Induction of TRPC6 Channel in Acquired Forms of Proteinuric Kidney Disease

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Injury to podocytes and their slit diaphragms typically leads to marked proteinuria. Mutations in the *TRPC6* gene that codes for a slit diaphragm–associated, cation-permeable ion channel have been shown recently to co-segregate with hereditary forms of progressive kidney failure. Herein is shown that induced expression of wild-type TRPC6 is a common feature of human proteinuric kidney diseases, with highest induction observed in membranous nephropathy. Cultured podocytes that are exposed to complement upregulate TRPC6 protein. Stimulation of receptor-operated channels in puromycin aminonucleoside–treated podocytes leads to increased calcium influx in a time- and dosage-dependent manner. Mechanistically, it is shown that TRPC6 is functionally connected to the podocyte actin cytoskeleton, which is rearranged upon overexpression of TRPC6. Transient *in vivo* gene delivery of TRPC6 into mice leads to expression of TRPC6 protein at the slit diaphragm and causes proteinuria. These studies suggest the involvement of TRPC6 in the pathology of nongenetic forms of proteinuric disease.

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Podocytes are specialized cells that reside in the kidney glomerulus and cover the outer surface of the filtering capillaries (1). Their foot processes form a complex interdigitating pattern with slits that are bridged by extracellular protein–protein contacts to form the slit diaphragm (2). Podocyte foot processes and slit diaphragms contribute to the formation of the glomerular filter, combining a seal to macromolecules with high hydraulic conductivity that is subject to regulation. These two characteristics both are perturbed in most glomerular kidney diseases, resulting in a leakage of proteins into the urine, which may give rise to progressive damage and decline in GFR (3). Mutations in genes that are specific for podocyte development and function have taken center stage in understanding glomerular kidney diseases that cause various degrees of proteinuria and, potentially, the progression to ESRD (4).

Recently, a mutation in the cation-permeable ion channel TRPC6 was identified as a culprit for a familial form of FSGS (5). Our group subsequently characterized TRPC6 as a component of the slit diaphragm, suggesting that it functions as critical regulator of normal renal function (6).

TRPC6 belongs to the TRP superfamily of ion channel-forming proteins. TRP channel genes encode subunits that form ion channels in many cell types (7,8). Members of the TRP superfamily have been identified on the basis of amino acid sequence and structural similarity and are classified into seven subfamilies (9). TRPC6 is a member of the TRPC subfamily and has been reported to be Ca^{2+} selective. Ca^{2+} entry through other TRPC channels has been shown to increase cytoplasmic Ca^{2+}, allowing for phosphorylation of signal transduction proteins and transcription factors or for the regulation of cytoskeletal dynamics (10). Mutations of TRP proteins produce many renal diseases, including Mg^{2+} wasting, hypocalcemia, and polycystic kidney diseases (11). The discovery of mutations in the TRPC6 gene as cause for hereditary FSGS as well as the localization of TRPC6 at the slit diaphragm open a whole series of questions about the physiologic and pathophysiologic role of this channel in podocytes. On the basis of our previous findings in hereditary FSGS, we hypothesized that also in acquired forms of kidney disease, TRPC6 expression levels and channel function may contribute to glomerular disease pathogenesis *via* a dysregulated Ca^{2+} influx at the slit diaphragm site. In this article, we provide evidence that, in addition to the effects of gain-of-function mutations in the *TRPC6* gene, elevated levels

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of wild-type TRPC6 protein in acquired glomerular diseases lead to podocyte dysfunction. We discuss a role of elevated TRPC6 levels in the reorganization of the podocyte actin cytoskeleton and the development of proteinuria.

