Trpm7 Protein Contributes to Intercellular Junction Formation in Mouse Urothelium*

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Background: Transient receptor potential melastatin 7 (Trpm7) is a Ca2+-permeable channel with a kinase domain that is implicated in cell migration and adhesion.

Results: Urothelium-specific Trpm7 knock-out caused immature intercellular junctions, inflammation, and smaller voided volume.

Conclusion: Trpm7 contributes to the intercellular junction formation in the urothelium.

Significance: These findings might provide the first evidence for the significance of Trpm7 in bladder function in vivo.

Trpm7 is a divalent cation-permeable channel that has been reported to be involved in magnesium homeostasis as well as cellular adhesion and migration. We generated urothelium-specific Trpm7 knock-out (KO) mice to reveal the function of Trpm7 in vivo. A Trpm7 KO was induced by tamoxifen and was confirmed by genomic PCR and immunohistochemistry. By using patch clamp recordings in primary urothelial cells, we observed that Mg2+-inhibitable cation currents as well as acid-inducible currents were significantly smaller in Trpm7 KO urothelial cells than in cells from control mice. Assessment of voiding behavior indicated a significantly smaller voided volume in Trpm7 KO mice (mean voided volume 0.28 ± 0.08 g in KO mice and 0.36 ± 0.04 g in control mice, p < 0.05, n = 6 – 8). Histological analysis showed partial but substantial edema in the submucosal layer of Trpm7 KO mice, most likely due to inflammation. The expression of proinflammatory cytokines TNF-α and IL-1β was significantly higher in Trpm7 KO bladders than in controls. In transmission electron microscopic analysis, immature intercellular junctions were observed in Trpm7 KO urothelium but not in control mice. These results suggest that Trpm7 is involved in the formation of intercellular junctions in mouse urothelium. Immature intercellular junctions in Trpm7 knock-out mice might lead to a disruption of barrier function resulting in inflammation and hypersensitive bladder afferent nerves that may affect voiding behavior in vivo.

Transient receptor potential (TRP)3 channels play critical roles in sensing environmental stimuli, including physical and chemical stimuli (temperature and pH), and conducting that information into cells (1 – 4). Trpm7 (transient receptor potential melastatin 7) and its closest homolog Trpm6 are unique members of the TRP channel family because they have a kinase domain within the same polypeptide (5). Trpm7 is a ubiquitously expressed non-selective cation channel that has been reported to be important for Mg2+ homeostasis. Although Trpm7 might indeed be involved in total body Mg2+ homeostasis (6), there is growing evidence suggesting that Trpm7 is not a determinant for intracellular Mg2+ content (7, 8). In contrast, it has been shown that Trpm7 has a crucial role in cancer cell migration, including formation of focal adhesions as well as adherence junctions (9 – 12). Nonetheless, the physiological significance of Trpm7 in vivo remains unknown.

To investigate the significance of Trpm7 in vivo, we focused on the epithelium in the urinary bladder (urothelium), the architecture of which is dynamically rearranged during the storage and emptying of urine (13, 14). Although the functional expression of Trpm7 has been reported in the urothelium (15), its physiological role remains unclear. We attempted for the first time to generate urothelium-specific Trpm7 knock-out (KO) mice, and we found that the endogenous acid-sensitive currents were derived at least in part from Trpm7. More importantly, Trpm7 KO mice exhibited a smaller voided volume of urine. Indeed, interstitial inflammation was observed in Trpm7 KO bladders. With electron microscopy, we observed that intercellular junctions were impaired in Trpm7 KO urothelium. These results suggest that Trpm7 is essential for the formation of barrier function. 

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3 The abbreviations used are: TRP, transient receptor potential; fl, floxed; eGFP, enhanced GFP; pF, picofarad.
mation of the intercellular junctions in the urothelium. Abnormal intercellular junctions could lead to a barrier disruption and inflammation that could impair bladder function in vivo.

