Human relevance of blocking the Rac1-TRPC5 pathway as a podocyte-protective strategy for progressive kidney diseases

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Abstract:

Background Podocyte injury and the appearance of proteinuria are key features of progressive kidney diseases including focal segmental glomerulosclerosis (FSGS). Genetic deletion or selective inhibition of TRPC5 channels with small-molecule inhibitors protects podocytes in rodent models of disease; however, less is known about the human relevance and translatability of TRPC5 inhibition into effective drug development programs. Here, we investigate the effect of TRPC5 inhibition in puromycin aminonucleoside (PAN)-treated human iPSC-derived podocytes and kidney organoids.

Methods A single i.p. injection of PAN (50mg/kg) was given to wild-type Sprague-Dawley rats (Male, 4-5 weeks, Charles River). AC1903 was administered twice a day for 7 days after PAN injection. 24-hour urine albumin levels were measured on day 7. Human iPS cells were used to generate podocyte and kidney organoid. PAN treatment was used to induce human podocyte injury in these in vitro model systems, and the effects of AC1903 were assayed by Western Blotting, immunofluorescence staining and confocal microscopy.

Results PAN treatment triggered the Rac1-TRPC5 injury pathway in human iPSC-derived podocyte cultures and kidney organoids. The TRPC5 inhibitor AC1903 reversed the effects of PAN-induced injury providing the first evidence for therapeutic applicability of TRPC5 inhibition to human podocytes in both 2D and 3D culture systems. Cross-validation in rats with PAN-induced nephrosis, an established model of podocyte injury and progressive kidney disease, confirmed

that inhibition of TRPC5 by AC1903 was sufficient to protect podocyte cytoskeletal proteins and suppress proteinuria.

Conclusion Taken together, our results confirmed the relevance of the TRPC5-Rac1 pathway in human kidney tissue thus highlighting the potential of this therapeutic strategy for patients.

Introduction

Progressive chronic kidney disease (CKD) is associated with increased risk of end-stage renal disease (ESRD), a condition that requires kidney transplantation or dialysis (1), and affects more than 850 million people worldwide (2). Despite increasing prevalence due to rising rates of obesity, hypertension, and diabetes, the therapeutic options available to slow or prevent disease progression are severely limited (3, 4). Nephrotic syndrome is a major determinant of CKD and is characterized by the presence of large amounts of albumin in the urine, due to impairment of the glomerular filtering unit of the kidney. An intact kidney filter is essential for retaining proteins in the blood and removing waste from the body. This size selective barrier is made up of endothelial cells, the basement membrane, and the podocyte foot process. Many chronic kidney diseases are associated with the loss of podocytes, critical post-mitotic, terminally differentiated cells that comprise the blood filtration barrier in the kidney and cannot be renewed once lost (5-9). Due to their limited capacity to proliferate, podocytes are especially vulnerable to various stimuli that lead to injury (10). Preventing podocyte injury remains a critical objective for the development of effective, targeted therapeutic strategies for kidney diseases.

Recent efforts using animal models demonstrated that a specific transient receptor potential canonical-5 (TRPC5) small molecule inhibitor, AC1903, administered after onset of severe proteinuria, but prior to elevated creatinine, could rescue podocytes and ameliorate the molecular and morphological changes associated with focal segmental glomerulosclerosis (FSGS). FSGS is a

glomerular disease characterized by sclerotic lesions and is associated with podocyte injury and loss, leading to damage of the kidney filter. Decreased expression of podocyte cytoskeletal proteins, including synaptopodin, nephrin, and podocin, is an early event in podocyte injury that results in disorganization of the cytoskeleton, the fusion of foot processes, and ultimately the development of proteinuria and subsequent kidney damage (11). Numerous studies indicate that dysfunction of the podocyte cytoskeleton contributes to various diseases (4, 12), such as FSGS, minimal change disease (MCD), and other progressive proteinuric kidney diseases.

A significant number of known mutations for FSGS result in excess Rac1 signaling in podocytes including mutations in *ARHGAP24* (13), *ARHGDIA* (14), and *ARHGEF17* (15). A small GTP-binding protein, Rac1 is closely associated with various proteinuric kidney diseases. Activation of Rac1 disrupts podocyte cytoskeletal proteins and causes podocyte injury and loss. Mechanistically, one result of Rac1 activation is the vesicular insertion of TRPC5 ion channels into the podocyte plasma membrane, further increasing Rac1 activation. Additional roles for Rac1 activation include increased ROS production and disruption of cytoskeletal protein remodeling (16).

Ion channels are critical to kidney function, and their involvement in kidney disease is an active area of investigation. Transient receptor potential (TRP) channels are receptor-operated, nonselective, Ca^{2+}-permeable, cationic channels that were first identified in *Drosophila* (17, 18). TRPC (TRP canonical) channels are a subgroup of this larger family that are particularly relevant to podocyte

biology (19) and have been shown to play an important role in the pathogenesis of kidney disease. TRPC channels are receptor operated meaning that they typically require activation of a membrane receptor such as a G-protein coupled receptor (GPCR) in order to open. The angiotensin II type 1 receptor (AT1R), a highly expressed GPCR in podocytes, activates TRPC channels and has been shown to induce apoptosis (21). TRPC5 activation in turn is known to Ca^{2+} through the cell membrane (20). Ca^{2+} ions elicit dynamic and tightly regulated biochemical responses activate Rac1. Rac1 activation leads to further vesicular insertion of TRPC5 into the plasma membrane thus making more TRPC5 channels available for activation and completing a feed-forward pathway. While the role of TRPC5 in an AT1R-Rac1-TRPC5 feedforward injury circuit in podocytes has been clearly defined, evidence implicating TRPC5 activity as a driver of progressive kidney diseases, especially in human kidney tissue, is an emerging area of interest. Mice lacking TRPC5 are protected from certain forms of kidney injury (22). Critically, data from three chemically distinct compounds that block TRPC5 activity (AC1903, ML204, and GFB-8438) have demonstrated beneficial effects when applied to rodent models of kidney disease (23). In addition to TRPC5, both gain-of-function and loss-of-function mutations in TRPC6 channel activity contribute to podocyte injury (24) further implicating TRPC channel activity in chronic kidney diseases.

The current study directly investigates the relevance of these findings to human biology by harnessing the technological advances afforded by human induced pluripotent stem cell (iPSC)-derived 2D podocyte cultures (iPodos) and

3D kidney organoids. We determined that human podocytes express functional TRPC5 channels and that TRPC5 inhibition protects podocytes from injury. Our data were cross-validated in the experimentally tractable PAN-induced nephrotic rat model, where we further demonstrate that Rac1 may serve as a biomarker of disease pathway activity. This work provides a strong rationale for ongoing efforts to move TRPC5 inhibitors into the clinic (NCT03970122; https://clinicaltrials.gov/) for the treatment of progressive proteinuric kidney diseases.

Results

Human iPSC-derived podocytes express functional TRPC5 channels

Generating human podocytes in 2D cultures offers a unique opportunity to conduct mechanistic studies *in vitro*. To generate human iPS cell-derived podocytes (iPodos) we adapted a previously published three-step protocol to induce differentiation into intermediate mesoderm, then into nephron progenitors, and finally, into mature podocytes (25). Mature iPodos exhibited typical *in vitro* mature podocyte morphology characterized by a large and flat cell body with a dense nucleus that highly resembled mouse and human immortalized podocytes (26, 27). The iPodos from this protocol expressed the major podocyte markers including podocin, synaptopodin, alpha actinin-4, and WT-1 (25).

We performed patch clamp electrophysiology, the gold standard in measuring ion channel activity, using iPodos, 12 to 14 days after induction. For whole-cell patch clamp recordings, a single iPodo was identified and the glass

pipette was moved adjacent to the iPodo nucleus to provide a more effective Giga-seal (Fig. 1A). Upon successful achievement of the whole-cell configuration, a strong outwardly rectifying current was observed upon application of a voltage ramp protocol, which decreased gradually within 30 seconds of perfusion. Englerin A, a compound known to be a nanomolar activator of TRPC4 and TRPC5, was applied once the baseline became stable. Large outward and inward currents were induced by 100 nM Englerin A, which could be blocked by the TRPC5 channel inhibitor AC1903 (Fig. 1B-C). The inhibitory effect of AC1903 was more prominent at negative potentials confirming that the baseline outwardly rectifying current did not correspond to a TRPC5 conductance (Fig. 1D). These data provide the first evidence that human podocytes express functional TRPC5 channels at baseline, without additional manipulation, indicating that TRPC5 channels may play a role in human podocyte physiology.

Inhibition of TRPC5 channel activity protects human iPodos from puromycin aminonucleoside (PAN)-induced injury

Although previous studies have indicated that TRPC5 activity is a major cause for podocyte injury in various *in vitro* and *in vivo* models, these studies were conducted in cells and kidney tissues from rodents (16, 22). To determine whether inhibition of TRPC5 channel activity could protect human podocytes from injury, we investigated the effect of the TRPC5 channel inhibitor AC1903 on mature human iPodos. We treated iPodos with puromycin aminonucleoside (PAN), which has been reported to cause podocyte injury through Rac1

activation and ROS production (28-30). In the context of previous studies in several pre-clinical animal models showing that Rac1 activation increases TRPC5 activity and subsequently switches on a Rac1-TRPC5 feedforward pathway contributing to podocyte injury, we hypothesized that PAN treatment would cause human iPodo injury by triggering the same Rac1-TRPC5 loop. In support of this hypothesis, incubation with PAN for 24 hours significantly increased iPodo intracellular ROS levels, which were reduced by co-treatment with AC1903 (Fig. 2A and B). At the protein level, PAN treatment significantly reduced the expression levels of the podocyte cytoskeletal proteins podocin and synaptopodin, but not the transcription factor WT1, which is consistent with previous studies (31). Co-treatment with AC1903 preserved podocin and synaptopodin expression levels (Fig. 2C and D). These results indicate that TRPC5 channel activity mediates PAN-induced Rac1-triggered human iPodo injury, and inhibition of TRPC5 channels by small molecule AC1903 can protect human iPodos from PAN-induced cytoskeletal degradation. PAN co-treated with AC1903 failed to induce ROS in mouse immortalized podocytes (data not shown), highlighting iPodos as a more clinically relevant *in vitro* model for studying podocyte biology and disease pathophysiology.