Materials and Methods

Animals and Treatments

All animal studies were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital. C57BL6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). The passive Heymann nephritis (PHN) model of experimental membranous nephropathy was induced in male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) by intraperitoneal injection (5 ml/kg body wt) of sheep antibody to Fx1A (12). Rats that received an injection of normal sheep serum (5 ml/kg body wt) served as controls for the anti-Fx1A antibody. Control and PHN rats were killed at 5, 11, and 28 d ($n = 4$ to 5 for each time point) for renal biopsies and glomerular isolation. For verification of efficiency of anti-Fx1A injection, urine protein excretion was determined using the sulfosalicylic acid method before control and PHN rats were killed (13) (data not shown). For assessment of TRPC6 mRNA levels and puromycin aminonucleoside (PAN)-induced albuminuria, rats were given three consecutive injections of PAN (50 mg/kg body wt) at days 0, 10, and 20. At days 27 and 34, rats were killed and kidneys were collected. Glomeruli were isolated using standard sieve technique. TRPC6 mRNA levels were analyzed by quantitative real-time reverse transcriptase–PCR according to standard protocols. Albuminuria was assessed by collection of urine from each animal for 24 h in metabolic cages (Nalgene, Rochester, NY) and measurement of the albumin concentration by ELISA using the Neph-Rat Kit (Exocell, Philadelphia, PA). For TRPC6 immunostainings, rats were given a single injection of PAN (50 mg/kg body wt) at day 0. At days 0, 4, 8, and 28, rats were killed and kidneys were collected. Kidneys were processed for immunostaining as described before (6).

Patients

Human kidney biopsies, obtained in a multicenter study for renal gene expression analysis (the European Renal cDNA Consortium), were processed as described (14). Informed consent was obtained according to the respective local ethical committee guidelines. Histologies were stratified by the reference pathologists of the European Renal cDNA Consortium. Predeveloped TaqMan assay reagent was used for TRPC6 and the internal standard 18S rRNA. Quantification of the given templates was performed according to the standard curve method. All measurements were performed in duplicate; controls that consisted of bi-distilled H_2O were negative in all runs. Microdissected glomeruli from 34 patients with proteinuric diseases and eight control subjects were analyzed. Patients were stratified according to their histologic diagnosis into FSGS ($n = 9$), minimal-change disease (MCD; $n = 13$), and membranous glomerulonephritis (MGN; $n = 28$). For control biopsies, renal tissue was derived from healthy poles of tumor nephrectomies (CON; $n = 9$).

Cell Culture

To determine the expression of TRPC6 in podocytes that were stimulated with complement *in vitro*, we used conditionally immortalized mouse podocytes (15). All studies were performed between days 15 and 17 of growth-restrictive conditions. For induction of sublytic C5b-9 attack, mouse podocytes were stimulated with sheep anti-mouse podocyte IgG and complement as described previously (16). In group C6+, podocytes were sensitized with medium that contained 0.5 mg/ml sheep anti-mouse podocyte IgG at 37°C for 30 min. Sensitized podocytes then were exposed to a sublytic amount of complement for 30 min, washed three times, and then incubated in growth medium for 48 h. In group C6−, which served as control for C5b-9–attacked cells, podocytes were sensitized with sheep anti-mouse podocyte IgG and exposed to complement that lacked C6 for 30 min. Whole-cell lysates were isolated in RIPA buffer, and Western blot analysis was performed according to standard procedures using anti-TRPC6 antibody and anti-actin antibody as a loading control.

Immunohistochemistry, Immunocytochemistry, and Electron Microscopy

Human glomerular biopsies were fixed in cold acetone and stained with anti-TRPC antibody (Abcam, Cambridge, MA) and monoclonal anti-synaptopodin antibody (15) following standard protocols. Immunocytochemistry on cultured podocytes was done on cells that were grown on coverslips as described previously (6). Actin cytoskeleton was visualized with the Alexa Fluor 594 phalloidin conjugate (Molecular Probes, Eugene, OR). Renal biopsies from PHN rats were fixed in methyl Carnoy's solution and embedded in paraffin. Indirect immunoperoxidase staining (16) was performed on 4-μm sections using TRPC6 primary antibody (omitted in controls). Glomeruli were scored from 1 to 4 on the basis of the percentage of positively stained podocytes in each glomerulus (0 to 25% positive = 1; 26 to 50% positive = 2; 51 to 75% positive = 3; 76 to 100% positive = 4). Scoring values were statistically analyzed by one-way ANOVA, and Bonferroni multiple comparison test was performed. At all time points, the increase in TRPC6 expression was statistically significant. Transmission electron microscopy and immunoelectron microscopy were performed according to standard protocols as described previously (6).

Calcium Flux Studies

Differentiated cultured podocytes were treated for 24 h with 10 or 50 μg/ml PAN. Cells then were loaded with the Fluo-4 calcium indicator in 0.1 mM Ca^{2+} for 1 h. Receptor-operated channels were activated by addition of 100 μM 1-oleoyl-2-acetyl-sn-glycerol, followed by changing the extracellular buffer to 2 mM Ca^{2+} to distinguish membrane-associated channel-dependent changes in calcium.

