Sphingomyelinase-Like Phosphodiesterase 3b Expression Levels Determine Podocyte Injury Phenotypes in Glomerular Disease

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ABSTRACT

Diabetic kidney disease (DKD) is the most common cause of ESRD in the United States. Podocyte injury is an important feature of DKD that is likely to be caused by circulating factors other than glucose. Soluble urokinase plasminogen activator receptor (suPAR) is a circulating factor found to be elevated in the serum of patients with FSGS and causes podocyte αVβ3 integrin-dependent migration in vitro. Furthermore, αVβ3 integrin activation occurs in association with decreased podocyte-specific expression of acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3b) in kidney biopsy specimens from patients with FSGS. However, whether suPAR-dependent αVβ3 integrin activation occurs in diseases other than FSGS and whether there is a direct link between circulating suPAR levels and SMPDL3b expression in podocytes remain to be established. Our data indicate that serum suPAR levels are also elevated in patients with DKD. However, unlike in FSGS, SMPDL3b expression was increased in glomeruli from patients with DKD and DKD sera-treated human podocytes, where it prevented αVβ3 integrin activation by its interaction with suPAR and led to increased RhoA activity, rendering podocytes more susceptible to apoptosis. In vivo, inhibition of acid sphingomyelinase reduced proteinuria in experimental DKD but not FSGS, indicating that SMPDL3b expression levels determined the podocyte injury phenotype. These observations suggest that SMPDL3b may be an important modulator of podocyte function by shifting suPAR-mediated podocyte injury from a migratory phenotype to an apoptotic phenotype and that it represents a novel therapeutic glomerular disease target.


Received November 21, 2013. Accepted April 22, 2014.

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Published online ahead of print. Publication date available at www.jasn.org.

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The pathogenesis of FSGS and diabetic kidney disease (DKD) is associated with podocyte injury.\textsuperscript{1–5} Acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3b) is a protein with homology to acid sphingomyelinase (ASMase), and it is involved in the sphingomyelin (SM) catabolic processes. SMPDL3b exists in two isoforms and is mainly localized in the lipid rafts of podocytes.\textsuperscript{6,7} We reported that decreased SMPDL3b expression is associated with actin cytoskeleton remodeling in podocytes, rendering them more susceptible to injury in recurrent FSGS.\textsuperscript{7} In addition, circulating factors are thought to play an important role in the pathogenesis of podocyte injury in both FSGS\textsuperscript{8–11} and DKD.\textsuperscript{12–16} In sera of patients with FSGS, increased levels of soluble urokinase plasminogen activator receptor (suPAR) were described.\textsuperscript{11} While increased suPAR levels are associated with other conditions, such as diabetes,\textsuperscript{17} cardiovascular diseases,\textsuperscript{18} inflammation,\textsuperscript{19} and different types of cancers,\textsuperscript{20} additional investigations are needed to establish the value of suPAR as a disease-specific biomarker.\textsuperscript{19,21} In FSGS, suPAR was found to cause lipid-dependent activation of αVβ3 integrin in podocytes,\textsuperscript{11,24} but whether this activation occurs in glomerular diseases other than FSGS remains to be established. Furthermore, a link between suPAR and SMPDL3b has not been established.

We hypothesized that suPAR and SMPDL3b functionally interact, and we investigated their respective roles in FSGS and DKD.

RESULTS

Differential Expression of SMPDL3b in FSGS and DKD Protects Podocytes from Injury

Microarray data obtained by glomerular transcriptome analysis of kidney biopsies from patients with FSGS and DKD indicate that SMPDL3b mRNA expression shows a trend to be decreased in patients with FSGS but is significantly increased in patients with DKD (Figure 1A). SMPDL3b protein expression was significantly decreased in podocytes cultured in the presence of high-risk FSGS patient sera (Concise Methods) compared with podocytes cultured in the presence of DKD patient sera or normal human sera (NHS) and upregulated in podocytes cultured in the presence of DKD patient sera compared with podocytes cultured in the presence of NHS (Figure 1B).

We previously described that podocytes exposed to sera of high-risk FSGS or DKD patients show disease-specific morphologic changes of the actin cytoskeleton\textsuperscript{7,15} (i.e., a disruption of stress fibers and cell blebbing, respectively). Interestingly, increased expression of SMPDL3b protected podocytes from high-risk FSGS sera-induced cytoskeletal changes (Figure 1C), whereas decreased expression of SMPDL3b protected podocytes from DKD sera-induced cytoskeletal remodeling (Figure 1D). Because podocyte apoptosis is a pathophysiological characteristic observed in DKD,\textsuperscript{2} we analyzed caspase-3 activity. Caspase-3 activity was significantly increased in DKD patient sera (DKD+)–treated podocytes and in podocytes cultured in the presence of sera from diabetic patients (DKD−) when compared to podocytes cultured in the presence of sera from healthy controls (NHS). DKD+ sera-induced apoptosis was abrogated in SMPDL3b knockdown (SMPDL3b KD) podocytes (Figure 1E).