**Experimental Procedures**

**RT-PCR**—Urinary bladder tissue or primary urothelial cells were extracted with a Sepasol Super G kit (20–40 mg/500 μl of solution, Nacalai, Japan). After homogenizing with a disposable Potter-type homogenizer, chloroform was added and incubated for 3 min. The mixture was then centrifuged at 10,000 × g for 15 min at 4 °C. The upper layer was treated with an equal volume of 2-propanol and incubated for 10 min. After centrifugation at 10,000 × g at 4 °C for 10 min, the pellet was washed with 75% ethanol and then dissolved with nuclease-free water. After measuring the absorbance, the total RNA solution (1 μg/μl) was stored at −80 °C prior to use. Reverse transcription was performed using a Superscript III kit (Invitrogen). One μg of the total RNA was mixed with 1 μl of oligo(dT)20 (50 μM) and 5 μl of dNTPs (2.5 mM) and incubated at 65 °C for 5 min. After the annealing, Superscript III reverse transcriptase was added in 4 μl of First-Strand buffer, 1 μl of DTT (0.1 M), and 1 μl of RNase OUT (Invitrogen) and incubated at 50 °C for 1 h. After heat inactivation (70 °C for 15 min), the single strand cDNA solution was stored at −20 °C until use.

The PCR was performed using an EmeraldAmp kit (Takara, Japan). As a template, the above mentioned cDNA sample was used. As a positive control, a vector construct with partial cDNA of each molecule was used with a 1:50 dilution. Primer sequences were as follows: Trpm7-forward, 5′-CCCTCATGGA-GACCATTTTCTAA, and Trpm7-reverse, 5′-ACACTGTTAACCTTCAACAG; β-actin-forward, TGGTACCACTCGGAGCAGC, and β-actin-reverse: AAGGAAGGCTGGA-AAAGAGC; Upk3a-forward, TCCACTGAGCACCATTTC, and Upk3a-reverse, AGCTTGCTGGAGAACACCCT; and Acta2 (smooth muscle actin)-forward, AGCTTGTGCGGAAACCTC, and Acta2-reverse, TGAAGTCAGTGTCGAAAGGC. The PCR mix contained the template or control vector (1 μl), EmeraldAmp PCR MasterMix (0.5 μl volume), and the above mentioned forward and reverse primers (1 μl each, 10 pmol/μl). The reaction cycle was as follows: after incubation at 94 °C for 2 min, 35 cycles of denaturing (94 °C for 30 s), annealing (49 °C for Trpm7, 63 °C for β-actin, and 53 °C for Upk3a and 51 °C for Acta2) for 30 s, and elongation (72 °C for 1 min). After the cycle, the sample was incubated at 72 °C for 10 min. These PCR fragments were then visualized by agarose gel electrophoresis.

**Trpm7 Knock-out Mice**—Trpm7-floxed mice were generated as described previously (7) and maintained on the 129/SvEvTac background. According to previous papers on Trpm7 KO mice (7, 8), global Trpm7 disruption in mice leads to early embryonic lethality. To obtain conditional Trpm7 KO mouse, the Cre-loxP system (16) was used. The Upk3a-GCE strain containing enhanced GFP-Cre-ERT2 expressed under the control of the Upk3a promoter was purchased from The Jackson Laboratory. Ten mg of tamoxifen (Sigma) was dissolved in 1 ml of corn oil (WAKO, Japan) at 65 °C for 1 h. Tamoxifen (50–100 μg/g body weight) was administered intraperitoneally to the control group (Trpm7a/+) or the Trpm7 KO group (Upk3a-Cre-Trpm7a/+) once a week for 4 weeks. All the experiments were performed at least 7 days after the last injection. All animal care and experimental procedures were performed according to the National Institutes of Health and National Institute for Physiological Sciences guidelines.

Genotyping PCR—The urothelial layer was collected from anesthetized, tamoxifen-treated mice, after which the tissue was incubated with a lysis buffer (10 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 0.2% SDS) containing proteinase K (0.5 μg/μl, Sigma) at 55 °C overnight with shaking. After inactivation at 95 °C for 10 min, the genomic DNA solution was stored at −20 °C until use. The genomic PCR was performed using an EmeraldAmp kit (Takara, Japan) with the following primers: Trpm7 exon 17-forward, 5′-GACCATCTCTCCTCTGTTTTT, and Trpm7 exon 17-reverse, 5′-GATAGACTATATACGAGTTTGGAGTGACCAGA, and 53 °C for 30 s, and elongation (72 °C for 1 min) were performed. These PCR fragments were then visualized by agarose gel electrophoresis.

