CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

CD147/Basigin Deficiency Prevents the Development of Podocyte Injury through FAK Signaling

Tomoki Yoshioka,^{*†} Tomoki Kosugi,^{*} Tomohiro Masuda,^{*} Tomoharu Watanabe,^{*†} Akihiro Ryuge,^{*†} Hiroshi Nagaya,^{*} Kayaho Maeda,^{*} Yuka Sato,^{*} Takayuki Katsuno,^{*‡} Noritoshi Kato,^{*} Takuji Ishimoto,^{*} Yukio Yuzawa,[§] Shoichi Maruyama,^{*} and Kenji Kadomatsu[†]

From the Departments of Nephrology* and Biochemistry,[†] Nagoya University Graduate School of Medicine, Nagoya; the Department of Nephrology and Rheumatology,[‡] Aichi Medical University, Nagakute; and the Department of Nephrology,[§] Fujita Health University School of Medicine, Toyoake, Japan

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Address correspondence to
Tomoki Kosugi, M.D., Ph.D.,
Department of Nephrology,
Nagoya University Graduate
School of Medicine, 65
Tsurumai-cho, Showa-ku,
Nagoya 466-8550, Japan.
E-mail: kosugi@med.nagoya-u.ac.jp.

Podocytes, which are susceptible to injury by various stimuli and stress, are critical regulators of proteinuric kidney diseases, regardless of the primary disease and pathogenesis. We further confirmed a significant correlation between urinary CD147/basigin (Bsg) levels and proteinuria in patients with focal segmental glomerulosclerosis. However, the molecular mechanism of podocyte injury involving Bsg is not fully understood. Here, the involvement of Bsg in the pathogenesis of podocyte injury was elucidated. Healthy podocytes rarely express Bsg protein. In two independent mouse models, including adriamycin-induced nephropathy and N^ω-nitro-L-arginine methyl ester (L-name)-induced endothelial dysfunction, Bsg induction in injured podocytes caused podocyte effacement, which led to development of proteinuria. Bsg silencing in cultured podocytes exposed to transforming growth factor- β suppressed focal adhesion rearrangement and cellular motility via the activation of β_1 integrin—focal adhesion kinase—matrix metalloproteinase signaling. In addition, induction of vascular endothelial growth factor and endothelin-1, which are implicated in podocyte-to-endothelial cross-communication, was lower in the supernatants of cultured Bsg-silenced podocytes stimulated with transforming growth factor- β . In this setting, Bsg may be involved in a physiological positive feedback loop that accelerates podocyte cell motility and depolarization. The current study thus suggests that Bsg silencing via suppression of β_1 integrin—focal adhesion kinase—matrix metalloproteinase signaling may be an attractive therapeutic strategy for the maintenance of podocytes in patients with proteinuric kidney diseases. (*Am J Pathol* 2019, 189: 1338–1350; <https://doi.org/10.1016/j.ajpath.2019.04.003>)

Proteinuria, the clinical manifestation of glomerular filtration barrier dysfunction, is a critical determinant of the progression of chronic kidney disease and cardiovascular diseases.^{1–4} Podocytes contribute to the maintenance of an adequate intracapillary environment by molecular signaling crosstalk with glomerular endothelial cells and the extracellular matrix.^{5,6} In particular, precise regulation of vascular endothelial growth factor (VEGF) derived from podocytes is essential for maintenance of endothelial function. The extracellular matrix serves as a storage compartment for signaling molecules that maintain a healthy glomerular balance. Matrix metalloproteinases (MMPs) promote disruption of the glomerular basement membrane (GBM), turnover of

extracellular matrix, and disruption of podocyte integrity.^{7–9} When intraglomerular homeostasis is disrupted, the skewed profile of cytokine expression causes loss of the size/charge selectivity of glomerular filtration, leading to development of persistent proteinuria. Therefore, further identification of these regulators, as well as an enhanced understanding of

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Table 1 Clinical Characteristics of Patients with FSGS

Clinical characteristics	FSGS (<i>N</i> = 95)	Pathologic control (<i>N</i> = 23)
Sex, male, %	54.7	39.1
BMI, kg/m ²	19.8 ± 5.1	17.6 ± 5.5
Systolic BP, mm Hg	137 ± 18.3	117 ± 20.4
Diastolic BP, mm Hg	80 ± 13.0	69 ± 12.4
eGFR, mL/min/1.73 m ²	64.2 ± 22.5	89.0 ± 24.8
UP/urine Cr	3.35 ± 3.06	0.10 ± 0.15
Hb, mg/dL	13.5 ± 2.14	13.9 ± 1.71

Data are expressed as means ± SD.

BMI, body mass index; BP, blood pressure; Cr, creatinine; eGFR, estimated glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; Hb, hemoglobin; UP, urine protein.

already established signaling, is required for therapeutic intervention in patients with proteinuric kidney diseases.

