

Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis

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Focal segmental glomerulosclerosis (FSGS) is a cause of proteinuric kidney disease, compromising both native and transplanted kidneys. Treatment is limited because of a complex pathogenesis, including unknown serum factors. Here we report that serum soluble urokinase receptor (suPAR) is elevated in two-thirds of subjects with primary FSGS, but not in people with other glomerular diseases. We further find that a higher concentration of suPAR before transplantation underlies an increased risk for recurrence of FSGS after transplantation. Using three mouse models, we explore the effects of suPAR on kidney function and morphology. We show that circulating suPAR activates podocyte β_3 integrin in both native and grafted kidneys, causing foot process effacement, proteinuria and FSGS-like glomerulopathy. Our findings suggest that the renal disease only develops when suPAR sufficiently activates podocyte β_3 integrin. Thus, the disease can be abrogated by lowering serum suPAR concentrations through plasmapheresis, or by interfering with the suPAR– β_3 integrin interaction through antibodies and small molecules targeting either uPAR or β_3 integrin. Our study identifies serum suPAR as a circulating factor that may cause FSGS.

Focal segmental glomerulosclerosis (FSGS) is a major cause of end-stage renal disease (ESRD)¹. It affects both native and transplanted kidneys^{2–4}, with recurrence after transplant occurring in about 30% of adult and pediatric FSGS patients⁵. FSGS in its early stages targets mainly podocytes in kidney glomeruli. These cells and their foot processes regulate the renal filtration barrier⁶. Generally, the effacement of podocyte foot processes marks the first ultrastructural step associated with the loss of plasma proteins into the urine⁷. Although podocyte gene defects are a known cause of human FSGS⁶, there are cases in which FSGS occurs in the absence of gene defects or in which proteinuria recurs within a few hours or days after kidney transplantation. These clinical observations have given rise to the idea that FSGS can be associated with a causative circulating factor, the so-called FSGS permeability factor⁸. This concept is supported by the recurrence of FSGS after transplantation⁹, by the response of proteinuria to therapy with plasmapheresis¹⁰ or immunoadsorption¹¹, and by a case of transient nephrotic syndrome in a newborn

whose mother had FSGS¹². The search for the circulating factor, however, has been long and painstaking^{13–17}.

We have recently defined a role for the podocyte urokinase receptor (uPAR; encoded by *PLAUR*) in glomerular disease¹⁸. uPAR is a glycosylphosphatidylinositol (GPI)-anchored three-domain (D_I , D_{II} and D_{III} , as numbered from the N terminus) protein, which has been identified as a cellular receptor for urokinase, but also as a versatile signaling orchestrator through association with other transmembrane receptors, including integrins^{19,20}. uPAR can be released from the plasma membrane as a soluble molecule (suPAR) by cleavage of the GPI anchor¹⁹. suPAR can be further cleaved in the linker region between domains D_I and D_{II} , thereby releasing, for example, the fragments D_I and $D_{II}D_{III}$. Thus, suPAR is a circulating protein ranging from 20 to 50 kDa, depending on the degree of glycosylation and proteolytic cleavage^{19,20}. suPAR is present under physiological conditions in low concentrations in human blood, and it has a known role as a circulating protein involved in neutrophil trafficking and stem cell mobilization^{19,20}.

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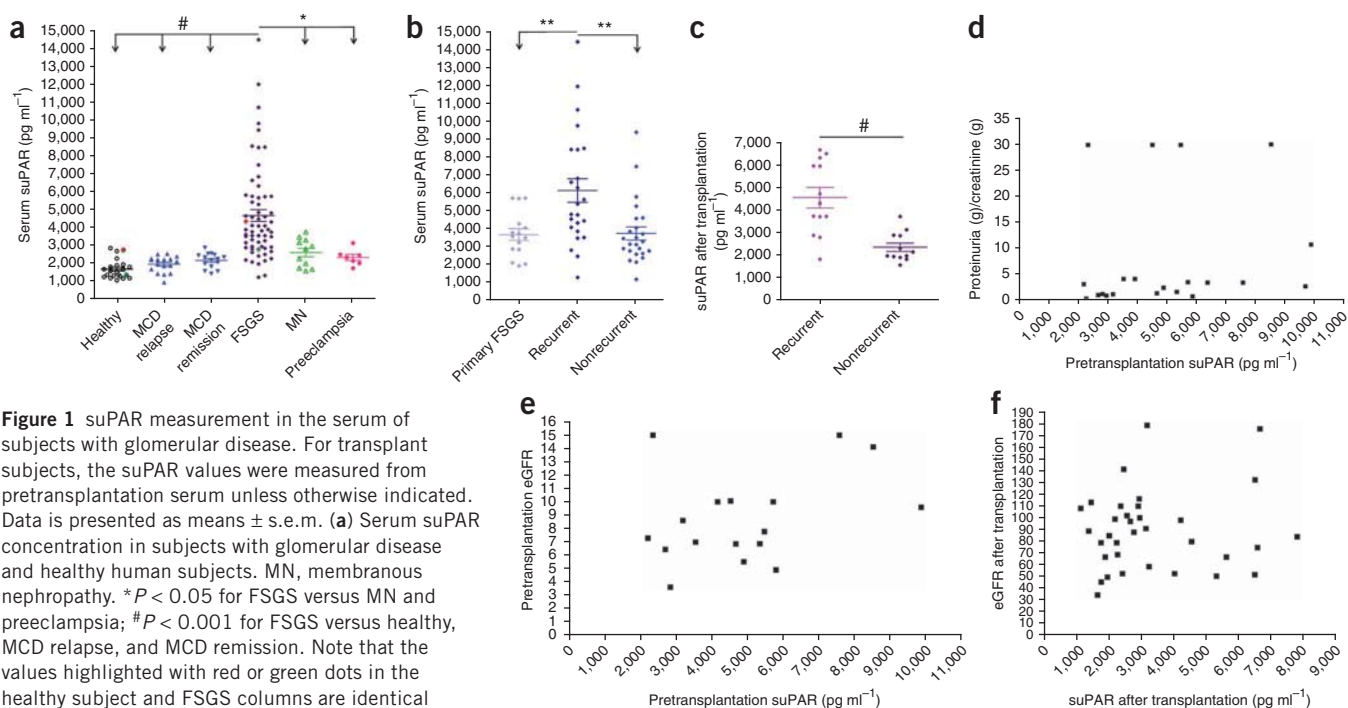


Figure 1 suPAR measurement in the serum of subjects with glomerular disease. For transplant subjects, the suPAR values were measured from pretransplantation serum unless otherwise indicated. Data is presented as means \pm s.e.m. (a) Serum suPAR concentration in subjects with glomerular disease and healthy human subjects. MN, membranous nephropathy. * $P < 0.05$ for FSGS versus MN and preeclampsia; # $P < 0.001$ for FSGS versus healthy, MCD relapse, and MCD remission. Note that the values highlighted with red or green dots in the healthy subject and FSGS columns are identical twin pairs; in each case, one is healthy and has a twin brother with FSGS. (b) Serum suPAR in different population of subjects with primary FSGS. ** $P < 0.01$ for recurrent FSGS versus nonrecurrent FSGS and nontransplant primary FSGS, respectively. (c) Serum suPAR concentrations after transplantation. # $P < 0.001$. (d) Correlation analysis of pretransplantation suPAR with proteinuria after transplantation. Pearson $r = 0.16$, $P = 0.50$. (e) Correlation analysis of pretransplantation suPAR with eGFR. Pearson $r = 0.36$, $P = 0.16$. (f) Correlation analysis of suPAR after transplantation with eGFR. Pearson $r = 0.10$, $P = 0.58$.