Circulating suPAR Levels Are Elevated in Sera from Patients with FSGS and DKD and Experimental Models of FSGS and DKD

Circulating suPAR levels are increased in a majority of patients with FSGS (primarily those patients at risk for FSGS recurrence after transplantation).\textsuperscript{25} We measured suPAR levels in the sera of the patients with DKD and compared them with the levels of patients with FSGS. We found elevated suPAR levels in high-risk FSGS patients compared with low-risk FSGS patients (Figure 2A) and high but comparable suPAR levels in patients with microalbuminuric or macroalbuminuric DKD compared with patients with normoalbuminuria and diabetes (Figure 2B, Table 1). In high-risk FSGS patients, suPAR levels were similar and significantly correlated with the decline in GFR within 1 year of follow-up (Figure 2C). In patients with diabetes, suPAR levels significantly increased in patients who progressed from normo- to microalbuminuria (Figure 2D) and patients who progressed from micro- to macroalbuminuria (Figure 2E) but not in patients who remained normo- or microalbuminuric in a 7-year longitudinal study (Figure 2, D and E). Of note, the DKD patients with elevated suPAR levels at baseline analysis had also normal GFR (Table 1, Figure 2B). Furthermore, suPAR levels significantly correlated with the yearly change in GFR (Figure 2F). In experimental animal models, suPAR levels were also significantly elevated in both

Table 1. Clinical characteristics of patients with type 1 diabetes at baseline

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\textsuperscript{a}Chronic Kidney Disease Epidemiology Collaboration. Median (interquartile range). AER, albumin excretion rate; m, male; f, female.
the adriamycin-induced (ADR) FSGS-like nephropathy model (Figure 2G) and diabetic db/db mice (Figure 2H) compared with their respective controls.

suPAR Does Not Modulate SMPDL3b Expression Levels but Modulates $\beta_3$ Integrin Activation in Cultured Human Podocytes

To determine if SMPDL3b expression is regulated by suPAR, we treated normal human podocytes with 0.5, 1, and 2 $\mu$g/ml suPAR (R&D Systems) for 3, 6, and 24 hours and determined SMPDL3b expression by quantitative real-time PCR. No significant change in SMPDL3b expression levels was observed (Figure 3A), indicating that the physiologic form of suPAR does not regulate SMPDL3b mRNA expression in podocytes. However, SDS-PAGE under nonreducing conditions followed by Western blot analysis using activated $\beta_3$ integrin (AP5) and total $\beta_3$ integrin (AP3) antibodies showed that $\beta_3$ integrin activation was significantly induced in normal human podocytes treated with suPAR in a dose- and time-dependent manner (Figure 3, B and C).

We previously reported that human podocytes that are cultured in the presence of sera from patients with DKD experience actin cytoskeleton remodeling in the form of cell blebbing (Figure 3D, top panel).\textsuperscript{15} To test if suPAR treatment of human podocytes is sufficient to induce the same phenotype, we treated normal and SMPDL3b overexpressing (SMPDL3b OE) podocytes with 1 $\mu$g/ml suPAR for 24 hours. As expected, suPAR treatment of normal human podocytes did not cause cytoskeleton remodeling in the form of cell blebbing. However, suPAR treatment of SMPDL3b OE podocytes led to cytoskeletal remodeling in the form of cell blebbing, indicating that increased SMPDL3b expression is necessary to cause an suPAR-mediated DKD-like phenotype in human podocytes (Figure 3D).
β3 Integrin Is Activated in ADR-Induced Nephropathy but Not db/db Mice

It was previously reported that suPAR can activate β3 integrin in rodent FSGS.11,26 We, therefore, investigated β3 integrin activation in glomeruli of ADR-treated and db/db mice. Fluorescence intensity of glomerular AP5 (a mAb against the activation-dependent conformation of β3 integrin) staining was significantly increased in glomeruli of ADR-treated mice compared with vehicle-treated controls. In contrast, no changes were observed in the glomeruli of db/db mice compared with db/m mice (Figure 4, A and B), indicating that suPAR-mediated activation of β3 integrin is blocked in this DKD model.

Decreased Ceramide Levels and Unchanged SMPDL3b Expression Occur in Kidney Cortices from ADR-Treated or db/db Mice

We analyzed Smpdl3b expression by quantitative real-time PCR in RNA isolated from kidney cortices of ADR-treated and db/db mice. Smpdl3b mRNA expression in kidney cortex was not significantly changed in ADR-treated (Figure 4C) or db/db mice (Figure 4D) compared with their respective controls. We analyzed the SM and ceramide lipid content in kidney cortices of ADR-treated and db/db mice. We were not able to detect any difference in the SM content in kidney cortices of ADR-treated or db/db mice compared with their respective controls (Figure

