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Further Evidence that the Soluble Urokinase Plasminogen Activator Receptor Does Not Directly Injure Mice or Human Podocytes

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Abstract

Background—The role of the soluble urokinase plasminogen activator (suPAR) in focal segmental glomerulosclerosis (FSGS) as the circulating factor or as a predictor of recurrence after transplantation remains controversial. Previously published studies in mice and isolated podocytes produced conflicting results on the effect of suPAR on podocyte injury, effacement of foot processes and proteinuria. These discordant results were in part due to diverse experimental designs and different strains of mice. The aim of our study was to determine the reasons for the

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inconsistencies of the previous studies results with suPAR by using uniform methods and studies in different strains of mice.

Methods—We utilized a primary culture of human podocytes and two mouse models, the wild type (WT) and the uPAR KO (uPAR−/−), in an attempt to resolve the reported conflicting results.

Results—In both wild-type and uPAR−/− mouse models, injection of recombinant uPAR, even at a high dose (100 μg), did not induce proteinuria, effacement of podocytes or disruption of the cytoskeleton. Injection of suPAR resulted in its deposition exclusively in the glomerular endothelial cells and not in the podocytes of WT mice, and was not detected at the uPAR KO mice. Kidneys from patients with recurrent FSGS had negative immunostaining for uPAR.

We also evaluated the effect of recombinant uPAR on primary culture of human podocytes. uPAR did not result in podocytes damage.

Conclusions—suPAR by itself is not the cause for direct podocyte injury, in vitro or in vivo These findings suggest a more complex and still poorly understood role of suPAR in FSGS.

Introduction

There is considerable evidence that one or more circulating plasma factors are involved in the pathogenesis of primary focal segmental glomerulosclerosis (FSGS) without podocyte gene mutations. The soluble identification of the circulating factor has been postulated for many years and has been elusive.[1–4] The soluble receptor of urokinase (suPAR) has been suggested as a possible causative factor in FSGS; however, the role of suPAR in FSGS and recurrent FSGS remains controversial.[5–8]

uPAR is a glycolipid-anchored cell surface receptor for urokinase plasminogen activator. The receptor is also involved in nonproteolytic pathways, mainly through its ability to form signaling complexes with other transmembrane proteins such as integrins, caveolin, and G protein-coupled receptors.[9] Through these signaling pathways, the versatile uPAR receptor has important roles in inflammation, adhesion, proliferation, and mobilization as well as in severe pathological conditions such as in malignancies.[10–15]

In the kidney, Wei et al showed that suPAR binds and activates the beta 3 integrin leading to downstream activation of GTPase that decrease podocyte stress fibers and results in effacement of podocytes and proteinuria.[5] In mice studies, suPAR infusion induced changes of FSGS, which could be prevented by co-administration of anti-uPAR antibodies. However, two well designed separate studies performed in mice failed to show that infusions of suPAR resulted in proteinuria and or effacement of podocytes.[21,22] A critique of these two studies was that suPAR was infused in wild type mice while the experiments performed by Wei, et al utilize uPAR KO recombinant mice (uPAR−/−). Therefore, we embarked on experiments to repeat the studies of Wei et al and use the uPAR−/− mice to determine if we could duplicate their observations on the role of suPAR on podocyte injury. We also extended the studies to evaluate the effect of suPAR on podocytes isolated from human glomeruli. Given that recent publications[16–19] showing synergistic podocytopathic effects of suPAR and anti-CD40 autoantibody, we also co-injected human suPAR and human CD40-autoantibody

isolated from the sera of patients with recurrent FSGS after kidney transplantation, into wild type mice, using the same injection regimen as previously used by Wei et al.[19]

Materials and Methods

All chemicals were manufactured by Sigma (St. Louis, MO), and cell culture and fluorescent detection reagents were obtained from ThermoFisher Scientific (Waltham, MA), unless otherwise stated. Solvents were of analytical grade or higher. uPAR antagonists for blocking shuPAR and stimulation with mouse/human uPAR were performed using recombinant proteins from R&D Systems (Minneapolis, MN). For high content analysis of podocytes health and morphology, we employed rabbit anti-vinculin (ThermoFisher Scientific, MA, 1:500) coupled to Alexa Fluor 647 conjugated goat anti-rabbit IgG, Alexa 488 conjugated phalloidin, and Hoechst 33342. AP5 mouse monoclonal antibody used to detect activated beta3 integrin was obtained from the BloodCenter of Wisconsin (Milwaukee, WI).

