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The small GTPase regulatory protein Rac1 drives podocyte injury independent of cationic channel protein TRPC5

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Abstract

Transient receptor potential canonical channels (TRPCs) are non-selective cationic channels that play a role in signal transduction, especially in G -protein-mediated signaling cascades. TRPC5 is predominantly expressed in the brain but also in the kidney. However, its role in kidney

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Author contributions

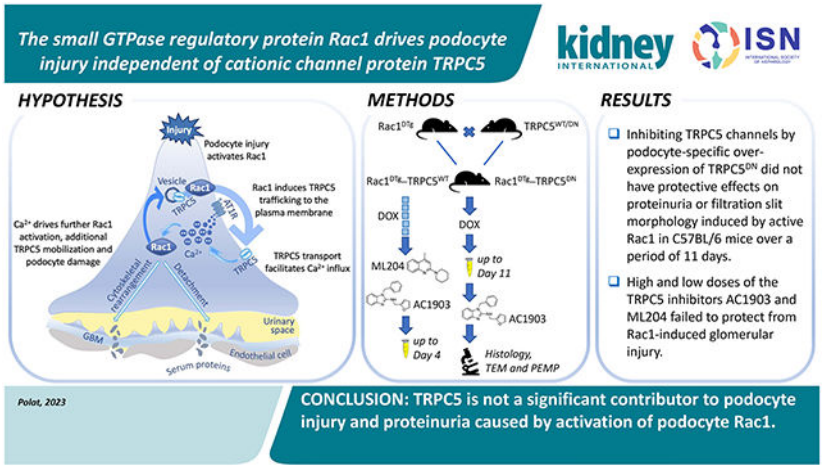
O.K.P., E.I., Y.R.S., B.K., K.Z., M.N., V.S.K., N.E., S.M., T.V.R., and B.S. performed experiments. C.W. and M.M.A. performed data analysis. S.E.D., S.S., A.S., and J.R. conceptualized, critiqued, commented, and wrote the paper. All authors commented, revised, and approved the manuscript.

Competing Interests

S.S. and J.R. are co-founders and shareholders of Walden Biosciences, a biotechnology company that develops novel kidney-protective therapies. N.E. serves as CEO and holds shares of NIPOKA GmbH. Other authors report no competing interests.

physiology and pathophysiology is controversial. Some studies have suggested that TRPC5 drives podocyte injury and proteinuria, particularly after small GTPase Rac1 activation to induce the trafficking of TRPC5 to the plasma membrane. Other studies using TRPC5 gain-of-function transgenic mice have questioned the pathogenic role of TRPC5 in podocytes. Here, we show that TRPC5 over-expression or inhibition does not ameliorate proteinuria induced by the expression of constitutively active Rac1 in podocytes. Additionally, single-cell patch-clamp studies did not detect functional TRPC5 channels in primary cultures of podocytes. Thus, we conclude that TRPC5 plays a role redundant to TRPC6 in podocytes and is unlikely to be a useful therapeutic target for podocytopathies.

Graphical Abstract



Keywords

focal segmental glomerulosclerosis; calcium

Introduction

Transient receptor potential canonical channels (TRPCs) are non-selective cationic channels that play a role in signal transduction, especially in G-protein-mediated signaling cascades. TRPC6, was the first TRPC channel identified in podocytes, and it was shown that gain-of-function mutations within *TRPC6* lead to focal segmental glomerulosclerosis (FSGS)[1,2] a form of chronic kidney disease (CKD) that frequently leads to end-stage kidney disease. Subsequent experiments have suggested that TRPC6 may also play a role in driving acquired forms of CKD[3,4,5]. While kidney disease associated with loss-of-function *TRPC6* mutations has also been reported[6], it is generally thought that chronic dysregulated Ca^{2+} influx from TRPC6 activity leads to podocyte injury underlying progressive CKD and familial FSGS[7].

TRPC5 is a receptor-regulated ion channel predominantly expressed in the brain[8], but also detectable in the kidney[9,10,11]. It has been suggested that TRPC5-mediated Ca^{2+} influx induces Rac1 activation in podocytes, leading to cytoskeletal dysregulation, foot process effacement, and proteinuria[12]. However, other reports showed that TRPC5 overexpression

does not cause or aggravate podocyte injury and proteinuria[13]. In contrast to TRPC5, activation of TRPC6 has been reported to drive RhoA activation and decrease cell motility *in vitro*. These observations have given rise to models whereby TRPC5 and TRPC6 work in tandem to regulate global actin cytoskeleton dynamics in podocytes[12,14]. While that model focuses on GTPases downstream of TRPC channels, it should be noted that small GTPases also function upstream of TRPC channels[4,12].