**Figure 2.** Serum suPAR in patients with and mouse models of FSGS and DKD. (A) Serum suPAR levels in 14 high-risk FSGS patients are significantly higher compared with low-risk FSGS patients (*P<0.05). (B) Serum suPAR levels are significantly increased in 34 microalbuminuric and 10 macroalbuminuric DKD patients compared with 30 patients with diabetes but normoalbuminuria (***P<0.01). Serum suPAR levels are further significantly increased in 10 macroalbuminuric compared with 34 microalbuminuric DKD patients (**P<0.01). (C) Serum suPAR levels significantly correlate with the renal disease progression in patients enrolled in the FSGS clinical trial cohort (P<0.01). (D) Longitudinal analysis in patients with diabetes showing that suPAR levels at baseline are not significantly different in the nonprogression group (normo-normo) compared with the progression group (normo-micro) but are significantly elevated in the progression group 7 years later (**P<0.01). (E) Longitudinal analysis in patients with diabetes shows that suPAR levels at baseline are not significantly different in the nonprogression group (micro-micro) compared with the progression group (micro-macro) but are significantly elevated in the progression group 7 years later (**P<0.01) and significantly increased compared with the nonprogression group (**P<0.01). (F) Serum suPAR levels significantly correlate with the renal disease progression in diabetic patients (P<0.01). (G and H) suPAR levels in a mouse model of FSGS (ADR-induced nephropathy) and a mouse model of DKD (db/db mice) are significantly increased compared with their respective controls (*P<0.05 ADR versus control; *P<0.05 db/db versus db/m).
However, ceramide content quantification revealed significant decreases in ceramide in kidney cortices of both db/db and ADR-treated mice compared with their respective controls (Figure 4F).

**SMPDL3b Interferes with the suPAR/Urokinase Plasminogen Activator Receptor and β3 Integrin Interaction**

Because SMPDL3b expression levels are conversely regulated in FSGS and DKD but suPAR serum levels are elevated in both diseases, we hypothesized that SMPDL3b may play a role in suPAR-mediated β3 integrin activation in podocytes. Co-immunoprecipitation experiments performed in human embryonic kidney 293 (HEK293) cells revealed an interaction between SMPDL3b and suPAR but not between SMPDL3b and β3 integrin (Figure 5A). This interaction between suPAR and SMPDL3b was further confirmed by endogenous immunoprecipitation using isolated glomeruli from C57Bl/6 mice injected with PBS or LPS (Figure 5B). Competitive Co-IP experiments using increasing amounts of GFP-SMPDL3b showed that SMPDL3b physically interacts with suPAR/urokinase plasminogen activator receptor (uPAR) but not with β3 integrin. More importantly, SMPDL3b binding to suPAR/uPAR interfered with suPAR/uPAR and β3 integrin interaction in a dose-dependent manner (Figure 5C).

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**Figure 3.** In vitro effects of suPAR treatment of human podocytes on SMPDL3b expression levels and β3 integrin activation. (A) Treatment of human podocytes with different concentrations of suPAR (0, 0.5, 1, and 2 μg/ml) for different time spans (3, 6, and 24 hours) indicates that suPAR concentrations and time of exposure to suPAR do not change SMPDL3b mRNA expression levels. (B) Western blot analysis followed by (C) quantitative analysis of normal human podocytes treated with different concentrations of suPAR (0, 0.5, and 1 μg/ml) for 6 and 24 hours reveal significant β3 integrin activation at 24 hours of treatment (*P<0.05). AP5 antibody was used to detect activated β3 integrin and AP3 antibody was used to detect total β3 integrin. (D) Normal human podocytes treated with the sera from patients with DKD (top panel) show a characteristic cytoskeletal remodeling phenotype in the form of cell blebbing. The same cytoskeletal phenotype was observed when SMPDL3b OE podocytes were treated with suPAR (bottom panel) but not when human podocytes expressing endogenous levels of SMPDL3b (middle panel) were exposed to suPAR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
SMPDL3b Regulates suPAR-Mediated Activation of $\beta_3$ Integrin and Downstream Signaling in Podocytes

To further assess the effect of differential SMPDL3b expression on $\beta_3$ integrin activation and dependent signaling, we performed Western blot analysis using antibodies to detect AP5 and a downstream integrin effector, Src. Treatment of normal human podocytes with suPAR significantly increased $\beta_3$ integrin activation as assessed by Western blot analysis (Figures 3, B and C and 6A) and immunofluorescence staining (Figure 6B). However, increased SMPDL3b expression (SMPDL3b OE) protected from suPAR-mediated $\beta_3$ integrin activation (Figure 6, A and B), whereas decreased expression of SMPDL3b (SMPDL3b KD) rendered podocytes more susceptible to suPAR-mediated $\beta_3$ integrin activation (Figure 6, A and B). Because the focal adhesion kinase/Src complex acts as a suppressor of RhoA activity downstream of integrin signaling, thus favoring integrin-mediated migration, we determined phospho-Src levels in suPAR-treated human podocytes. As expected, the phospho-Src/Src ratio was significantly increased in suPAR-treated normal podocytes and SMPDL3b KD podocytes but remained unchanged in suPAR-treated SMPDL3b OE podocytes (Figure 6A).