Inhibition of TRPC5 channel activity reduces proteinuria and protects podocytes from injury in PAN treated rats

Although we confirmed the relevance of TRPC5 inhibition in iPodos, a human *in vitro* model system, full clinical studies targeting TRPC5 would still

require tractable *in vivo* models to guide confident lead selection. Studies have shown that PAN injection administered to rats causes progressive renal injury that resembles minimal change disease (MCD) or focal segmental glomerular sclerosis (FSGS). The extent of damage depends on the amount and frequency of the PAN injection (32-33). Studies have indicated that many molecules, including TRPC6 channels, are associated with PAN-induced nephrosis in rats (34-37). Of interest, a recent study showed little to no protective effects in the early phase of PAN treatment in rats with genetic deletion of TRPC6 channels (38), suggesting that another molecule mediates at least the early phase of disease. Our previous studies have shown that inhibition of TRPC5 protects podocytes from injury and loss in the early phases of disease in several rodent models, suggesting a more clinically relevant role for TRPC5 inhibition (rather than TRPC6 inhibition). To investigate the role of TRPC5 in PAN nephrosis, we administered a single dose of PAN (50 mg/kg body weight) to rats, which induced a significant amount of urine albumin 7 days after the injection. In contrast, co-administration of the TRPC5 channel inhibitor AC1903 twice per day significantly reduced the urine albumin level compared with nephrosis in rats 7 days after PAN injection (Fig. 3A). Hemotoxylin and eosin staining results showed no obvious morphological changes in both glomeruli and tubules from all groups (Fig. 3B). However, transmission electron microscopy graphs showed extensive foot process effacement (FPE) with neither glomerular basement membrane (GBM) nor mesangial cell injury in PAN rats (Fig. 3C), resembling the clinical manifestations of Minimal Change Disease. Statistical analysis of rat podocyte

FPs showed that treatment with the TRPC5 channel inhibitor AC1903 preserved FP number and protected FPs from effacement in the PAN-treated rats (Fig. 3D and E). These results indicate that TRPC5 channels *in vivo* play an important role in inducing injury by PAN.

Pharamacological inhibition of TRPC5 *in vivo* protects podocyte cytoskeletal proteins from PAN-induced nephrosis

We further characterized the role of several important podocyte proteins using immunofluorescence and Western blot experiments in PAN-induced nephrosis rats. Immunofluorescence showed that the expression levels of two podocyte cytoskeletal proteins, podocin and synaptopodin, were reduced in the PAN-treated rat kidneys, while the expression levels of podocyte transcription factor WT1 was not affected by PAN treatment, indicating that PAN at this concentration caused alterations in podocyte cytoskeletal structure but not in cell death or loss. Intriguingly, treatment with the TRPC5 channel inhibitor AC1903 successfully restored the PAN-induced depletion of podocin and synaptopodin in PAN-treated rat kidneys (Fig. 4A). Western blotting from the same rat kidneys was consistent with the IF experiment for podocin, synaptopodin, and WT1 (Fig. 4B). Although we observed some protective effect on nephrin by AC1903, there was no statistical difference between kidneys treated with PAN and those receiving co-administration of PAN and AC1903 (Fig. 4C). This discrepancy was most likely due to the different physiological functions between podocin, synaptopodin, and nephrin, since nephrin is a major component of the slit

diaphragm, which may be more sensitive to PAN treatment and more refractory to recovery from injury. Taken together, these results demonstrate that inhibition of TRPC5 channel activity can protect podocyte cytoskeletal proteins in the PAN nephrosis rat model, thereby reducing FPE. The accelerated timeline of injury in PAN-induced nephrosis increases the translatability of this model in the context of pre-clinical studies, especially in comparison to AT1R transgenic and DahI Salt-sensitive spontaneous hypertensive rat models which require at least a month for podocyte injury and proteinuria to be established.

Systemic TRPC5 inhibition reduces PAN-induced glomerular channel activity

To understand the detailed molecular mechanism of TRPC5 channel involvement and contribution to PAN-induced podocyte injury, we performed TRPC5 single-channel recordings from acutely isolated rat kidney glomeruli according to our previously reported protocol and procedures (16). A single dose of PAN treatment successfully increased TRPC5 single channel activity in response to the TRPC5 agonist riluzole, while systemic co-administration of AC1903 with PAN in rats significantly reduced TRPC5 activity from isolated glomeruli (Fig. 5A). PAN-treated rats showed a higher NPo value, which is the product of channel number and open-channel probability, while AC1903-treated rats exhibited a lower NPo value (Fig. 5B). We hypothesize that systemic AC1903 administration significantly lowered the number of TRPC5 channels inserted in the podocyte plasma membrane, resulting in a low NPo. This result

shows that, similar to AT1R transgenic and Dahl spontaneous hypertensive rat models, AC1903, when administered systemically, protects podocytes from injury by reducing TRPC5 channel activity.

Inhibition of TRPC5 channel activity reduces Rac1 activity in PAN rats

Previous studies have demonstrated that inhibition of TRPC5 activity decreases Rac1 activation and cytosolic ROS production in LPS-treated mice, AT1R transgenic, and Dahl spontaneous hypertensive rat models (16, 22). In light of these studies, and given the fact that PAN is an experimentally tractable, easy to use animal model (a single injection of PAN is sufficient to induce proteinuria in rats; see Methods), we investigated whether TRPC5 inhibition could decrease Rac1 activity and suppress ROS production in PAN-induced nephrosis rats. We isolated PAN rat glomeruli 7 days after PAN injection with or without AC1903 treatment together with PBS treated rat glomeruli. We found that the abundance of Rac1-GTP (the active form of Rac1) was highly increased in PAN treated glomeruli compared to that of the PBS treated rat glomeruli. Furthermore, AC1903 successfully decreased the levels of Rac1-GTP (Fig. 6A). More than 60% of Rac1 was changed to the active form after PAN treatment, while AC1903 treatment kept the level of active Rac1 below 50% (Fig.6 B). These results indicate that inhibition of TRPC5 channel activity could decrease ROS levels by reducing Rac1 activity. We further examined the correlation of 24-hour urine albumin level and Rac1-GTP level in these PAN rats. Intriguingly, we found a strong correlation between urine albumin levels and Rac1-GTP levels

(Fig. 6C), which not only supported that Rac1 activity is important to PAN-induced podocyte injury, but also suggested that AC1903 could protect podocytes from this injury by reducing Rac1 activity. Taken together these data indicate the potential to develop Rac1 activity as a biomarker for progression of proteinuric kidney diseases.

TRPC5 inhibition preserves podocin, synaptopodin, and nephrin levels in PAN-treated human kidney organoids

To evaluate the effect of AC1903 in human kidney tissue, we took advantage of the human iPSC-derived kidney organoid model. The human kidney organoid contains self-organized nephron-like structures composed of early glomerular structures connected to tubular cells including proximal tubules, loops of Henle and distal tubules. These organoids thus hold the potential to be excellent *in vitro* models for preclinical drug screening to enable the exploration of drug effects on multiple kidney cell types (39-41). Using immunofluorescence, we confirmed that PAN treatment reduced podocin, synaptopodin, and nephrin but not WT1 expression levels (Fig. 7A and Suppl Fig. 1). Co-treatment with AC1903 preserved podocin and synaptopodin signals similar to the results observed in iPodos and PAN rats. Interestingly, AC1903 also preserved nephrin signals in PAN-treated human kidney organoids, which was not the case in PAN-treated rat kidneys (Fig. 4B). This was potentially due to early intervention in the TRPC5-Rac1 pathway by AC1903 *in vitro*, further suggesting that nephrin may be more sensitive to PAN treatment. Similarly, the Western blots confirmed that

AC1903 protected podocin and synaptopodin abundance in PAN treated kidney organoids (Fig. 4B-C). Taken together, our data suggest that inhibition of TRPC5 channel activity can protect human podocytes from PAN-induced injury in an *in vitro* 3D model of the human kidney.

Discussion

Traditionally, the majority of studies investigating podocyte biology have used rodent models and human immortalized podocyte cell lines. Although these cell lines faithfully express podocyte markers such as podocin, synaptopodin, nephrin, WT1, and respond to many stimuli, maintenance of such cell lines creates technical challenges, such as de-differentiation, resulting in variability. Moreover, these approaches fall short of recapitulating the crosstalk and interactions between podocytes and other cell types of the kidney. Recently, successful generation of 2D human podocyte cultures (iPodos) and 3D kidney organoids *in vitro* from human iPSCs has offered a unique opportunity to study podocyte-associated kidney diseases in a human-specific and potentially more reproducible system (25, 40-42). Our results show that PAN treatment causes podocyte injury in kidney organoids through the activation of the Rac1-TRPC5 pathway and disruption of cytoskeletal protein dynamics. Inhibition of TRPC5 channel activity protected podocyte cytoskeletal degradation, establishing the podocyte-protective effects of small-molecule inhibitors of TRPC5 channels in human kidney organoids. Identical results in human iPSC-derived podocytes confirm the human relevance of the Rac1-TRPC5 disease-associated pathway.

Our previous data demonstrated that a specific TRPC5 small-molecule inhibitor, AC1903, can rescue podocytes and attenuate the progression of kidney diseases in angiotensin II type 1 receptor transgenic, and spontaneous hypertensive rat models (16, 22). These findings provided a mechanistic rationale for therapeutically targeting TRPC5 channels in the treatment of progressive chronic kidney diseases. To date, there has been no evidence describing functional TRPC5 channels in human podocytes. In this study, we generated human iPSC-derived podocytes and used patch clamp electrophysiology to demonstrate their response to the TRPC4/5 channel agonist, Englerin A, which was blocked by the TRPC5-selective inhibitor AC1903. To our knowledge, this is the first demonstration that human podocytes express functional TRPC5 channels, even at basal conditions, further strengthening the notion that these channels play an important role in progressive kidney diseases.