**Immunohistochemistry and Immunocytochemistry**—The bladder tissue was resected from anesthetized mice and fixed in 10% buffered formaldehyde (Wako, Osaka, Japan). After further fixation for 24 h at 4 °C, the tissue was incubated twice with 30% sucrose in PBS for 24 h at 4 °C and then embedded with OCT compound (Sakura, Japan). An 8-μm section was stored at −80 °C until use. The sections were cut with a cryostat (CM3050S, Leica, Germany) with a 7-μm thickness. Primary cultures of mouse urothelial cells were fixed with 10% buffered formaldehyde for 10 min and incubated twice with 30% sucrose in PBS for 24 h at 4 °C. The treated primary culture and the tissue sections were then washed three times with PBS and treated with 5% normal goat serum with 0.05% Triton X-100 (Sigma) in PBS for 30 min at room temperature. After the blocking treatment, the sections and the cells were treated with diluted rabbit anti-human TRPM7 antibody (from Dr. Yasuo Mori, Kyoto University, Japan). The amino acid sequence of the compound (Sakura, Japan). The tissue block was stored at −80 °C until use. The sections were cut with a cryostat (CM3050S, Leica, Germany) with a 7-μm thickness. Primary cultures of mouse urothelial cells were fixed with 10% buffered formaldehyde for 10 min and incubated twice with 30% sucrose in PBS for 24 h at 4 °C. The treated primary culture and the tissue sections were then washed three times with PBS and treated with 5% normal goat serum with 0.05% Triton X-100 (Sigma) in PBS for 30 min at room temperature. After the blocking treatment, the sections and the cells were treated with diluted rabbit anti-human TRPM7 antibody (from Dr. Yasuo Mori, Kyoto University, Japan). The amino acid sequence of the epitope is identical between human and mice. It was used at a 1:50 dilution for immunohistochemistry and 1:50 for immunocytochemistry. Rabbit anti-eGFP antibody (Life Technologies, Inc.) was used at a 1:1000 dilution. Mouse anti-human cytokeratin 7 (CK7) antibody (CK7, Monosan, Netherlands) was used at a 1:50 dilution. After incubation with the primary antibody overnight at 4 °C, the sections or cells were washed three times with PBS containing 0.05% Triton X-100 and treated with secondary antibody (Alexa Fluor-conjugated, Molecular Probes) for 2 h at room temperature under dark conditions. DAPI was used for nuclear staining. After a 10-min incubation, sections and cells were washed and mounted with Fluoromount (DBS) and visualized by fluorescent microscopy (BZ9000, KEYENCE, Japan). The signal was semi-quantified by ImageJ software.

**Primary Culture of Mouse Urothelial Cells**—Following anesthesia, whole bladders were separated, and urothelial cells were prepared by the methods described previously with slight mod-
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ifcations (17, 18). The everted bladder was incubated with papain (6 μl/ml) for 25 min at 37 °C with just one tapping for 15 min after incubation. The isolated urothelial cells were seeded on 12-mm diameter coverslips (Matsumani, Japan) coated with fibronectin (AGC Techno Glass Co., Ltd., Japan) and incubated in urothelial-dedicated culture medium (CNT-16; CELN-TEC, Switzerland). Immunocytochemistry with an epithelial marker (cytokeratin 7) was performed to ascertain whether the cultured cells were urothelial cells (19).

