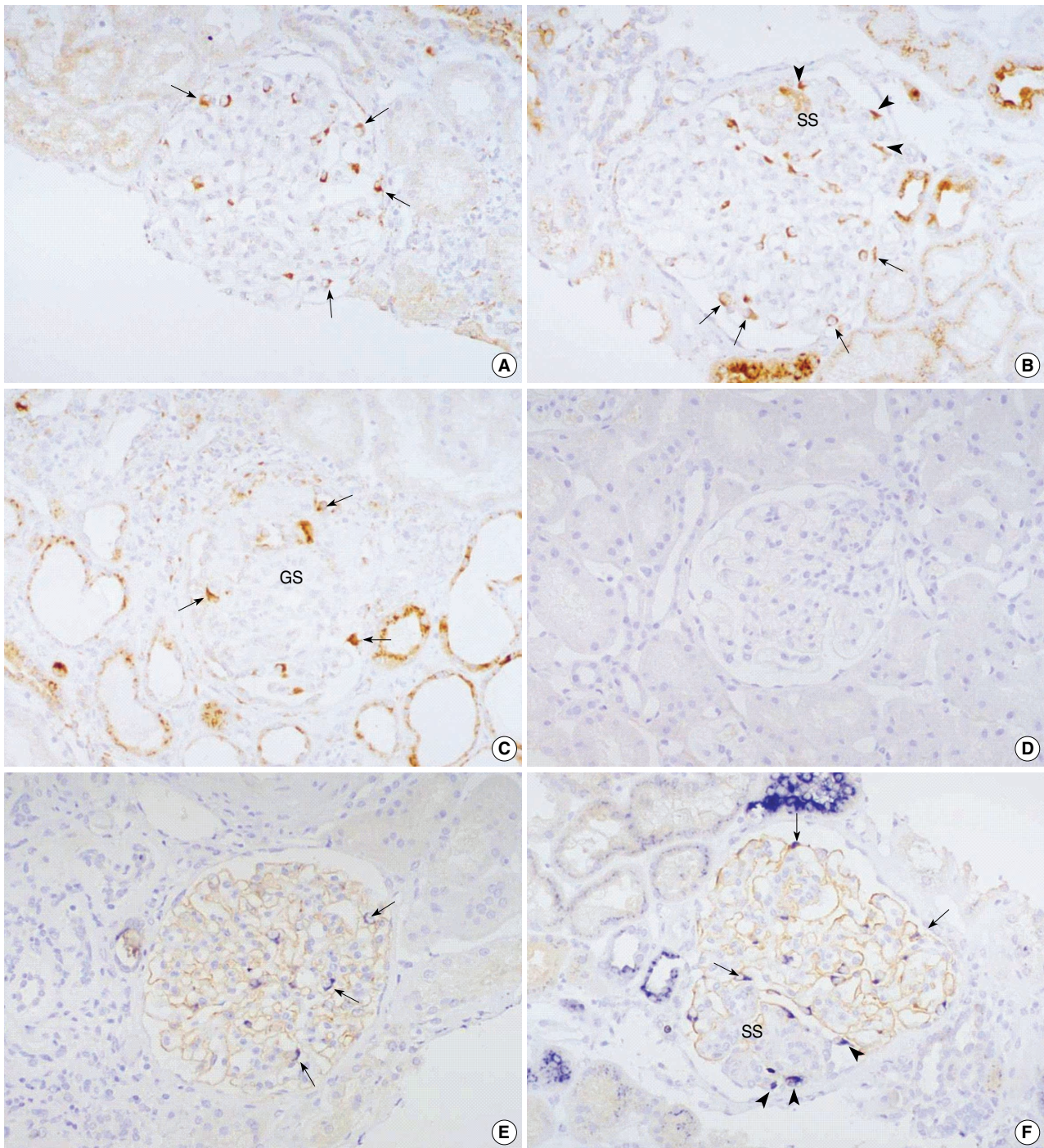


Fig. 1. Immunohistochemistry of osteopontin (OPN) expression in glomeruli from patients with focal segmental glomerulosclerosis (FSGS) (A-C) compared to normal controls (D). (A) A hypertrophic, non-sclerotic glomerulus showing many podocytes expressing OPN (arrows). (B) A segmentally sclerotic glomerular tuft (SS) showing few podocytes expressing OPN (arrowheads), and non-sclerotic glomerular tufts showing many podocytes expressing OPN (arrows). (C) A globally sclerotic glomerulus (GS) showing few podocytes expressing OPN (arrows). (D) A normal glomerulus from the normal controls showing no OPN expression. Double immunohistochemical staining for synaptopodin (DAB)-OPN (NBT/BCIP) expression in glomeruli from patients with FSGS (E, F). (E) A hypertrophic non-sclerotic glomerulus showing complete synaptopodin expression along the capillary tufts and many podocytes expressing OPN (arrows). (F) SS showing few podocytes with synaptopodin loss (arrowheads) and non-sclerotic glomerular tufts showing many podocytes expressing OPN (arrows).

glomerular capillary tufts in patients with FSGS (Fig. 1E, F).

DISCUSSION

Segmental glomerular sclerosis is a key pathological feature of human idiopathic FSGS.⁹ Non-sclerotic hypertrophic glomerular tufts provide a morphological clue suggesting the progression of segmental glomerular sclerosis.¹⁰ The detachment of podocytes (urinary shedding) from the glomerular basement membrane plays a key role in FSGS progression. However, it is still unclear how many podocytes must lose their attachment to the glomerular basement membrane to induce segmental glomerular sclerosis in an individual glomerulus.¹¹ Wiggins¹² suggested that more than a 20% podocyte loss results in segmental glomerular tuft scarring.