Bsg encodes CD147/basigin (*Bsg*), which is an extracellular MMP inducer and a highly glycosylated, transmembrane, immunoglobulin superfamily member protein.¹⁰ *Bsg* is expressed in many cell types, such as hematopoietic, epithelial, and endothelial cells, and is critically important in processes such as invasion by cancer cells, spermatogenesis, viral infection, and immune system regulation.^{11–15} Generation of *Bsg*-deficient mice (*Bsg*^{−/−}) in our laboratory^{16,17} showed that *Bsg* induces cytokine production and regulates neutrophil and macrophage migration in models of ischemic renal injury and renal fibrosis.^{18,19} In addition to a role in eliciting chemotactic activity, *Bsg* also serves as a negative immune modulator in IL-17—producing T-cell differentiation in lupus nephritis.²⁰ In a series of clinical studies,^{21–23} a striking increase in *Bsg* in the circulating blood and urine was observed in patients with acute kidney injury, lupus nephritis, and persistent kidney diseases with proteinuria. Regardless of the primary kidney disease or pathogenesis, a significant relationship between proteinuria and urinary CD147/*Bsg* levels was observed in proteinuric kidney diseases. However, the involvement of *Bsg* in the molecular mechanism of proteinuric kidney diseases has not yet been elucidated in detail.

In accordance with previous reports, *Bsg* is involved in the production of VEGF, MMPs, and hyaluronan, and in the interaction with β_1 integrin, all of which increase our understanding of the complex scenario regarding glomerular filtration barrier dysfunction.^{24–27} In this study, we focused on *Bsg* functions in proteinuric kidney diseases induced by doxorubicin hydrochloride (ADR) or N^ω-nitro-L-arginine methyl ester (L-name). It was then investigated whether *Bsg* deficiency reduced podocyte cell motility by inhibiting focal adhesion turnover, thereby preventing the development of proteinuria.

Materials and Methods

Patients and Procedures

From 2008 to 2014 at Nagoya University and affiliated hospitals, 95 adults who underwent a kidney biopsy as part of standard of care for the differential diagnosis of various renal diseases (52 males; 43 females) were enrolled. These

95 patients received a final diagnosis of focal segmental glomerulosclerosis (FSGS) because they satisfied the criteria of the Kidney Disease Improving Global Outcomes guidelines. Patients diagnosed with cancer, severe infections, and other renal disorders were excluded. Patients underwent biopsy before any medical treatment began. Also, between 2008 and 2014, 23 pathologic control patients (9 males; 14 females) with only microhematuria or minimal proteinuria, which are not classified as kidney diseases according to clinical and pathologic criteria, were enrolled. All procedures were performed as described previously.²³

This study was conducted as stated by the principles of the Declaration of Helsinki, the Japanese National Ethical Guidelines (approval number 1135), and the institutional review boards of Nagoya University Hospital and affiliated hospitals. Before participating in the study, each patient provided written informed consent.

Animals and Experimental Design

Mice deficient in *Bsg* (*Bsg*^{−/−}) were rarely born following ordinary mating, and thus, a protocol was established for producing sufficient numbers of pups.¹⁶ *Bsg*^{+/-} mice on the 129/SV background were backcrossed with C57BL/6J mice to produce F1 hybrid offspring (reverse F1 hybrid). By intercrossing these mice, mixed reverse F2 mice were generated and used in this study. For the adriamycin model, 8- to 12-week-old male mice weighing 25 to 30 g were administered a single intravenous injection of ADR (Wako Pure Chemical, Osaka, Japan) at a dosage of 16 mg/kg body weight.²⁸ An equal volume of 0.9% saline was injected into mice as a control. Mice were sacrificed 2 weeks after treatment. For the L-name model, 8- to 12-week-old male mice weighing 20 to 30 g were given 1 mg/mL L-name (Sigma-Aldrich, St. Louis, MO) in their drinking water for 12 weeks²⁹ and then sacrificed. Blood and urine samples and kidneys were removed for examination. Serum creatinine (Cr), the ratio of albumin to Cr in urine, and systolic blood pressure were measured as described previously.^{30,31} Plasma and urinary *Bsg* levels were measured using commercial enzyme-linked immunosorbent assay kits according to the respective instructions from the manufacturers (R&D Systems, Minneapolis, MN). Measured levels in urine were

then normalized to urinary Cr levels. All experiments with mice were conducted as specified by the animal experimentation guidelines of Nagoya University School of Medicine.

Renal Histology

Kidneys were incubated in 10% formalin, embedded in paraffin, and then sectioned at a thickness of 1 μ m (periodic acid-Schiff reagent staining) or 4 μ m (immunohistochemistry). The sections were stained with mouse monoclonal anti-human CD147 antibody (Ab; Abcam, Cambridge, MA), rabbit monoclonal anti-human β_1 integrin Ab (Abcam), goat anti-mouse Bsg Ab (R&D Systems), and rabbit anti-mouse WT-1 Ab (Santa Cruz Biotechnology, Dallas, TX), followed by detection using a peroxidase-conjugated secondary antibody (Nichirei, Tokyo, Japan). The staining was visualized with 3,3'-diaminobenzidine (Dako, Carpinteria, CA), which produces a brown color. Podocytes positive for WT-1 were counted by examining all glomeruli of the cortex under a microscope at high magnification. Quantitation was performed by two experts (T.M. and K.M.) in nephropathology who were blinded to the experimental conditions.

