SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Cell lines and cell culture

Conditionally immortalized wild-type human podocyte (ciPods) and conditionally immortalized human glomerular endothelial cells (ciGEnCs) developed by Saleem et al.,[1] and Satchell et al.,[2] respectively were used in this study. ciPods were cultured in RPMI 1640 (Sigma, R8758) supplemented with 10% foetal bovine serum (FBS) (Sigma, F9665) and 1% penicillin/streptomycin (Sigma P4333). ciGEnCs were cultured in EBM-2 media (Lonza, CC-3156) supplemented with EGM-2 Endothelial Cell Growth Medium-2 BulletKit (Lonza, CC-4147). Both cells were cultured at the permissive temperature (33°C) for 3-4 days or until 70-80% confluency and subsequently moved to non-permissive temperature (37°C) for 10-14 days to allow differentiation before being used experimentally. All cells were tested negative for mycoplasma infection.

Patient plasma samples

Plasma samples were processed and carried out as previously described.[3] (Supplementary Table S1)

Podocyte cell treatments

ciPods were cultured in 6 well plates and serum-starved for 3 hours prior to treatment. Relapse and paired remission plasma were diluted to 15% in serum-free RPMI-1640 media and treated to the cells for 15 minutes at 37°C. The cells were also treated with 15 uM of PAR-1 selective agonist peptide (Thr-Phe-Leu-Leu-Arg-NH2 (TFLLR-NH2)) (Sigma, S1820) and 10 ng/mL of TGF-β1 (Sigma, T7039) at different time points before cell lysis generation. In the experiment with PAR-1 inhibition, ciPods were pre-treated with four PAR-1 antagonists, 10

uM RWJ56110 (Tocris, 2614), 150 nM SCH79797 (Sigma, SML1939), 100 nM Vorapaxar (A12832-5, Generon), and 15 uM FR171113 (SML0028, Sigma) for 30 minutes and subsequently stimulated by PAR-1 agonist peptide (PAR-1 AP) or SRNS relapse and paired remission plasma for 15 minutes. For inhibition of TGF-β1 receptor, the cells were pre-treated with 5 and 10 nM of SB431542 (Tocris, 1614), a specific inhibitor of TGF-βRI for 30 minutes before induction with 10 ng/mL of TGF-β1 or SRNS relapse and paired remission plasma for 15 minutes.

Generation of GlomSpheres and Treatments

Our group has developed a 3D glomerulus-like structure spheroid model (GlomSpheres) which is a co-culture of ciPods and ciGEnCs using magnetic nanoparticles to induce self-organization (manuscript submitted). Spheroid formation generates a mature GBM layer and results in a massive upregulation of key podocyte proteins such as nephrin and podocin. ciPods and ciGEnCs were first cultured in monolayers in a T-75 culture flask as described above. The cells were then magnetized with NanoShuttle particles (n3D Biosciences) overnight. The following day, in order to generate a sequential spheroid, the magnetized ciGEnCs were first seeded at 5,000 cells/well in an ultra-low attachment 96-well plate. A magnetic drive was placed underneath the plate and incubated at 33°C for 1 hour. The magnetic drive was then removed, and the magnetized ciPods were added at 5,000 cells/well in a suspension. The magnetic drive was placed back on and incubated at 33°C overnight. The magnet was then removed to allow them to self-form a sequential spheroid whereby peripheral podocytes wrapped a core of endothelial cells. For treatments, GlomSpheres were treated with 15% of relapse and paired remission plasma at different time points (5, 15, 30, 45, and 60 minutes) at 37°C to investigate whether circulating factor could stimulate the same signalling pathways as in ciPods. Additionally, the 3D co-culture of GFP-tagged podocytes and

ciGEnCs spheroids was used for the investigation of the effect of circulating factor on podocyte loss. The GFP-tagged spheroids were treated with 15% of SRNS relapse and paired remission plasma for 7 days at 37°C and imaged on the Leica DM IRB microscope and images captured using Zeiss Axiocam ERc 5S camera for fluorescence intensity analysis.