It can be elevated in some malignant neoplasms (for example, ovarian cancer²¹) as well as in HIV infection²². On the basis of our recent report showing that induced uPAR expression in podocytes can cause podocyte foot process effacement and proteinuria¹⁸, we hypothesized that suPAR might be a candidate circulating factor in FSGS. Thus, we analyzed a multicenter collection of sera from glomerular disease patients to investigate suPAR concentrations in cases of FSGS. We found significantly elevated suPAR concentrations in subjects with primary and recurrent FSGS. Mechanistically, enhanced circulating suPAR deposits into the glomeruli, allowing activation of podocyte β_3 integrin. This activation is sufficient to drive podocyte foot process effacement, proteinuria and initiation of FSGS. Moreover, suPAR-induced glomerular disease can be blocked by expression of a suPAR point mutant that is strongly reduced in β_3 integrin binding, or by use of neutralizing suPAR antibodies. In conclusion, our study suggests circulating suPAR as a previously undescribed cause for both primary and recurrent FSGS.

RESULTS

suPAR is increased in serum of subjects with FSGS

We found that suPAR serum concentrations are significantly elevated in people with FSGS when compared to healthy subjects (Fig. 1a). In contrast, we did not observe any significant variance of suPAR in subjects with minimal change disease (MCD)—either in relapse or remission—or in people with membranous nephropathy or preeclampsia (Fig. 1a). We then stratified the FSGS cases into three different subpopulations: primary FSGS, recurrent FSGS in the allograft and FSGS without recurrence after transplantation. We found the highest suPAR concentrations in pretransplantation blood from subjects with FSGS who later developed recurrent FSGS after transplantation (Fig. 1b). Thus the pretransplantation suPAR serum concentration may be a predictor of heightened risk of recurrent FSGS after transplantation.

We also compared suPAR serum concentrations in transplanted FSGS patients 1 year after transplantation and found significantly higher suPAR serum concentrations in patients that developed recurrent FSGS than in FSGS patients who received kidney transplants and then had normal renal function (Fig. 1c). We found that suPAR concentrations correlated with the presence but not with the degree of proteinuria (Fig. 1d), and they were unrelated to the pretransplantation estimated glomerular filtration rate (eGFR) (which was low) or eGFR after transplantation (which was high), (Fig. 1e,f).

We also carried out a longitudinal analysis to evaluate serum suPAR concentrations in subjects with recurrent and nonrecurrent FSGS by measuring pretransplantation suPAR serum concentrations and comparing them with suPAR serum concentrations in the same subjects for up to 1 year after kidney transplantation. We noticed that the subjects with recurrent FSGS had sustained higher suPAR serum concentrations over the course of 1 year when compared to those in which no transplant FSGS occurred (Supplementary Fig. 1a,b). In 8 of 13 subjects that developed recurrent FSGS, we found that suPAR was further increased during the course of the 1-year interval, a finding that was in contrast to the suPAR concentrations we observed in subjects with no recurrence after kidney transplantation. To better define a cutoff for suPAR-associated FSGS, we analyzed the variance in suPAR serum concentrations and found suPAR concentrations of 3000 pg ml^{-1} or above in 45 out of 63 subjects with FSGS, but only in 4 out of 11 subjects with membranous nephropathy, 1 out of 7 subjects with preeclampsia and in none of 25 subjects with MCD (Supplementary Table 1). In summary, our data show that suPAR is increased specifically in FSGS but not in other analyzed glomerular diseases with podocyte involvement, such as MCD and membranous nephropathy, nor in preeclampsia, a proteinuric disease that is caused largely by endothelial dysfunction²³.

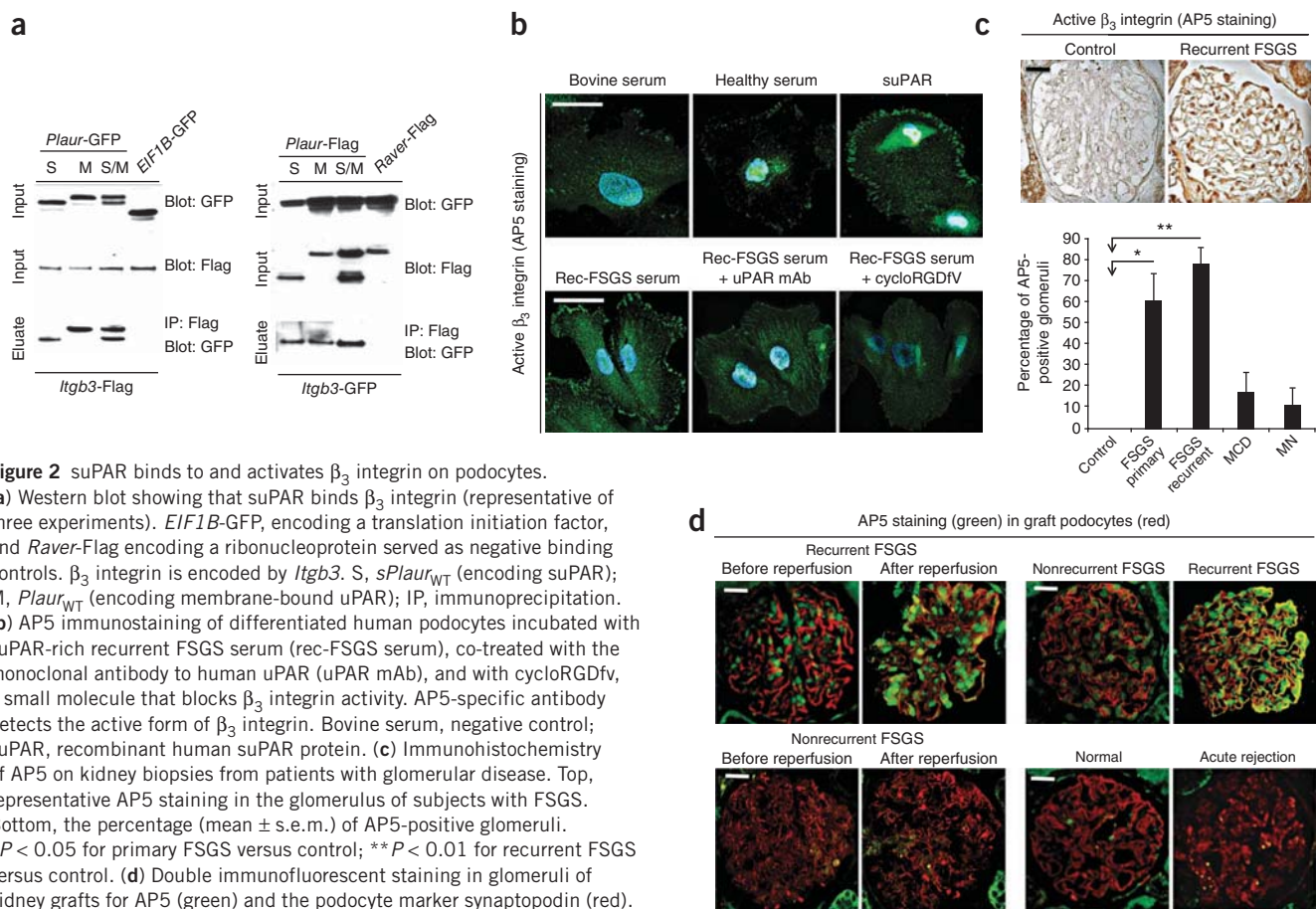


Figure 2 suPAR binds to and activates β_3 integrin on podocytes.