A recent model has proposed a pathogenic positive feedback loop whereby podocyte injury activates Rac1, which induces exocytotic trafficking of TRPC5 channels to the plasma membrane (Fig. 1A)[15,16]. This, in turn, leads to an increase in TRPC5-mediated Ca^{2+} influx, which drives further Rac1 activation and additional TRPC5 mobilization[15-17]. This model predicts that disrupting the connection between TRPC5 and Rac1, for example, by inhibiting TRPC5, should terminate this positive feedback loop, thereby protecting podocytes from further damage (Fig. 1A). The purpose of the present study was to test this model experimentally.

Short Methods

Mice:

All animal experiments and protocols were approved by the Rush University Institutional Animal Care and Use Committee. Food and water were available *ad libitum*. TRPC5-wild-type ($TRPC5^{WT}$) and TRPC5-dominant negative ($TRPC5^{DN}$) over-expressing mice were generated as described earlier[13]. Podocyte-inducible Rac1 mice ($Rac1^{Dtg}$)[18] were a gift from Dr. Andrey S. Shaw (Washington University, St. Louis, MO). $Rac1^{Dtg}$ and $TRPC5^{WT/DN}$ mice were crossed to obtain $Rac1^{Dtg}$–$TRPC5^{WT}$ and $Rac1^{Dtg}$–$TRPC5^{DN}$ double transgenic mice. All mice in this study are on a C57BL/6 background. Mouse genotypes were confirmed with genomic PCR.

Proteinuria induction:

Proteinuria was evoked by the expression of a constitutively active form of Rac1 induced by the introduction of doxycycline (DOX)-laced chow (2000 ppm) *ad libitum* for 4-11 days, depending on the nature of the experiment, as described previously[18].

Urine collection:

Urine samples were collected from each mouse within each group every 24 h for 4-11 days. Urine samples were stored at −80°C until further use.

Albumin to creatinine ratio (ACR):

Urinary ACR was measured by Albumin ELISA kit (Bethyl Laboratories) and Creatinine assay kit (Cayman Chemicals). Urine samples were diluted 1:20 (for the creatinine assay) and 1:5000 (for the albumin assay) and assayed based on the manufacturer's recommendations. Resulting albumin and creatinine values were converted to mg/ml and g/ml, respectively, and ratioed.

Genomic PCR:

Tails of each mouse were cut and placed in a digestion buffer (MCG buffer, 10% Triton-X, β-mercaptoethanol, and dH_2O). After 3 min of boiling at 93°C, the tails were allowed to cool to room temperature, followed by the addition of Proteinase K. This mixture was left to digest overnight at 60°C. Proteinase K was neutralized by heat inactivation (95°C for 5 min) and 1 μl crude DNA lysate was amplified with the *Taq-Blue* PCR kit according to the instructions from the manufacturer. The DNA was amplified with the following gene-specific primers:

TRPC5 WT/DN F-primer: *5′- GCCTCTGCAGATCTCTTTGG -3′*

TRPC5 WT/DN R-primer: *5′- CAAACATCGTAGCTCCCACA -3′*

Rac1 F-primer: *5′- AAGTTCATCTGCACCACCG-3′*

Rac1 R-primer: *5′- TCCTTGAAGAAGATGGTGCG-3′*

NRTTA F-primer: *5′- GAAGCAGCAGAATGAGTTCAGACTGG-3′*

NRTTA R-primer: *5′- ACTTTGCTCTTGTCCAGTCAGACAT-3′*

Amplified products were separated on 1% agarose gel and visualized using SYBR- safe DNA gel stain.

TRPC5 inhibitors:

DOX-dependent proteinuria was induced as described above. TRPC5 inhibitors, 0.3 mg/kg (low dose) and 1 mg/kg (high dose) of ML204 or 0.75 mg/kg (low dose) and 1.5 mg/kg (high dose) of AC1903 were dissolved in dimethylsulfoxide (DMSO) and injected into mice 48 and 76 hrs after DOX-chow addition to establish the effect of TRPC5 inhibition on ACR. ML204 and AC1903 were obtained from Sigma-Aldrich. Control mice received only the vehicle.