Immunofluorescence Staining of GlomSpheres

Treated and untreated GlomSpheres were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature (RT), thereafter, washed with 1% Triton-X 100 in PBS for 20 minutes at RT, X3. The spheroids were then blocked with 5% BSA in 0.1% Triton X-100 in PBS overnight at 4°C. Next, the spheroids were incubated with following primary antibodies diluted in 5% BSA in 0.1% Triton X-100 in PBS: anti-podocin (ab50339, Abcam, 1:100), anti-CD31 (PECAM-1) (3528S, Cell Signalling Technology, 1:100), anti-phospho-VASP S157 (ab58555, Abcam, 1:100), anti-phospho-JNK1/JNK2 (44-682G, Invitrogen, 1:100), or anti-fibronectin (ab2413, Abcam, 1:100) for 48 hours at 4°C. After washing, the spheroids were incubated with appropriate Alexa Fluor conjugated secondary antibodies diluted in 5% donkey serum albumin in 0.1% Triton X-100 in PBS: donkey anti-rabbit Alexa Fluor 488 (IgG H&L; A-21206, Invitrogen, 1:400), donkey anti-rabbit Alexa Fluor 594 (IgG H&L; A-21207, Invitrogen, 1:400), donkey anti-mouse Alexa Fluor 555 (IgG H&L; A-31570, Invitrogen, 1:400), donkey anti-mouse Alexa Fluor 647 (IgG H&L; A-31571, Invitrogen, 1:400), or anti-phalloidin Alexa Fluor 568 (IgG H&L; A-12380, Invitrogen, 1:400) overnight at 4°C. Cell nuclei were eventually counterstained by DAPI (D1306, Invitrogen) diluted 1:5000 in PBS for at least 30 min at room temperature. The stained spheroids were visualized by wide-field microscopy (Leica DM IRB linked to Zeiss Axiocam ERc 5S camera) or confocal microscopy (Leica SP5II attached to a Leica DMI 6000). The fluorescence intensity of stained cells was analyzed on the obtained images using Image J (NIH, USA).

Culture of human iPSCs

Human Episomal iPSC Line (A18945, ThermoFisher Scientific) was used to generate kidney organoids in this study. Human iPSCs were maintained in Essential 8 medium (A1517001, ThermoFisher Scientific) on Matrigel (354277, Corning) at 37°C. Cells were passaged every 3 to 4 days using 0.5mM EDTA (15575020, Gibco). The human iPSC line was tested negative for mycoplasma infection.

Generation of kidney organoids from human iPSCs and Treatments

The stepwise differentiation of human iPSCs to kidney organoids was performed as previously reported protocol from the work of Takasako et al.[4] with slight modifications. Briefly, the iPSC cells were dissociated into single cells by TrypLE Select (12563-029, ThermoFisher Sceintific), and plated at 10,000 cells/cm^2 in Essential 8 medium supplemented with RevitaCell Supplement (A2644501, ThermoFisher Sceintific) at the first 24 hours of differentiation. Then, the cells were treated with 6uM CHIR99021 (4423, Tocris) in TeSR™-E6 medium (05946, Stemcell Technologies) for 4 days followed by 200ng/mL FGF9 (273-F9, R&D Systems) and 1ug/mL heparin (H4784-250MG, Sigma Aldrich) in TeSR™-E6 medium for 2 days. At day 7 of differentiation, the cells were formed aggregates in which 150,000 cells were centrifuged at 1,800 rpm for 3 mins, 3 times with 180° flip to make a pellet and placed on a six-well Transwell filter membrane plate (CLS3450, Corning). The aggregates were pulsed with TeSR™-E6 medium contained 5uM CHIR99021 for 1 hour at 37 °C to stimulate nephrogenesis. Then, the media was changed to TeSR™-E6 medium supplemented with 200ng/mL FGF9 (and 1ug/mL heparin for 5 days. Subsequently, the organoids were cultured in growth factor-free TeSR™-E6 medium supplemented with 5uM ATRA (R2625-100MG, Sigma Aldrich) for an additional 13 days. Medium was changed every other day. At day 25 of differentiation, the kidney organoids were treated with 15% nephrotic plasma in TeSR™-E6 medium for either 15

minutes at 37 °C for signalling pathway experiments or 24 hours at 37 °C for podocyte injury investigation.