(a) Western blot showing that suPAR binds to β_3 integrin (representative of three experiments). *EIF1B*-GFP, encoding a translation initiation factor, and *Raver*-Flag encoding a ribonucleoprotein served as negative binding controls. β_3 integrin is encoded by *Itgb3*. S, *sPlaur*_{WT} (encoding suPAR); M, *Plaur*_{WT} (encoding membrane-bound uPAR); IP, immunoprecipitation. (b) AP5 immunostaining of differentiated human podocytes incubated with suPAR-rich recurrent FSGS serum (rec-FSGS serum), co-treated with the monoclonal antibody to human uPAR (uPAR mAb), and with cycloRGDFV, a small molecule that blocks β_3 integrin activity. AP5-specific antibody detects the active form of β_3 integrin. Bovine serum, negative control; suPAR, recombinant human suPAR protein. (c) Immunohistochemistry of AP5 on kidney biopsies from patients with glomerular disease. Top, representative AP5 staining in the glomerulus of subjects with FSGS. Bottom, the percentage (mean \pm s.e.m.) of AP5-positive glomeruli. * $P < 0.05$ for primary FSGS versus control; ** $P < 0.01$ for recurrent FSGS versus control. (d) Double immunofluorescent staining in glomeruli of kidney grafts for AP5 (green) and the podocyte marker synaptopodin (red). Top and bottom left, AP5 in the graft glomerulus 2 h after reperfusion in recurrent and nonrecurrent transplant biopsies ($n = 2$ per group). Top right, AP5 signal in recurrent transplant biopsies ($n = 3$) and nonrecurrent grafts ($n = 5$). Bottom right, normal kidney sections ($n = 2$) and biopsies from acute T cell-mediated rejections ($n = 3$) served as controls. Scale bars, 30 μ m.

As multiple forms of suPAR have been attributed to domain cleavage or alternative splicing^{19,24,25}, we further defined which forms of suPAR exist in the blood of subjects with FSGS. We did immunoprecipitation on FSGS serum samples with a uPAR-specific antibody and found a predominant suPAR fragment at ~ 22 kDa, and the other two forms at ~ 45 and 40 kDa respectively, albeit at much lower expression levels (Supplementary Fig. 2a). In contrast, healthy subjects do not show strong serum expression of suPAR (Supplementary Fig. 2a). Next, we tested whether suPAR is albumin bound or freely circulating in the blood. Although we could detect adiponectin, an albumin-bound protein²⁶, we did not detect suPAR in the albumin immunoprecipitates under the same experimental conditions (Supplementary Fig. 2b). Furthermore, immunoprecipitation of FSGS serum with a monoclonal uPAR-specific antibody, followed by immunoblotting with an antibody specific to human albumin, did not detect albumin from the precipitants (Supplementary Fig. 2c), thereby suggesting that suPAR in the blood of subjects with FSGS is largely not bound to albumin.

Concentrations of the ligand of uPAR, urokinase (uPA), are often elevated in certain types of cancers that also present with elevated suPAR concentrations in various body fluids²⁷. Thus, we measured serum uPA concentrations in the groups within our glomerular disease cohort. Notably, and unlike suPAR, we found no difference in the serum uPA concentrations among the groups (Supplementary Fig. 3). These findings, together with the data obtained from previous mouse experiments in *Plaur*^{-/-} mice¹⁸ suggest that, in contrast

to cancer, uPA does not seem to be crucial for suPAR-mediated noninflammatory glomerular injury, such as FSGS.

suPAR binds to and activates β_3 integrin in podocytes

In podocytes, uPAR binds to β_3 integrin¹⁸. In addition, suPAR is known to be associated with β_1 and β_2 integrins²⁸. Thus, we investigated whether suPAR can also bind to β_3 integrin. Using coimmunoprecipitation of suPAR and β_3 integrin, we observed that suPAR interacted with β_3 integrin (Fig. 2a), similarly to membrane-bound uPAR (Fig. 2a and ref. 18).

We hypothesized that suPAR could activate β_3 integrin in a similar manner to membrane-bound uPAR in podocytes¹⁸. The activity of β_3 integrin is typically measured using the activation epitope-recognizing antibodies such as the β_3 integrin-specific antibody AP5 (refs. 29,30). We used human differentiated podocytes³¹ and incubated them either with FSGS serum that contains high concentrations of suPAR or with recombinant suPAR, in the absence or presence of a blocking antibody to uPAR or with the β_3 integrin small molecule-inhibitor cycloRGDFV¹⁸. After 24 h, we used immunofluorescent staining to analyze the expression and localization of the AP5 signal that corresponds to activated β_3 integrin (Fig. 2b). We found that human podocytes show low-level activation of β_3 integrin when they are grown in bovine serum or in serum from healthy subjects (Fig. 2b). In contrast, we found that incubation with serum from subjects with recurrent FSGS (high in suPAR) or with recombinant suPAR strongly induces the AP5 signal in a pattern highlighting areas of focal adhesions; these