Isolation of glomeruli and primary podocyte cultures:

Mice were euthanized by CO_2 overdose followed by cervical dislocation. The kidneys were immediately collected, rinsed, and decapsulated. The cortex was minced with a steel blade on a Petri dish filled with ice-cold sterile PBS. The resulting minced slurry was passed through three-stack metal sieves with 100, 70, and 53 μm meshes. The slurry was washed thoroughly with ice-cold PBS, and the material that remained in the 53 μm mesh was collected. The collected material was then centrifuged at 1200 rpm for 8 min. The pellet was resuspended in an appropriate volume of primary podocyte medium (RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin). Glomeruli were left undisturbed with intermittent medium changes for 14 days, during which time primary podocytes migrated onto the culture dish.

Western Blot:

Podocytes from $Rac1^{Dtg}$ and $TRPC5^{WT}$ mice were isolated as described above. To activate Rac1, podocytes were treated with DOX (Sigma, D3072, 10 μg/mL for 48 h). The samples were then homogenized by using a Dounce homogenizer, and 30-50 μg of protein was loaded onto Novex Bis-Tris 4-12% gels. Bands were visualized using a customized TRPC5 antibody (GenScript, 1:1000) that has been described previously[13]. GAPDH was detected by a GAPDH antibody (Cell Signaling, 5147, 1:10,000).

Histology:

Periodic Acid-Schiff (PAS) staining was performed at the histology core of the University of Illinois at Chicago (UIC), according to standard methods.

Electron microscopy (TEM):

Kidneys were fixed, prepared, and analyzed at an in-house EM facility at Rush University Medical Center.

Super-resolution microscopy (SIM):

Kidneys were prepared as previously described[19]. The filtration slit density (FSD) was measured at NIPOKA GmbH (Greifswald, Germany). Briefly, paraffin sections of the kidney (2.5 μm) were stained using rabbit anti-podocin (1:150; IBL Hamburg, Germany) and mouse anti-integrin α3 (1:750; Santa Cruz), overnight at 4°C. After intensive washing, the secondary antibodies anti-rabbit Alexa Fluor 488-conjugated IgG (1:450; ChromoTek, Planegg, Germany), anti-mouse Cy3-conjugated IgG antibody (Jackson ImmunoResearch, 1:600) were used as well as DAPI (1:100). Z-stacks (19 planes with a z-step size of 0.12 μm) were acquired using an N-SIM microscope (Nikon, Tokyo, Japan) equipped with a 100× silicone objective. Images were converted into maximum intensity projections (MIPs). Manual selection of podocyte borders was done followed by the automatized identification of the filtration slit. The filtration slit density (FSD) was calculated as the ratio of the total filtration slit per podocyte foot process area. The FSD of 20 glomeruli was determined per animal.

Electrophysiology:

Isolated podocytes were cultured for 14 days and analyzed using cell-attached patch-clamp recording methods as described previously[20]. In brief, bath and pipette solutions were (in mM) 135 NaAsp, 1 $CaCl_2$, 10 HEPES, 2 $MgCl_2$, 10 glucose; pH 7.4., and 126 NaCl, 1.5 $CaCl_2$, 10 HEPES, 10 glucose, respectively, pH 7.4. Current-voltage (I-V) relationships were obtained by monitoring channel activity at applied pipette voltages (−Vp) from 0 to +100 mV with a step of 20 mV for at least 180 s.

Statistics:

Significance tests were analyzed using the Kruskal-Wallis test utilizing public access software implemented at www.vassarstats.net.

Results

In order to test the proposed model whereby a Rac1-induced TRPC5 feed-forward loop drives proteinuria (Fig. 1A), we generated double-transgenic mice that over-express either $TRPC5^{WT}$ or the dominant-negative pore mutant $TRPC5^{DN}$, together with podocyte-specific and inducible expression of a constitutively-active form of Rac1 denoted as $Rac1^{Dtg}$. In this model, $Rac1^{Dtg}$ expression is controlled by the DOX promoter.