Whole-cell Patch Clamp Recording in Primary Urothelial Cells—Whole-cell patch clamp recordings were performed 3–8 h after seeding freshly isolated urothelial cells. Primary urothelial cells on 12-mm diameter coverslips were mounted in a chamber. The control bath solution (300–310 mosmol/kg H2O) contained 143 mM NaCl, 5 mM KCl, 5 mM HEPES, 10 mM glucose, 2 mM CaCl2, and 1 mM MgCl2 (pH 7.4). The solution for observing the activity of Trpm7 contained 125 mM NaCl, 20 mM HEPES, 10 mM glucose, 5.1 mM CaCl2, 0 mM MgCl2, 5 mM EGTA, and 30 mM mannitol (pH 7.4). The solution for observing the response to an acid (pH 4) contained 125 mM NaCl, 140 mM CsCl, 10 mM HEPES, 10 mM glucose, 0.1 mM CaCl2, 0 mM MgCl2, 5 mM EGTA, and 30 mM mannitol (pH 4). To observe the effects of Mg2+, 10 mM MgCl2 was added to the bath while maintaining a constant osmolality by reducing the concentration of mannitol. The free Mg2+ or Ca2+ concentration was calculated with Max chelator software. The pipette solution (300–305 mosmol/kg H2O) contained 140 mM CsCl, 10 mM O2-bis-(2-aminophenylethyl)ethleneglycol-N,N,N′,N″-tetraacetic acid, and 10 mM HEPES (pH 7.3). The resistance of the pipette was around 7 megohms. Data were sampled at 10 kHz and filtered at 4 kHz for analysis (Axon 700B amplifier with pCLAMP software, Axon Instruments, Molecular Devices, Japan). Membrane potential was clamped at −60 mV, and voltage ramps pulses from −100 to +100 mV (500 ms) were applied every 5 s. All experiments were performed at room temperature. Recording started at about 2 min after making a whole-cell configuration. When the increase of the current seemed to reach a plateau, 10 mM Mg2+ was applied for 30 s, and the inhibition of the current was analyzed as a magnesium-inhibitable current. For the measurement of the response to an acid, an acidic solution (pH 4) was applied for 30 s when the increase of the current seemed to reach a plateau. The data at −60 mV before and after application of the acidic solution were analyzed. Regarding the measurement of the Mg2+ inhibition in response to the acidic solution, the data before and after application of 10 mM Mg2+ were analyzed.

Study of Voiding Behavior—A study of voiding behavior was performed with free-moving mice that were awake. They were housed in modified metabolic cages (type 304 stainless steel, TECNIPLAST, Italy). Each mouse was placed in an individual metabolic cage under 12-h light-dark conditions. They could freely access water and food. The adaptation period was 4–7 days. Cages were located above an electronic balance (GX-06, A&D Instruments, Japan), and the weight of urine was directly measured by this device in real time. Most of the excrement was trapped by two metal filters. These filters were treated with a water-repellent finish so that almost all the urine could pass through these filters. Data were continuously recorded for 3–7 days with Powerlab software and analyzed with Labchart7 software. Total weight, total amount of urine for 24 h, and the weight of voided volume per micturition were calculated.

Histological Analysis—Tissue blocks were prepared as described under “Immunohistochemistry and Immunocytochemistry.” The sections were made with a cryostat (CM3050, Leica, Germany) with a thickness of 9 μm. The sections were fixed in 10% buffered formaldehyde (Wako, Japan) for 10 min, washed three times with PBS, and stained with Meyer’s hematoxylin solution (Wako, Japan) and eosin solution (Wako, Japan), followed by dehydration with 30, 70, 90, and 100% ethanol and then 100% xylene, each for 2 min. The dehydrated sections were then mounted with Permoun (Fisher) and dried overnight. The tissue sections were analyzed by microscopy (BZ9000, KEYENCE, Japan). An edema score was determined for each section as described previously (20).