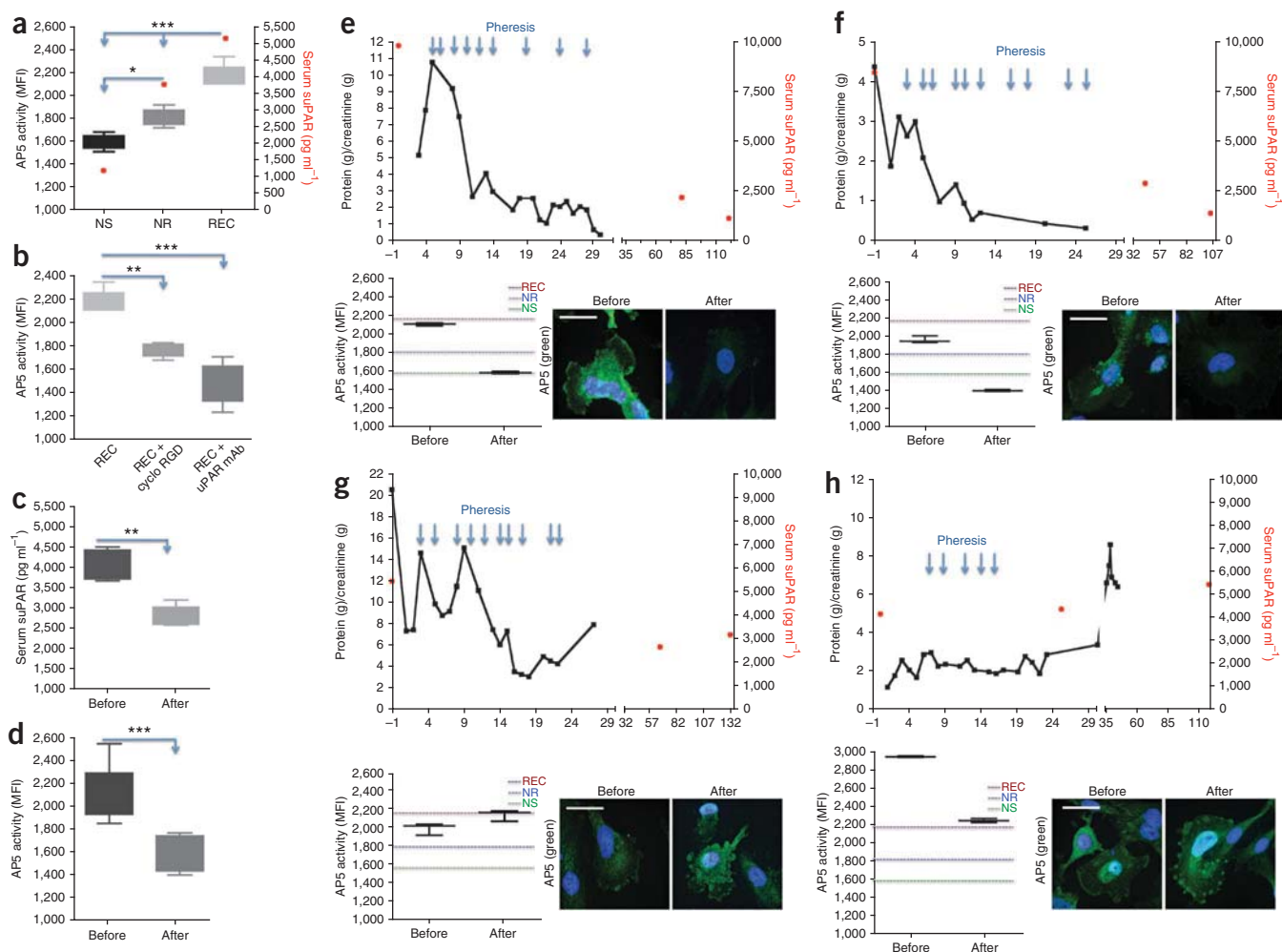


Figure 3 suPAR serum concentrations and podocyte β_3 integrin activity determine treatment response to plasmapheresis in recurrent FSGS. **(a)** Human podocytes incubated with different pooled serum samples and assayed for β_3 integrin activity. MFI, mean fluorescence intensity. * $P < 0.05$ for nonrecurrent FSGS versus normal subjects, *** $P < 0.001$ for recurrent versus nonrecurrent FSGS or versus healthy subjects. The respective suPAR concentration of the pooled sera is marked in red. NS, normal (healthy) subject; NR, nonrecurrent FSGS; REC, recurrent FSGS (representative of three experiments). **(b)** Pharmacological modulation of β_3 integrin activity in podocytes. ** $P < 0.01$ for cycloRGDfv co-treated cells versus recurrent FSGS serum alone; *** $P < 0.001$ for uPAR-specific mAb co-treated cells versus recurrent FSGS serum alone. **(c)** suPAR in serum from subjects with recurrent FSGS ($n = 4$) before and after a course of plasmapheresis. ** $P < 0.01$. **(d)** Effect of plasmapheresis on β_3 integrin activity in podocytes incubated with recurrent FSGS serum ($n = 6$), collected before and after serial treatment with plasmapheresis. *** $P < 0.001$. **(e–h)** Clinical cases of recurrent FSGS. Top graphs show serum suPAR, urine protein/creatinine ratio (g/g) and individual plasmapheresis treatment as indicated by arrows and plotted over time **(d)** from before (–1) to after transplantation. Bottom graphs and images show podocyte β_3 integrin activity measured by FACS (left) and immunofluorescence (right) as a result of incubation with pretransplantation serum, or with the after-transplantation serum collected after repetitive plasmapheresis treatments. As a reference, the mean concentration of AP5 from **a** is marked as a dashed line. **(e, f)** Patients who obtained full remission after pheresis. **(g, h)** Patients who did not achieve remission after pheresis. Scale bars, 30 μm . Whiskers in plots of AP5 activity and serum suPAR show minimum to maximum.

adhesions are known to be the location of β_3 integrin³². We also found that this effect could be blocked by a blocking antibody specific to uPAR or by cycloRGDfv (**Fig. 2b**).

Next, we studied β_3 integrin activity in human kidneys affected by glomerular disease by analyzing a patient biopsy cohort. We found induced glomerular AP5 staining in 7 of 9 idiopathic FSGS patients, and in all patients with recurrent FSGS (**Fig. 2c**). In contrast, we observed no or only weak AP5 signal in glomeruli of healthy kidneys or in kidneys affected by MCD and membranous nephropathy (**Fig. 2c**), suggesting that induced podocyte β_3 integrin activity is a specific feature of FSGS.

To show that circulating suPAR affects the transplanted kidney by activating podocyte β_3 integrin, we used double immunofluorescent staining with synaptopodin, a podocyte marker³³, to analyze after-transplantation graft biopsies for the presence of AP5 signal in

podocytes. We found that β_3 integrin activity is low in graft podocytes before reperfusion, whereas it is markedly increased 2 h after reperfusion in recurrent FSGS, but not in nonrecurrent FSGS (**Fig. 2d**). Moreover, we found that the AP5 signal was high in the after-transplantation biopsies from subjects with recurrent FSGS but not in subjects with nonrecurrence, nor in subjects with acute T cell-mediated rejection episodes (**Fig. 2d**). Taken together, these findings suggest that increased podocyte β_3 integrin activity is a feature of both native and recurrent FSGS.

suPAR and β_3 integrin activity during plasmapheresis

To further define the relationship between suPAR and podocyte β_3 integrin activity, we did fluorescence-activated cell sorting (FACS) analysis for β_3 integrin activity in cultured human podocytes