For transmission electron microscopic analysis, kidneys were fixed in formalin, embedded in epoxy resin, sectioned, and then stained with uranyl acetate and lead citrate. As described by Koop et al,³² the total length of the GBM and the number of slit pores were evaluated in the free capillary wall, and then the average foot process width was examined by calculating the ratio of the total length of the GBM to the total number of slits.

In Situ Hybridization

The mice were perfused and fixed with G-Fix (GenoStaff Co., Tokyo, Japan), and kidneys were dissected, embedded in paraffin with G-Nox (GenoStaff Co.), and then sectioned at 8 μ m. Paraffin-embedded sections were hybridized with sense (<https://www.ncbi.nlm.nih.gov/nucore>; accession number NM_009768.2) and antisense *Bsg* probes. *In situ* hybridization was performed with the ISH Reagent kit according to the manufacturer's instructions (GenoStaff Co.). The sections were incubated with anti-digoxigenin alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany), and color reactions were developed with NBT/BCIP solution (Sigma-Aldrich). For immunostaining as the second stain, the sections were incubated with rabbit anti-

mouse WT-1 Ab and incubated with biotin-conjugated goat anti-rabbit IgG (Dako), followed by addition of peroxidase-conjugated streptavidin (Nichirei). Peroxidase activity was visualized with 3,3'-diaminobenzidine.

Real-Time PCR

Mouse glomeruli were dissected from frozen renal sections using the LMD CM 1950 system (Leica Biosystems, Nussloch, Germany).³³ The RNeasy Micro Kit (Qiagen, Hilden, Germany) was used to isolate total RNA from these samples. Real-time PCR was conducted using the Applied Biosystems Prism 7500HT sequence detection system and TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The TaqMan probes and primers were: *Bsg* (Mm01144228_g1), endothelin (ET)-1 (*Edn1*: Mm0043656_m1), transforming growth factor (TGF)- β (*Tgfb1*: Mm01178820_m1), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*: Mm99999915_g1). Applied Biosystems Sequence Detection software version 1.3.1 was used for analysis.

Western Blot Analysis

Mouse renal tissues and podocytes grown *in vitro* were lysed in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology). Western blot analysis was conducted as described.³⁴ After separation of proteins and transfer to membranes, the membranes were incubated with goat anti-mouse nephrin Ab (R&D Systems), goat anti-human Bsg Ab, mouse anti-human focal adhesion kinase (FAK) Ab (BD Biosciences, San Diego, CA), and mouse anti-human cellular fibronectin (FN) Ab (Abcam). Membranes were washed and incubated with peroxidase-conjugated anti-goat IgG, mouse IgG, or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). An enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK) was used to visualize Ab-bound proteins. The density of each band was quantitated using ImageJ software version 1.48 (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>).

Cell Culture

Conditionally immortalized human podocytes obtained from Prof. Richard J. Johnson at the University of Colorado were maintained as described previously.³⁵ Cells were incubated at 37°C for 7 days to induce differentiation and then transfected using lipofectamine (Thermo Fisher

Figure 1 CD147/basigin expression in patients with focal segmental glomerulosclerosis (FSGS). Scatter plots display the data for plasma (A) and urinary CD147/basigin (B) values in individual patients and median values. C: Correlation between plasma and urinary CD147/basigin levels. Linear approximation line, coefficient of determination (R^2), and *P* value are shown. D–G: Correlation between proteinuria and plasma (D), and proteinuria and urinary (E) CD147/basigin values. Correlation between estimated glomerular filtration rate (eGFR) and plasma (F), and eGFR and urinary (G) CD147/basigin levels. Linear approximation lines, coefficients of determination (R^2), and *P* values are shown. H: Representative periodic acid-Schiff reagent (PAS) staining in the glomeruli, and immunohistochemical staining of CD147 expression in the glomeruli and tubulointerstitium of patients with FSGS and in a pathological control. Arrows indicate CD147-positive cells. I: Representative images of β_1 integrin and CD147 expression in serial sections from an FSGS patient. Arrows indicate β_1 integrin expression; arrowheads indicate CD147-positive podocytes. *n* = 95 (A and B, FSGS); *n* = 23 (A and B, pathological controls). ***P* < 0.01. Scale bars: 100 μ m.

Scientific, Waltham, MA) with siRNA for *BSG* (GE Healthcare) or negative control siRNA. After 3 days of transfection, the medium was changed to fresh RPMI 1640 medium (Thermo Fisher Scientific) containing 5 ng/mL recombinant TGF- β (R&D Systems) for 0, 12, 24, 36, or 48 hours in the time-course study. The total experimental period from the start of transfection to the end of stimulation was 120 hours. Supernatants from cultured podocytes were collected after the different times, and the ET-1 and VEGF levels were measured using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (R&D Systems).