SMPDL3b Regulates suPAR-Mediated Podocyte Migration and Apoptosis

Using transwell migration assays, we showed that suPAR-induced cell migration is more prominent in SMPDL3b KD podocytes compared with normal human podocytes, whereas SMPDL3b OE podocytes are protected from suPAR-induced cell migration (Figure 6C). Because small guanosine 5’-triphosphatases are
associated with podocyte motility\textsuperscript{24} and pathogenesis of DKD,\textsuperscript{28} we analyzed Rac1 (Figure 6D) and RhoA (Figure 7A) activity in suPAR-treated podocytes with differential expression of SMPDL3b. Rac1 activity was significantly higher in suPAR-treated SMPDL3b KD podocytes compared with normal podocytes, whereas SMPDL3b OE podocytes were protected from suPAR-induced Rac1 activation (Figure 6D).

In contrast, RhoA was activated in SMPDL3b OE but not SMPDL3b KD podocytes (Figure 7A), and SMPDL3b KD podocytes were protected from DKD sera-induced increases in RhoA expression (Figure 7B). These findings suggest that SMPDL3b binding to suPAR leads to differential activation of small guanosine 5’-triphosphatases and that a Rac1-dependent migratory phenotype may occur in FSGS, whereas a more stationary and RhoA-dependent phenotype may occur in DKD. Because podocytopenia is a feature of both FSGS and DKD and may result from either excessive migration or apoptosis, we also investigated how the differential expression of SMPDL3b in FSGS and DKD affects podocyte susceptibility to apoptosis. We found that suPAR significantly increased caspase-3 activity in SMPDL3b OE podocytes, whereas SMPDL3b KD podocytes are protected from suPAR-induced apoptosis (Figure 7C). Because increased SMPDL3b expression prevented β3 integrin activation, we investigated
whether inhibition of $\beta_3$ integrin signaling in the presence of suPAR would also be sufficient to induce apoptosis. Interestingly, normal human podocytes simultaneously treated with suPAR and an $\alpha V\beta_3$ integrin inhibitor (cyclo RGD) were characterized by significantly increased apoptosis (Figure 7D). This activation occurred in the absence of SMPDL3b mRNA modulation (Figure 7E) and further shows that inactive $\alpha V\beta_3$ integrin is necessary to promote suPAR-mediated apoptosis, similar to what we observed in suPAR-treated SMPDL3b OE podocytes.

**Figure 6.** Decreased SMPDL3b expression is associated with $\beta_3$ integrin activation, resulting in a migratory FSGS-like phenotype in podocytes. (A) Human podocytes were incubated for 24 hours with or without suPAR (1 $\mu$g/ml). suPAR treatment (S) significantly increased AP5 and phospho-Src protein expression in podocytes, and these changes are more prominent in suPAR-treated human SMPDL3b KD podocytes compared with control podocytes. However, human SMPDL3b OE podocytes are protected from suPAR-induced increases in AP5 (activated $\beta_3$ integrin) and Src expression (*P<0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Immunofluorescence staining using antibodies for AP5 (green) and AP3 (red) reveals that human SMPDL3b KD podocytes are characterized by activation of $\beta_3$ integrin at baseline compared with normal podocytes. Furthermore, although $\beta_3$ integrin can be activated by suPAR in control podocytes, human SMPDL3b OE podocytes are protected from suPAR-induced $\beta_3$ integrin activation (*P<0.05; **P<0.01). MFI, mean fluorescence intensity. (C) Migration assay showing that suPAR-induced cell migration is significantly increased in control human podocytes and even more accentuated in human SMPDL3b KD podocytes (*P<0.05; **P<0.01). However, suPAR does not increase migration in human SMPDL3b OE podocytes. (D) Rac1 activity is significantly increased in suPAR-treated human SMPDL3b KD podocytes compared with control podocytes and SMPDL3b OE podocytes (*P<0.05, control versus SMPDL3b KD podocytes; **P<0.05, control versus SMPDL3b OE podocytes).

**ASMase Inhibitor Aggravates Podocyte Injury in ADR-Induced Nephropathy but Protects from Podocyte Injury in db/db Mice**

We previously showed that decreased SMPDL3b expression occurs in parallel with decreased ASMase activity in podocytes treated with sera from patients with recurrent FSGS. On the basis of our in vitro observations, we hypothesized that treatment with an ASMase inhibitor would worsen the glomerular phenotype of mice with an FSGS-like podocyte injury, whereas mice with a DKD-like phenotype would be protected from podocyte injury.
As expected, ASMase inhibitor treatment of mice with ADR-induced nephropathy worsened proteinuria (Figure 8A), increased segmental sclerosis (Figure 8B), and increased glomerular b3 integrin and Src activation (Figure 8C) compared with ADR alone.