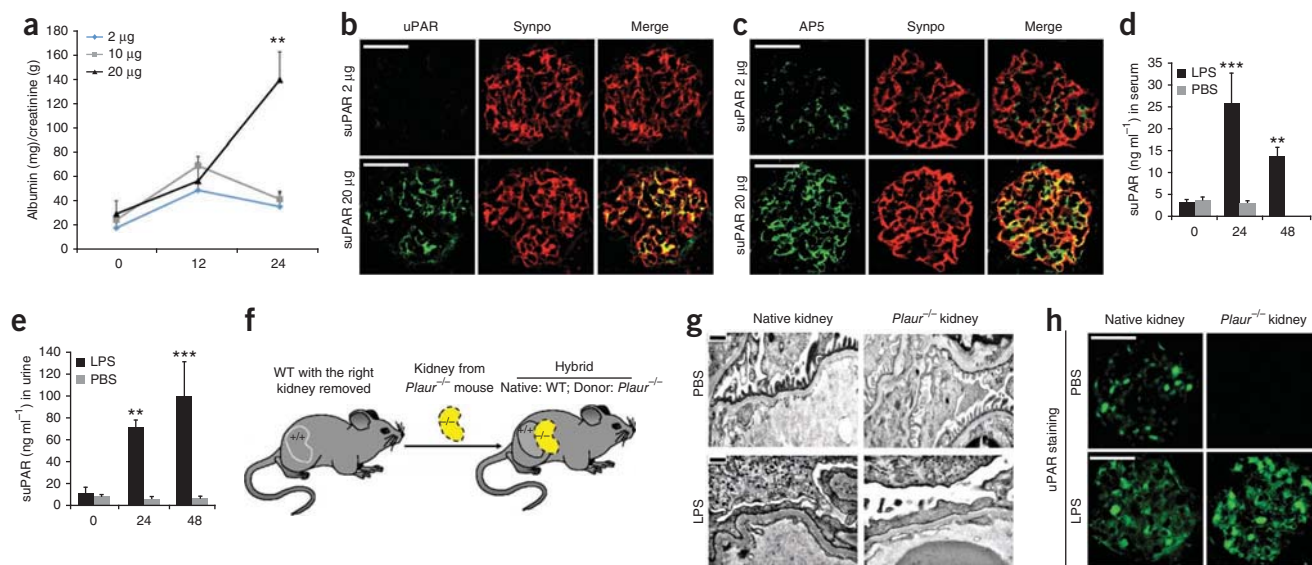


Figure 4 suPAR activates β_3 integrin and causes foot process effacement in *Plaur*^{-/-} mouse kidneys and albuminuria in *Plaur*^{-/-} mice. **(a)** Injection (i.v.) of high doses of recombinant mouse suPAR into *Plaur*^{-/-} mice ($n = 4$ per group) induces proteinuria. $^{**}P < 0.01$ for mice injected with 20 μ g of suPAR at 24 h versus mice injected with other doses or versus other time points. **(b)** Injection (i.v.) of high doses of recombinant suPAR deposits into podocytes. Green, uPAR; red, synaptopodin (Synpo). **(c)** AP5 activity induced in the podocytes of high-dosage suPAR-injected *Plaur*^{-/-} mice ($n = 4$). Green, AP5; red, Synpo. **(d,e)** LPS induced endogenous suPAR in wild-type mice ($n = 6$). **(d)** Serum suPAR concentrations in LPS-treated mice. $^{***}P < 0.001$ for LPS-injected mice at 24 h versus PBS control, and versus LPS-injected mice at 0 h. $^{**}P < 0.01$ for LPS-injected mice at 48 h versus at 0 h. **(e)** Urinary suPAR concentrations. $^{***}P < 0.001$ for LPS-injected mice at 48 h versus 0 h, and versus PBS control at any time point. $^{**}P < 0.01$ for LPS-injected mice at 24 h versus 0 h. **(f)** Generation of a hybrid-kidney mouse model. **(g)** Electron microscope analysis of the PBS ($n = 3$) or LPS ($n = 5$) treated hybrid kidney. **(h)** uPAR expression in the native or *Plaur*^{-/-} kidneys from the hybrid-kidney mice with or without LPS treatment. Scale bars, 30 μ m in **b,c** and **h**; 250 nm in **g**. Error bars, means \pm s.e.m. in **a**; means \pm s.d. in **d,e**.

incubated with serum from healthy subjects ($n = 5$) or with pretransplantation serum from subjects with nonrecurrent ($n = 10$) and recurrent FSGS ($n = 15$). We found that incubation with recurrent FSGS pretransplantation serum significantly elevated β_3 integrin activity compared to serum from subjects with nonrecurrent FSGS or from healthy subjects (Fig. 3a). In general, we found that suPAR concentrations correlate well with the activity of podocyte β_3 integrin (Fig. 3a). We then explored whether inhibiting suPAR could lower AP5 activity on podocytes. Indeed, we found that co-incubation of serum from subjects with recurrent FSGS with cycloRGDFV or with antibodies specific to uPAR resulted in a significant reduction of podocyte β_3 integrin activity (Fig. 3b).

The current standard of care for treating recurrent FSGS is (repetitive) plasmapheresis, in which each treatment usually consists of a 1.5-liter plasma volume that is pheresed before replacement with 5% (vol/vol) albumin³⁴. To test whether suPAR could be removed by plasmapheresis, we collected serum from subjects with recurrent FSGS immediately before and after a single course of plasmapheresis and analyzed suPAR concentrations. We found that plasmapheresis could significantly reduce suPAR serum concentrations in subjects with FSGS (Fig. 3c). We then studied the effects of before- and after-pheresis serum samples from subjects with FSGS on podocyte β_3 integrin activity by measuring AP5 signal. We found that plasmapheresis could significantly lower podocyte β_3 integrin activity caused by incubation of podocytes with serum from subjects with FSGS (Fig. 3d). To further understand the effects of plasmapheresis on patient clinical outcome, we studied four clinical cases of recurrent patients with FSGS who received plasmapheresis after transplantation (Fig. 3e-h). All patients had elevated suPAR serum concentrations before transplantation. After serial plasmapheresis treatments, we found

that two patients reached a clinical remission; their serum suPAR concentrations fell below 2,000 pg ml⁻¹, and, notably, their serum also lost the capacity to induce podocyte β_3 integrin activity (Fig. 3e,f). In contrast, the other two patients (Fig. 3g,h) remained in recurrence despite plasmapheresis. Their serum suPAR concentrations remained elevated and their sera still caused strong podocyte β_3 integrin activity (Fig. 3g,h). These findings suggest that the disease-stabilizing effects of plasmapheresis depend on lowering individual serum suPAR to levels that sharply decrease podocyte β_3 integrin activity.

Mouse models showing that suPAR causes proteinuria and FSGS

To determine whether suPAR is a cause or a consequence of FSGS, we established three different mouse models: (i) uPAR-knockout (*Plaur*^{-/-}) mice injected with recombinant suPAR, (ii) hybrid-transplant mice modeling endogenous suPAR release and (iii) genetically engineered wild-type mice that drive expression of a suPAR plasmid in the skin, leading to increased serum suPAR concentrations.

First, we examined whether exogenous circulating suPAR could deposit into kidneys and cause albuminuria. We used *Plaur*^{-/-} mice and injected escalating doses of recombinant mouse suPAR protein intravenously into *Plaur*^{-/-} mice. We found that low-dose injection at 2 and 10 μ g did not cause albuminuria, which is consistent with the physiological low concentrations of suPAR we observed in the blood of healthy subjects (Fig. 4a). However, we found that doses of 20 μ g and greater led to induction of albuminuria within 24 h (Fig. 4a); albuminuria resolved within 2–3 days (data not shown). When we studied the kidneys of suPAR-injected *Plaur*^{-/-} mice, we observed a prominent deposition of suPAR along the podocytes of the *Plaur*^{-/-} mice that had received 20 μ g of suPAR, but we did not see this in the mice that received only 2 μ g (Fig. 4b). Moreover, we found