Prior work has shown that Rac1 activation is a nodal event in a spectrum of glomerular diseases, while inhibition of Rac1 activity ameliorates podocyte injury in response to various noxious stimuli (14, 28-30, 43). In this study, we investigated the contribution of the Rac1-TRPC5 feedforward pathway in a PAN-induced nephrosis rat model. A single dose of PAN was sufficient to induce podocyte injury and proteinuria in rats within a week, which was consistent with the results from PAN-treated human podocytes and kidney organoids. Inhibition of TRPC5 by AC1903 was sufficient to protect podocyte cytoskeletal proteins and suppress proteinuria in PAN-induced nephrosis rats within a week. Interestingly, we found a strong correlation between Rac1 activity and rat albumin levels in the

urine, which suggests that follow-up studies in patients should investigate whether Rac1 levels may serve as a biomarker for patient stratification in clinical trials and/or target engagement.

Taken together, these data indicate that inhibition of TRPC5 channel activity blocks the Rac1-TRPC5 feedforward pathway and protects podocytes from PAN-induced injury. Our data also highlight the use of human iPodos, kidney organoids, and the PAN-induced nephrosis rat model as useful tools not only for studying disease pathways in podocytes, but also for the preclinical development of TRPC5 channel inhibitors. This diverse set of validated models spans both human relevant *in vitro* systems conducive for mechanistic studies and experimentally tractable *in vivo* disease models with physiological readouts. In sum, this study bolsters the human relevance and scientific rationale for a targeted therapeutic strategy that is imminently being tested in the clinic.

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Conflict of interest statement

A.G. has a financial interest in Goldfinch Biopharma, which was reviewed and is managed by Brigham and Women's Hospital and Partners HealthCare and the Broad Institute of MIT and Harvard in accordance with their conflict of interest policies.

Methods

Animals

Wild-type Sprague-Dawley rats (Male, 4-5 weeks, Charles River) were housed under a controlled environment with a 12-hour light-dark cycle and access to food and water ad libitum. All animal experiments were performed in accordance with the guidelines established and approved by the Animal Care and Use Committee at Brigham and Women's Hospital, Harvard Medical School. After wild-type Sprague-Dawley rats were acclimated for a week in the BWH CCM animal facility. A single dose of puromycin aminonucleoside (50 mg/kg, PAN group) was given i.p. to rats to induce nephrosis, and PBS was given as control. Following the PAN injection, vehicle or AC1903 (50 mg/kg) was administered twice daily (at 9 am and 9 pm) for seven days. 24-hour urine albumin levels were measured on day 0, 3 and 7. Rats were euthanized after the metabolic collection on Day 8. Both kidneys were collected for downstream experiments. In most cases, one kidney was used for acute glomeruli isolation and the other was stored in -80°C for subsequent experiments. In combination, we have studied 34 rats (PBS group n = 6, PAN group n = 15, PAN + AC1903 group n = 13).

Chemical preparation and IP administration

All chemicals were purchased from Sigma-Aldrich unless described otherwise. AC1903 was synthesized, purified and prepared by C.H. as previously published (16). Immediately prior to injections, AC1903 solution was placed on a heated shaker at 48˚C and 800 rpm. Vehicle was prepared in the same fashion. Injection

amount was determined by body weight (2mL vehicle/compound per kg body weight). Body weight was measured at the time of injection.

Metabolic collection and urine albumin assay

Rats were housed individually in a metabolic cage supplied with adequate amounts of food and water. Urine was collected into a 50 mL Falcon tube for 24 hours. Total urine volume was measured and then centrifuged at 3,200x g for 10 min at 4°C. Albumin quantification was done according to our previously published protocol (16). Coomassie Brilliant Blue stained gels of urine samples were quantified by densitometry with albumin standards using Image J software.

Human iPSC culture

Human Episomal iPSC Line (ThF) (ThermoFisher, #A18945) was maintained in mTeSR1 medium (Stem Cell Technologies, #85870) in T25 flasks pre-coated with Matrigel (Stem Cell Technologies, #354277). Cells were passaged using Gentle Cell Dissociation Reagent (Stem Cell Technologies, #7174). iPSCs were confirmed to be karyotype normal and maintained below passage 10 and all the cell lines were routinely checked and were negative for mycoplasma.

Differentiation into human iPSC-derived podocytes (iPodos)

Human iPSC-derived podocytes (iPodos) were generated using the cited protocol with a few modifications (25). A total number of 3.75 X 10^5 ThF human iPSCs were seeded in a Matrigel-coated T25 flask in mTeSR1 medium (Stem

Cell Technologies, #85870) with ROCK inhibitor, Y-27632 (10 µM, Stem cell Technologies, #72304). After 24h cells were treated with a 1:1 mixture of DMEM/F12 + GlutaMAX (Life Technologies, #10565-018) and Neurobasal media, supplemented with N2 and B27 (Life Technologies, #21103049), CP21R7 (1 µM, Cayman Chemical, #20573), and BMP4 (25 ng/mL, Peprotech, #AF-120-05ET), for three days. On day four, the medium was replaced with STEMdiff APEL2 medium (Stem Cell Technologies, #05270) supplemented with FGF9 (200 ng/mL, Peprotech, #100-23), BMP7 (50 ng/mL, Peprotech, #120-03), and Retinoic Acid (100 nM, Sigma-Aldrich, #R2625) for two days. On day six, cells were dissociated with Accutase (Stem Cell Technologies, #07920) and 2 X 10^5 cells were seeded on Type I Collagen-coated 6-well dishes and cultured until day fourteen in DMEM/F12+GlutaMAX medium supplemented with 10% FBS (Life Technologies, #16140071). Vitamin D3 (100 nM, Tocris Bioscience, #4156), and Retinoic Acid (100 µM, Sigma-Aldrich, #R2625) were added every other day. Cells were fully differentiated and ready to use from Day 12 to Day 14.

Kidney organoid differentiation

Kidney organoids were generated using a previously described protocol (39) with slight modifications. A total number of 3.75 X 10^5 ThF iPSCs were plated in a T25 flask in the mTeSR1 medium with ROCK Inhibitor Y-27632 (10 µM, Stem cell Technologies, #72304). After 24 hours, cells were treated with CHIR99021 (8 µM, R&D systems, #4423/10) in the STEMdiff APEL2 medium (Stem Cell Technologies, #05270) for four days, followed by recombinant human FGF-9

(200 ng/mL, Peprotech, #100-23) and heparin (1 µg/mL, Sigma-Aldrich, #H4784) for an additional three days. At day seven, cells were dissociated into single cells using AccutaseTM (Stem Cell Technologies, #07920). 5 X 10^5 cells were pelleted at 350x g for 2 min and transferred onto a 6-well transwell membrane (Stem Cell Technologies, #3450). Pellets were incubated with CHIR99021 (5 µM) in the APEL2 medium for one hour at 37°C. Then the medium was changed to the APEL2 medium with FGF-9 (200 ng/mL) and heparin (1 µg/mL) for an additional five days, and an additional two days with heparin (1 µg/mL). Medium was changed every other day. The organoids were maintained in APEL2 medium with no additional factors until day 25. Then kidney organoids were treated with PBS, PAN (150 µg/mL) with or without AC1903 (30 µM) for 72 h before the downstream experiments.

ROS assay

Human iPSC-derived podocytes (iPodos) were treated with either PBS, PAN (150 µg/mL), or PAN with 30 µM AC1903 for 24 h. Intracellular production of ROS was measured using a cell-permeable fluorescent ROS indicator (Invitrogen, #C10444) following the official protocol. Briefly, cells were incubated with 5 µM CellRox Green at 37°C for 30 min in Hanks' balanced salt solution (ThermoFisher Scientific, #14025092). Cells were then washed with PBS and fixed with 4% PFA. Fluorescence images were taken under a confocal microscope Olympus FV-1000. ROS signal intensities were quantified using ImageJ software.

Rat kidney western blotting and immunofluorescence

Rats were euthanized and perfused with PBS. The kidney was quickly removed and cut into half. One half was flash frozen in liquid nitrogen, and the other was fixed in 4% PFA overnight and stored in PBS for follow-up experiments. For Western blotting experiments, rat kidney cortex was homogenized at 4°C in CHAPS lysis buffer with 1x protease inhibitor (ThermoFisher Scientific, #78441B). The homogenates were subjected to centrifugation at 12,000 g for 5 min. Total protein from supernatant was quantified with a BCA assay kit (ThermoFisher Scientific, #23225). Proteins were resolved in 4-15% Mini-PROTEAN TGX gels (Bio-Rad, #4568091) and transferred to PVDF membranes. Rabbit anti podocin (ThermoFisher Scientific, #PA5-61987), guinea pig anti synaptopodin (Progen, GP94-C), rabbit anti WT-1 (Santa Cruz, #SC-192), Goat anti Nephrin (R&D Systems, #AF3159), and mouse anti GAPDH (Sigma Aldrich, #G8795) were used at a dilution of 1:1000. Goat anti rabbit and guinea pig IgG HRP-conjugated secondary antibody (ThermoFisher Scientific, #G-21234) and donkey anti goat IgG HRP-conjugated secondary antibody (Abcam, #ab97110) were used at a dilution of 1:5000. For immunofluorescence, Kidney tissues were sectioned at 6 µm thickness and blocked with 3% BSA at room temperature for 1 h. The rabbit anti podocin, guinea pig anti synpo, goat anti Nephrin, and rabbit anti WT-1 antibodies were used at a dilution of 1:200. The Alexa goat anti rabbit and guinea pig IgG 488 and Alexa donkey anti goat IgG 594 were used at a dilution of 1:200. Fluorescence images were taken with a confocal microscope Olympus FV-1000.

Rac1 activity assay

Rac1 activity was measured using the Rac1 activity assay kit (Cell Biolabs, STA-401-1) as previous described (44). 500 mg total protein freshly isolated from rat glomeruli was incubated with PAK1-conjugated agarose beads at 4°C for 1h with gentle agitation. The beads were then centrifuged at 14,000x g for 30sec and washed with 1x assay buffer three times. The activated GTP-bound forms of Rac1 were recovered in SDS buffer by boiling at 95°C for 5min and separated in 4-15% Mini-PROTEAN TGX gels (Bio-Rad, #4568091) and transferred to PVDF membranes. Membranes were probed with primary antibodies directed against Rac1 (Cell Biolabs, STA-401-1). The input total proteins were also blotted as total Rac1.