On the contrary, a 3-month treatment of db/db mice with the same ASMase inhibitor showed a significant reduction of proteinuria compared with untreated mice (Figure 9A). This was associated with a preservation of the glomerular surface area in treated db/db mice that was comparable with that in db/+ mice (Figure 9B). Increased apoptosis (Figure 9C), as shown by increased caspase-3 activation, and RhoA activation (Figure 9D), as shown by immunostaining, were detected in db/db mice compared with control mice or ASMase inhibitor-treated mice.

DISCUSSION

Our study provides three novel findings. First, podocyte SMPDL3b levels are contrasted in FSGS (low) versus DKD (high); second, serum suPAR levels are similarly elevated in FSGS and DKD, and third, increased SMPDL3b expression shifts suPAR-mediated podocyte injury from a migratory to an apoptotic phenotype.

We recently described that decreased SMPDL3b expression occurs in glomeruli from patients with recurrent FSGS. Using microarray analysis, we showed that SMPDL3b expression is increased in glomeruli from patients with DKD and decreased (although not significantly) in a cohort of patients with FSGS and variable degrees of progression to ESRD. In addition, we showed that FSGS sera-treated podocytes exhibit decreased SMPDL3b expression, increased cortical actin, and loss of stress fibers, whereas DKD sera-treated podocytes show increased SMPDL3b expression associated with actin reorganization in cell blebs (Figure 1).

Increased and similar suPAR levels were detected in the sera from patients with FSGS and DKD and correlate with the yearly change in GFR. Increased levels of suPAR were also observed in mouse models of FSGS and DKD (Figure 2). Because SMPDL3b levels were differentially regulated, whereas suPAR levels were similarly elevated in patients with FSGS and DKD, we hypothesized that SMPDL3b is an important regulator of
suPAR-induced activation of \( \alpha V \beta_3 \) integrin signaling in podocytes.

SM is a critical component of the plasma membrane and abundant in lipid rafts.\(^6\) Because SMPDL3b is a protein with homology to ASMase, it may activate SM metabolic pathways, leading to the redistribution of SM and cholesterol from the lipid raft domains to intracellular compartments. Such concomitant loss of SM and cholesterol is often observed during apoptosis and may be associated with membrane blebbing.\(^{29,30}\) It is also possible that increased SMPDL3b levels (as observed in DKD) lead to increased cellular sphingosine, which is a ceramide metabolite. This finding could explain why we detected decreased cellular ceramide levels in kidney cortices from \( db/db \) mice (Figure 4). In support of this possibility, an increased sphingosine content in glomerular mesangial cells in \( db/db \) mice was previously described,\(^{31}\) and increased sphingosine levels in the presence of decreased SM and ceramide levels were observed in adipocytes of \( ob/ob \) mice.\(^{32}\) Thus, increased sphingosine production could explain why we observed increased apoptosis, cell blebbing, and RhoA activation in suPAR-treated SMPDL3b OE podocytes (Figures 3 and 7).

Rho kinase was previously shown to play an important role in the pathogenesis and progression in DKD, and Rho kinase inhibition decreases glomerular injury in diabetic animal models.\(^{28,33}\) Sphingosine-1-phosphate (S1P), another metabolite of ceramide, was shown to affect RhoA and Rac1 activity.\(^{34}\) In vascular smooth muscle cells, S1P binding to the receptor S1P3 leads to RhoA activation.\(^{34}\) In contrast, in human and mouse endothelial cells, S1P-induced cell migration was shown to occur by activation of Rac1.\(^{35}\) Increased S1P production as a result of increased ceramide metabolism could, therefore, explain the migratory phenotype observed in

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**Figure 8.** ASMase inhibitors (AIs) worsen the phenotype in a mouse model for FSGS. (A) Urine albumin (Alb)-to-creatinine ratio is significantly higher in ADR mice compared with control mice, and AI treatment significantly increases proteinuria in ADR mice (*\( P < 0.05 \), ADR versus control; \( \# P < 0.05 \), ADR versus ADR+AI). (B) Segmental sclerosis assessed by periodic acid-Schiff staining is significantly worse in ADR mice than control mice (*\( P < 0.05 \)), and the extent of segmental sclerosis is further significantly increased by AI treatment (**\( P < 0.01 \)). (C) Glomerular AP5 (activated \( \beta_3 \) integrin) and phospho-Src protein expressions are significantly higher in ADR mice, and these changes are markedly increased after AI treatment (*\( P < 0.05 \); \( \# P < 0.05 \)). cRGD, cyclo-(Arg-Gly-Asp) peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
SMPDL3b KD podocytes (Figure 6) and the decreased ceramide levels observed in kidney cortexes of ADR-treated mice (Figure 4).