Flow Cytometry Analysis

Isolated immortalized human podocytes were stained for flow cytometry with the following Abs: mouse anti-human β_1 integrin (BD Biosciences, Piscataway, NJ) or mouse IgG (BD Biosciences), followed by AF488-conjugated goat anti-mouse IgG1 (Thermo Fisher Scientific). 7AAD (BioLegend, San Diego, CA) was added before flow cytometry to label dead cells. All flow cytometry data were acquired on a BD Canto II (Becton, Dickinson and Company, Franklin Lakes, NJ) and analyzed with FlowJo software version 10 (Tree Star, Ashland, OR).

Migration Assay

Cell culture inserts with a pore size of 8 μ m (BD Biosciences, Franklin Lakes, NJ) were placed in the lower compartment, and RPMI 1640 was added. Podocytes (7×10^3) transfected with negative control siRNA or *BSG* siRNA were placed in 24-well inserts and incubated for 10 hours at 37°C to allow migration. Cells that had not migrated were removed from the upper membrane. The number of cells that had migrated were counted in the middle of the membrane.

Wound Healing Assay

For each experiment, 2.5×10^4 podocytes treated with or without siRNA for *BSG* were seeded overnight on FN-

coated coverslips in 96-well plates. Each well was then scratched with an InCuCyte wound maker (Essen BioScience, Ann Arbor, MI) and washed with phosphate-buffered saline, and then fresh medium was added. At 10 hours after scratching, the numbers of cells that had migrated into the same-sized square fields were counted.

Gelatin Zymography

Gelatin zymography¹⁹ was performed with the same amount of conditioned medium from 2.0×10^4 podocytes treated with or without siRNA for *BSG* on 30-mm plastic dishes to assess MMP-mediated proteolytic activity. Samples of condition medium were loaded onto a 10% gelatin zymogram protein gel (Thermo Fisher Scientific). Proteins were separated with electrophoresis, and then the gel was treated with 2.5% Triton X-100 at room temperature for 30 minutes with gentle shaking, followed by incubation at 37°C for 24 hours in buffer containing calcium chloride Tris-HCl for development. The gel was stained with Coomassie Blue, and the density of the bands was quantitated with ImageJ software version 1.48 (NIH).

Statistical Analysis

Data are the means $\pm$ SEM. For single comparisons, the unpaired *t*-test or the nonparametric *U*-test was used. For multiple comparisons, analysis of variance was used, followed by post hoc least significant difference tests if the initial analysis of variance was significant. Spearman correlation coefficients were calculated to determine the strength of the association between two variables. *P* < 0.05 was considered statistically significant.

Results

Clinicopathological Characteristics of Patients with FSGS

Proteinuria in 28 patients with FSGS showed a significant correlation with urinary CD147/*Bsg* levels, but not plasma CD147/*Bsg* values.²³ Because the previous study was small, 95 patients with FSGS were enrolled in the present study.

Figure 2 *Bsg* deficiency ameliorates podocyte injury in doxorubicin hydrochloride (ADR)-treated mice. **A:** Representative images of *Bsg* mRNA expression in the glomeruli of healthy mice, using *in situ* hybridization (left panel). Double staining for *Bsg* mRNA expression with *in situ* hybridization and WT-1 expression with immunostaining (right panel). Colocalization of *Bsg* (black arrows) and WT-1 expression (white arrows) in "healthy" glomeruli. **B:** Time course of albuminuria-to-creatinine ratio in *Bsg*^{+/+} and *Bsg*-deficient (*Bsg*^{-/-}) mice after ADR treatment. Closed circles, *Bsg*^{+/+} mice; open circles: *Bsg*^{-/-} mice. **C:** Correlations between the urinary albumin-creatinine ratio and *Bsg* levels (left panel), and the urinary albumin/creatinine ratio and serum *Bsg* levels (right panel) in ADR-treated *Bsg*^{+/+} mice. **D:** Representative images of periodic acid-Schiff reagent–stained glomeruli at 14 days in ADR-treated *Bsg*^{+/+} and *Bsg*^{-/-} mice. Arrows, podocyte swelling. **E:** Immunostaining for *Bsg* protein in the glomeruli of "healthy" or ADR-treated wild-type (*Bsg*^{+/+}) mice. Black arrows, positive areas. **F:** Glomerular *Bsg* mRNA expression in ADR-treated mice. **G:** Immunohistochemistry for WT-1 expression. Black arrowheads, WT-1–positive cells. **H:** Evaluation of the rate of decline in WT-1–positive cells in the glomeruli of ADR-treated mice. Black bars, *Bsg*^{+/+} mice; white bars, *Bsg*^{-/-} mice. **I:** Nephron expression in the kidneys at 14 days after ADR treatment as determined by Western blot analysis. **J:** The intensities of the nephron bands following normalization to β -actin. **K:** Electron micrograph showing the development of foot process effacement in ADR-treated *Bsg*^{+/+} mice. **L:** Quantification of foot process width. **M:** Endothelin (ET)-1 and transforming growth factor (TGF)- β mRNA expression in the glomeruli of ADR-treated mice. Data are expressed as means $\pm$ SEM (**B**, **F**, **H**, **J**, **L**, and **M**). *n* = 9 to 11 (**C**); *n* = 7 to 11 (**F**, **H**, **J**, **L**, and **M**). **P* < 0.05, ***P* < 0.01. Scale bars: 50 μ m (**A**, **D**, **E**, and **G**); 5.0 μ m (**K**, left panels); 0.5 μ m (**K**, right panels). IHC, immunohistochemistry; ISH, *in situ* hybridization.