In Vivo *Gene Delivery*

FLAG-TRPC6 plasmids were introduced into C57BL6 mice using the TransIT *in vivo* gene delivery system (Mirus Bio, Madison, WI). In brief, 15 μg of plasmid DNA was mixed with 15 μl of Mirus polymer solution and 170 μl of endotoxin-free H_2O. It then was topped with 1.8 ml of Mirus delivery solution before injection through tail vein. Fifteen hours after gene delivery, proteinuria was assessed by Bradford protein assay (Bio-Rad, Hercules, CA). Mice that received an injection showed significant proteinuria as compared with baseline urine protein excretion ($P = 0.0275$). For the luciferase activity assay, kidneys were harvested from eight mice 12 h after gene delivery of 10 μg of pGL3 luciferase reporter plasmid, and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI). We also tested kidneys from eight control mice that did not receive an injection. Light intensity was measured on a plate reader luminometer.

Statistical Analyses

Unless stated otherwise, statistical analyses were performed by using the unpaired t test, and the null hypothesis was rejected at the 0.05 level. Values are presented as means ± SD.

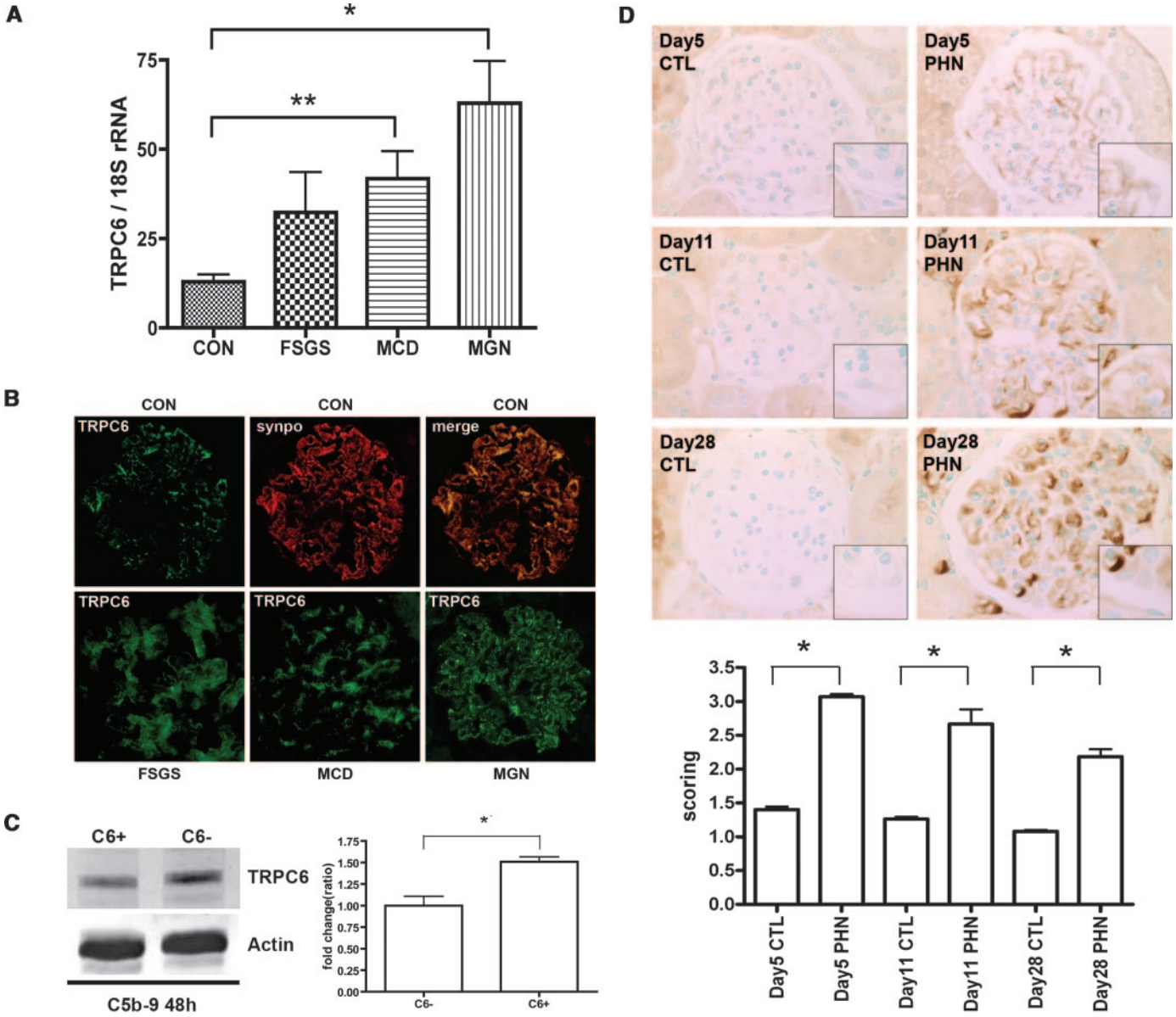

Figure 1. TRPC6 mRNA and protein expression in human glomerular disease. (A) The ratio of human TRPC6 mRNA *versus* 18S ribosomal RNA was determined by quantitative real-time reverse transcriptase–PCR. Compared with control kidneys (CON; $n = 9$), TRPC6 mRNA was statistically significantly upregulated in kidneys from patients with minimal-change disease (MCD; $^{**}P = 0.0063$; $n = 13$) and with membranous glomerulonephritis (MGN; $^{*}P = 0.0238$; $n = 28$). In kidneys from patients with FSGS ($n = 9$), the increase in TRPC6 mRNA did not reach statistical significance. (B) Kidneys were examined by indirect immunofluorescence using a primary antibody against TRPC6. Double labeling with the podocyte marker synaptopodin leads to a yellow overlap (merge). In FSGS and MCD kidneys, TRPC6 staining was induced and appeared clustered. TRPC6 intensity was stronger in FSGS than in MCD. TRPC6 staining was strongest and appeared granulated in glomeruli during MGN. TRPC6 levels are increased in models of MGN. (C) Cultured podocytes were differentiated for 15 to 17 d and stimulated with antipodocyte antibody and C6− or C6+ serum. Western blot analysis shows that the expression of TRPC6 increased in cells that were stimulated with C6+ serum when compared with cells that were stimulated by C6− serum (left). Densitometric analysis of three Western blots revealed a significant increase of TRPC6 in podocytes that were stimulated with C6+ serum compared with C6− serum (right; $P < 0.05$). (D) Immunostaining for TRPC6 shows increased glomerular TRPC6 expression levels at days 5, 11, and 28 of passive Heymann nephritis (top). Although TRPC6 expression predominantly followed a podocyte staining pattern, few endothelial also were positively stained. Semiquantitative scoring of glomerular TRPC6 immunostaining (score from 1 to 4) of the percentage of positively stained podocytes was performed (bottom). Scoring values were statistically analyzed by one-way ANOVA. At all time points, the increase in TRPC6 expression was statistically significant.