Animals, Maintenance, and Euthanasia

All animal studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication number 85–23, revised 1985) and in accordance with a University of California, San Francisco (UCSF; San Francisco, CA) Institutional Animal Care and Use Committee protocol. WT C57BL/6 mice (6–9 weeks old) were obtained from Harlen Laboratories. uPAR deficient mice[20] were kindly donated by Dr. Thomas Bugge, NIH. Mice were kept in specific pathogen-free conditions under constant environmental conditions (22°C, 12 h light/dark cycles) and fed with standard laboratory chow and 3% sucrose enriched water. Euthanasia of the sedated mice was performed by cervical dislocation.

Injection of recombinant muPAR into WT mice or uPAR−/− mice

C57BL/6 mice were injected with 0, 20 or 100 μg/mouse of smuPAR-Fc chimera (mouse uPAR linked to a human IgG1) i.v. as described before.[5,21,22] uPAR deficient mice were injected with 100 μg/mouse of recombinant mouse smuPAR-Fc protein. As negative controls, mice were injected with Fc and vehicle PBS. Experiment design is illustrated in Figure 1A. Blood and urine were collected at 0, 10 and 24 hr. and analyzed for smuPAR, albumin and creatinine levels (Exocell Inc assays). smuPAR-Fc concentrations in the serum and blood were determined using a smuPAR ELISA. A standard curve was generated by adding the indicated amounts of purified recombinant smuPAR-Fc protein to serum or urine from uPAR−/− mice as described before.[22] mice were sacrificed 24 hours after injection and kidneys were retrieved.

Injection of human CD40 auto-antibody, and human suPAR into C57BL/6 mice

As suPAR injection in wild type mice did not show proteinuria, we further examined if “priming” with CD40 auto-antibody (isolated from sera of patients with recurrent FSGS after kidney transplantation), could result in proteinuria after suPAR injection. To examine this we used the injection protocol previously used by Wei et al for both agents and human CD40 auto-antibody was injected daily for 6 doses followed by a single dose of suPAR.[19]

Immunohistochemistry (uPAR, IgG1, Integrin Beta 3) and electron microscopy on mouse kidneys

Immunostains for uPAR were performed on formalin-fixed paraffin-embedded (FFPE) mouse kidneys using standardized immunoperoxidase protocol with goat anti-mouse uPAR antibody (AF534, R&D System) following antigen retrieval at 100°C in pH 8.0, a secondary antibody Rabbit anti-goat HRP, and BOND Polymer detection RTU KIT with mouse HRP Polymer (Abcam) and DAB for detection. The IgG1 immunostain was performed on mouse FFPE kidneys with an anti-human IgG1 antibody (RevMAb Biosciences) using a standardized indirect immunofluorescence technology; human FFPE tonsil tissue was used as positive control. Immunostains against Integrin Beta 3 were performed on mouse frozen kidneys with an anti-Integrin Beta 3 (AP-5) antibody (Richard H. Aster, Blood Center of Wisconsin) using a standardized immunofluorescence protocol. All immunostains were preformed on a Leica Bond RX Autostainer platform. For electron microscopy, ultrathin (80 nm) sections of the glutaraldehyde-fixed, Epon-embedded mouse kidneys were stained with 2% uranyl acetate. Sections were examined in a Tecnai G212 transmission electron microscope at 80 kV, with images obtained with a Hammamatsu camera model Orca HR.

Immunohistochemistry (uPAR) on human kidney biopsies

Immunostains for uPAR were performed on human frozen kidney biopsies using a mouse monoclonal antibody to uPAR (DAKO, M7294) using standardized indirect immunofluorescence and immunoperoxidase protocols on a Leica Bond RX Autostainer platform. Study groups included native biopsies with FSGS (n=10), early post-Transplant recurrent FSGS prior to (n=15) and post plasmapheresis (n=7). Native biopsies with membranous nephropathy (MN) (n=10) and minimal change disease (MCD (n=5), and normal 6 month post-Transplant protocol biopsies [n=10] served as kidney controls. Human lung and endometrial cancer specimens were used as positive controls.