Table 2 General Characteristics of ADR-Treated Mice

General characteristics	Control mice		ADR mice	
	<i>Bsg</i> ^{+/+}	<i>Bsg</i> ^{-/-}	<i>Bsg</i> ^{+/+}	<i>Bsg</i> ^{-/-}
Body weight, day 0, g	27.7 ± 1.0	28.6 ± 0.6	27.4 ± 0.5	27.5 ± 0.6
Body weight, day 14, g	29.1 ± 0.8	30.1 ± 0.3	28.1 ± 0.9	25.8 ± 0.9
Kidney weight/body weight, %	0.69 ± 0.04	0.90 ± 0.01	0.77 ± 0.02	0.87 ± 0.04
Serum Cr, mg/dL	0.16 ± 0.01	0.13 ± 0.01	0.19 ± 0.02	0.15 ± 0.01
Serum albumin, mg/dL	3.30 ± 0.12	3.42 ± 0.06	3.00 ± 0.16	3.31 ± 0.13

Data are expressed as means ± SEM.

ADR, doxorubicin hydrochloride; Cr, creatinine.

Their characteristics are summarized in [Table 1](#). All participants were Japanese, and slightly more than half (54.7%) were male. Both plasma and urinary CD147/Bsg levels were almost twice those of pathologic control patients ([Figure 1](#), A and B). A close relationship between plasma and urinary CD147/Bsg levels was observed ([Figure 1C](#)). Of note, both CD147/Bsg values were closely correlated with proteinuria, but the association with the estimated glomerular filtration rate was lower ([Figure 1](#), D–G). These data suggest that plasma and urinary CD147/Bsg levels may not be strongly affected by glomerular filtration function. Immunohistochemical staining of kidney sections from patients with FSGS showed CD147/Bsg protein expression in the glomeruli as well as infiltrating inflammatory cells in the interstitium, but no CD147/Bsg expression in the glomeruli of pathologic controls ([Figure 1H](#)). Following staining of serial sections, β_1 integrin and Bsg expression was observed in injured podocytes including adhesions to the Bowman capsule ([Figure 1I](#)).

Bsg Deficiency Ameliorates the Development of Proteinuria and Podocyte Injury in ADR-Treated Mice

Previously, Bsg protein expression was not detected in the glomeruli of kidney sections from nontreated mice.²² In the present study, Bsg mRNA expression was observed within the glomeruli as well as the tubular epithelium and vascular endothelium with *in situ* hybridization (data not shown). Podocyte Bsg expression was determined *in vivo* by co-labeling with Bsg and WT-1, a podocyte cell marker. Bsg mRNA expression was colocalized with WT-1 protein expression by immunohistochemistry in normal podocytes ([Figure 2A](#)).

To investigate the role of Bsg in the pathogenesis of proteinuric kidney diseases, the degree of glomerular injury was evaluated in *Bsg*^{+/+} and *Bsg*^{-/-} mice at 14 days after ADR treatment. ADR-treated *Bsg*^{+/+} mice tended to show a worse kidney function compared with *Bsg*^{-/-} mice, but the differences were not significant ([Table 2](#)). Until 14 days after ADR injection, albuminuria was gradually greater in *Bsg*^{+/+} mice than in *Bsg*^{-/-} mice ([Figure 2B](#)). Similar to the profile of patients with FSGS, plasma and urinary Bsg levels showed a significant association with albuminuria ([Figure 2C](#)). In addition, *Bsg*^{+/+} mice with proteinuria appeared to show

segmental abnormalities in glomerular architecture and podocyte swelling ([Figure 2D](#)). No obvious differences between *Bsg*^{+/+} and *Bsg*^{-/-} mice were observed in the tubulointerstitium (data not shown). Healthy podocytes rarely expressed Bsg protein, but they began to express Bsg in the glomerulus upon activation by ADR ([Figure 2E](#)). Bsg mRNA expression was also increased in the glomerulus of ADR-treated mice ([Figure 2F](#)). The number of macrophages that infiltrated into the glomerulus appeared to be augmented in *Bsg*^{+/+} mice (data not shown).