uPAR, a glycosylphosphatidylinositol-anchored protein, is a cellular receptor for urokinase and signals through association with other transmembrane receptors, including integrin. Previous reports showed that uPAR translocates to the lipid raft domain in podocytes after LPS stimulation and that blocking of its localization to the lipid raft by cyclodextrin can prevent the β3 integrin activation. Co-IP experiments in transfected HEK293 cells revealed interaction of SMPDL3b with uPAR but not with β3 integrin, and SMPDL3b/uPAR interaction was further confirmed in glomeruli isolated from C57Bl/6 mice. Using competitive Co-IP, we show that SMPDL3b interferes with binding of suPAR/uPAR and β3 integrin, attenuating β3 integrin activation and signaling (Figure 5). We show that, in SMPDL3b KD podocytes, suPAR/uPAR-mediated β3 integrin activation and signaling are functional, whereas they are impaired in SMPDL3b OE podocytes (Figure 6). Our results suggest that, in the absence of SMPDL3b, suPAR can activate β3 integrin signaling (FSGS), whereas in the presence of SMPDL3b, SMPDL3b binds to suPAR, preventing β3 integrin activation (DKD). The mechanistic model for podocyte damage suggested by our findings is shown in Figure 10.

Furthermore, our study shows that suPAR increases Rac1 activity in SMPDL3b KD podocytes (Figure 6) and that an ASMase inhibitor worsens proteinuria in a model of FSGS (Figure 8), which may be because of decreased Smpdl3b expression in podocytes. On the contrary, suPAR increases RhoA activity in SMPDL3b OE podocytes (Figure 7) and diabetic animals, and an ASMase inhibitor ameliorates proteinuria in

Figure 9. ASMase inhibitors (AIs) ameliorate the phenotype in a mouse model for DKD. (A) Urine albumin (Alb)-to-creatinine ratio is significantly higher in db/db mice compared with db/m mice (**P<0.01), and AI treatment significantly abrogates proteinuria in db/db mice compared with untreated db/db mice (**P<0.05). (B) Glomerular surface area is increased in db/db mice compared with db/m mice (*P<0.05); however, glomerular surface area is significantly smaller in ASMase-treated db/db mice compared with untreated db/db mice (**P<0.05) and seems to not be significantly changed compared with db/m mice. (C) Glomerular cleaved caspase-3 expression is significantly higher in db/db mice compared with db/m mice (**P<0.05), and expression is significantly reduced by AI treatment (**P<0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (D) Glomerular active RhoA expression is increased in db/db mice (**P<0.01) and attenuated by AI treatment (**P<0.05). MFI, mean fluorescence intensity.
SMPDL3b to suPAR, thus preventing suPAR in the presence of high SMPDL3b expression leads to competitive binding of and ultimately causing a migratory podocyte phenotype. In DKD, increased circulating a
creased circulating suPAR together with low or absent SMPDL3b expression lead to

Figure 10. Mechanistic model of podocyte injury in FSGS and DKD. In FSGS, increased circulating suPAR together with low or absent SMPDL3b expression lead to aV3 integrin activation, resulting in increased Src phosphorylation and Rac1 activity and ultimately causing a migratory podocyte phenotype. In DKD, increased circulating suPAR in the presence of high SMPDL3b expression leads to competitive binding of SMPDL3b to suPAR, thus preventing aV3 integrin activation but allowing for RhoA activation and increased apoptosis. an in vivo model of DKD, possibly because of increased Smpdl3b expression in podocytes (Figures 4 and 9). However, we were not able to detect regulation of Smpdl3b in RNA isolated from kidney cortices of these mice, which may be explained by the fact that podocytes represent only a very small percentage of the cells present in the kidney.

In summary, our study suggests an important role for podocyte SMPDL3b expression in the pathogenesis of FSGS and DKD. We show that high circulating suPAR levels are necessary but not sufficient to induce podocyte injury in FSGS and DKD. We also show that, in the presence of high suPAR levels, reduced podocyte SMPDL3b expression leads to activation of β3 integrin signaling and Rac1 and induction of a migratory, FSGS-like podocyte phenotype. In contrast, high suPAR in the presence of increased podocyte SMPDL3b levels will not allow for β3 integrin activation but will lead to RhoA activation and podocyte apoptosis. The fact that ASMase inhibition partially prevented proteinuria and the increase in glomerular surface area (within limitations of the method used) underlines the potential role of SMPDL3b in DKD.

To our knowledge, this study shows, for the first time, how suPAR can affect the same cell through a different effector pathway, thus challenging the concept that targeted interventions to treat FSGS are also suitable for the treatment of DKD. Furthermore, our study suggests that, in addition to the removal or neutralization of suPAR, therapeutic targeting of

CONCISE METHODS

Affymetrix Gene Chip Analyses of Human Glomeruli and Microarray Data in Human Glomeruli

Renal biopsy samples from healthy pretransplant kidney donors (n=32) and patients with primary FSGS (n=18) were obtained from the European Renal cDNA Bank following the guidelines of the respective local ethics committees. For glomerular mRNA expression profiles specific to patients with DKD, kidney biopsy specimens were procured from 70 Southwestern American Indians enrolled in a randomized, placebo-controlled clinical trial to evaluate the renoprotective efficacy of losartan in type 2 diabetes. Informed consent was obtained from all patients. The biopsy tissue specimens were manually microdissected, and glomerular gene expression profiling was performed as previously described.