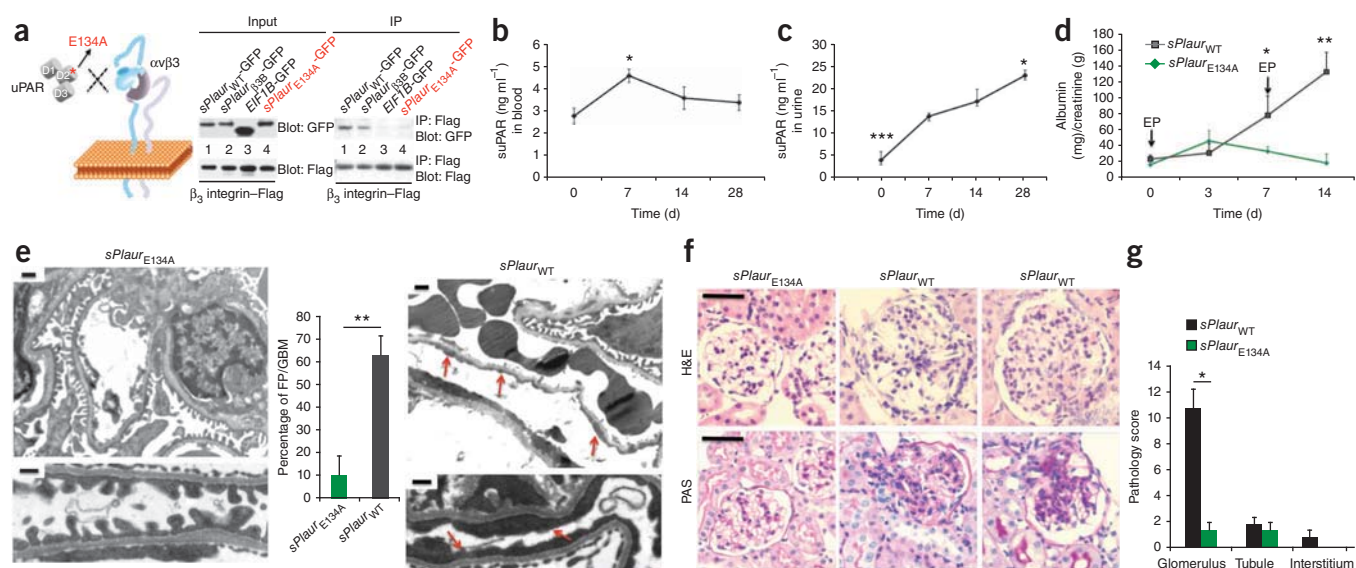


Figure 5 Sustained overexpression of suPAR in the blood of wild-type mice leads to an FSGS-like glomerulopathy. (a) Generation of β_3 integrin binding-deficient suPAR mutants. (b) Serum suPAR concentrations in the *sPlaur_{WT}* engineered mice. * $P < 0.05$ at day 7 versus day 0 (before initial electroporation) (c) Urinary suPAR in *sPlaur_{WT}* engineered mice. *** $P < 0.001$ for days 7, 14 and 28 versus day 0; * $P < 0.05$ for day 28 versus day 7. ($n = 4$ in each group). (d) Albuminuria in *sPlaur_{WT}* and *sPlaur_{E134A}* mice. * $P < 0.05$ for *sPlaur_{WT}* mice at day 7 versus before treatment or versus *sPlaur_{E134A}* mice at day 7. ** $P < 0.01$ for *sPlaur_{WT}* engineered mice at day 14 versus before treatment or versus *sPlaur_{E134A}* treated mice at day 7 or 14. (e) Kidney EM analysis of *sPlaur* engineered mice. Podocyte damage is reflected by the length of effaced foot process (FP) to the total length of the glomerular basement membrane (GBM) analyzed. Scale bars, 1 μm for upper image, 250 nm for lower image. ** $P < 0.01$. (f) Histochemistry and light microscopy of the kidney from *sPlaur* engineered mice. PAS, periodic acid–Schiff. Scale bars, 30 μm . (g) Histopathological alteration of the kidneys was semiquantitatively scored. * $P < 0.05$. Error bars, means \pm s.e.m. in d; means \pm s.d. in b, c, e and g.

that this deposition was associated with an increase in β_3 integrin activity in podocytes, as shown by increased AP5 labeling that, again, is suPAR dose dependent (Fig. 4c). Next, we studied whether an increase of endogenous suPAR causes kidney disease in wild-type mice. Lipopolysaccharide (LPS) has been shown to increase suPAR in the blood of human subjects through release from monocytes³⁵. Thus, we tested whether LPS could also enhance suPAR concentrations in the blood of mice. Indeed, we found that LPS injection causes a strong increase of suPAR in mouse serum (Fig. 4d) and urine, up to fivefold greater than concentrations observed in PBS-injected control mice (Fig. 4e).

Second, we generated kidney hybrid mice in which we removed one kidney from wild-type mice and engrafted a *Plaur^{-/-}* kidney (Fig. 4f). These mice fully recovered within 14 d after surgery and had normal renal function (data not shown) and structure (Fig. 4g). We injected five hybrid-kidney mice with a single low dose of LPS to stimulate suPAR release from circulating blood cells into the serum. Twenty-four h later, we found suPAR in glomeruli of the *Plaur^{-/-}* kidneys, thereby showing that entry from extrarenal sources can occur after transplantation (Fig. 4h). Moreover, we showed that there is prominent podocyte foot process effacement in both the *Plaur^{-/-}* and the wild-type kidneys (Fig. 4g). Because *Plaur^{-/-}* mice are generally protected from LPS-induced proteinuria and podocyte effacement¹⁸, we suggest that the podocyte effacement of the *Plaur^{-/-}* graft is best explained by deposited suPAR that stems from the wild-type host, thus leading to excessive podocyte β_3 integrin activation in the graft.

Third, to explore whether prolonged elevation of suPAR in the serum of mice causes a progressive glomerulopathy, we engineered wild-type mice that drive expression of suPAR in the skin. We generated a mouse plasmid (*sPlaur_{WT}*) based on a known coding sequence for secreted suPAR²⁶ that contains the D_I and D_{II} domains.

We delivered this plasmid into mice by *in vivo* electroporation into the skin. As a control, we generated a β_3 integrin binding-deficient suPAR mutant, *sPlaur_{E134A}*. This mutant has a point mutation (E134A) in the D_{II} domain (Fig. 5a). Both forms of mouse suPAR express equally well in the skin of mice after electroporation (data not shown). Notably, we found that suPAR concentrations in serum and urine start to rise 2 d after electroporation (Fig. 5b,c). We repeated electroporation once a week to achieve a sustained elevation of blood suPAR concentrations over the course of the analyzed time period (Fig. 5b). Coinciding with the rise of suPAR in mouse serum, we observed an induction of albuminuria that persisted over the course of the analyzed 4 weeks (Fig. 5d). Of note, we found that the mice that expressed *sPlaur_{E134A}* did not become albuminuric, suggesting that binding of suPAR to β_3 integrin is an important characteristic of suPAR-induced renal injury (Fig. 5d).

We next studied the ultrastructure of podocytes after 4 weeks and noted prominent foot process effacement consistent with glomerular disease; however, we only observed this in mice that expressed suPAR capable of binding β_3 integrin (Fig. 5e). To study whether the suPAR-induced glomerulopathy behaves more like MCD or FSGS, we analyzed the kidneys by light microscopy and histochemistry. We observed abnormalities in kidney morphology as early as 2 weeks after initial suPAR gene overexpression and found that they were aggravated by 4 weeks. By light microscopy, we found features of a progressive glomerulopathy, including hypercellularity, mesangial expansion, mesangiolysis and occasional tuft adhesions (Fig. 5f). Of note, we did not detect immune-complex deposition in any of the mice analyzed. The blinded, semiquantitative histopathological scoring³⁶ revealed indices of a progressive glomerulopathy reminiscent of early FSGS (Fig. 5g). Notably, we found that these changes were absent in mice expressing *sPlaur_{E134A}*, which is incapable of β_3 integrin binding (Fig. 5f,g).