Isolation, culture and automated microscopy of primary human podocytes

Primary human podocytes were isolated from fresh normal tissues taken from surgically removed kidneys and cultured by a method employed previously.[23] We used previously described procedures to plate, process and analyze podocytes using high content analysis.[23]

Human podocyte injury via examination of morphology and viability

Known triggers of podocyte injury, LPS and puromycin aminonucleoside, were used as positive podocyte injury control. Culture medium was used for negative control. Injury was measured using high content analysis, which compared changes following serial dilutions of shuPAR on podocyte morphology. This was quantified by the adherent cell count and the morphology of the podocyte nucleus (Figure 3A–F).

Beta3 integrin activation of human podocyte primary culture

Human podocytes were pretreated with suPAR 1–0.0039 μg/ml for 24 hours or with 50mM EDTA for 1 hour as positive control. The activated beta3 integrin was detected by the AP5 antibody. The AP5 antibody was diluted in PBS without Ca^{2+} or Mg^{2+}, cells were independently labeled using the AP5 mouse monoclonal antibody at a 1:100 dilution of the

stock concentration followed by an Alexa 488 conjugated goat anti-mouse antibody. The Spot Detector BioApplication was used to measure total AP5 Ab signal/cell in peripheral adhesions as integrated brightness of all AP5 foci per cell.

Statistical analysis

Quantitative variables were expressed as the mean ± std. error of Mean, and the qualitative variables were expressed as a proportion. Groups' distributions were compared utilizing an exact version of the Wilcoxson rank sum test. Statistical significance was defined as $p < 0.05$.

Results

Recombinant smuPAR did not induce proteinuria in WT mice and did not cause proteinuria in uPAR deficient mice

We administered (i.v) a commercially available uPAR-Fc chimera (smuPAR linked to a human IgG1 Fc, R&D Systems) to both WT C57BL/6 and uPAR−/− mice.[20] WT mice were injected with two different doses of smuPAR-Fc chimera, 20 or 100 μg/mouse, and the uPAR deficient mice were injected with 100 μg /mouse. As controls, mice were injected with Fc (human IgG1 Fc, R&D Systems) and vehicle PBS. Experimental design is illustrated in Figure 1A. The functionality of the injected smuPAR-Fc was verified by the ability of the receptor recognition of its ligand, uPA, and the functionality of the bound ligand by enzyme assay. The enzymatic activity of the bound uPA was measured by fluorogenic assays using serial dilutions (0–625 ng/ml) of mouse uPA (Active Mouse Urokinase, Molecular Innovations, Inc., Figure 1B). No increase in urine protein excretion at 10 or 24 hr. was detected by urine albumin/creatinine ratio (ACR) analysis of WT smuPAR–Fc mice relative to controls (Figure 1C). Injection of 100 μg smuPAR-Fc chimera to uPAR−/− mice lead to proteinuria however, injection of Fc by itself was sufficient to induce proteinuria in those mice (Figure 1C). Circulating mouse uPAR concentrations in serum and urine were measured by ELISA (Figure 1D–E) against a recombinant smuPAR-Fc standard curve in serum or urine, respectively (Figure 1C) as described by Spinale et al.[22] Priming with human anti-CD40 autoantibody followed by an injection of human suPAR did result in a modest increase in proteinuria (Figure 1F).

Infused smuPAR-FC did not localize to podocytes

Immunohistochemistry (IHC) revealed weak baseline endothelial expression of uPAR in control WT mice. Stronger uPAR signal in the glomerular endothelial cells was observed following injection of smuPAR-FC to WT mice compared to controls (Figure 2A). The strength of the IHC signal correlated with the smuPAR-FC dose with stronger uPAR signal in the mice injected with 100 μg compared with those injected with 20 μg. uPAR IHC was negative in the kidneys of uPAR knockout mice and remained negative following injection of 100 μg of suPAR or Fc control (Figure 2A). The anti-IgG1 stains in WT FFPE mouse kidneys from animals injected with 100 ug msuPAR-Fc or with Fc alone were negative.