Measurements of suPAR

Circulating suPAR levels were determined in the sera from 53 patients with FSGS from the FSGS clinical trial25 and 30 patients with type 1 diabetes and normoalbuminuria, 34 patients with type 1 diabetes and microalbuminuria, and 10 patients with type 1 diabetes and macroalbuminuria from the FinnDiane study cohort using Quantikine Human uPAR Immunoassay (R&D Systems) performed following the manufacturer’s protocol. Among those patients, only 40 patients had two sample collections for longitudinal determination of suPAR, and the clinical characteristics of these patients are shown in Table 1. Patients belonging to the high-risk group of FSGS were defined by an onset age of <15 years or progression to a GFR<60 ml/min per 1.73 m2 within 6 months after onset of disease. Urinary albumin excretion rate was assessed from two of three urine collections as normal albumin excretion rate (<30 mg/24 h), microalbuminuria (≥30 to <300 mg/24 h), and macroalbuminuria (≥300 mg/24 h).

Animal Studies and Glomerular Isolation

All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee. Thirty-two 7- to 8-week-old BALB/C mice were used. FSGS-like lesions were induced by single intravenous ADR (Sigma-Aldrich, St. Louis, MO) injection (12 mg/kg). Eight mice were injected with ADR, eight mice were injected with ADR and desipramine hydrochloride, eight mice were injected with desipramine hydrochloride, and eight mice without treatment served as a control group. Desipramine hydrochloride, an ASMas inhibitor (10 mg/kg; Sigma-Aldrich) was injected intraperitoneally
every other day for 4 weeks. Mice were euthanized after 4 weeks. Sixteen B6.Cg-m<sup>m</sup>/? Lepr<sup>db</sup>/Lepr<sup>db</sup> (db/db) and sixteen B6.Cg-m<sup>m</sup>/? Lepr<sup>db</sup>/ (db/+ ) female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). At 8 weeks of age, eight db/db and eight db/+ mice were injected intraperitoneally with 10 mg/kg ASMA inhibitor every other day for 12 weeks. At euthanasia, mice were perfused, kidneys were fixed, and glomerular protein lysates were prepared as described. Circuiting suPAR levels were measured in serum using the DuoSet ELISA Development Kit (DY531; R&D Systems) according to the manufacturer’s recommendations. Values are expressed as picograms suPAR per milliliter serum. Periodic acid–Schiff staining of 3-μm-thick slides was performed for the analysis of segmental sclerosis in ADR mice as previously described. For glomerular volume determination, the glomerular surface was delineated, and the mean surface area was then calculated as previously described. Determination of the lipid content in kidney cortices was determined by the Wake Forest University Bioanalytical Laboratory, Mass Spectrometry Core Laboratory, Wake Forest School of Medicine, Winston-Salem, NC, according to their standard protocols.

Co-IP, Competitive Co-IP, and Endogenous Immunoprecipitation
Co-IP experiments were performed following the previous published protocols. For Co-IP experiments, HEK-293 cells were transfected with 1 μg plasmid containing GFP-SMPDL3b and FLAG-empty vector, FLAG-uPAR/suPAR, or FLAG-β3 integrin. For competitive Co-IP experiments, HEK-293 cells were transfected with increasing amounts of GFP-tagged SMPDL3b cDNA or a plasmid containing a GFP-tag without any fused cDNA (empty vector) together with 1 μg each FLAG-suPAR/uPAR and GFP-β3 integrin plasmid in HEK-293 cells. For endogenous immunoprecipitation experiments, C57Bl/6 mice were injected with LPS (250 μg) or vehicle (PBS), and glomeruli were prepared by sieving technique 24 hours later. To immunoprecipitate endogenous protein complexes, protein lysates of the isolated mouse glomeruli were prepared in radioimmunoprecipitation assay buffer. One milliliter milliliter glomerular extract solution was incubated for 1 hour with the dynabeads M-270 Epoxy (Invitrogen) coupled with antibodies (SMPDL3b; Genway or uPAR; R&D Systems) at 4°C under rotation. Immune complexes were eluted in 60 μl elution buffer. Both eluates and protein lysates (input) were loaded for Western blot analysis.

Podocyte Culture, Protein Extraction, and Western Blotting
Normal human podocytes were cultured as described. Stable SMPDL3b OE or SMPDL3b KD podocyte cell lines were previously described. Differentiated podocytes were serum-restricted for 24 hours followed by 24 hours of treatment with recombinant suPAR (1 μg/ml; R&D Systems) and cyclo-RGD, a cyclo-(Arg-Gly-Asp) peptide (1 μg/ml; Enzo Life Sciences) with 4% patient serum from the FinnDiane study cohort of patients with DKD or pretransplant serum from patients with high risk for FSGS recurrence after transplant if not indicated otherwise; 22 pretransplant FSGS patient sera, five age-matched control sera, and 30 patient sera from the FinnDiane study were used as previously described.