Electrophysiology

iPodo whole-cell patch clamp was performed using an Axopatch 700B and Digidata 1550A (Molecular Devices). Bath solution contained (in mM) 135 CH_3SO_3Na, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose adjusted with NaOH to pH 7.4. Pipette solution contained (in mM) 135 CH_3SO_3Cs, 10 CsCl, 3 MgATP, 0.2 EGTA, 0.13 $CaCl_2$, and 10 HEPES adjusted with CsOH to pH 7.4. For glomerular single-channel recording, acutely isolated glomeruli were prepared as previously published (16). Single-channel recordings were carried out using an Axopatch 200B and Digidata 1550A (Molecular Devices). Bath and pipette solutions for glomerular single-channel recording contained (in mM) 135

CH_3SO_3Na, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose adjusted with NaOH to pH 7.4. Once the inside-out configuration was achieved, the bath solution was replaced by an intracellular solution, containing (in mM) 135 CH_3SO_3Cs, 10 CsCl, 3 MgATP, 0.2 EGTA, 0.13 $CaCl_2$, and 10 HEPES adjusted with CsOH to pH 7.4. Patch pipettes, with a resistance of 4-6 MΩ, were prepared using a two step-protocol (Sutter Instrument, P-97). Pipettes were fire-polished before use with a microforge (Narishige, MF-9). For glomerular single-channel recording, data was acquired at 10 kHz sampling frequency, and filtered with low-pass filtering at 1 kHz. Holding membrane potential was at -60 mV. Single-channel analysis was carried out using Clampfit 10.4 software (Molecular Devices). NPo were analyzed for 10 sec before and after the application of TRPC5 agonist riluzole (Sigma, R116).

Statistical analysis

All the data were presented as Mean ± SEMs unless described otherwise. Microsoft Office Excel, Origin 6.0 and Graphpad Prism 6 were used for statistical analysis and creation of the graphs. For statistical analysis of differences, an unpaired t-test and a one-way ANOVA followed by Bonferroni or Tukey Correction were used. P value less than 0.05 was considered to be significant.

Figure Legend

Figure 1. Functional TRPC5 channel activity recorded in human iPSC-derived podocytes (iPodos). (**A**) Representative image of a human iPodo patch clamp recording in the whole-cell configuration. P: Glass pipette, N: iPodo nuclear, C: iPodo cytosol. (**B**) Representative diary plots of whole-cell currents from iPodos in response to TRPC5 channel agonist Englerin A (100 nM) in the absence or presence of TRPC5 channel inhibitor AC1903 (30 µM). Currents shown are from +100 mV and -100 mV of a ramp protocol. (**C**) Representative TRPC5 channel current-voltage (I-V) curves from iPodo whole-cell recording. (**D**) Statistical analysis of I-V curves from iPodos treated with Englerin A in the absence or presence of AC1903.

Figure 2. TRPC5 channel inhibitor AC1903 reduces cytosolic ROS and protects cytoskeletal proteins in PAN-treated iPodos. (**A**) Representative cytosolic ROS images in iPodos after 24-hour treatment with PAN (150 µg/mL) with or without AC1903 (30 µM). (**B**) Statistical analysis of the ROS signal intensities. (**C**) Representative Western blots for iPodo cytoskeletal proteins podocin and synaptopodin with podocyte marker protein WT1 after 24 h treatment of PAN (150 µg/mL) with or without AC1903 (30 µM). (**D**) Statistical analysis of podocin, synaptopodin and WT1 protein abundance.

Figure 3. AC1903 reduces proteinuria and protects podocytes from injury in a PAN nephrosis rat model. (**A**) 24-hour urine albumin levels from PBS, PAN

and PAN + AC1903 treated rats on day 0, 3 and 7. PAN 50 mg/kg, AC1903 50 mg/kg. PBS n = 6; PAN n = 15; PAN + AC1903 n = 13. ***p < 0.001, ###p < 0.001. (**B**) Representative PAS staining images of PBS, PAN and PAN + AC1903 treated rats on day 7. Scale bar 20 µm. (**C**) Representative TEM images of podocyte foot processes (FPs) from PBS, PAN and PAN + AC1903 treated rat on day 7. Scale bar 1 µm. (**D, E**) Quantification of podocyte FPEs using the FP number (**D**) and width (**E**) on 1 µm glomerular basement membrane from PBS, PAN and PAN + AC1903 treated rats on day 7. *p < 0.05, ***p < 0.001.

Figure 4. Inhibition of TRPC5 channel activity protects podocytes in PAN rats. (**A**) Representative immunostaining images of podocyte cytoskeletal and marker proteins podocin, synaptopodin, and WT1 from PBS, PAN and PAN +AC1903 treated rats on day 7. Scale bar 20 µm. (**B**) Representative Western blotting results of podocin, synaptopodin, nephrin and WT1 in PBS, PAN and PAN + AC1903 treated rats on day 7. (**C**) Statistical analysis of Western blotting results. *p < 0.05, **p < 0.01.

Figure 5. Single-channel recordings from acutely isolated glomeruli show that systemic treatment with AC1903 abrogates TRPC5 activity. (**A**) Representative TRPC5 single-channel current traces from PBS, PAN and PAN + AC1903 treated rats in response to TRPC5 channel agonist Riluzole (30 µM). (**B**) Quantification of TRPC5 single-channel activity by analysis of NPo values. *p < 0.05, ***p < 0.001.

Figure 6. Inhibition of TRPC5 channel activity reduces Rac1 activity in PAN rats. (**A**) Representative Rac1 activity assay from PBS, PAN and PAN + AC1903 treated rat glomeruli. (**B**) Statistical analysis of normalized Rac1 activity (Rac1-GTP/total Rac1). *$p < 0.05$. (**C**) Correlation of normalized Rac1 activity and 24-hour urine albumin levels in PBS, PAN and PAN + AC1903 treated rats.

Figure 7. The Rac1-TRPC5 pathway mediates injury in human kidney organoids. (**A**) Representative immunostaining images of podocyte cytoskeletal and marker proteins, nephrin (Green) and WT1 (Red) in PBS, PAN and PAN + AC1903 treated human kidney organoids. Scale bar 20 µm. (**B**) Representative Western blot for podocin, synaptopodin, and WT1 in PBS, PAN and PAN + AC1903 treated human kidney organoids. (**C**) Statistical analysis of Western blotting results. *$p < 0.05$, **$p < 0.01$.

Supplementary Figure 1. Inhibition of TRPC5 activity protects podocin and synaptopodin in kidney organoids treated with PAN. (A, B) Immunostaining for podocin (A) and synaptopodin (B) in PBS, PAN and PAN + AC1903 treated kidney organoids. Scale bar 20 µm.

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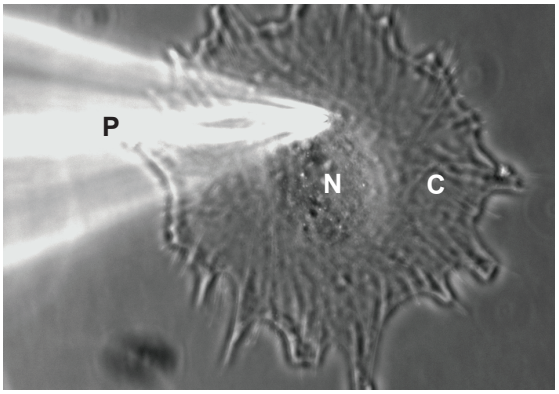
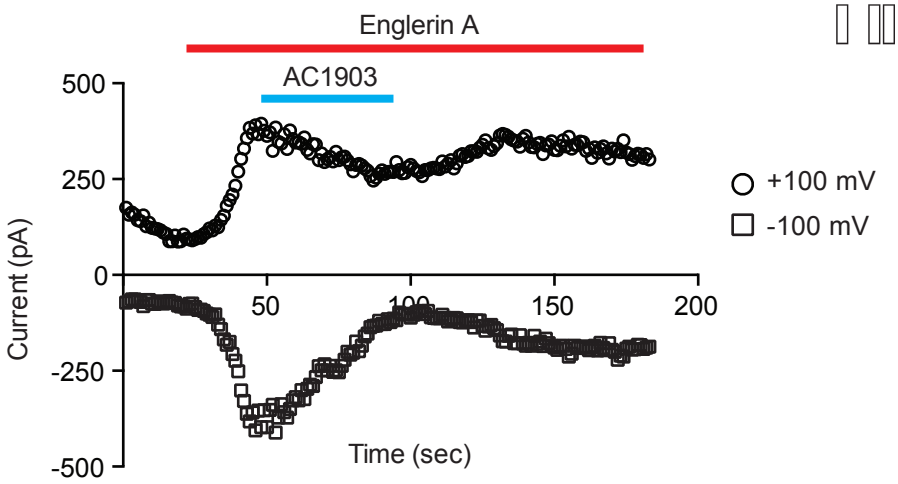
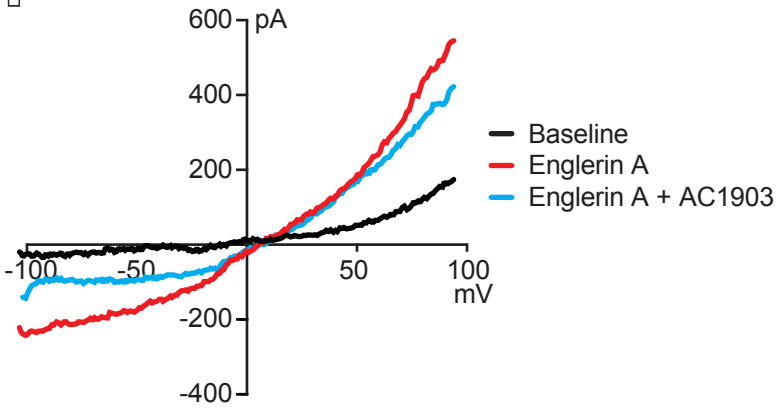

P
N
C


Englerin A
AC1903
500
250
0
-250
-500
Current (pA)
50
100
150
200
Time (sec)
+100 mV
-100 mV


600
pA
400
200
-200
-400
-100
-50
50
100
mV
Baseline
Englerin A
Englerin A + AC1903