The results of the AP5 stains showed strong glomerular positivity with 100 ug dose and weak positivity with the 20 ug dose. However, the precise cellular localization of AP5 could

not be determined due to freezing artifacts and therefore it is not clear if a super high dose of uPAR-Fc is required to demonstrate AP5, a marker of ß3 integrin activation, or it reflects nonspecific staining. Electron microscopy analysis of the smuPAR-FC injected WT mouse kidney revealed minimal focal foot process effacement. No damage was detected in the uPAR −/− kidneys (Figure 2B).

Recombinant human suPAR (shuPAR) did not cause injury to primary human podocytes

It has been reported that suPAR alters podocyte morphology and function in cell culture and in animals. To evaluate whether suPAR directly mediates podocyte injury, we compared the effect of shuPAR to other known triggers of podocyte injury such as LPS and puromycin aminonucleoside or culture medium as the negative control (Figure 3). No difference was observed in cell viability between shuPAR-treated and untreated control podocytes by an MTS assay (Figure 3A). Injury was also measured using high content analysis comparing the changes following serial dilutions of shuPAR on podocyte morphology as measured by high content analysis of adherent cell count and nuclear morphology (Figure 3B–C). The positive controls, LPS and PAN, did affect podocyte viability, morphology and adhesion as expected. F-actin rearrangement and AP5 activation were not observed in the shuPAR treated podocytes (Figure 3D–F).

uPAR immunostaining of human kidneys

The uPAR immunostain in kidneys from patients with native kidney FSGS as well as in patients with early recurrence of FSGS (with demonstrated effacement of podocytes by EM) were negative. uPAR staining was also negative in patients with MCD and MN.

Discussion

The initial excitement associated with the publication of the study by Wei et al that suPAR was the circulating factor associated with recurrent FSGS has been replaced by skepticism and frustration.[5] The clinical findings of selective elevation of suPAR in FSGS patients and their ability to predict recurrence after transplantation has not been confirmed in several studies.[22] However, several factors could potentially account for the discordant results, including the degree of renal failure of the patients since the glomerular filtration rate is an important determinant of suPAR levels, ethnicity, the heterogenicity of the disease itself and methodology for measuring suPAR levels (although most, if not all studies have used the commercially available ELISA assay). In fact a critique of the suPAR studies is that the commercially available ELISA assay does not differentiate the different forms of circulating suPAR.[24] In a recently published study from our lab, we used a time-resolved fluorescent immunoassay (TR-FIA) to measure the different fragments of circulating suPAR in patients with recurrent FSGS to determine if a pathological fragment was present and could be associated or predictive of recurrence.[24] Similar to the results obtained with the ELISA assay, the TR-FIA of the free fragment of suPAR were not found to be predictive of post transplant recurrence.[24] Furthermore, concerns have been raised on results of the elegantly performed experiments in mice by Wei et al that showed a robust causal relationship between suPAR and the development of histologic and functional injury to the podocytes in

vivo. Two independent studies in mice failed to duplicate the findings of Wei et al that infusion of suPAR induces podocyte injury and proteinuria.

Cathelin D et al used two well-characterized recombinant forms of mouse suPAR produced by eukaryotic cells that were administered over the short and long term to WT mice.[21] suPAR was deposited in the glomeruli of mice but did not alter the podocytes histologically (i.e. foot effacement) or functionally (i.e. proteinuria). Spinale JM et al used the commercially available Fc-mouse suPAR used by Wei et al at the same concentration of 20 μg in WT mice.[22] The injected mice had a 6 to 12 fold increase in serum suPAR levels over 4 to 24 hours but did not develop proteinuria. An inducible transgenic mouse model that maintained elevated serum suPAR levels for six weeks did not also injure the glomeruli or induce proteinuria. Were the results of these two studies negative because both groups of investigators used WT rather than uPAR−/− mice? The investigators reasoned that unlike the uPAR −/−mice utilized in all the experiments by Wei et al, the WT mice are more physiologic and represent a better experimental model.

In a different set of experiments, Alfano et al showed that infusion of 20 μg of recombinant murine suPAR using uPAR−/− mice induced deposits of suPAR in the glomeruli and increased proteinuria.[25] In addition, Alfano et al observed down modulation of nephrin. The investigators also reported similar findings in vitro using immortalized podocytes: suPAR down regulated nephrin expression and this effect was blocked by adding an antagonist to the αVβ3 integrin, supportive evidence that downstream signaling requires suPAR binding to the αVβ3 integrin. These studies add more complexities to understanding the suPAR effect. Is the suPAR mediated injury then only reproducible in uPAR−/− mice and WT mice are resistant to the effect of high circulating levels of suPAR?