$Rac1^{Dtg}$ mice did not exhibit proteinuria prior to feeding with a DOX-supplemented diet (Fig. 1B, 0 days). The majority of $Rac1^{Dtg}$, $Rac1^{Dtg}$–$TRPC5^{WT}$, and $Rac1^{Dtg}$–$TRPC5^{DN}$ mice developed varying degrees of proteinuria, which peaked at day 4, consistent with the published literature[18]. Peak proteinuria subsequently diminished even though animals were continued on the DOX diet for up to 11 days, with proteinuria not fully resolved on day 11 (Fig. 1B). The mechanism of the decline in proteinuria is not known, but it occurred in all of the DOX-treated groups[18]. However, no statistically significant differences were observed between $Rac1^{Dtg}$–$TRPC5^{WT}$, and $Rac1^{Dtg}$–$TRPC5^{DN}$ on any day tested. We note that $Rac1^{Dtg}$–$TRPC5^{WT}$, and $Rac1^{Dtg}$–$TRPC5^{DN}$ had significantly more proteinuria on days 4, 7, and 9, but because this was also seen in animals expressing the dominant-negative form, it cannot be attributed to increased Ca^{2+} influx through TRPC5. We also note that on day 0, the ACR in $Rac1^{Dtg}$ mice was statistically significantly greater in $Rac1^{Dtg}$–$TRPC5^{DN}$ or $Rac1^{Dtg}$–$TRPC5^{WT}$ mice, although urine albumin excretion was not pathologically significant in any group at day 0 (Fig. 1B).

To further examine a possible role for TRPC5 in Rac1-induced proteinuria, we also examined whether the TRPC5 inhibitors ML204 and AC1903 could inhibit Rac1-dependent proteinuria as previously suggested[15,21]. We did not observe a statistically significant protective effect of low or high doses of either ML204 or AC1903 (Fig. 1C). In fact, there was a trend toward increased ACR in animals treated with higher doses of AC1903. We also confirmed that TRPC5 channels were expressed in the relevant transgenic animal models using immunoblot and electrophysiological measurements. Thus, we detected comparable amounts of TRPC5 protein in cultured $Rac1^{Dtg}$ podocytes compared to cultured podocytes that do not express $Rac1^{Dtg}$ (Supplementary Figure S1). Because protein levels of ion channels do not indicate their activity, we carried out single-channel recordings to access the functional activity of TRPC5 channels. In podocytes from mice over-expressing $TRPC5^{WT}$ (n=8) we detected two distinct types of channels based on their unitary slope conductance. One channel had a unitary conductance of ~38±1 pS, whereas a second had a unitary conductance of ~25±1 pS. These correspond to the conductances expected for TRPC5 and TRPC6, respectively[20,22-24]. By contrast, only the lower-conductance channel was detected in podocytes from $TRPC5^{DN}$ (n=9) and Rac-1^{Dtg} mice (n=7) (Figs. 1D,E) which most likely represents the activity of TRPC6 channels.

We also examined whether the proteinuria observed in DOX-fed mice was associated with podocyte foot process (FP) effacement as is typically seen in CKD. PAS-stained sections did not reveal any aberrant gross morphological changes (Supplementary Figure S2). Some degree of FP effacement was detected by transmission electron microscopy (TEM) in kidney sections from $Rac1^{Dtg}$–$TRPC5^{WT/DN}$ mice fed with DOX but not from mice fed with

normal chow (Figs. 2A-F, Supplementary Figure S3). Surprisingly, foot process effacements were more severe in mice treated with drugs previously used as TRPC5 inhibitors, which may be due to an off-target toxic effect of the inhibitors on podocytes. In order to assess the impairment of the filtration apparatus, we analyzed filtration slit density (FSD). FSD levels were used to quantify the extent of FP effacement using super-resolution-microscopy combined with software-driven image analysis (Podocyte Exact Morphology Measurement Procedure, PEMP) as previously described[19] (Figs. 2G,H). The FSD value from control chow-fed Rac1-TRPC5^{WT} mice was 4.00 ± 0.24 μm^{-1} (Fig. 2A). DOX treatment to induce expression of Rac1^{Dtg} had a minimal effect on the FSD value, which was 3.97 ± 0.16 μm^{-1} in those animals (Fig. 2C). Injection of the TRPC5 inhibitor AC1903 led to a significantly lower FSD value of 2.82 ± 0.55 μm^{-1} indicating a shortening of the FPs, which typically occurs during podocyte disease (Fig. 2E). Rac1-TRPC5^{DN} mice followed a similar trend with average FSD values of 3.99 ± 0.35 μm^{-1}, 3.92 ± 0.50 μm^{-1}, and 3.41 ± 0.60 μm^{-1} for control chow-fed, DOX-fed, and DOX-fed +TRPC5 inhibitor-treated mice, respectively (Figs. 2B,D, and F). All values of the length measurements are shown in Fig. 2H. These data show that TRPC5 inhibition fails to protect against FP effacement induced by Rac1 and instead may even exacerbate FP effacement.