Glomeruli or cells were lysed in radioimmunoprecipitation assay (RIPA) or 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic (CHAPS) acid buffer. Western blot analysis was performed as previously described using the following primary antibodies: anti-AP5, anti-AP3 (1:1000; Gen-Probe, Wakukeha, WI), antiphospho-Src, anti-Src (1:1000; Cell Signaling Technology, Temecula, CA), anti-cleaved caspase-3 (1:250; Cell Signaling Technology), anti-Gapdh (1:5000; Calbiochem), anti-FLAG (1:1000; Sigma-Aldrich), anti-GFP (1:1000; Abcam, Inc.), and anti-SMPDL3b (1:1000; Novus Bio). Anti-mouse IgG HRP (1:10,000; Promega) or anti-rabbit IgG HRP (1:10,000; Promega) was used as secondary antibody.

Immunofluorescence Staining
A standard immunofluorescence protocol was followed. AP5 and AP3 monoclonal mouse antibodies (Gen-Probe), polyclonal anti-rabbit synaptopodin (Santa Cruz Biotechnology), or active RhoA mouse mAb (New East Biosciences) were used. Alexa-conjugated secondary antibodies from Invitrogen were used. Images were acquired by confocal microscopy.

Transwell Migration Assay
Transwell migration assay was performed as previously described using normal, SMPDL3b KD, or SMPDL3b OE podocytes untreated or treated with recombinant human suPAR (1 μg/ml; R&D Systems).

Apoptosis Analyses
Apoptosis in human podocytes treated with the sera from patients with FSGS, DKD, or suPAR was assessed as previously described using the Caspase 3/PP32 Colorimetric Assay Kit (BioVision, Inc.) or the Caspase 3 HTS Kit (Biotium). Absorbance values were expressed as fold change to controls.

Rac1 and RhoA Activity Assay
Rac1 and RhoA activity was assessed using the Rac1 Activation Assay Kit (Cell Biolabs) and RhoA pull-down assay (Thermo Fisher Scientific) following the manufacturers’ protocols. After 24 hours of recombinant suPAR (1 μg/ml) treatment, podocyte lysates were incubated at 4°C for 1 hour with agarose beads specific for the p21-binding domain of p21-activated protein kinase (Rac1 pull-down assay) or glutathione-sepharose beads coupled with glutathione-S-transferase–Rhodekin fusion protein for determination of RhoA activity. Expression analysis was performed by Western blotting using anti-Rac1 and RhoA antibody.

Statistical Analyses
Values are expressed as means and SDs. Statistical analysis was performed using the statistical package SPSS for Windows, version 11.0 (SPSS Inc., Chicago, IL). Results were analyzed using the Kruskal–Wallis nonparametric test for multiple comparisons. Significant differences were confirmed by the Mann–Whitney U test. Wilcoxon
signed rank test was performed to determine changes in suPAR levels between baseline and 7 years in the FinnDiane study group. Pearson correlation analysis was used to clarify the relationship between suPAR levels and GFR decline slope in FSGS patients. P values <0.05 were considered statistically significant.

ACKNOWLEDGMENTS

Nurses A. Sandelin and J. Tuomikangas at the Folkhalsan Institute of Genetics are acknowledged for their contribution to this work. We also thank the FinnDiane Study Group for their valuable contributions.

This study was supported, in part, by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Part of this project was supported by Grant 1UL-1TR00046 (University of Miami Clinical and Translational Science Institute) from the National Center for Advancing Translational Sciences and the National Institute on Minority Health and Health Disparities. This study was supported by Yonsei University College of Medicine Faculty Research Grant 6-2013-0143 (to T.-H.Y.) for 2013. The study was also supported by the Wilhelm and Else Stockmann Foundation (M.L. and P.-H.G.), the Novo Nordisk Foundation (M.L. and P.-H.G.), the Folkhalsan Research Foundation (P.-H.G.), and Academy of Finland Grant 134379 (to P.-H.G.). J.R. is supported by National Institutes of Health Grants R01-DK089394, R01-DK073495, and R01-DK101350. G.W.B., A.F., and S.M. are supported by National Institutes of Health Grant DK090316. A.F. and S.M. are supported by the Diabetes Research Institute Foundation, the Nephcure Foundation, the Peggy and Harold Katz Family Foundation, and the Diabetic Complications Consortium (DiaComp). S.M. is supported by a Stanley J. Glaser Foundation Research Award.

DISCLOSURES

C.W., J.R., G.W.B., A.F., and S.M. are inventors on pending or issued patents aimed to diagnose or treat proteinuric renal diseases. They stand to gain royalties from their future commercialization. A.F. is a consultant for Hoffman-La Roche and Mesoblast on subject matters that are unrelated to this publication.

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