In this study, the mean number of glomeruli [$N_{(\text{glom})}$] in patients with FSGS was significantly lower than that of normal controls. This difference was not caused by technical problems but was due to tubulointerstitial attenuation. The mean glomerular area and volume [mean $A_{(\text{glom})}$] [mean $V_{(\text{glom})}$] increased significantly in patients with FSGS compared to those in normal controls, as described previously ($p < 0.01$) (Table 2).¹⁰ However, the morphometric analysis had technical problems regarding the counts of the podocyte area and volume. In normal glomeruli, the morphological features of podocytes showed that they were tightly attached to the glomerular basement membrane. Under pathological conditions, slightly detached podocytes with podocyte foot process effacement and their morphological features could be easily mistaken for podocyte hypertrophy. In this study, we did not evaluate the mean area and volume of podocytes in patients with FSGS in normal controls.

Therefore, the molecular markers for non-sclerotic glomerular tufts restricted to the effects of FSGS progression might provide more significant clinical prognostic clues rather than counting podocyte loss. Recently, Bennett *et al.*¹³ reported that OPN was highly expressed at the mRNA level in the glomeruli of patients with FSGS; however, OPN expression was not localized to specific glomerular cells. The present study demonstrated that OPN expression was localized in the podocytes from hypertrophic non-sclerotic glomeruli in patients with FSGS (Fig. 1). Additionally, the number of OPN positive podocytes per glomerular area [$N_{(\text{OPN}^+ \text{ GVEC})}/A_{(\text{glom})}$] increased in patients with FSGS compared to normal controls ($p < 0.01$) (Table 2).

It is still unknown whether OPN expression represents an adaptive process or a process associated with decompensation.