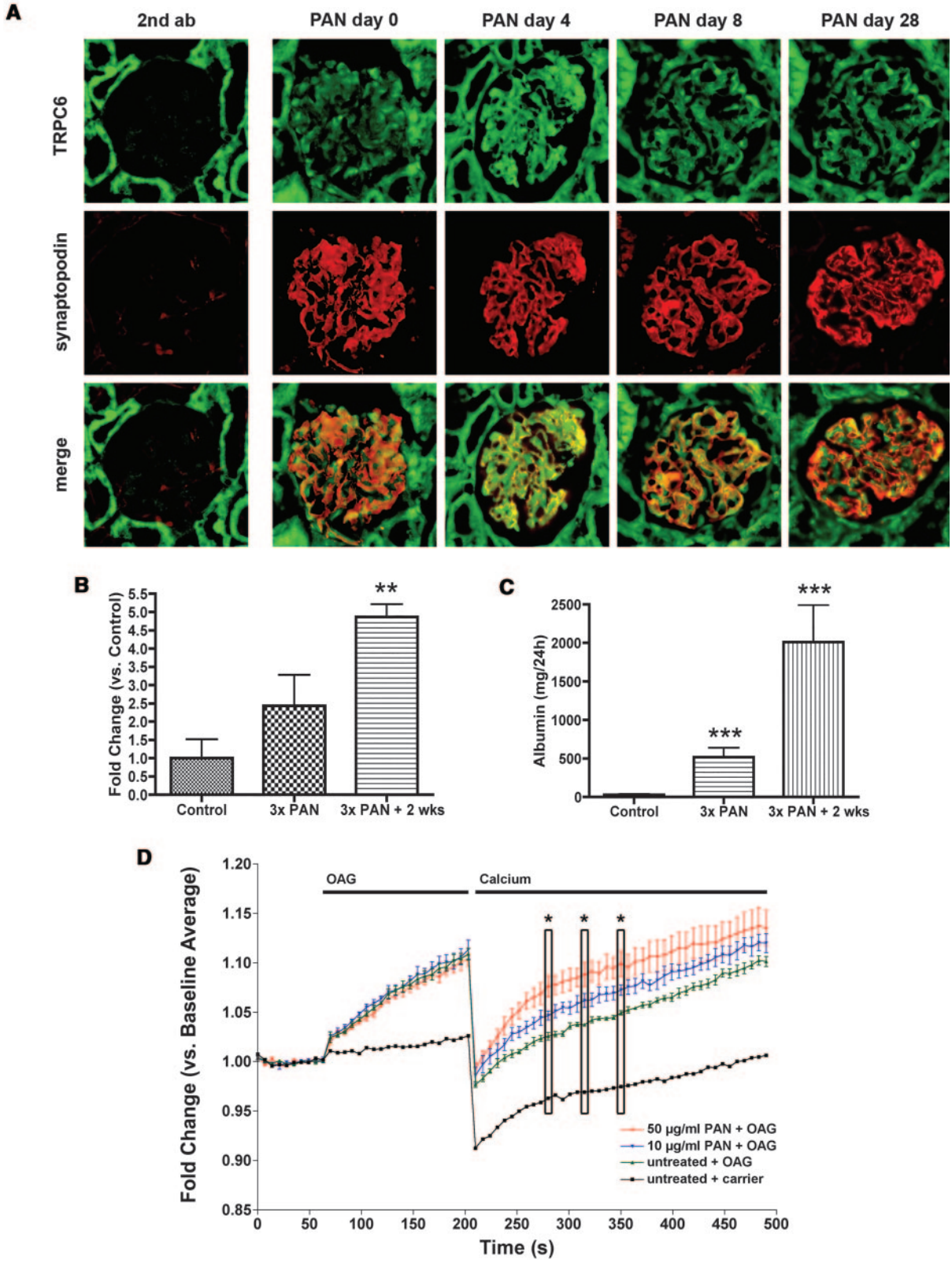

Figure 2. TRPC6 levels are induced in podocytes *in vivo* after treatment of rats with puromycin aminonucleoside (PAN). (A) Frozen rat kidney sections were stained using antibodies that were specific for TRPC6 and the podocyte marker synaptopodin. When primary antibodies were omitted in the staining procedure (second antibody), we could detect a high background signal for TRPC6 in tubules but no signal in glomeruli and no signal for synaptopodin. In the glomeruli of untreated rats (PAN day 0), TRPC6 is expressed predominantly in podocytes as shown by a partial overlap with synaptopodin. Four days after PAN treatment, in the peak phase of PAN-induced stress, TRPC6 expression is induced dramatically in podocytes. Eight days after

Results and Discussion

In this study, we first examined the expression of TRPC6 in nongenetic forms of human proteinuric kidney diseases. We analyzed TRPC6 mRNA expression in isolated glomeruli from patients with FSGS, MCD, and MGN (Figure 1A). Control samples were obtained from the healthy kidney poles of individuals who underwent tumor nephrectomies. By real-time quantitative PCR, we determined the ratio of TRPC6 mRNA to 18S ribosomal RNA (17). We found significantly increased levels of TRPC6 mRNA in patients with MCD ($n = 13$, $P = 0.006$) and MGN ($n = 28$, $P = 0.024$) as compared with control patients ($n = 9$). In patients with FSGS ($n = 9$), the increase in TRPC6 mRNA did not reach statistical significance. Immunostaining of TRPC6 was performed on human kidney biopsies of normal tissue and of glomeruli during FSGS, MCD, and MGN (Figure 1B). In control glomeruli, TRPC6 labeling was found throughout the glomerulus and in particular at the outer aspects of capillary loops (Figure 1B, CON). Double labeling with the podocyte marker synaptopodin showed that glomerular TRPC6 was localized in podocytes represented by a yellow overlap (Figure 1B, CON). In FSGS, TRPC6 staining appeared clustered and aggregated as well as segmentally more intense (Figure 1B, FSGS). TRPC6 staining in MCD displayed a similar pattern as FSGS but with less intensity (Figure 1B, MCD). In MGN, glomerular TRPC6 expression was granular and dramatically induced in podocytes (Figure 1B, MGN).