We performed our mice studies to specifically determine if any differences exist in response to suPAR infusions between WT and uPAR−/− mice. We administered smuPAR-Fc to mice with the same dose used in previous studies, 20 μg, as well as the higher dose of 100 μg. Neither dose produced effacement of podocytes or proteinuria in WT or uPAR−/− mice. By IHC the infused suPAR deposited within the glomeruli but only in the endothelium and not in podocytes and only in WT mice. We also could not demonstrate a difference in viability or morphology of the cytoskeleton of human podocytes treated with suPAR in contrast to the podocyte cytoskeleton disruption observed with LPS and puromycin. In contrast to previous reports, activation of Beta 3 integin signaling has not observed when shuPAR was added to human podocytes as AP5 antibody labeling did not show increased signals in focal adhesions (Figure 3F). Interestingly, a small surge in proteinuria was seen after suPAR was co-injected with anti CD40 autoantibody, isolated from the plasma of patients with recurrence FSGS, following a short course of prior priming with this antibody, which supports prior observations of multiple pathways possibly acting in synergy to drive podocyte injury. The exact role and pathogenetic mechanism of podocyte injury of CD40 antibodies require additional investigation. Delville et al showed that the presence of anti-CD40 antibodies predicted recurrence of FSGS post transplant and in vitro podocyte injury.[16] In fact a clinical trial with Bleselumab a humanized anti-CD40 monoclonal antibody which presumably neutralizes the pathogenic CD40 autoantibodies is underway in FSGS patients to prevent recurrence of the disease (Clinicaltrials.gov).

Our observation of a lack of immunostaining for uPAR in kidney diseases characterized by podocyte effacement further complicates our understanding of the role of suPAR on podocyte injury in FSGS.

Our studies in mice and in patients do not necessarily relegate suPAR to irrelevancy in FSGS. A follow up study in WT mice performed by Wei et al showed that podocyte effacement and proteinuria occurred when suPAR was co-administered with anti-CD40 antibodies isolated from plasma of patients with recurrent FSGS but not when only suPAR was infused. In fact, neither, suPAR or the anti-CD40 alone could produce podocyte injury. [19] In summary, suPAR's role in FSGS and recurrent FSGS as well as in experimental models is more complex than initially suggested. The synergistic dual effect of suPAR and anti-CD40 antibodies require additional studies. Before therapeutic interventions are initiated, a better understanding of the role of suPAR in FSGS and recurrent FSGS will be required.

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Abbreviations Page

ACR	albumin/creatinine urine ratio
FSGS	focal segmental glomerulosclerosis
IHC	immunohistochemistry
LPS	lipopolysaccharide
MTS	cell viability assay
PAN	puromycin aminonucleoside
uPAR	urokinase plasminogen activator receptor
shuPAR	soluble human recombinant uPAR
smuPAR-Fc	soluble mouse recombinant uPAR-Fc chimera
suPAR	soluble urokinase plasminogen activator receptor