Whole-mount kidney organoids immunofluorescence

The immunofluorescence protocol was adapted from the protocol previously described.[4] Briefly, the organoids were fixed with 2% PFA at 4 °C for 20 minutes. Then, the organoids were blocked with 5% donkey serum in PBTX (0.3% TritonX-100 in PBS) at 4 °C overnight or at room temperature for 2 hours. Thereafter, the organoids were incubated with primary antibodies diluted in blocking buffer: anti-nephrin (AF4269, R&D system 1:300), anti-GATA3 (5852S, Cell Signalling Technology, 1:300), anti-ECAD (610181, BD Biosciences, 1:300), Biotin conjugated LTL (B-1325, Vector Laboratories, 1:300), ant-WT1 (sc-7385, Santa Cruz Biotechnology, 1:200), anti-podocin (ab50339, Abcam, 1:200), anti-collagen IV pan (ab6586, Abcam, 1:200), anti-collagen IV alpha1 (7070, Chondrex, 1:200), anti- collagen IV alpha3 (7076, Chondrex, 1:200), ant-Laminin alpha5 (ab77175, Abcam, 1:200), anti-phospho-VASP S157 (ab58555, Abcam, 1:200), or anti-phospho-JNK1/JNK2 (44-682G, Invitrogen, 1:200) overnight at 4°C. After incubation, the organoids were washed 6 times with PBTX for 10 minutes each with gentle shaking. After washing, the organoids were incubated with appropriate Alexa Fluor conjugated secondary antibodies diluted in blocking buffer: donkey anti-rabbit Alexa Fluor 488 (IgG H&L; A-21206, Invitrogen, 1:400), donkey anti-mouse Alexa Fluor 647 (IgG H&L; A-31571, Invitrogen, 1:400), donkey anti-sheep Alexa Fluor 555 (IgG H&L; A-21436, Invitrogen, 1:400), Streptavidin Alexa Fluor 405 (S32351, Life technologies, 1:400), or goat-anti-rat Alexa Fluor 633 (IgG H&L; A-21094, Invitrogen, 1:400) overnight at 4°C. The organoids were then washed 3 times with PBS for 10 minutes each. Cell nuclei were eventually counterstained by DAPI (D1306, Invitrogen) diluted 1:5000 in PBS for at least 30 min at room temperature. The stained organoids were visualized by confocal microscopy (Leica SP5II attached to a Leica DMI

6000). The fluorescence intensity of stained cells was analyzed on the obtained images using Image J (NIH, USA).

Protein Extraction and Western Blot Analysis

Podocyte cells were lysed in SDS-extraction buffer containing 50 mM Tris-HCL (pH 6.8), 10% glycerol, and 5% (w/v) SDS with protease inhibitor (Sigma, P8384) and phosphatase inhibitor cocktail 2 (Sigma, P5726) and 3 (Sigma, P0044) (1:500). The protein extraction protocol for kidney organoids was performed as previously described.[5] The lysates were diluted in sample buffer (240 mM Tris-HCl (pH 6.8), 8% SDS, 5% β-mercaptoethanol, 0.04% bromophenol blue, and 40% glycerol), and heated at 95°C for 5-10 minutes. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The blots were subsequently incubated with the following primary antibodies: anti-phospho-VASP S157 (ab58555, Abcam), anti-VASP (3132S, Cell signalling Technologies), anti-phospho-SAPK/JNK (9255S, Cell signalling Technology), anti-SAPK/JNK (9252S, Cell signalling Technology), anti-phospho-ATF-2 (5112S, Cell signalling Technology), ant-ATF-2 (35031T, Cell signalling Technology), anti-phospho-c-Jun S73 (9164S, Cell signalling Technology), anti-c-Jun (9165T, Cell signalling Technology), anti-phospho-SMAD2 (44-244G, Invitrogen), anti-SMAD2 (18338S, Cell signalling Technology), anti-phospho-SMAD3 (44-246G, Invitrogen), anti-SMAD2/3 (5678S, Cell signalling Technology), anti-GAPDH (5014S, Cell signalling Technology), anti-nephrin (BP5030, Origene), or anti-WT1 (sc-7385, Santa Cruz Biotechnology) followed by incubation with secondary antibodies, anti-mouse IgG peroxidase antibody (A9044, Sigma), anti-rabbit IgG peroxidase antibody (A6667, Sigma), or anti-guinea pig IgG peroxidase antibody (AP108P, Millipore). The blots were then developed using Clarity™ and Clarity Max™ Western ECL Blotting Substrates (Biorad, USA) to create luminescence before imaging with an Amersham imager 600 system (GE Healthcare, USA).