Given our observation that TRPC6 mRNA and protein were strongest in MGN, we next exposed cultured differentiated mouse podocytes to antipodocyte antibody and serum with or without complement. Treatment of cultured podocytes with sublytic amounts of C5b-9 mimics the effects of the complement system as a potent mediator of glomerular injury in membranous glomerular disease. Consequences of sublytic C5b-9 attack include the release of calcium and the activation of signaling pathways in the podocyte (16). Western blot analysis of TRPC6 in treated cells showed significantly increased TRPC6 expression in cells that were stimulated with C6+ serum when compared with cells that were stimulated by C6− serum that lacked functional complement ($n = 3$, $P < 0.05$; Figure 1C).

To test the hypothesis that C5b-9 induces podocyte TRPC6 expression *in vivo*, we used the PHN model of experimental membranous nephropathy (12). In the PHN model, rats are administered an injection of anti-Fx1A antibody, inducing formation of subepithelial immune deposits, the activation of the C5b-9 membrane attack complex of complement, and the development of severe proteinuria. Sprague-Dawley rats that received an injection of normal sheep serum served as controls for the anti-Fx1A antibody. Control and PHN rats were killed at 5, 11, and 28 d ($n = 4$ to 5 for each time point) for immunohistochemical analysis of glomeruli. TRPC6 protein expression was strongly induced in glomeruli in rats with PHN (Figure 1D). While induced TRPC6 staining was observed predominantly along capillary loops that resembled a podocyte staining pattern, we also found noticeable expression in glomerular endothelial cells.

Next, we explored TRPC6 expression levels in the PAN experimental model of podocyte injury and nephrotic syndrome (18). TRPC6 levels were induced significantly in the glomeruli of PAN-injected rats 4 d after treatment at the peak of PAN-mediated injury (Figure 2A). Induced TRPC6 protein was detected mainly in podocyte as shown by double labeling with the podocyte marker synaptopodin (Figure 2A). Eight days after PAN treatment, TRPC6 expression levels still were upregulated slightly. Twenty-eight days after PAN treatment, which represents the recovery from PAN-mediated injury, TRPC6 expression levels returned to baseline (Figure 2A). By real-time quantitative PCR, we determined an upregulation of TRPC6 mRNA levels in rats upon PAN-mediated injury (Figure 2B). The increase in TRPC6 mRNA correlated with the development of albuminuria in these rats (Figure 2C).

To understand better whether induction of wild-type TRPC6 is associated with changes in intracellular calcium, we examined the calcium influx in cultured podocytes after injury with PAN (18) (Figure 2C). Twenty-four hours after the application of PAN on cultured podocytes, receptor-operated calcium channels were activated by addition of the diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol. We observed elevated calcium influx in PAN-treated podocytes compared with untreated control cells, suggesting that increased influx of calcium is a component of podocyte injury, possibly mediated by TRPC6 channels (Figure 2C).

In the next step, we focused on the molecular mechanisms that may account for the pathogenic effect of induced TRPC6 protein. TRP channels are known to be associated with the actin cytoskeleton (19), the proper organization of which is essential for normal podocyte function (20). Therefore, we first tested whether the disruption of the actin cytoskeleton in cultured podocytes (15) would affect the localization of TRPC6. After

PAN treatment, TRPC6 levels still seem to be elevated, and 28 d after PAN treatment, which represents the recovery from PAN-induced stress, TRPC6 expression returns to baseline levels. The upregulation of TRPC6 mRNA in isolated glomeruli correlates with the development of albuminuria in PAN-treated rats. (B) Rats were administered injections of three consecutive doses of PAN. Two weeks after the third injection, TRPC6 mRNA levels were 4.9-fold elevated in isolated glomeruli ($P < 0.01$). (C) Rats were administered injections of three consecutive doses of PAN. One week after the third injection, the average amount of albumin in the urine was determined to e 519 mg/24 h ($P < 0.001$). Two weeks after the third injection, the average amount of albumin in the urine was determined to be 2009 mg/24 h ($P < 0.001$). PAN treatment is correlated with increased calcium influx in cultured podocytes. (D) Differentiated podocytes were treated for 24 h with 10 or 50 μg/ml PAN. Cells then were loaded with Fluo-4 in 0.1 mM Ca^{2+} for 1 h. Receptor-operated channels were activated by addition of 100 μM 1-oleoyl-2-acetyl-sn-glycerol, followed by changing the extracellular buffer to 2 mM Ca^{2+} to distinguish membrane-associated channel-dependent changes in calcium. Differences in intracellular calcium concentration were statistically significant at the time points indicated. *$P < 0.05$.