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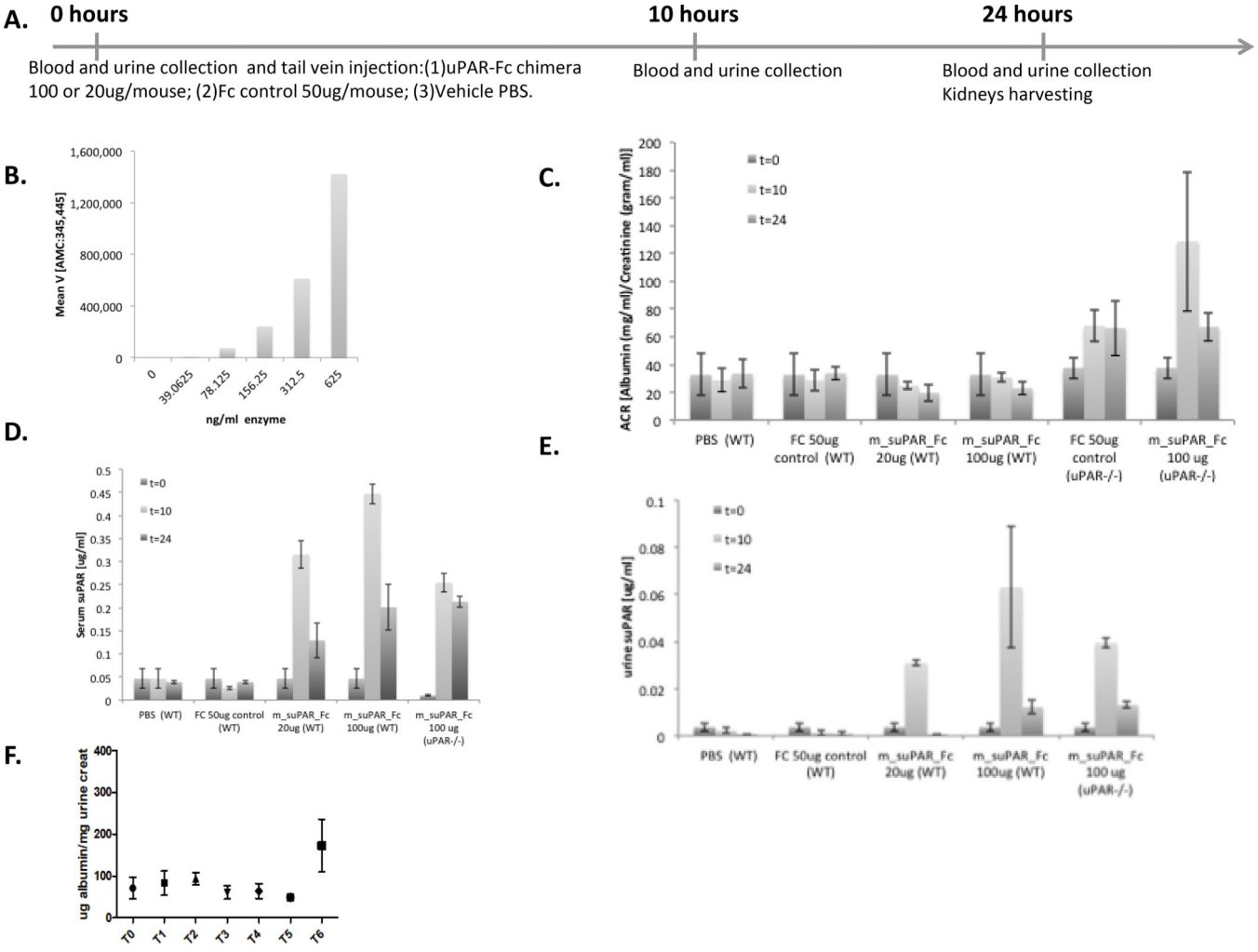

Figure 1: Administration of soluble murine urokinase-type plasminogen activator receptor (smuPAR-Fc) does not induce proteinuria in WT mice

(A) Scheme of experimental design. Recombinant smuPAR-Fc (mouse uPAR linked to a human IgG1, R&D Systems; 20 μg or 100 μg) was administered through tail vein injections to C57BL6 WT mice, As a control, mouse were injected with 100 μl of 50 μg of Fc and vehicle PBS. (B) SmuPAR-Fc injected to mice. Functionality was validated by the receptor ability to bind its ligand mouse urokinase (uPA) and the enzymatic activity of the bound uPAR. (C) Proteinuria was evaluated by analysis of urine albumin/creatinine ratio (ACR) at 10 and 24 hr. post smuPAR-Fc injection. Mean smuPAR-Fc concentrations were assayed using smuPAR ELISA in serum (D) and urine (E) of WT mice. (F) Mean urine albumin post co-injection of human suPAR and human CD40-autoantibody isolated from the sera of patients with recurrent FSGS after kidney transplantation, into wild type mice C57BL/6.