Cytokine Profiling—Total RNA from bladder was isolated, and cDNA was synthesized as described under “RT–PCR.” Real time PCR was performed with 10 μl of SYBR Master Mix Reagent (Takara, Japan). 0.25 μM specific primer sets (Tnfα, Il6, Il10, II12, Il15, Il18, and Tgfb1) as follows: mTnfa-F, 5′-CCCCAAGGGATGAAAGGT, and mTnfa-R, 5′-GGTGGGTTAGGAGACGTAGT; mIl1b-F, 5′-GGATGGAGGACATGAGACCATT; and mIl1b-R, 5′-AGGCCACAGGTTATGCAGGC; mIl6-F, 5′-TGGTCAAATGCGAATTCTGAT; and mIl6-R, 5′-GGAAAATTGGGTAGGAGAGGA; mIl1o10-F, 5′-TGGTATGTCTGCCTGCTTCA; and mIlL10-R, 5′-TCCACCCAGGGAATTC; and mL12b-F, 5′-TGACACGCTTGAAGAAGATG; and mL12b-R, 5′-TCAAGGGAACGTACTGCT; mL15F, 5′-CATTTGGCCGTGTGCAGT; and mL15-R, 5′-TCTCCCTCCAGCTCCTACAT; and mL18-F, 5′-ACAACTTGGGCGACTTCAG; and mL18-R, 5′-TGGATCCATTTCCTCAAAAGG; and mTGFb1-F, 5′-TGAAGTGCTGTCTTGTACG; and mTGFb1-R, 5′-TCTCTCGTGAGGCGA; and 1 μl of the prepared cDNA. Real time PCRs were performed using a StepOne analyzer (Life Technologies, Inc.). The temperature profile consisted of 40 cycles of denaturation at 95 °C for 15 s, annealing for 30 s, and elongation at 72 °C for 1 min. To discriminate specific and nonspecific amplifications, melting curve analysis was performed at the end of each PCR assay. To determine the starting cDNA amount, purified PCR products with known concentrations were serially diluted and used as standards.

Transmission Electron Microscopy—Transmission electron microscopic analysis was carried out as described previously with some modifications (21, 22). In the transmission electron microscopy study, female mice were used. Following anesthesia, a midline abdominal incision was performed and the bladder was exposed. A polyethylene catheter, the end of which had side holes (SP 10, Natsume Seisakusho, Japan), was inserted through the urethra and the contents inside the bladder were drained. Ten minutes after emptying the bladder, a perfusion fixation was performed through the left ventricle with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion fixation, the bladder was cut into small pieces for embedding and fixed further for 1 h at room temperature in the same fixative. After washing with 0.1 M PB, post-fixation with 2% osmium tetroxide in 0.1 M PB was
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FIGURE 1. Generation of the urothelium-specific Trpm7 KO mouse. A, this diagram shows our targeting strategy of the homologous recombination for the disruption of the Trpm7 gene. Exon 17 of the Trpm7 allele was flanked with two Lox P sites (arrowheads). Exon 17 encodes the protein sequence preceding the first transmembrane segment. The Cre-mediated deletion of exon 17 leads to a frameshift to translation without the channel and the kinase domain. The Upk3a promoter drives expression of the membrane protein called uroplakin 3a that is expressed only in urothelium. Thus, one can obtain urothelium-specific recombination by using this construct. Moreover, this construct also contains enhanced green fluorescent protein (eGFP), so in the transgenic mice, GFP should be expressed in cells that express uroplakin 3a. These mice were mated with the Trpm7 flox mice to generate the double-positive mice. B, to confirm whether this Cre-loxP system worked correctly, PCR assessment of urothelial genomic DNA with or without tamoxifen was performed. In this PCR, the band of floxed Trpm7 allele should be detected at around 1000-bp size, and the band of the null allele should be around 200 bp. Only the band of WT allele was found without tamoxifen (1st lane, ctl). After two or more administrations of tamoxifen, the band of the null allele was observed (2nd to 5th lanes). C–E, eGFP-positive cells were observed by immunohistochemistry using an anti-eGFP antibody to confirm the expression of Cre recombinase. Red signals indicate eGFP, and blue signals show DAPI. Signals were mainly found in the superficial layer of the urothelium with higher magnification (E). D shows a representative result without anti-eGFP antibody (Ab). Scale bars, 100 μm (C and D) and 25 μm (E). L, lumen.

Results

We first generated urothelium-specific Trpm7 KO mice to reveal the functional significance of Trpm7 in the urothelium. We used the promoter for the uroplakin 3a gene (Upk3a), the expression of which had been reported to be restricted to the urothelium (23, 24). Uroplakin is known to be a major component in the umbrella cells and makes a tight urothelial barrier (23, 25). First, we analyzed the actual promoter activity of the Upk3a gene in Upk3a-Cre mice by using an anti-eGFP antibody (Fig. 1A). The signal was indeed the highest in the superficial layer of the urothelium, modest in the intermediate, and lowest in the basal layer (Fig. 2, C–E). This outcome seemed to be suitable for the Trpm7 KO because of their spatial expression patterns; by RT-PCR analysis, Trpm7 mRNA was detected in the urothelium (Fig. 2A). When assessed by immunohistochemistry, Trpm7 protein was also observed in the smooth muscle layer. However, the expression level in the smooth muscle was lower than that in the urothelium (Fig. 2, B–D). In the urothelium, Trpm7 protein was predominantly expressed in the superficial layer as reported previously (Fig. 2D) (26). This layer is composed of “umbrella cells” with long narrow rectangular shapes (13, 14). Trpm7 protein was observed in the intermediate and basal layers of the urothelium as well, but expression levels were lower than in the superficial layer (Fig. 2D).