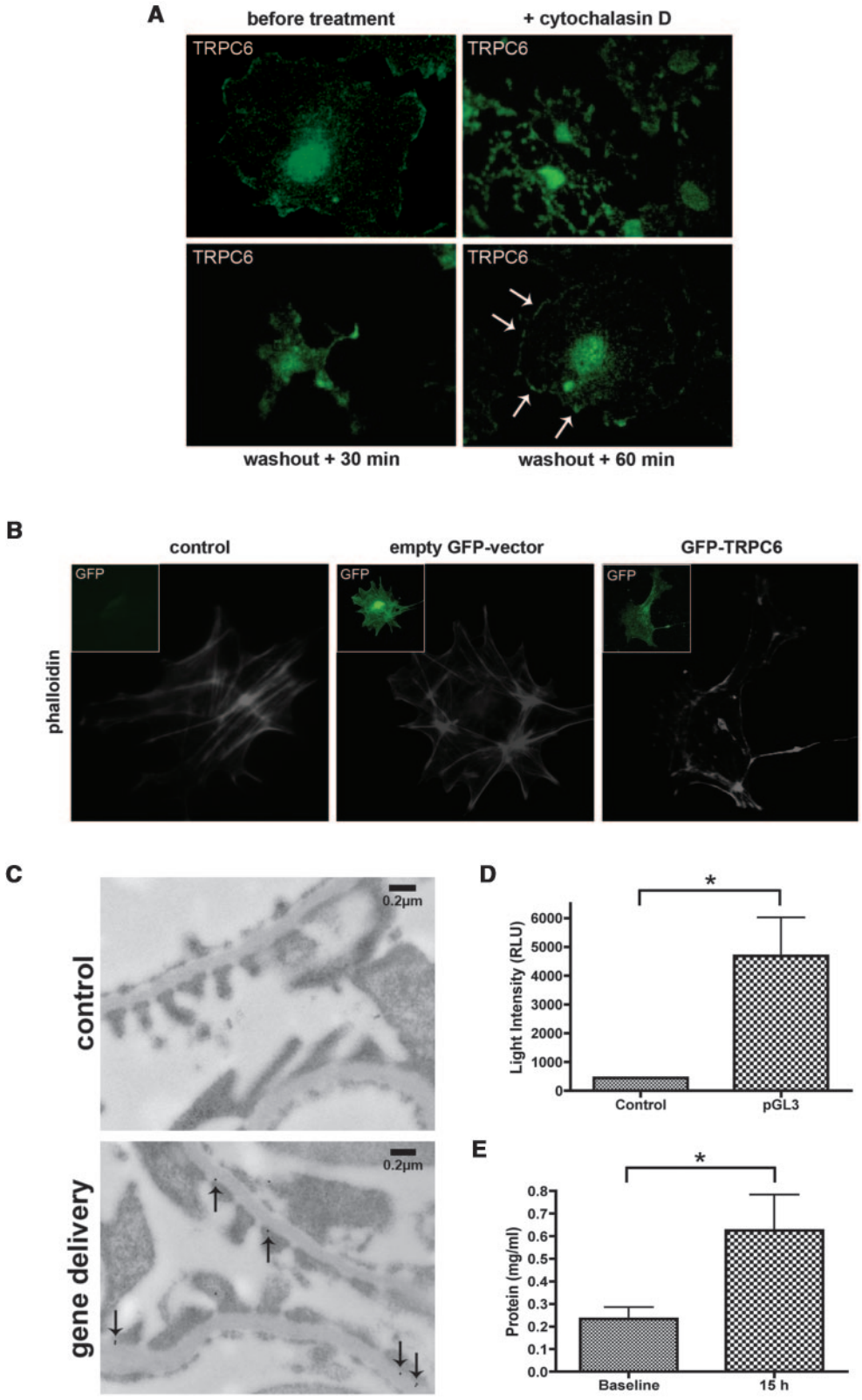

Figure 3. TRPC6 is associated with the podocyte actin cytoskeleton. (A) Effect of cytochalasin D treatment on TRPC6 subcellular localization. Six hours after cytochalasin D incubation, TRPC6 localizes with depolymerized actin clusters within the cell body (top right). Thirty minutes after washout of cytochalasin (partial recovery), TRPC6 starts to redistribute within the cell (bottom left). Sixty minutes after washout (full recovery), most of TRPC6 is re-localized at the cell membrane (bottom right, arrows). (B) TRPC6 overexpression leads to the loss of stress fibers in podocytes. Differentiated podocytes were transfected with a green fluorescence protein (GFP)-TRPC6 fusion protein (green) and stained with phalloidin to visualize actin stress fibers. Cells were analyzed by confocal microscopy. Cells that were transfected with GFP-TRPC6 (right) show a significant loss of stress fibers and exhibit a more