Bsg deficiency prevented the decline in the number of WT-1–positive cells and down-regulation of nephrin expression in the kidneys ([Figure 2](#), G–J). In support of these findings, electron microscopy of the glomerular ultrastructure revealed that foot process effacement outside the GBM was present in *Bsg*^{-/-} mice to a lesser extent than in *Bsg*^{+/+} mice ([Figure 2](#), K and L). Adequate barrier function in the GBM is maintained by vasoactive factors such as VEGF and ET-1 produced by podocytes.⁵ Glomerular ET-1 (*EDN1*) mRNA expression increased in *Bsg*^{+/+} mice, consistent with the profile of proteinuria and podocyte injury ([Figure 2M](#)). These results suggest that Bsg deficiency suppressed the degree of glomerular injury, which leads to the development of proteinuria in ADR-treated mice.

Albuminuria Is Also Suppressed in L-name–Treated *Bsg*^{-/-} Mice

Further experiments were performed to determine whether Bsg deficiency ameliorates podocyte injury and albuminuria in another mouse model representing endothelial dysfunction and hypertension. Bsg depletion in a novel model of glomerular sclerosis induced by L-name, a nitric oxide synthase inhibitor, attenuated the progression of albuminuria ([Figure 3A](#)). Although systolic blood pressure in *Bsg*^{+/+} and *Bsg*^{-/-} mice increased during the experimental period, no significant differences between the two genotypes were observed until 8 weeks after treatment ([Figure 3B](#)). At 12 weeks after L-name treatment, blood pressure in *Bsg*^{-/-} mice was slightly lower than that in *Bsg*^{+/+} mice. The glomeruli of both *Bsg*^{-/-} and *Bsg*^{+/+} mice showed endothelial dysfunction as reported previously.^{36,37} The degrees of podocyte injury and glomerular

Figure 3 L-name-treated $Bsg^{-/-}$ mice show a similar profile as doxorubicin hydrochloride (ADR)-treated $Bsg^{-/-}$ mice. Time course of albuminuria (**A**) and systolic blood pressure (BP) (**B**) in L-name-treated $Bsg^{+/+}$ and $Bsg^{-/-}$ mice for 12 weeks after uninephrectomy. **Filled circles** represent L-name-treated $Bsg^{+/+}$ mice; **open circles** represent L-name-treated $Bsg^{-/-}$ mice. Representative periodic acid-Schiff reagent (**C**) and WT-1 (**D**) staining of $Bsg^{+/+}$ or $Bsg^{-/-}$ glomeruli treated with L-name at 12 weeks after uninephrectomy. **Arrowheads**, WT-1-positive cells. **E**: Evaluation of the rate of decline in WT-1-positive cells based on the podocyte number in the respective control mice. Black bar represents $Bsg^{+/+}$ mice; white bar represents $Bsg^{-/-}$ mice. **F**: Electron micrograph showing foot process effacement in L-name-treated mice. Data are expressed as means $\pm$ SEM (**A**, **B**, and **E**). $n = 11$ (**A** and **B**). * $P < 0.05$ and ** $P < 0.01$ versus $Bsg^{-/-}$ mice; $^{\dagger}P < 0.05$ versus $Bsg^{+/+}$ BP at pretreatment; $^{\ddagger}P < 0.05$ versus $Bsg^{-/-}$ BP at pretreatment. Scale bars: 5.0 μ m.

sclerosis were suppressed in $Bsg^{-/-}$ mice consistent with the profile of albuminuria (Figure 3, A, C–F).

Podocytes with Silenced Bsg Show Suppression of FAK Activation and Cell Motility

Podocyte cell spreading and migration are required for the development of proteinuria and focal adhesion in

glomerular architecture.^{2,38} Therefore, in cultured mouse podocytes with silenced Bsg, β_1 integrin and phosphorylated FAK expression was evaluated because these proteins play an integral role in cell movement secondary to focal adhesion turnover.^{39,40} The expression of Bsg in cultured podocytes transfected with *BSG* siRNA was gradually reduced in a time-dependent manner (Figure 4A).

Figure 4 Basigin silencing inhibits directional podocyte movement via focal adhesion kinase phosphorylation (p-FAK). **A:** Time course of Bsg expression in podocytes transfected with control or BSG siRNA as seen with Western blot analysis. **B:** The frequency of β₁ integrin⁺ podocytes transfected with control or BSG siRNA after 5 ng/mL TGF-β stimulation was determined with flow cytometry. **C:** Western blot analysis indicates the time course of p-FAK (Y397) in podocyte cells transfected with control or BSG siRNA after 5 ng/mL TGF-β stimulation. **D:** The intensity of p-FAK bands was normalized to total FAK. Black bars represent control podocytes; gray bars represent BSG siRNA-transfected podocytes. **E:** Representative images of migrated podocyte cells exposed to TGF-β for 10 hours in the migration assay. **F:** Quantitative analysis of the number of migrated cells. **G:** Representative images from the wound healing assay. White dashed lines indicate the periphery of the scratch areas. **H:** Quantification of the results in G. Data are expressed as means ± SEM (B, D, F, and H). *n* = 3 (B, C, and F, independent experiments); *n* = 5 (D, independent experiments). **P* < 0.05, ***P* < 0.01. Scale bar, 100 μm.