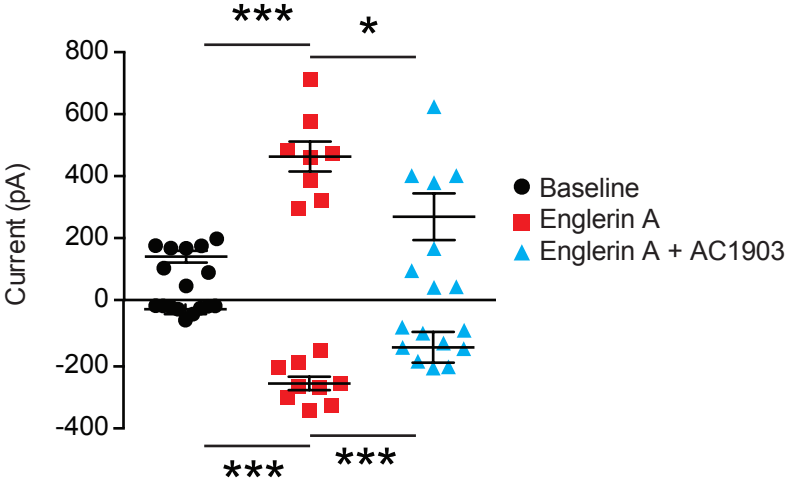
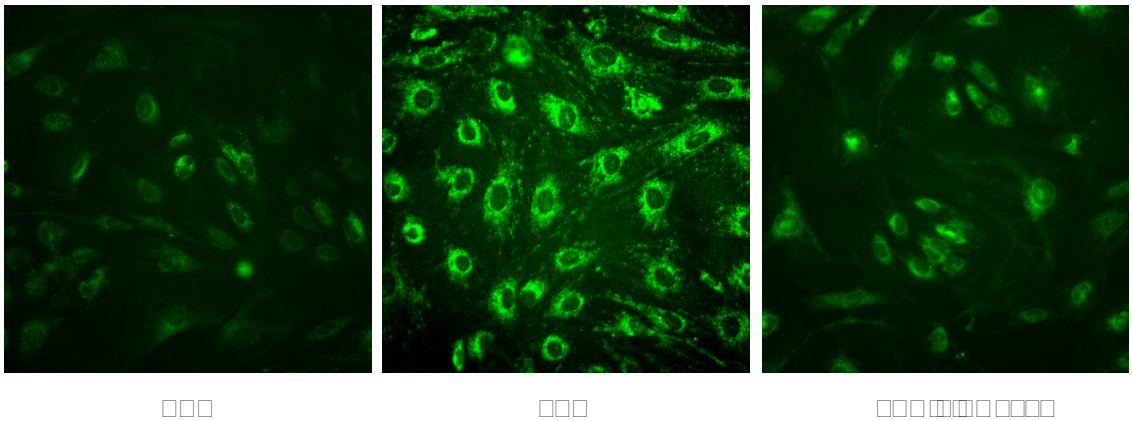
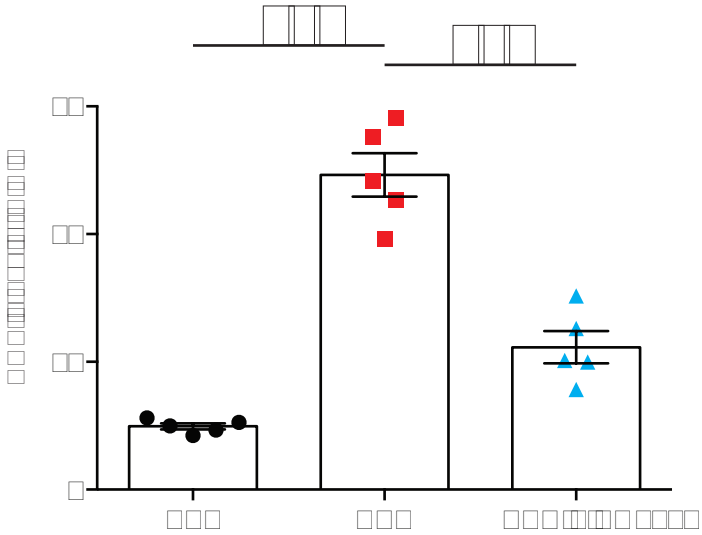

***
*
800
600
400
200
0
-200
-400
Current (pA)
***
***
Baseline
Englerin A
Englerin A + AC1903

Fig.2

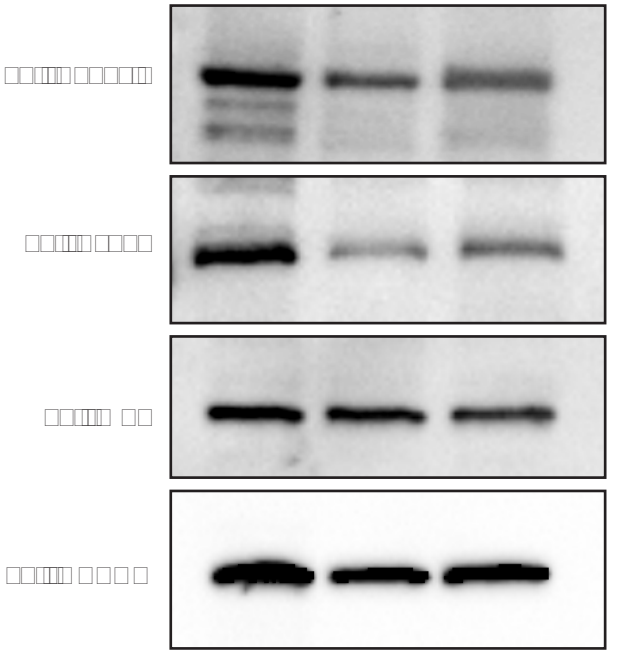
A



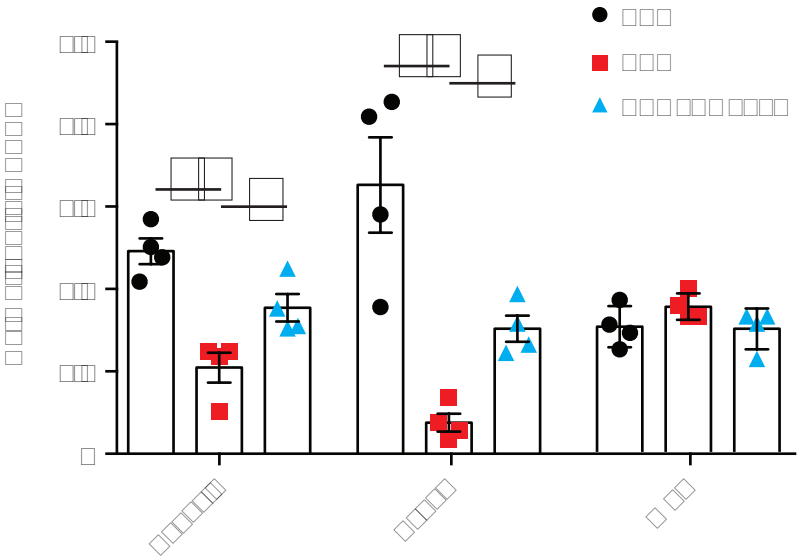
B

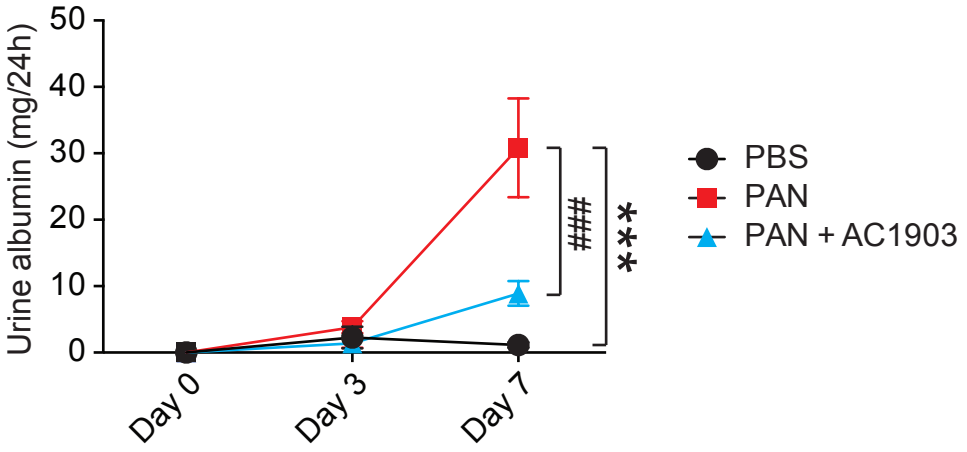


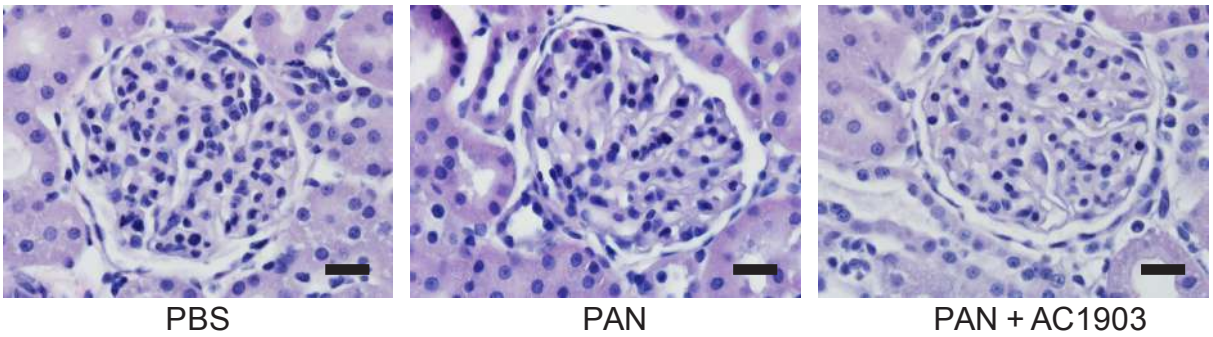
C



D




Urine albumin (mg/24h)
50
40
30
20
10
0
Day 0
Day 3
Day 7
###
***
PBS
PAN
PAN + AC1903