depolymerization of F-actin in cultured podocytes using cytochalasin D (18), we found TRPC6 closely associated with depolymerized clusters of actin (Figure 3A, top right). The removal of cytochalasin D allows the reorganization of the actin cytoskeleton; consequently, TRPC6 started to redistribute to its original localization at the cell membrane (Figure 3A, bottom).

To study whether TRPC6 overexpression affects the actin cytoskeleton directly, we overexpressed green fluorescence protein (GFP)-tagged TRPC6 (6) in cultured differentiated podocytes (Figure 3B). Cells that expressed GFP-TRPC6 displayed loss of actin stress fibers (Figure 3B, right) that normally are found in untransfected control cells (Figure 3B, left). Transfection of the empty GFP vector had no effect on the podocyte actin cytoskeleton (Figure 3B, middle). These studies suggest that TRPC6 is functionally associated with the actin cytoskeleton in podocytes and that induction of TRPC6 channel directly affects cytoskeletal organization in these cells.

If such a functional association of TRPC6 with podocyte actin cytoskeleton were true, we wondered, then, whether we could observe a phenotype in mice when overexpressing wild-type TRPC6 in podocytes. Therefore, we used a transient gene delivery approach to deliver wild-type FLAG-tagged TRPC6 in mouse kidneys (21). With this approach, we found gene-transferred FLAG-TRPC6 in podocyte foot processes often in close vicinity to the slit diaphragm 12 h after gene injection (Figure 3C, arrows). When mice were administered an injection of the pGL3 luciferase reporter vector, we were able to detect luciferase activity in whole-kidney lysates 12 h after gene injection (Figure 3D). Functionally, mice that were administered an injection of FLAG-TRPC6 displayed transient proteinuria 15 h after gene injection (Figure 3E).

The discovery that mutations in the TRPC6 gene cause hereditary FSGS together with the finding that TRPC6 is a component of the slit diaphragm multiprotein complex raises questions regarding the mechanisms that are involved in the physiologic and pathophysiologic role of this channel in podocytes. In this study, we provide evidence that, in addition to the effects of gain-in-function mutations in *TRPC6*, pathophysiologically elevated expression levels of TRPC6 are detected in acquired glomerular diseases. Our data in cultured podocytes suggest that the consequences of elevated TRPC6 include reorganization of the podocyte actin cytoskeleton and altered calcium regulation. Importantly, overexpression of wild-type TRPC6 seems to be sufficient to cause proteinuria in healthy mice. Taken together, these data indicate that TRPC6 plays a pivotal role in nongenetic forms of glomerular disease, suggesting that functional TRPC6 blockade may provide an effective treatment for genetic and acquired forms of proteinuric kidney disease, in particular in MGN.

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Disclosures

None.

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cortical actin cytoskeleton than untransfected control cells (left) or cells that were transfected with empty GFP vector (middle) displaying a regular actin cytoskeleton. TRPC6 *in vivo* gene delivery leads to transient overexpression of exogenous TRPC6 in podocytes and is associated with the development of proteinuria. (C) A construct coding for a FLAG-TRPC6 fusion protein was gene-delivered into mice *via* tail-vein injection. Twelve hours after gene transfer, mouse kidneys were processed for immunogold analysis with anti-FLAG antibody. Immunogold labeling of FLAG-TRPC6 was detected in podocyte foot processes in close vicinity of the slit diaphragm site. (D) Gene delivery of the pGL3 luciferase reporter vector leads to the detection of luciferase activity in kidneys 12 h after gene transfer ($P = 0.0158$). (E) FLAG-TRPC6 was gene-delivered into mice and proteinuria was assessed by Bradford assay. Before gene delivery, baseline urine protein excretion averaged to 0.23 mg/ml. Fifteen hours after gene delivery, mice developed proteinuria with an average urine protein excretion of 0.63 mg/ml ($P = 0.0275$).

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