TGF-β plays a critical role in the pathogenesis of glomerular sclerosis. An *in vivo* study showed no obvious difference between *Bsg*^{-/-} and *Bsg*^{+/+} mice in glomerular TGF-β expression (Figure 2M). After transfection with BSG siRNA, β₁ integrin expression and FAK phosphorylation were suppressed in BSG-silenced podocytes stimulated with 5 ng/mL TGF-β compared with control transfected cells (Figure 4, B–D). To further clarify podocyte migration, podocytes transfected with control siRNA or BSG siRNA were seeded onto the insert filters, and the cells were exposed to 5 ng/mL TGF-β. Podocyte migration in response to TGF-β was significantly blocked by BSG silencing (Figure 4, E and F). The experiment of podocyte migration was expanded by performing a wound-healing assay, and the number of cells

that migrated into the scratched area were calculated. BSG silencing and exposure to TGF-β appeared to inhibit directional podocyte movement to fill the wound compared with control podocytes (Figure 4, G and H).

The Transition of Cytokines Involved in Intraglomerular Homeostasis

In this setting, a variety of cytokines and chemokines that podocytes produce are involved in the pathogenesis of proteinuria leading to progressive glomerular sclerosis. The expression of VEGF and ET-1, which are implicated in podocyte-to-endothelial cross-communication, was examined in the presence or absence of Bsg. Induction of VEGF

Figure 5 Basigin silencing in podocytes contributes to suppression of several cytokines for disruption of intraglomerular homeostasis following transforming growth factor (TGF)- β stimulation. Time course of induction of endothelin (ET)-1 (**A**) and vascular endothelial growth factor (VEGF)-A (**B**) in the supernatants of cultured podocyte cells transfected with control or *Bsg* siRNA after exposure to 5 ng/mL TGF- β . Black bars, control podocytes; gray bars, *Bsg* siRNA-transfected podocytes. **C**: Zymographic analysis demonstrates matrix metalloproteinase (MMP)-2 activity in the culture medium of podocytes treated with control and *Bsg* siRNA following TGF- β stimulation. **D**: The intensity of active MMP-2 bands. **E**: Representative Western blot analysis of fibronectin expression in cultured podocytes. **F**: The intensity of fibronectin bands normalized to β -actin. Data are expressed as means $\pm$ SEM (**A**, **B**, **D**, and **F**). $n = 3$ (**F**); $n = 4$ (**D**, independent experiments); $n = 5$ (**A** and **B**, independent experiments). * $P < 0.05$, ** $P < 0.01$.

and ET-1 expression was lower in the supernatants of cultured *Bsg*-silenced podocytes exposed to TGF- β (Figure 5, A and B).

MMP-2 promotes disruption of the GBM and interruption of podocyte integrity. *Bsg* silencing after exposure to TGF- β activated MMP-2 to a lesser degree than in control cells that expressed *Bsg* (Figure 5, C and D). In addition to MMPs, FN is involved in podocyte dedifferentiation and integrin-linked kinase-mediated cross-communication with the GBM.⁴¹ Similar to the profile of the above cytokines, cellular FN expression was less marked in *Bsg*-silenced cultured podocytes versus control cells (Figure 5, E and F).

Discussion

Podocyte injury is caused by maladaptation to various types of stress including mechanical stress, oxidative stress, and biochemical responses, followed by the spread of injury to unique cellular components of glomerular filtration units.³⁸ Glomerular endothelial dysfunction also causes podocyte injury by disruption of cross-communication.⁵ According to prior and present clinical studies, healthy podocytes rarely express *Bsg* protein, but they begin to express *Bsg* upon

activation (Figure 2E). Both urinary and plasma levels of CD147/*Bsg* showed a closer association with proteinuria in patients with podocytopathy, suggesting that CD147/*Bsg* induction may be a common feature in human proteinuric kidney diseases. It was therefore assessed how *Bsg* may be involved in the development of proteinuria. In the present *in vivo* study using two independent rodent models including ADR-induced nephropathy and L-name-induced endothelial dysfunction, *Bsg* expressed in injured podocytes was shown to cause podocyte effacement, leading to proteinuria and podocyte injury. *Bsg* silencing in cultured podocytes suppressed cellular motility and focal adhesion rearrangement via the activation of β_1 integrin-FAK signaling. However, *Bsg* silencing in glomerular endothelial cells did not affect several molecules related to the maintenance of intraglomerular homeostasis such as VEGF receptor 1 and 2 (data not shown). The collective data supported the idea that CD147/*Bsg* in podocytes may be a critical molecule in proteinuric kidney diseases.