Endlich *et al.*¹⁴ demonstrated that podocytes are mechanosensitive and easily from mechanical stretch-induced radial actin stress fibers, which are similar to podocyte actin cytoskeleton rearrangements in response to glomerular capillary hypertension.⁴ An *in vitro* study showed that OPN is the most significantly up-regulated gene in podocytes, in response to mechanical stretch.³ A few *in vivo* studies have shown that OPN is highly expressed at the protein and mRNA levels, in the glomeruli of an experimental spontaneous renal hypertension model.⁶ In this study, OPN expression was frequently noted in non-sclerotic hypertrophic glomerular tufts rather than in sclerotic glomerular tufts. Furthermore, OPN expression also increased in the podocytes from glomeruli in patients with FSGS compared to the podocytes in normal controls (Fig. 1). An OPN-integrin interaction induces cell-matrix adhesion, and OPN expression is more reliable as an early “adaptive” adhesive process before mechanical stretch-induced podocyte foot process detachment *in vitro*.³

OPN is an acidic 70 kD glycoprotein, which yields 24 and 45 fragments.¹⁵ OPN is thought to bind primarily to $\alpha v \beta 3$ integrin heterodimers through its RGD sequence; however, other OPN receptors have been described. The integrin heterodimers $\alpha v \beta 1$, $\alpha v \beta 3$, and $\alpha v \beta 5$ have affinity for the OPN RGD motif, and OPN contains a cryptic binding sequence (SVVYGLR) recognized by the $\alpha 9 \beta 1$ and $\alpha 4 \beta 1$ integrin heterodimers.¹⁶ Injury derived intracellular Ca^{2+} signaling may regulate cell adhesion through OPN-integrin interaction¹⁷ and increased soluble integrins regulate intracellular Ca^{2+} influx.¹⁸ Mechanical stretch-induced intracellular Ca^{2+} influx in podocytes may be capable of responding to changes in glomerular capillary pressure.¹⁹ Moreover, OPN is also capable of binding the CD44 cell surface proteoglycan. However, standard or variant CD44 expression was not found in any of the glomerular cells in normal controls in a previous study (data not shown).²⁰ A few human and animal studies have reported that parietal epithelial cells expressing OPN-CD44 contribute to cellular crescent formation.^{21,22} The autocrine occupation of CD44 by its ligand OPN, induces the loosening of cell-matrix adhesion and contributes to the cellular migration via Rho family GTPase and Rac1 activation.²³

OPN expression increases in the parietal epithelial cells around sclerotic glomerular tufts from the early to late phases in an adriamycin-induced experimental model.²⁴ However, in this study, OPN expression increased mainly in the glomerular visceral epithelial cells and scarcely in the attenuated glomerular parietal epithelial cells around the hypertrophic glomerular tufts in patients with idiopathic FSGS, which results from an enlarged

Bowman's capsule (data not shown). Additionally, parietal progenitor epithelial cell hyperplasia with collapsing glomerulopathy has been reported²⁵ but not with perihilar sclerotic FSGS or glomerular tip lesions in humans; the glomeruli are not related to an animal model of spontaneous glomerular hypertension.⁶

OPN expression is enhanced in the atrophic or attenuated distal tubules and thick loops of Henle in normal adult kidney compared to that in normal childhood kidney.²⁶ After long-term treatment with cyclosporine A, OPN is expressed in the tubular epithelial cells in humans and in experimental models.²⁷ However, cyclosporine A-induced podocyte injury is not initiated by mechanical stretch but by cytoskeletal remodeling.^{3,28} OPN expression is due, in part, to the adaptive process to mechanical stretch; however, another portion of OPN expression is due to hypoxic injury in age-related peritubular capillary damage induced tubulointerstitial disease.²⁹ Furthermore, we have also observed OPN expression in the dilated distal tubules from surgically resected kidney specimens.³⁰

In this study, OPN was strongly expressed in podocytes from the non-sclerotic hypertrophic glomerular tufts in patients with FSGS compared to the podocytes in normal controls. These results suggest that OPN might contribute to an early podocyte response to the mechanical stress caused by glomerular hypertrophy preceding FSGS. These results also suggest that OPN might contribute to the increase in transient survival of podocytes before detachment and podocyte migration to the denuded glomerular basement membrane surface.

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