We then injected tamoxifen into these Upk3a-Cre;Trpm7fl/fl double-positive mice to induce a urothelium-specific Trpm7 KO (Fig. 1A). As shown in the genomic PCR with whole urothelium in Fig. 2B, intraperitoneal injections of tamoxifen indeed deleted exon 17 of the Trpm7 gene in the urothelium, although the intact Trpm7 genomic band was still present because we used whole urothelium. This deletion reportedly results in the production of a truncated Trpm7 protein lacking both channel and kinase domains (7). We also confirmed that the quantity of Trpm7 protein in the urothelium was significantly smaller after the application of tamoxifen but not in the detrusor, the smooth muscle layer (Fig. 2, E–I). These results indicated that urothelial Trpm7 proteins were decreased by tamoxifen treatments.

Next, we established primary cultures of the urothelial cells. However, umbrella cells could not be maintained because they did not attach to culture dishes. However, we successfully isolated Upk3a-positive smooth muscle actin (Acta2)-negative cells that were most likely derived from the intermediate and basal layers of the urothelium (Fig. 3, A and B). In this culture, almost all of the cells were positive for cytokeratin 7 (CK7), an epithelial cell marker (Fig. 3D), but negative for cytokeratin 20 (CK20), an umbrella cell marker, on the 1st day of culture but

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Dehydration in a graded ethanol series was followed by embedding samples in epoxy resin (Durcupan). Serial 70-nm-thick sections were cut from the sample surface orthogonally oriented to the epithelial cell layer and were collected in ploioform-coated single slot copper grids. Thin sections were stained with uranyl acetate and lead citrate and then observed with a JEM1010 transmission electron microscope (JEOL Co., Japan). Three images that had the widest intercellular junctions were selected from each mouse and the length between the surfaces of lateral cellular membranes was measured in each image.

Statistical Analysis—Data were analyzed using an unpaired t test or Mann-Whitney rank sum test. Values are shown as means ± S.E. p values < 0.05 were considered significant.

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partially positive on the 2nd and 3rd days of the culture (data not shown). This strongly suggested that these cells were urothelial cells from the intermediate or basal layer of the urothelium. Moreover, Trpm7 protein was colocalized with CK7 (Fig. 3, C–E), and Trpm7 protein signals were confirmed to be reduced by tamoxifen treatments without changes in CK7 signals (Fig. 3, F–H).

Using this culture system, we performed whole-cell patch clamp recordings. Because it had been reported that physiological concentrations of Mg^{2+} inhibited Trpm7 activity, we used...
extracellular and intracellular Mg\textsuperscript{2+}-free conditions (27). After starting incubation with 0 mM Mg\textsuperscript{2+} and 100 \(\mu\)M Ca\textsuperscript{2+}, membrane currents with an outwardly rectifying property were significantly increased, and these currents were gradually increased further during the incubation with the same bath solution (Fig. 4, A and B). The increase of these currents usually lasted \(\sim\)5 min. All these characteristics were similar to those reported for Trpm7 currents (Mg\textsuperscript{2+}-inhibitable current) (27). Indeed, these currents were significantly smaller when cells isolated from tamoxifen-treated mice were used (Fig. 4, C–E). Immunocytochemistry of primary urothelial cells isolated from Trpm7 KO mice with tamoxifen treatments. The TRPM7 signals were significantly reduced without changes in CK7 signals.