PBS
PAN
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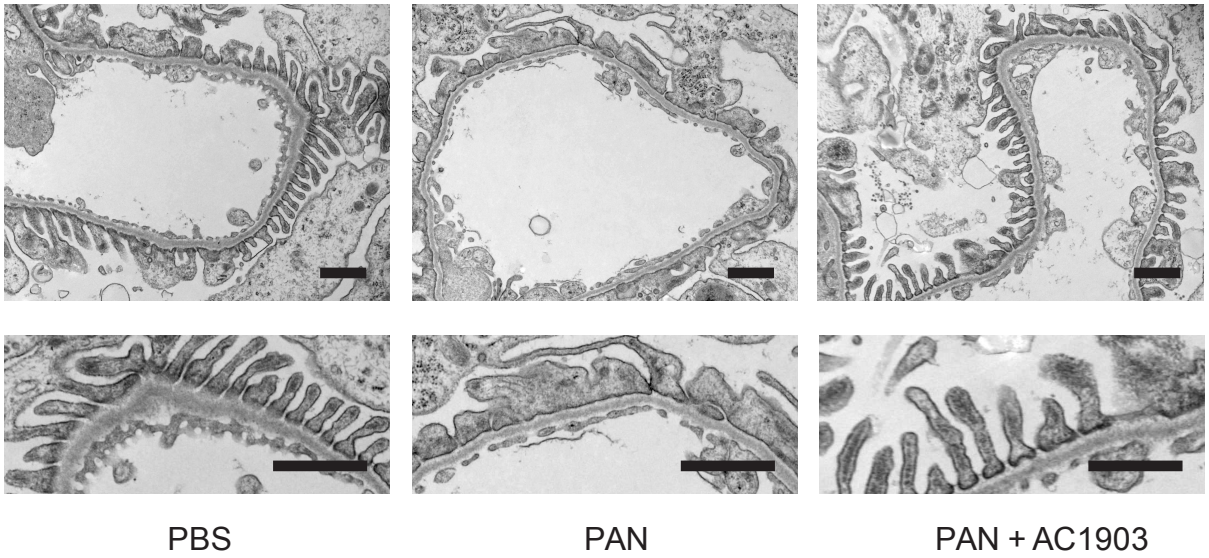
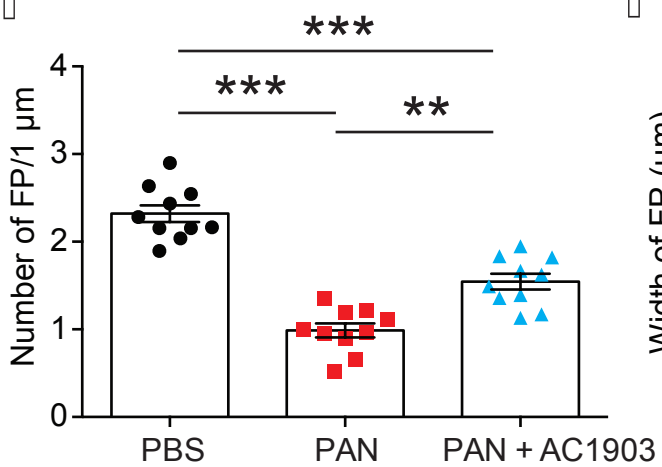

PBS
PAN
PAN + AC1903


Number of FP/1 μm
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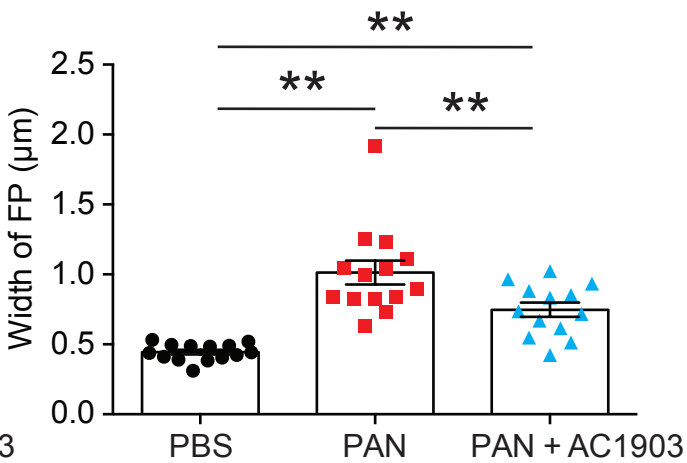
3
2
1
0
***
***
**
PBS
PAN
PAN + AC1903


Width of FP (μm)
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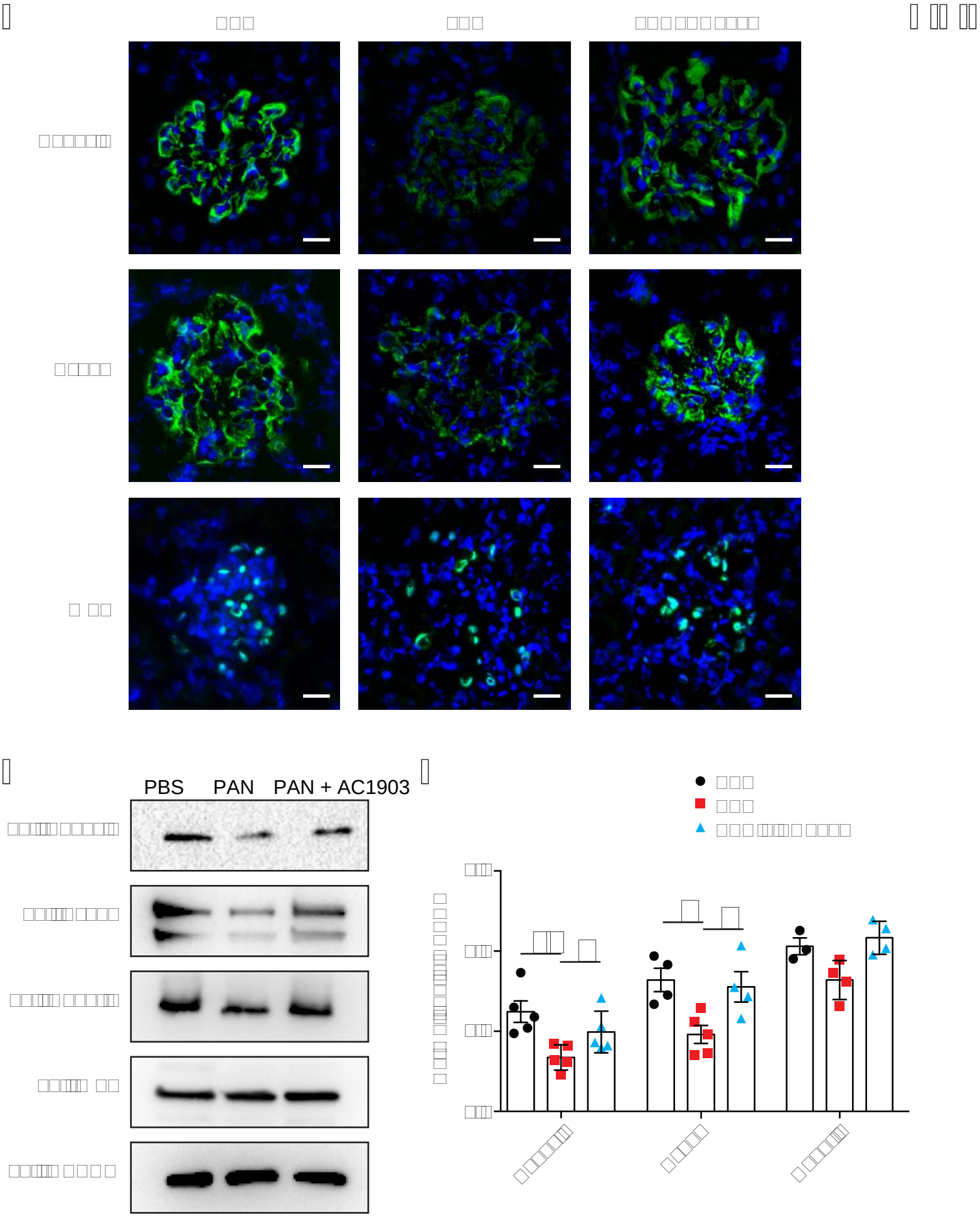
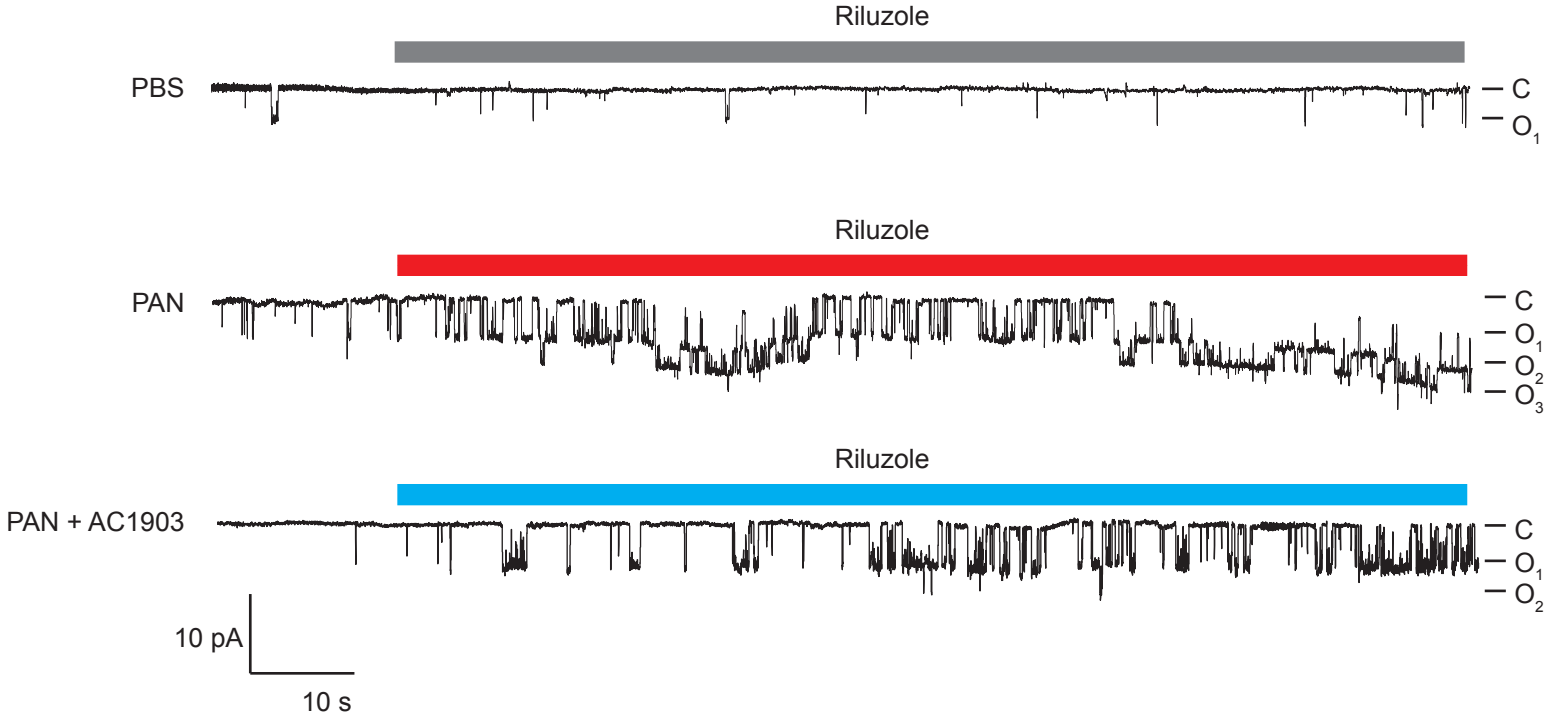
1.5
1.0
0.5
0.0
**
**
**
PBS
PAN
PAN + AC1903