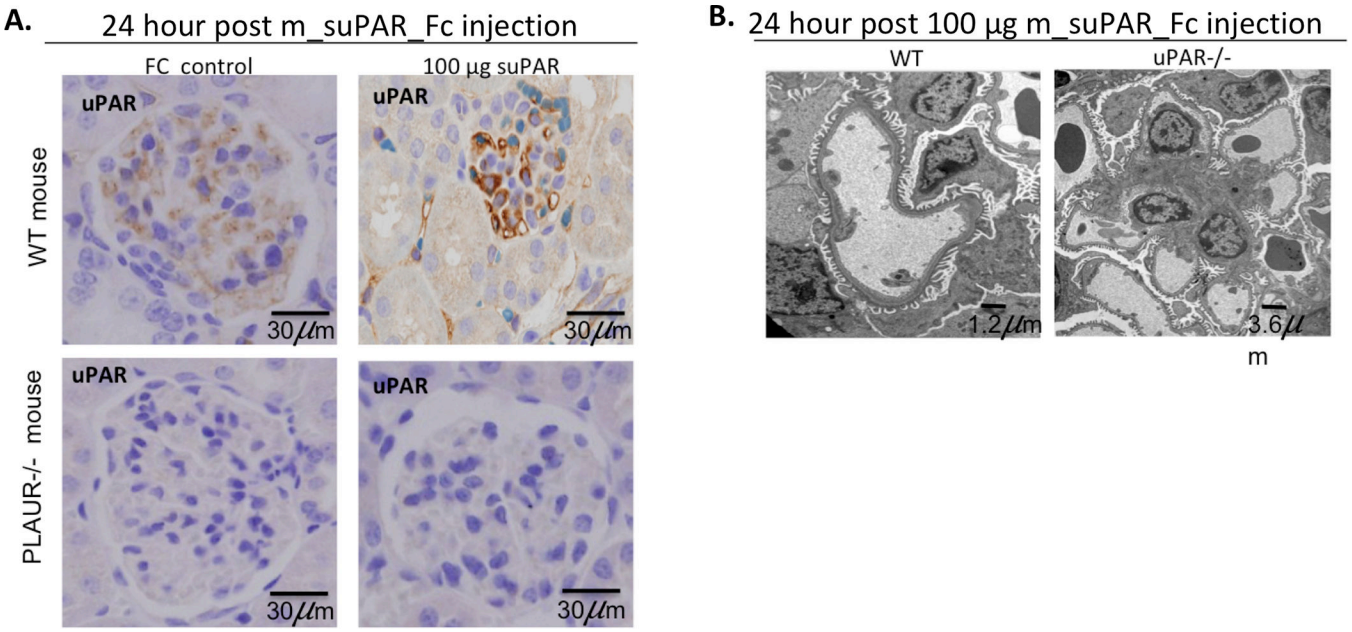

Figure 2: Immunohistochemical and Electron microscopic analysis of mouse kidneys

(A) Immunohistochemical staining of uPAR shows weaker glomerular endothelial positivity in WT mice injected with Fc vs. 100 µg of suPAR (x 400). (B) Electron microscopic analysis of 100 µg of suPAR treated kidney in WT and uPAR−/− mice (X 4000). Each group contained 4–5 mice aged 7–10 weeks. All values are expressed as mean±Std. Error of Mean $P<0.05$.