Statistical Analysis

Each experiment was carried out in triplicate. Graphs illustrating mean and standard deviation (SD) bars were generated by GraphPad Prism 8.4.2 (GraphPad Software, Inc, CA, USA). The one-way ANOVA followed by Tukey's Multiple Comparison Test was used to evaluate the statistical significance where there were more than two groups to compare. T-test was used to determine the significance between the means of two groups. A P-value < 0.05 was indicated to be significant.

REFERENCES

1. Saleem MA, O'Hare MJ, Reiser J, et al. A Conditionally Immortalized Human Podocyte Cell Line Demonstrating Nephrin and Podocin Expression. *J Am Soc Nephrol*. 2002;13(3):630-638.

2. Satchell SC, Tasman CH, Singh A, et al. Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney Int*. 2006;69(9):1633-1640.

3. Harris JJ, McCarthy HJ, Ni L, et al. Active proteases in nephrotic plasma lead to a podocin-dependent phosphorylation of VASP in podocytes via protease activated receptor-1. *J Pathol*. 2013;229(5):660-671.

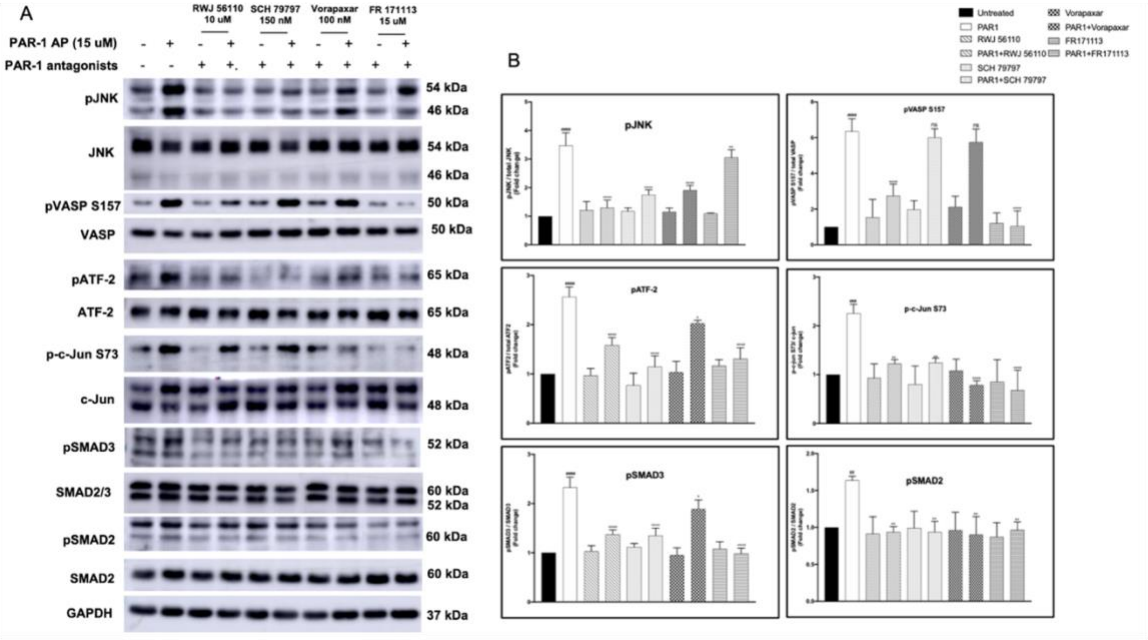
4. Takasato M, Er PX, Chiu HS, Little MH. Generation of kidney organoids from human pluripotent stem cells. *Nat Protoc*. 2016;11(9):1681-1692.

5. Hale LJ, Howden SE, Phipson B, et al. 3D organoid-derived human glomeruli for personalised podocyte disease modelling and drug screening. *Nat Commun*. 2018;9(1):5167.