PBS
PAN
PAN + AC1903

A

Fig.5


Riluzole
PBS
C
O1
Riluzole
PAN
C
O1
O2
O3
Riluzole
PAN + AC1903
C
O1
O2
10 pA
10 s

B

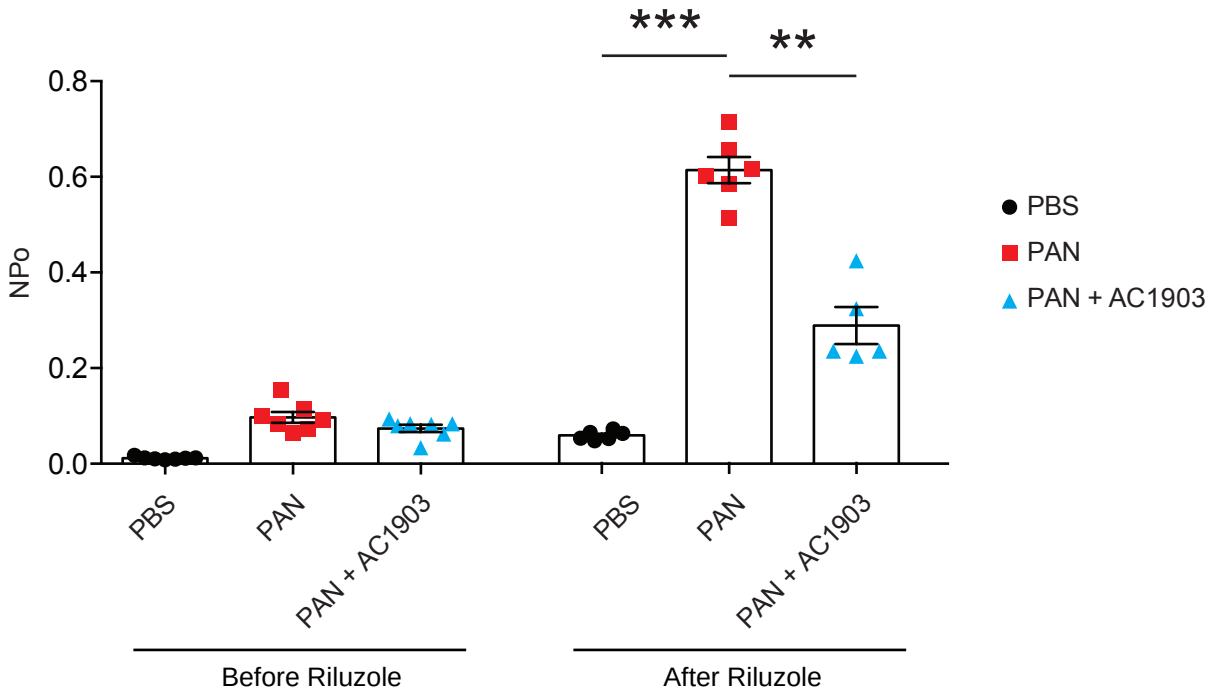
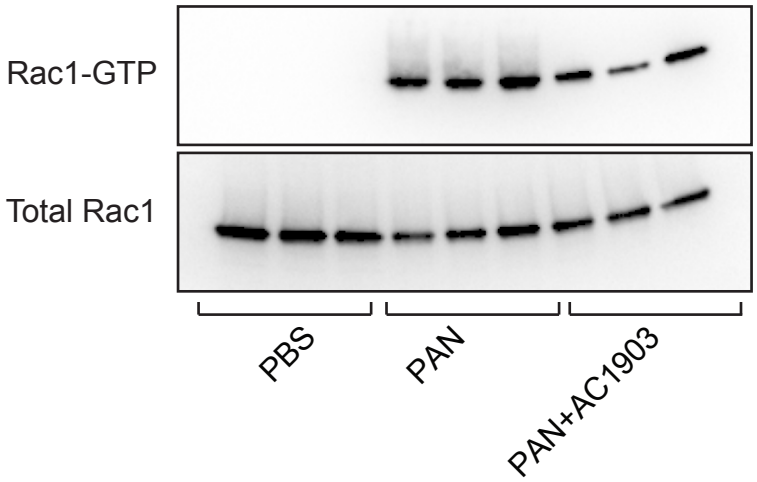
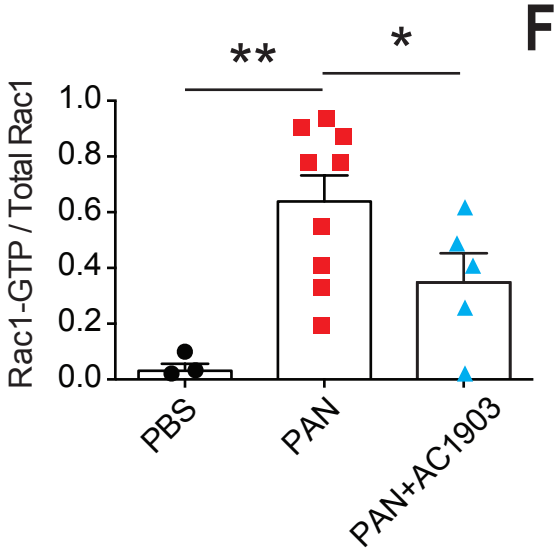

***
**
0.8
0.6
0.4
0.2
0.0
NPo
PBS
PAN
PAN + AC1903
PBS
PAN
PAN + AC1903
Before Riluzole
After Riluzole
PBS
PAN
PAN + AC1903