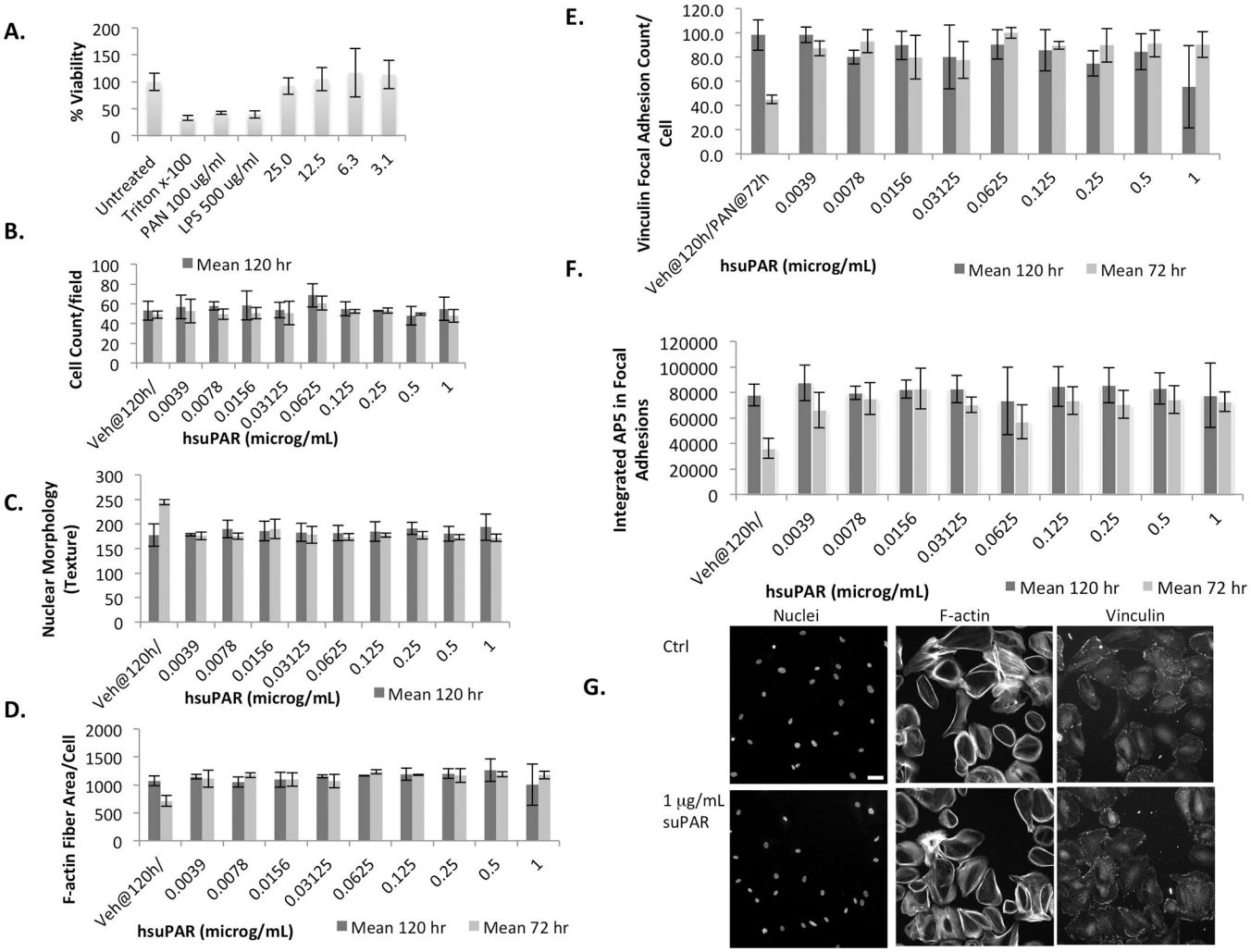

Figure 3: Treatment with recombinant shuPAR of primary human podocytes does not induce detectable specific podocyte injury

Podocytes cultured on sterile glass coverslips coated with type I collagen and were exposed to recombinant human uPAR. (A) 24 hr treatment of shuPAR (3.1–25 µg /ml, 24 h) does not affect human primary culture podocyte viability at any concentration from 3 to 25 µg/ml as evaluated by a colorimetric method for determining the number of viable cells, the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS assay. The effect of shuPAR (3.1–25 µg /ml, 24 h) was compared to other known triggers of podocyte injury such as LPS (1 µg/ml, 24 h) and puromycin aminonucleoside (PAN, 100 µg/ml, 24 h) or culture medium as the negative control. ShuPAR (3.9 ng/mL-1 µg/mL for 72 and 120 h) appears not to cause significant damage to human primary podocytes as evidenced by high content analysis of (B) adherent cell count, (C) nuclear morphology, (D) F-actin fiber area, (E) vinculin focal adhesion count and (F) integrated brightness of AP5 in focal adhesions. Graph bars indicate mean ± SD values from three independent experiments. (G) Representative immunefluorescent staining for nucleus (right panels), actin (middle panels) and vinculin (right panels) of primary human podocytes with podocytes, showing prominent actin stress fibers in the podocyte cell

body, however, no difference between media control or 1μg/ml shuPAR post (120 hr of treatment; Bar = 66 μm). Confocal images were obtained with the Leica TCS SP2 confocal system (Leica Microsystems, Wetzlar, Germany), using a 63× water immersion lens. The digital images were processed and grouped using Adobe Photoshop version 7.0.1 (Adobe Systems, San Jose, CA) and Auto-Quant software (Media Cybernetics, Bethesda, MD).