Activation of β_1 integrin-FAK signaling causes disruption of cell-substrate interactions between podocytes and the GBM because of cellular retraction and movement.^{39,40} Indeed, up-regulation of β_1 integrin expression was found in the early phase of diabetic kidney disease.⁴² A redistribution

Figure 6 Schematic diagram showing the possible relationship between basigin and podocyte injury, which disrupts maintenance of intraglomerular homeostasis. ECM, extracellular matrix; ET-1, endothelin-1; FAK, focal adhesion kinase; GBM, glomerular basement membrane; MMP, matrix metalloproteinase; TGF- β , transforming growth factor beta; VEGF, vascular endothelial growth factor.

of β_1 integrin leading to impaired adhesion may be involved in the pathogenesis of podocytopathy.⁴³ An elegant study by Ma and colleagues demonstrated that inhibition of podocyte FAK ameliorates the development of proteinuria and foot process effacement. In addition, FAK activation activates MMPs.^{44,45} The *in vitro* study also demonstrated a beneficial effect of *Bsg* silencing through suppression of β_1 integrin and FAK activation in the presence of TGF- β stimulation. Mesenchymal molecules such as MMP-2 and FN in cultured podocytes were reduced by *Bsg* silencing, similar to the profile of phosphorylated FAK, suggesting that *Bsg* may participate in a phenotypic conversion during podocyte injury that is characterized by defects in epithelial features. During the process of carcinoma metastasis, *Bsg* interacts with β_1 integrin and then activates downstream FAK/PI3K signaling.²⁷ In this setting, interestingly, depolarization of the epithelium appears to be caused by *Bsg*.⁴⁶ The function of *Bsg* in hepatocellular carcinoma progress may be partially related to the spread of podocyte injury. Some investigators have documented that podocytes undergo a phenotypic transition after injury.^{41,47} Considering that MMPs activate TGF- β and that *Bsg* is a strong inducer of MMPs,^{19,48} a molecular circuit may be present for podocyte injury that includes signaling molecules such as *Bsg*, TGF- β , β_1 integrin, FAK, MMPs, and FN. The molecular details by which *Bsg* induces mesenchymal transition in podocytes require further elucidation. In support of these data and consistent with previous reports, *Bsg* plays a deleterious role in podocyte injury by activating FAK-MMP signaling in proteinuric kidney diseases.

Growing evidence supports the idea that signaling molecules mediate the maintenance of intraglomerular function in an autocrine or paracrine manner. Inappropriate regulation of VEGF and ET-1 derived from podocytes can lead to

endothelial dysfunction, including endothelial cell swelling and loss of the glycocalyx.^{5,6,38} Indeed, ET-1 release from podocytes causes mitochondrial dysfunction in adjacent endothelial cells and eventually results in the exacerbation of podocyte apoptosis and albuminuria.⁴⁹ In the present study, both expression of VEGF and ET-1 was higher in cultured podocytes without *Bsg* silenced that were treated with TGF- β . Glomerular endothelial injury precedes podocyte apoptosis in endothelial nitric oxide synthase-deficient mice,⁵⁰ which show similar characteristics to L-name-treated mice in this experiment. Podocytes may be susceptible to injury by various stimuli and types of stress, regardless of the primary or secondary glomerular diseases. Thus, targeting the cross-communication between podocytes and the endothelium as well as the various signaling molecules may represent new therapeutic opportunities.

Clinical studies of a wide range of kidney diseases with proteinuria, including lupus nephritis, acute kidney injury, IgA nephropathy, FSGS, and diabetic kidney disease, have demonstrated the induction of CD147/*Bsg* protein in glomerular components in proportion to the progress of disease activity.^{21–23} Here, it was demonstrated that *Bsg* production in the early phase of podocytopathy causes podocyte effacement, which promotes proteinuria. In this setting, *Bsg* may be involved in a physiological positive feedback loop that accelerates podocyte cell motility and depolarization (Figure 6).

This study has a few limitations. Podocyte-specific deletion of *Bsg* in mice was not performed, and primary cultured podocytes were not used. Because one phenotype of *Bsg*-deficient mice is male and female infertility, creating and maintaining a conditional knockout mouse strain is difficult. Because the mice used are a mixed strain that includes 129/SV, susceptibility to ADR may be higher compared with other mouse strains. In a preliminary study, higher dosages of ADR resulted in very low survival rates accompanied by marked glomerular injuries such as severe global glomerulosclerosis (data not shown). To investigate a critical role for *Bsg* in the early phase of proteinuric kidney diseases, the dosage administered to these mice was therefore set at 16 mg/kg body weight in the present study.

Conclusions

In conclusion, the current study suggests that *Bsg* silencing may be an attractive therapeutic strategy for the maintenance of podocytes via suppression of β_1 integrin–FAK–MMP signaling. These results may open new avenues in the treatment of patients with proteinuric kidney diseases.

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