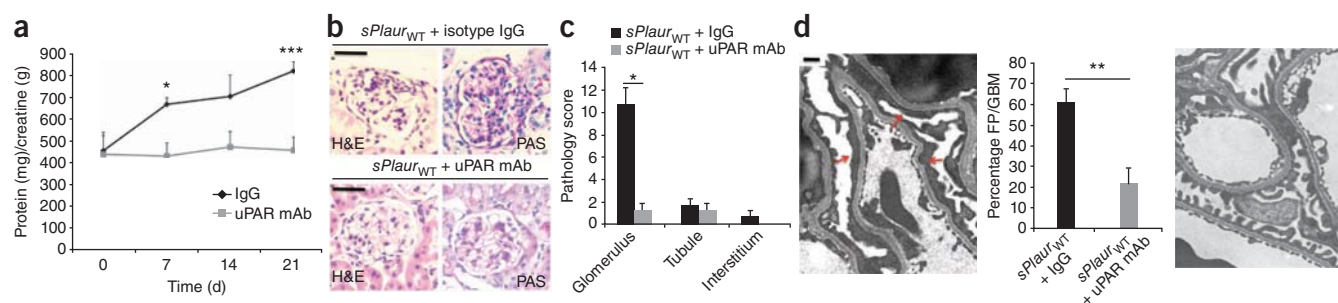


Figure 6 Administration of blocking antibody to uPAR ameliorates suPAR-caused kidney damage. ($n = 4$ in each group). **(a)** Proteinuria in the antibody treated *sPlaur_{WT}* mice. * $P < 0.05$ for *sPlaur_{WT}* mice receiving isotype control at day 7 versus before initial electroporation at day 0 or versus mice treated with antibody to uPAR at day 7; *** $P < 0.01$ for *sPlaur_{WT}* mice receiving isotype control at day 21 versus at day 0 or versus antibody to uPAR-treated mice at day 21. **(b)** Morphological examination of the antibody treated *sPlaur_{WT}* kidney. Scale bars, 30 μm . **(c)** Pathology score. * $P < 0.05$. **(d)** Electron microscopic analysis of the antibody treated kidney from *sPlaur_{WT}* engineered mice. ** $P < 0.01$ for IgG isotype control versus uPAR-specific antibody-treated *sPlaur_{WT}* engineered mice with respect to the ratio of effaced foot process (FP) to total GBM length measured. Scale bar, 360 nm. Error bars, means \pm s.e.m.

To further study the disease-causing effects of suPAR, we also carried out experiments that blocked suPAR action. We administered an uPAR-specific monoclonal antibody to mice expressing *sPlaur_{WT}* and found protection of proteinuria whereas proteinuria was high when using an IgG isotype control (Fig. 6a). Our examination of the kidneys in mice that received 4 weeks of uPAR-specific antibody treatment indicated improved morphology and histopathology scores compared to those animals that received isotype control antibodies (Fig. 6b,c). Moreover, semiquantitative electron-microscopic analysis showed significantly improved podocyte foot process structures in the uPAR-specific antibody treatment group, in contrast to the *sPlaur_{WT}* mice that received control IgG and that developed foot process effacement (Fig. 6d). Taken together, this data suggests that neutralization of suPAR action can improve suPAR-induced renal injury.

DISCUSSION

The present study identifies suPAR as a circulating, causative FSGS factor that is elevated in the serum of approximately two-thirds of primary FSGS patients. suPAR-mediated activation of β_3 integrin on podocyte foot processes is the mechanism of injury caused by high suPAR blood concentrations. Since the first clinical description of nephrotic syndrome recurrence after kidney transplantation³⁷, there has been mounting evidence suggesting the presence of a circulating permeability factor both for native and transplant FSGS^{8–17}. Although others have proposed the existence, in subjects with FSGS, of a 30- to 50-kDa glycoprotein that could be removed by plasmapheresis³⁸, the molecular identity and the mechanisms of action have not yet been elucidated. On the basis of our previous work, in which we showed that podocyte-produced membrane-bound uPAR is induced in FSGS and diabetic nephropathy to pathologically activate β_3 integrin, thereby causing foot process effacement and proteinuria¹⁸, we examined the role of circulating suPAR in idiopathic FSGS. The analysis of human serum samples in a glomerular disease cohort showed elevated serum concentrations of suPAR in a population of pediatric and adult FSGS patients. Furthermore, our studies of mouse models with engineered serum suPAR overexpression showed the development of a renal disease characteristic of FSGS. High pretransplantation serum suPAR concentrations are associated with the presence of native FSGS and also constitute a significantly increased risk for recurrent FSGS after transplantation. One year after kidney transplantation, suPAR concentrations remained significantly elevated in patients who developed FSGS recurrence.

The amount of podocyte β_3 integrin activity that is driven by circulating systemic suPAR depends on the amount of individual serum

suPAR and, possibly, also on suPAR post-translational modifications (such as glycosylation status). In addition, podocyte β_3 integrin activity can also be driven by augmented podocyte uPAR expression, which is sufficient to initiate podocyte foot process effacement and proteinuria¹⁸. Podocyte β_3 integrin activity seems to be independent of total serum uPA concentrations; this is in contrast to the suPAR-uPA associations in some forms of cancer^{19,27}. The exact differences of FSGS-causing suPAR and cancer-associated suPAR will likely be a key focus of future studies.

Several modes of interference can protect from suPAR-mediated podocyte injury: (i) blockade of suPAR using a blocking antibody specific to suPAR; (ii) protecting β_3 integrin from increased activation by cycloRGDFv or β_3 integrin-specific antibody¹⁸; (iii) blocking suPAR- β_3 integrin interaction by modulating the suPAR- β_3 integrin binding site (E134A) and (iv) removing suPAR by plasmapheresis to levels that decrease podocyte β_3 integrin activity.

Using assays that measure all suPAR forms, we noted that ~70% of subjects with primary FSGS presented with significantly elevated concentrations of serum suPAR before transplantation when compared to other primary glomerulopathies. In addition, we found that total suPAR concentrations remained significantly elevated after kidney transplantation in people who have developed recurrent FSGS compared to those with proper renal function. On the basis of these clinical observations, we created mouse models that could explore the cause or effect nature of suPAR and demonstrate the kidney pathogenicity of elevated systemic suPAR. Notably, we found different forms of suPAR that correspond to different domain fragments in the serum of subjects with FSGS, with molecular weights ranging from 22 to 45 kDa. This is close to the molecular range (30 to 50 kDa) of the factor predicted by others¹⁵.

Our study provides the rationale for a more measurable prediction of FSGS risk in subjects with FSGS before and after transplantation. Approximately 70% of subjects with FSGS have elevated concentrations of suPAR compared to other glomerular diseases such as membranous nephropathy, MCD or preeclampsia. This further separates FSGS from other glomerulopathies involving phospholipase A2 receptor-specific antibodies in membranous nephropathy³⁹ and factors such as angiotensin-like⁴⁰ or c-mip in MCD⁴¹. Because suPAR is detectable both in healthy human subjects and normal mice, physiological suPAR concentrations or physiological suPAR domain combinations do not seem to be harmful. It is also important to note that there might be species differences with respect to the pathogenic strength of various suPAR domain combinations. Future studies with new and more

specific suPAR domain-specific antibodies should clarify this question and focus more on the role of suPAR glycosylation in FSGS.