Supplementary Table S1: Patient clinical details

Patient	Age at onset of disease (yrs)	Gender	Age at transplant (yrs)	Transplant No.	Timing of recurrence	Relapse			Remission			Immunosuppression used for treatment of recurrence
						Alb (g/L)	Pr:Cr (µg/ml)	Cr (µmol/L) (eGFR)	Alb (g/L)	Pr:Cr (µg/ml)	Cr (µmol/L) (eGFR)	
1	26	Male	14	1	<1wk	35	8000	396 (16)	37	924	170 (38)	HDS; Ritx
2	8	Male	15	1	<1wk		6118	85 (78)		119	71 (90)	HDS; Ritx
3	2	Male	15	1	2m	33	567	142 (47)	26	27	130 (51)	HDS
4	2	Male	16	1	4m	28	1640	191 (36)	46	20	202 (35)	HDS
5	10	Male	14	1	<1wk	26	1686	79 (82)	30	629	70 (92)	HDS; Ritx; CP
6	11	Female	15	1	9m	26	1482	88 (71)	33	10	106 (59)	HDS
7	12	Female	15	1	<2wk	25	215	859 (67)		85		Corticosteroids; Tacrolimus; MMF
8	6	Female		1	10m	32	529	89 (51)	33	300	46 (98)	Ciclosporin; Cuclophosphamide; Corticosteroids
9		Female	38	1	<1m	35	642	178 (32)	37	191	170 (33)	HDS
10	8	Male	11	1	1m	33	350	63 (94)	35	50	84 (70)	MMF
11	6	Male	13	1	1m	21	2915	57	31	9	43	

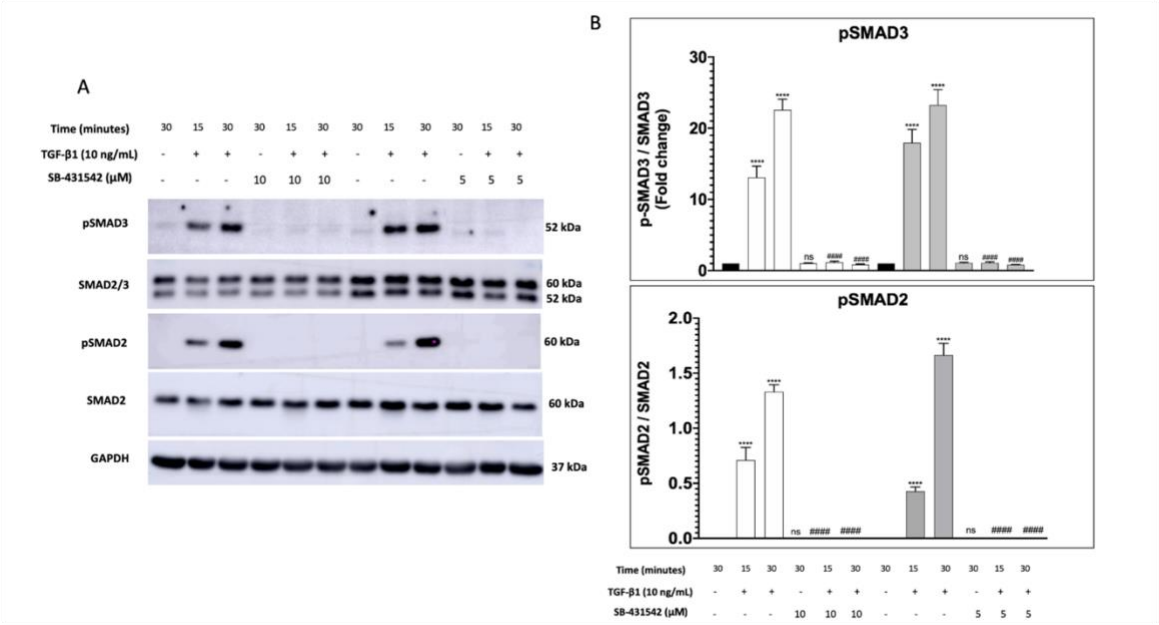
Supplementary Figure S1: Effect of PAR-1 antagonists on JNK, VASP, ATF-2, c-Jun, SMAD3, and SMAD2 phosphorylation activation by PAR-1 agonist peptide



Supplementary Figure 1: Effect of PAR-1 antagonists on JNK, VASP, ATF-2, c-jun, SMAD3, and SMAD2 phosphorylation activation by PAR-1 agonist peptide