Another characteristic of Trpm7 currents is its pH dependence (28, 29). Trpm7 currents are reportedly activated by extracellular acid loading due to the competition between H\textsuperscript{+} and Mg\textsuperscript{2+}, and this effect was observed only in inward currents (29). In our hands, the increase in inward currents by acid (Fig. 4, F and G) and their inhibition by Mg\textsuperscript{2+} (10 mM) (Fig. 4, K–M) were confirmed. Importantly, these acid-induced inward currents were significantly smaller when cells isolated from tamoxifen-treated mice were used (Fig. 4, H–J), suggesting that endogenous acid-sensitive currents were derived at least in part from Trpm7. To exclude the possibility that acid-evoked currents were derived from other channels such as acid-sensing ion channels, TRPV1, TRPA1 or TRPV4, we first performed Ca\textsuperscript{2+} imaging with application of capsaicin (TRPV1 agonist) or allyl isothiocyanate (TRPA1 agonist). There was no significant increase in intracellular Ca\textsuperscript{2+} concentration (data not shown). These results suggested there might be a small contribution of TRPV1 or TRPA1.
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A

B

C

D

E

F

G

H

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J

K

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Next, we investigated why the voided volume was decreased. We analyzed the voiding behavior of free-moving mice by using metabolic cages (30) and found a significant difference in the urine voided volume between control (tamoxifen-injected Trpm7K/O mice) and Trpm7 KO mice (tamoxifen-injected Upk3a-Cre;Trpm7K/O mice). The voided volume was significantly smaller in Trpm7 KO mice than controls (mean voided volume 0.28 ± 0.08 g in KO mice versus 0.36 ± 0.04 g in control mice, p = 0.03, n = 6–8, Mann-Whitney test) (Fig. 5A). There was no significant difference in total micturition number (p = 0.65, n = 6–8), urine weight (p = 0.36, n = 6–8), or water and food intake (p = 0.22 and 0.16, n = 6–8) between the two genotypes (Fig. 5, B–E). There was no difference in urine osmolarity either (1651.6 ± 268.9 mosM/kg H2O in controls, 2156.7 ± 222.5 mosM/kg H2O in Trpm7 KO mice, p = 0.24, n = 6, Mann-Whitney test).

Next, we investigated why the voided volume was decreased. We performed histological analysis of hematoxylin and eosin-stained tissues. As shown in Fig. 6B, partial but substantial edema in the submucosal layer was observed in tamoxifen-injected Trpm7 KO bladders but not in control (Fig. 6A). We found that the edema scores were significantly higher in the Trpm7 KO mice (2.43 ± 0.43 versus 0.75 ± 0.37, p < 0.05, n = 7–8) (Fig. 6C). These results suggested that interstitial inflammation led to the decreased voided volume. Moreover, we performed quantitative PCR analysis of the expression of various cytokines (Tnfa, Il1b, Il6, Il10, Il12, Il15, Il18, and Tgfb1). Among these cytokines, mRNA expression of proinflammatory cytokines Tnfa and Il1b was significantly higher in Trpm7 KO bladders than in the bladders from Cre-negative tamoxifen-treated control mice (Fig. 6, D and E). These results supported our idea that loss of urothelial Trpm7 resulted in submucosal inflammation.

We hypothesized that Trpm7 was important to the formation of intercellular junctions (9–12) and that Trpm7 KO might lead to the disruption of intercellular junctions, resulting in impaired epithelial barrier function and inflammation. Therefore, we investigated the microarchitecture of the urothelium by electron microscopy. We found that the intercellular junctions of the superficial layer of the urothelium differed between control and Trpm7 KO mice (Fig. 7, A–D). When focusing on the apical junction complex with surrounding intracellular dense filaments, which were most likely adherens junctions, the extra space between the urothelial cells in Trpm7 KO mice was significantly larger compared with controls (43.2 and 17.1 nm, respectively, p < 0.01, n = 12–15, Fig. 7E). It seemed that the tightness of the intercellular junction in the superficial layer of the urothelium was looser in Trpm7 KO mice than in control mice. No changes in other structures were observed, including intracellular vesicles. These results suggested that Trpm7 was involved in the formation of intercellular junction in mouse urothelium.