Discussion

This study shows that TRPC5 is not a significant contributor to podocyte injury and proteinuria caused by overexpression of Rac1. TRPC channels, especially TRPC5 and TRPC6, are typically activated in receptor-mediated signal transduction cascades, including G-protein coupled receptors that drive PIP_2 depletion and the production of diacylglycerol (DAG). This can occur, for example, through the activation of AT1 angiotensin receptors and P_2Y purinergic receptors[7,20,25]. It has been suggested that P_2Y and TRPC6 contribute to pathogenic Ca^{2+} influx in podocytes *in vivo*[7,20,25].

The influx of Ca^{2+} through active TRPC channels is thought to induce actin cytoskeleton remodeling mediated in part by the Rho family GTPases (e.g., RhoA and Rac1)[26,27]. Rac1 and RhoA typically function antagonistically to promote or constrict lamellipodia and filopodia[26,27]. It has been suggested that Rac1 is specifically activated downstream of TRPC5 whereas RhoA activation is a downstream target of TRPC6[12]. This concept formed the idea that TRPC5 itself is a driver or propagator of podocyte injury and proteinuria, and earlier reports have suggested inhibition of TRPC5 by various means to reduce podocyte injury and proteinuria in certain disease models[15,16,21]. On the other hand, we have previously reported that over-expression of TRPC5 channels in mice does not cause or aggravate glomerular barrier injury and proteinuria[13], in marked contrast to what occurs with over-expression of TRPC6[28,29].

The key results of the present study are that experimental manipulations that inhibit TRPC5 channels, including podocyte-specific over-expression of TRPC5^{DN} and sustained administration of the TRPC5 inhibitors ML204 and AC1903, had no protective effect on proteinuria or filtration slit morphology induced by constitutive activation of Rac1 in C57BL/6 mice over a period of 11 days. The 11 day-protocol used here was selected based on a previous study[18]. These results are consistent with our previous publication showing

that activation of TRPC5 does not cause or aggravate other animal models of glomerular disease[18]. Indeed, one significant result of the present study is that some exacerbation of proteinuria occurred with podocyte-specific overexpression of either $TRPC5^{DN}$ or $TRPC5^{WT}$. It is possible that this reflects a type of unfolded protein or endoplasmic reticulum stress response, or it could reflect an effect of enhanced trafficking of TRPC6 channels to the cell surface. In the present study, we also observed that low and high doses of the TRPC5 inhibitors AC1903 and ML204 failed to produce protection from Rac1-induced glomerular injury. Indeed, by certain measures, AC1903 had a detrimental effect. Recent work has shown that AC1903 affects multiple TRP channels, including TRPC3, TRPC6, and TRPV4[30], which might explain these adverse effects.

In contrast to the predictions of recent models[15,16], podocyte-specific over-expression of constitutively active Rac1 by itself did not lead to a detectable increase in TRPC5 activity. Unitary currents corresponding to TRPC5 were only seen in mice that over-expressed both $TRPC5^{WT}$ and $Rac1^{Dtg}$. Collectively, these data suggest that TRPC5 channel activity does not synergize with Rac1 to induce podocyte injury. It should also be noted that sustained activation of Rac1 would be expected to produce many other effects in cells, for example, activation of NADPH oxidases NOX2 and NOX4 and consecutive generation of reactive oxygen species (ROS), activation of TRPC6[31], and possibly other cationic channels[7]. There is a substantial body of literature showing that TRPC6 knockout and inactivation is protective in multiple models of kidney disease[32,33].