(A) Representative immunoblots of PAR-1 agonist-induced the phosphorylation of VASP, JNK, ATF-2, c-Jun, SMAD3, and SMAD2 along with in ciPods at different time points (5, 15, 30, 45, and 60 minutes). (B) Relative amount of JNK (pJNK/JNK), VASP (pVASP/VASP), ATF-2 (pATF-2/ATF-2), SMAD3 (pSMAD3/SMAD3), SMAD2 (pSMAD2/SMAD2), and c-Jun (p-c-Jun/c-Jun). * statistically significant with $p<0.05$, ** statistically significant with $p<0.01$, *** statistically significant with $p<0.001$, **** statistically significant with $p<0.0001$; one-way ANOVA, Tukey's Multiple Comparison Test; each experiment performed in triplicate. Results were represented as means ± SD.

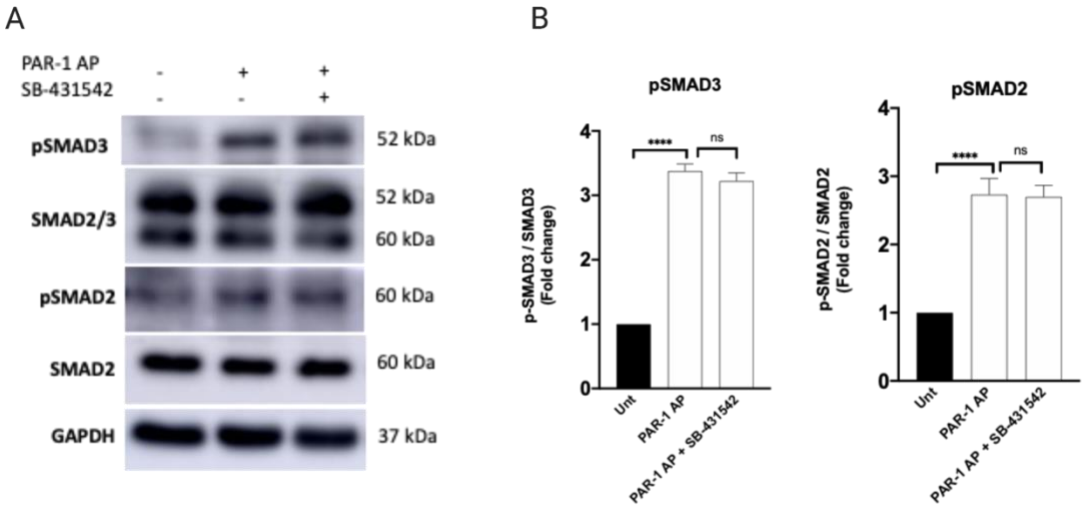
Supplementary Figure S2: SB-431542 efficiently inhibits SMAD phosphorylation induced by TGF-β1 in podocytes.



Supplementary Figure S2: SB-431542 efficiently inhibits SMAD phosphorylation induced by TGF-β1 in podocytes.