Another interesting question is why a few FSGS patients without elevated suPAR still develop FSGS as well as recurrent FSGS. An obvious answer would be that suPAR can act in concert with podocyte uPAR¹⁸ and this might drive FSGS even in the absence of high suPAR concentrations. Another reason might be that native FSGS is caused by a mutation in a podocyte gene⁶. Also, the current ELISA assay for serum suPAR is likely to measure all suPAR domains, and thus it might be possible that FSGS subjects with low total suPAR do have a higher portion of pathological suPAR fragments that current tests cannot readily detect. Once new reagents are developed, even more subjects with FSGS might test positive for pathological suPAR, thereby further increasing the clinical prediction of the test. Alternatively, there is the possibility of the presence of yet-to-be-identified additional permeability factor candidates¹⁷ or the absence of protective podocyte factors⁴².

Podocyte β_3 integrin expression and activation responses must also be evaluated further. Future studies will have to focus more on the expression of the β_3 integrin-encoding gene (*ITGB3*) in the graft⁴³ or consider genetic polymorphisms of *ITGB3* such as the platelet antigen 2 (PI^{A2}) polymorphism⁴⁴. The latter has been shown to facilitate β_3 integrin activation⁴⁴. It will have to be tested to see whether the presence of this polymorphism also contributes to the development of recurrent FSGS. Notably, recipients of kidney transplants that are positive for the PI^{A2} polymorphism have been identified as carrying extra risk for acute renal graft rejection, thus affecting short-term graft survival⁴⁵. Moreover, the prevalence of this polymorphism is increased in dialysis patients⁴⁶, suggesting that PI^{A2} status may have a general role in renal diseases.

In conclusion, we show that suPAR is a circulating factor that can cause FSGS before and after transplantation. Our studies will allow better risk stratification of patients with FSGS by measuring serum and urine concentrations of suPAR, and they will provide the conceptual framework for refined treatment options that remove or neutralize suPAR to a level insufficient to activate podocyte β_3 integrin. Regardless of the source of the stimulant (podocyte or systemic), a pathological activation of podocyte β_3 integrin is emerging as a key event for the initiation of proteinuric glomerular disease; it is likely to be important in some forms of secondary FSGS, such as diabetic nephropathy¹⁸, as well. Accordingly, pharmacological modulation of excessive podocyte β_3 integrin activation is a promising target for achieving protection from renal disease.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

J.R. conceived the study. J.R. and C.W. designed the experiments, coordinated the study, analyzed the data and wrote the manuscript. C.W., S.E.H., J.L., D.M., Q.Z., B.N., P.D., V.G. performed the experiments. A.F., N.G., G.B., J.S., S.A.K., H.-K.Y., M.Saleem, A.C., E.S., A.T., M.Salifu, M.M.S., F.S., C.M., V.S., M.Z., D.R., M.P.R., P.R., J.R. contributed to clinical samples and clinical information. M.P.R. and P.R. provided pathology service.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

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ONLINE METHODS

Human subjects. We studied 78 human subjects with FSGS, 25 with MCD, 7 preeclampsia, 16 with membranous nephropathy and 22 healthy subjects (**Supplementary Tables 2 and 3, Supplementary Methods**). Study samples were provided by the following institutions and sample collection was approved by the participating Institutional Review Boards, either by informed consent (Boston University Medical Center/Boston Medical Center, Beth Israel Deaconess Medical Center, Stanford University, National Healthcare Groups (Domain-Specific Review Boards), and SUNY Downstate Medical Center) or by obtaining a consent waiver (University of Miami, Massachusetts General Hospital).

Serum suPAR measurement. We measured serum suPAR with the Quantikine Human suPAR Immunoassay (R&D Systems) as well as with an in-house ELISA kit⁴⁷.

Injection of recombinant suPAR into *Plaur*^{-/-} mice. We injected different doses of recombinant mouse suPAR protein (R&D Systems) i.v. into female *Plaur*^{-/-} mice, and collected urine before and after suPAR injection for albumin and creatinine analysis. Twenty-four hours after injection, we killed the *Plaur*^{-/-} mice and snap-froze the kidneys for immunofluorescence assays. Animal experiments were carried out at Massachusetts General Hospital and/or the University of Miami with prior approval by the Subcommittee on Research Animal Care (Massachusetts General Hospital) or the Institutional Animal Care and Use Committee (University of Miami).

Hybrid-kidney mouse, transplantation and LPS-mediated suPAR release. To determine if endogenously induced suPAR cause podocyte injury, we established a hybrid-kidney transplantation mouse model ($n = 10$). The right kidney was harvested *en bloc* from female *Plaur*^{-/-} mice, and we designated this *Plaur*^{-/-} kidney as the donor kidney. We used the female wild-type mice with the native right kidney removed as the recipients. Fourteen days after surgery, we observed no rejection in any of the transplanted mice. We killed two mice to analyze native and transplanted kidneys. We treated five hybrid-kidney mice with LPS (Sigma) i.p. at 10 mg kg⁻¹ body weight, to induce elevated suPAR concentrations in the blood, whereas three hybrid-kidney mice received the same amount of PBS (Boston BioProducts) as controls. Twenty-four hours after LPS treatment, we killed the hybrid-kidney mice and cut out both the native and transplant kidneys for analysis.

Sustained suPAR overexpression model. To investigate whether sustained elevation of suPAR causes FSGS, we expressed a plasmid, *sPlaur*_{WT} encoding mouse suPAR (domain DI-DII, Genbank accession no. BC010309) in female wild-type mice using *in vivo* gene delivery. With mice under anesthesia, we injected *sPlaur*_{WT} plasmid (40 µg in PBS) intradermally into the leg, followed by *in vivo* electroporation with Derma Vax DNA delivery system (Cyto Pulse Sciences). As control we generated a plasmid, *sPlaur*_{E134A}, which expresses a suPAR point mutant deficient in binding β₃ integrin (**Supplementary Methods**). We did gene delivery once a week for up to 4 weeks. We collected blood and urine before and after each gene delivery for analysis.

Immunohistochemistry and immunofluorescence. To analyze the activity of glomerular β₃ integrin in humans, we did immunohistochemistry with the active β₃ integrin-specific murine monoclonal antibody AP5 (GTI Diagnostics, cat. no. GTI-N7P, 1:50) on kidneys affected by primary FSGS ($n = 9$), recurrent FSGS ($n = 6$), MCD ($n = 5$), membranous nephropathy ($n = 5$) and on the healthy pole of tumor-nephrectomized kidneys ($n = 3$). For immunofluorescence assays of cultured human podocytes or the cryosection of mouse kidneys, we followed previously described procedures¹⁸.

Immunoprecipitation and western blotting. Coimmunoprecipitation and western blotting were done to examine the interaction between suPAR and β₃ integrin according to our previously established protocols¹⁸.

Light microscopy and histochemistry. Mouse kidney tissues were semi-quantitatively analyzed in a blinded fashion (**Supplementary Methods**)³⁶.

Statistical analyses. We did statistical analyses by one-way analysis of variance (ANOVA) or Student's paired or nonpaired *t* test. We rejected the null hypothesis at a *P* value of 0.05. Values are presented as means ± s.d. unless otherwise stated.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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