A limitation of the present study is that it has not examined actions of Rac1 upstream of channels. Another limitation is that the experimental designs have only tested the role of TRPC5 downstream of Rac1 activation and have not examined other insults such as angiotensin II or salt load[18]. Nevertheless, based on these observations and those in our previous paper using LPS-treated proteinuric mice[13], in which Rac1 activation is expected, we conclude that TRPC5 is unlikely to be a useful therapeutic target for podocytopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Statement

In recent years, TRPC5 inhibitors have been developed with the idea to combat proteinuria in glomerular diseases such as diabetic nephropathy and focal segmental glomerulosclerosis. However, published data obtained in rodent models and through cell culture studies are discrepant. In this paper, we tested the key hypothesis that TRPC5 activity is in response to activation of podocyte Rac1 using genetic mouse models. Data shows that TRPC5 expression and activity are redundant for Rac1 mediated proteinuria, questioning TRPC5 inhibition to be of benefit in glomerular diseases.

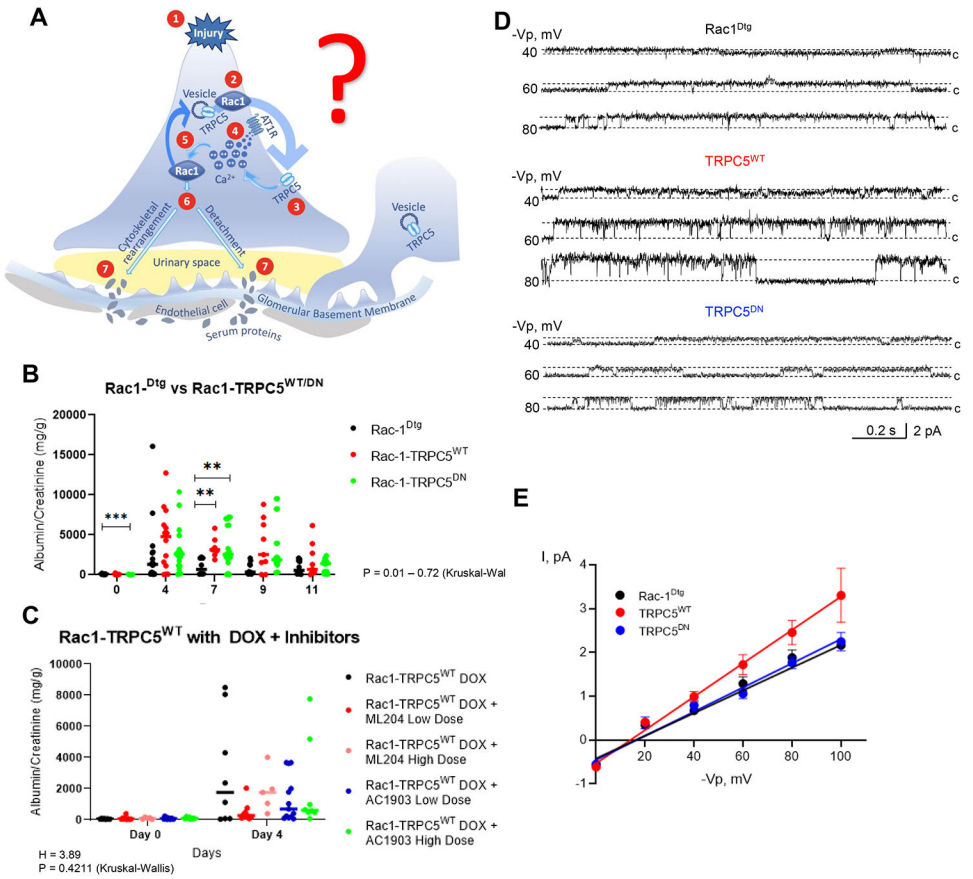

Figure 1: TRPC5 activity does not influence Rac1-induced proteinuria.