Fig.6

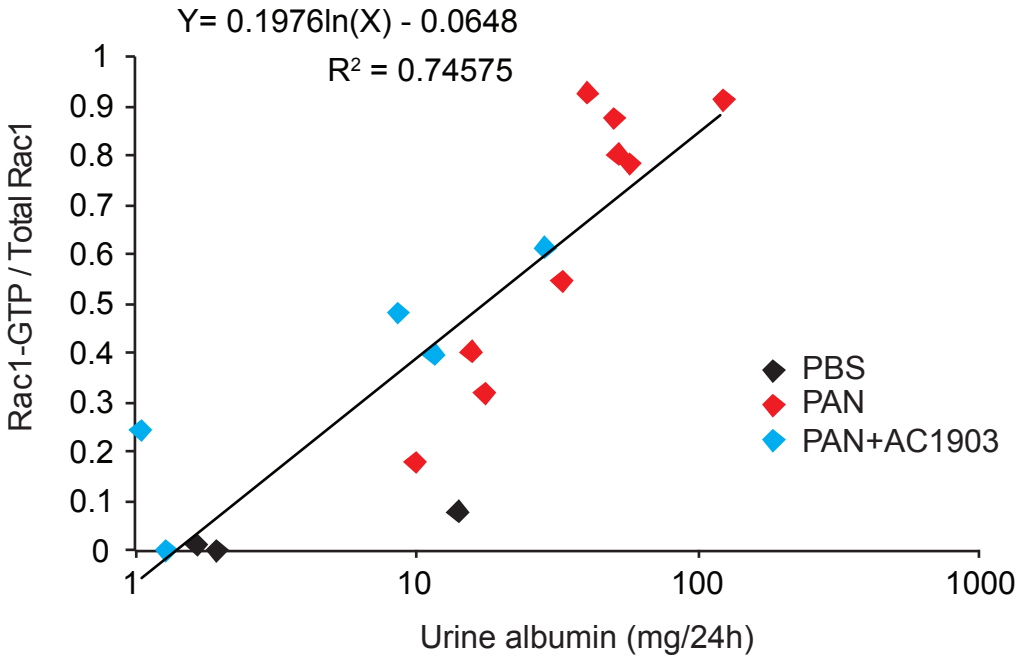
A

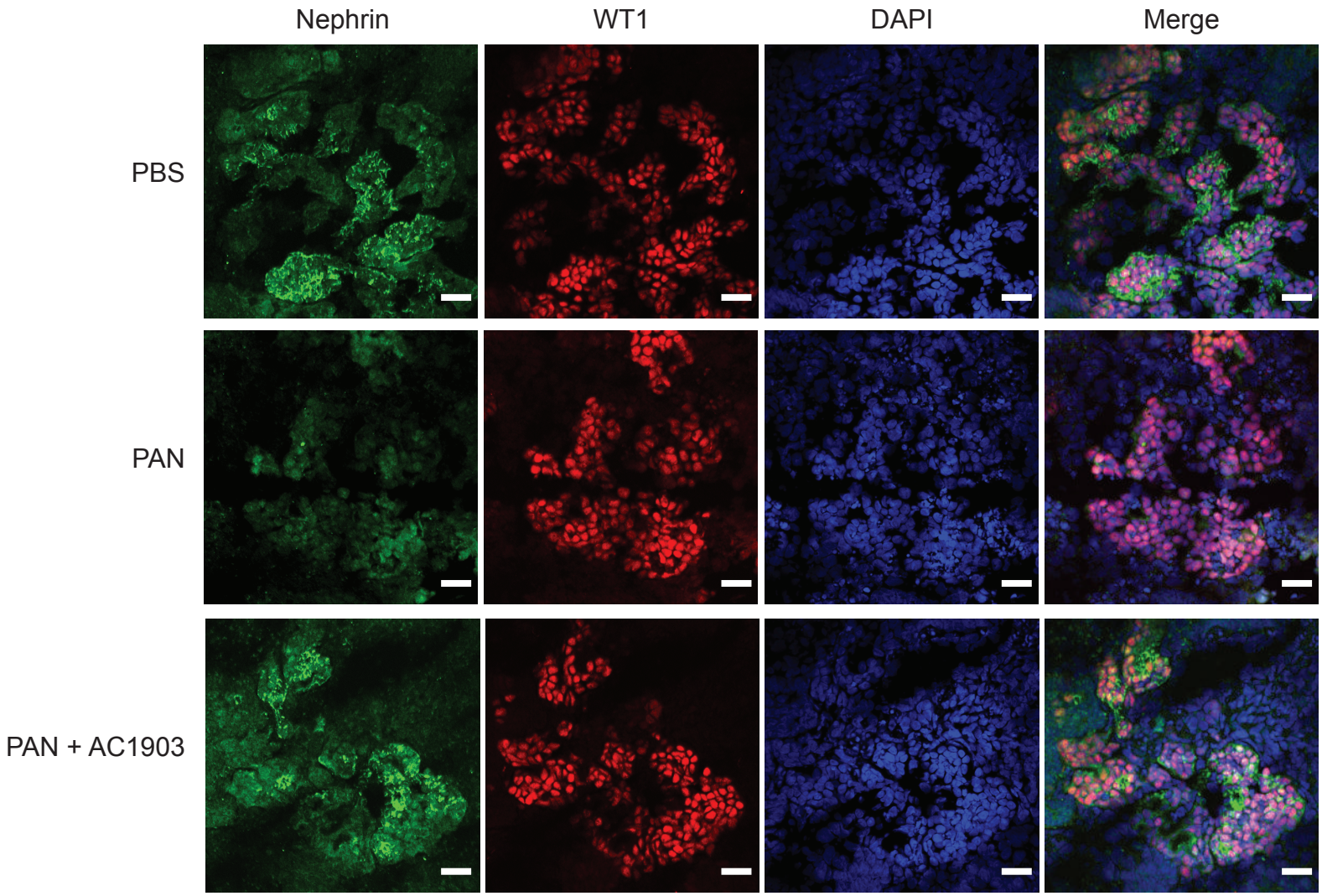

Rac1-GTP
Total Rac1
PBS
PAN
PAN+AC1903

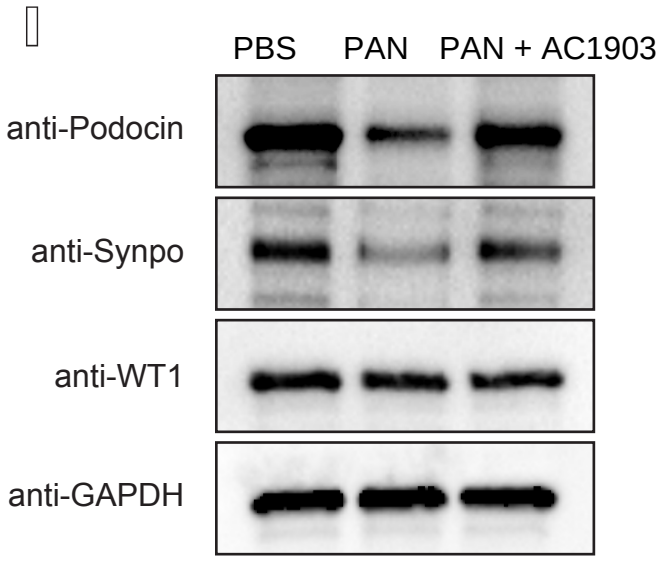
B

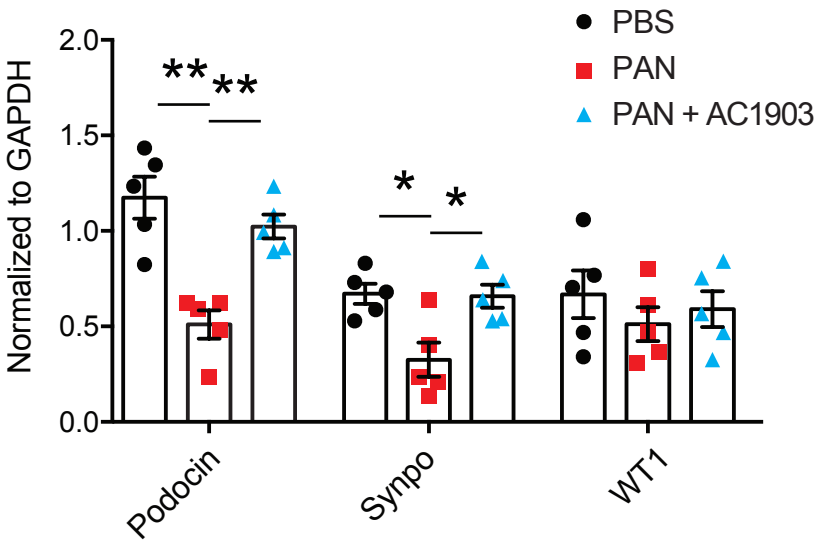

**
*
Rac1-GTP / Total Rac1
1.0
0.8
0.6
0.4
0.2
0.0
PBS
PAN
PAN+AC1903

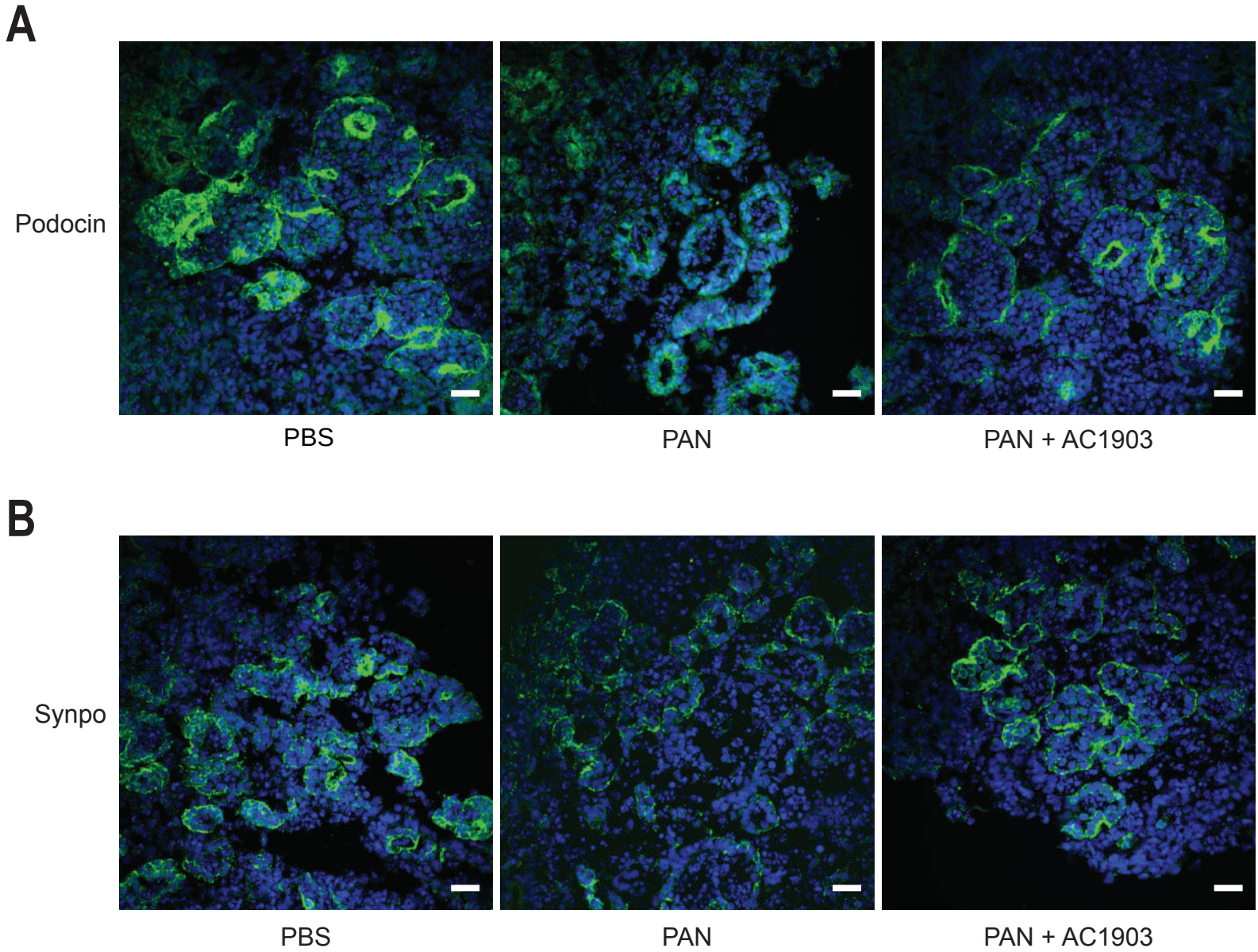
C


Y= 0.1976ln(X) - 0.0648
R² = 0.74575
Rac1-GTP / Total Rac1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
10
100
1000
Urine albumin (mg/24h)
PBS
PAN
PAN+AC1903


Nephrin
WT1
DAPI
Merge
PBS
PAN
PAN + AC1903


PBS
PAN
PAN + AC1903
anti-Podocin
anti-Synpo
anti-WT1
anti-GAPDH


PBS
PAN
PAN + AC1903
Normalized to GAPDH
2.0
1.5
1.0
0.5
0.0
**
**
*
*
Podocin
Synpo
WT1


A
Podocin
PBS
PAN
PAN + AC1903
B
Synpo
PBS
PAN
PAN + AC1903