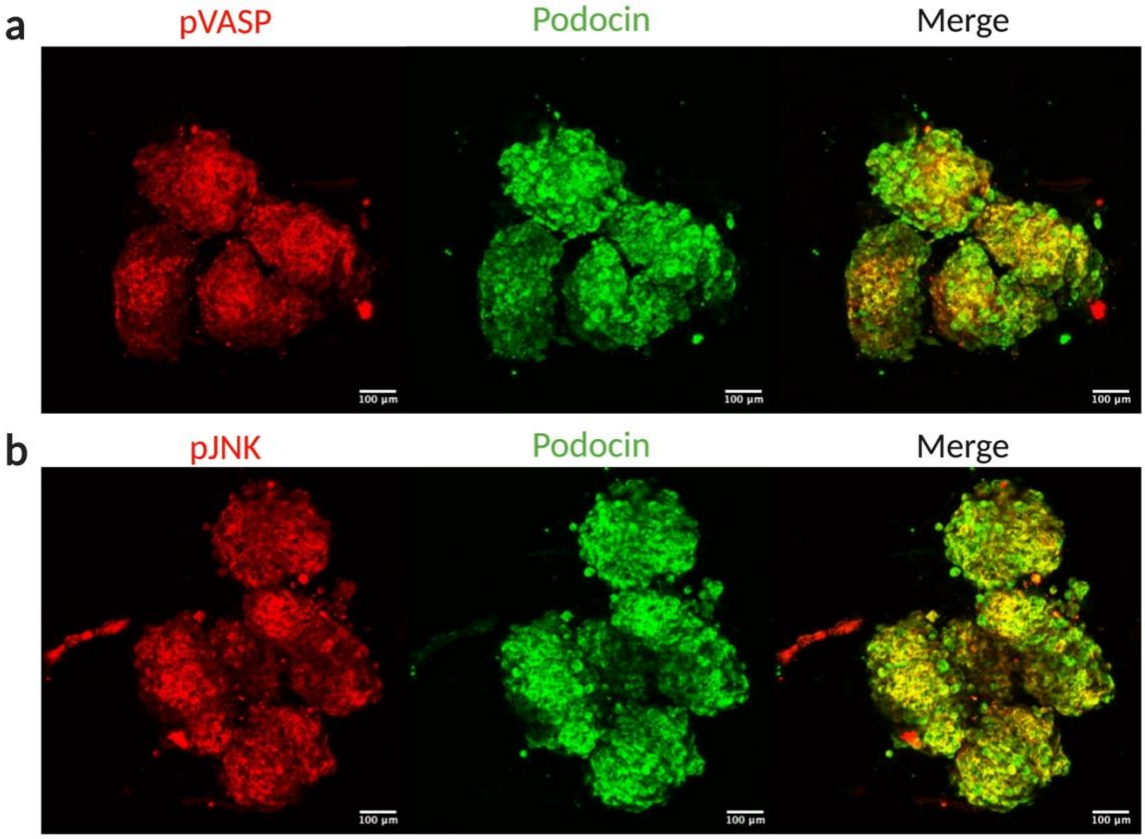
(A) ciPods were pre-treated for 30 minutes with 10 and 5 nM of SB-431542, a TGF-β1 receptor inhibitor and then induced with 10 ng/mL for 15 and 30 minutes. The phosphorylation of SMAD3 and SMAD2 was analysed by Western blotting. The immunoblots shown are representative blots. (B) Densitometry analysis of the phosphorylation of SMAD3 (pSMAD3/SMAD3), and SMAD2 (pSMAD2/SMAD2). * statistically significant with $p<0.05$, ** statistically significant with $p<0.01$, *** statistically significant with $p<0.001$, **** statistically significant with $p<0.0001$ compared with control (untreated); #### statistically significant with $p<0.0001$ compared with TGF-β1 treatment; one-way ANOVA, Tukey's Multiple Comparison Test; each experiment performed in triplicate. Data are represented as means ± SD.

Supplemental Figure 3: TGF-β1 receptor inhibitor does not inhibit SMAD phosphorylation induced by PAR-1 agonist peptide in podocytes.



Supplementary Figure S3: TGF-β1 receptor inhibitor does not inhibit SMAD phosphorylation induced by PAR-1 agonist peptide in podocytes.
(A) ciPods were pre-treated for 30 minutes with 10 nM of SB-431542 and then induced with 15 uM of PAR-1 agonist peptide for 15 minutes. The phosphorylation of SMAD3 and SMAD2 was analysed by Western blotting. The immunoblots shown are representative blots. (B) Densitometry analysis of the phosphorylation of SMAD3 (pSMAD3/SMAD3), and SMAD2 (pSMAD2/SMAD2). * statistically significant with $p<0.05$, ** statistically significant with $p<0.01$, *** statistically significant with $p<0.001$, **** statistically significant with $p<0.0001$ compared with control (untreated); one-way ANOVA, Tukey's Multiple Comparison Test; each experiment performed in triplicate. Data are represented as means ± SD.

Supplemental Figure 4: Phospho-VASP and phosphor-JNK mainly expresses in podocyte compartment of GlomSpheres.



Supplementary Figure S4: Phospho-VASP and phosphor-JNK mainly expresses in podocyte compartment of GlomSpheres.
Immunostaining of GlomSpheres show the colocalization of pVASP and podocin (a) and pJNK and podocin (b). The expression of pVASP and pJNK are mainly observed in podocyte compartment of GlomSpheres.