(A) A model adapted from Chung and Shaw[16]. The model depicts the endless feedback loop between TRPC5 and Rac1. The sequence of events includes: (1) Injury due to external or internal factors; (2) Activation of Rac1; (3) Movement of TRPC5 to the cell surface due to the presence of Rac1; (4) Ca^{2+} influx; (5) Ca^{2+}-dependent Rac1 activation; (6) Rac1 signaling to other mediator molecules; and (7) Podocyte damage. **(B)** Albumin (mg) to creatinine (g) ratio (ACR) is shown on the y-axis with the progression of proteinuria in various groups of mice as indicated. The black color plot indicates $Rac1^{Dtg}$ mice, the red color shows $Rac1^{Dtg}$–$TRPC5^{WT}$ and the green color indicates $Rac1^{Dtg}$–$TRPC5^{DN}$ mice. Error bars denote SEM. Statistical significance between groups was assessed by the Kruskal-Wallis test (*, $p< 0.05$; **, $p<0.01$; NS, $p>0.05$). **(C)** ACR measurements of mice injected with DMSO (black bars), ML204 low dose (LD) (red), ML204 high dose (HD) (Orange), AC1903 LD (dark blue), and AC1903 HD (green). Low doses of ML204 and AC1903 were 0.3 and 0.75 mg/kg, and high doses were 1 mg/kg and 1.5 mg/kg, respectively. Error bars denote SEM. No significant difference was detected by the Kruskal-Wallis test. **(D)** Representative traces of unitary currents demonstrating activity from $Rac1^{Dtg}$, $Rac1^{Dtg}$–$TRPC5^{WT}$, and $Rac1^{Dtg}$–$TRPC5^{DN}$ podocytes, respectively. Membrane potentials are shown. C: closed state. **(E)** Current-voltage (I/V) relationships for identified channels. Two types of channels were detected in cells from mice overexpressing TRPC5 (n=8), with unitary slope conductances of 38±1 pS, and 25±1 pS. Only the 25 pS channels were detected in mice overexpressing $TRPC5^{DN}$ (n=9) and Rac1 (n=7).

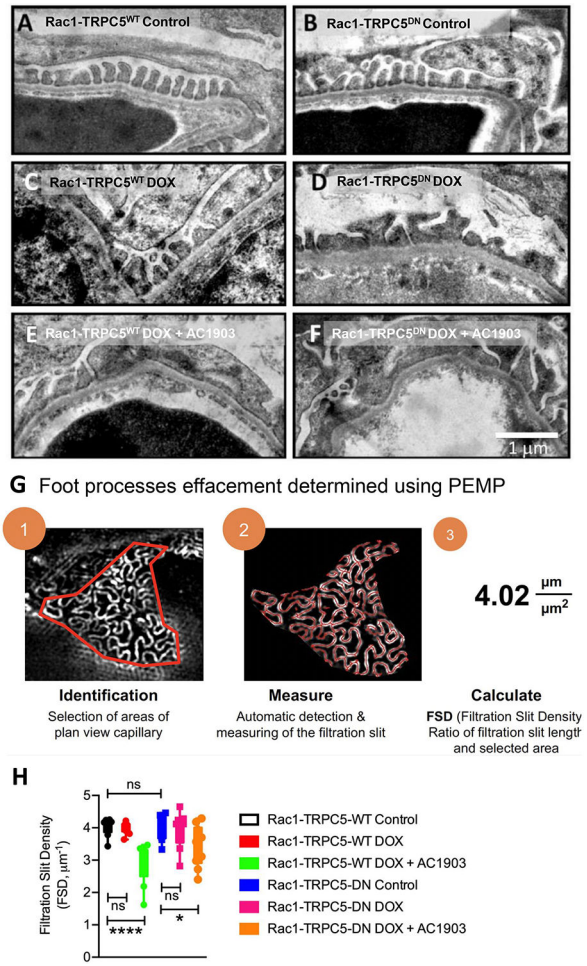

Figure 2: TRPC5 channels have no effect on podocyte foot process (FP) morphology.

Representative TEM images of podocyte FPs from control double transgenic (Dtg) $Rac1^{Dtg}$–$TRPC5^{WT}$mice **(A)** and $Rac1^{Dtg}$–$TRPC5^{DN}$ **(B)** that were fed a normal diet. DOX induction of Rac1 resulted in visible foot process effacement in both **(C)** $Rac1^{Dtg}$–$TRPC5^{WT}$ and **(D)** $Rac1^{Dtg}$–$TRPC5^{DN}$. Treatment with the TRPC5 inhibitor AC1903 did not improve FP morphology in either **(E)** $Rac1^{Dtg}$–$TRPC5^{WT}$ or **(F)** $Rac1^{Dtg}$–$TRPC5^{DN}$ mice. Scale bar: 1 µm. Foot slit density measurement methodology **(G)** for the quantification of foot process lengths **(H)**. Statistical significance between groups was assessed by *one-way* ANOVA test (*, $p< 0.05$; ****, $p<0.0001$; NS, $p>0.05$).