Discussion

In this study, we found that Trpm7 was expressed in the urothelium, especially the superficial layer, and that endogenous Trpm7 elicited acid-evoked currents. When Trpm7 in the superficial layer of the urothelium was knocked out, we observed immature intercellular junctions in the superficial layer, substantial inflammation in the submucosal layer, and significantly smaller voided volume. Overactive bladder that is defined as urine storage symptoms, including urgency and frequency, has been attributed to urothelial, smooth muscle, or neurogenic mechanisms. In terms of the urothelial mechanism, although it has been believed that several transmitters (e.g. ATP and NO) secreted from urothelial cells play a role in the over-activation of the urinary bladder via its primary sensory neurons, the detailed mechanism is still unknown. By using in vivo analysis, we found a decreased voided volume in Trpm7 KO urothelial cells under these conditions. Next, we carried out patch clamp recordings to further investigate acid-evoked currents. Neither amiloride (acid-sensing ion channel inhibitor) nor HC067047 (TRPV4 antagonist) inhibited acid-evoked currents (Fig. 4, N and O, for amiloride and data not shown for HC067047). These results supported our hypothesis that Trpm7-derived acid-sensitive currents are present in native urothelial cells.

Among these cytokines, mRNA expression of proinflammatory cytokines Tnfa and Il1b was significantly higher in Trpm7 KO bladders than in the bladders from Cre-negative tamoxifen-treated control mice (Fig. 6, D and E). These results supported our idea that loss of urothelial Trpm7 resulted in submucosal inflammation.

We hypothesized that Trpm7 was important to the formation of intercellular junctions (9–12) and that Trpm7 KO might lead to the disruption of intercellular junctions, resulting in impaired epithelial barrier function and inflammation. Therefore, we investigated the microarchitecture of the urothelium by electron microscopy. We found that the intercellular junctions of the superficial layer of the urothelium differed between control and Trpm7 KO mice (Fig. 7, A–D). When focusing on the apical junction complex with surrounding intracellular dense filaments, which were most likely adherens junctions, the extra space between the urothelial cells in Trpm7 KO mice was significantly larger compared with controls (43.2 and 17.1 nm, respectively, p < 0.01, n = 12–15, Fig. 7E). It seemed that the tightness of the intercellular junction in the superficial layer of the urothelium was looser in Trpm7 KO mice than in control mice. No changes in other structures were observed, including intracellular vesicles. These results suggested that Trpm7 was involved in the formation of intercellular junction in mouse urothelium.

Discussion

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mice. We hypothesize that the smaller voided volume is due to the formation of immature tight junctions, resulting in an increased urothelial permeability, which in turn could cause inflammation and an overactive bladder.

One example of this kind of phenomenon is observed in autoimmune responses against urothelial proteins (31) or umbrella cell-specific proteins (32) that could cause submucosal inflammation. Other reports showed that immature intercellular junctions caused by p120 catenin KO (33) or dominant-negative N-cadherin (34) affected epithelial barrier function followed by inflammation in the small intestine. We hypothesize that our urothelial Trpm7-knockdown mice follow a similar pathogenic course.

A remaining question is the mechanism by which the lack of Trpm7 leads to abnormal intercellular junction formation. Previously, it was reported that TRPV4 was involved in intercellular junction formation in mouse epidermal keratinocytes (35).
TRPV4 is another non-selective cation channel that can be activated by warm temperature or hypo-osmolarity. Sokabe et al. (35) showed that TRPV4 activation triggered intracellular Ca²⁺ increase, followed by the activation of Rho GTPase, which is a key component for keratinocyte differentiation, resulting in actin organization and intercellular junction formation. When TRPV4 was inhibited by ruthenium red, keratinocytes formed weaker stress fibers. Similar to the case of keratinocyte TRPV4, we speculate that the differentiation process of umbrella cells might be attenuated in the Trpm7 KO.

In the case of TRPV4, warm temperature could be a good candidate for the environmental stimulus that induces epidermal keratinocyte differentiation. Thus, one can ask what kind of stimulus activates Trpm7 to induce umbrella cell differentiation. Our hypothesis is that environmental (urinary) acid or hypotonic stress stimulates Trpm7 to induce the maturation of umbrella cells. The turnover rate of umbrella cells has been reported to be extremely long (3–6 months) (13). However, surprisingly, when cells in the intermediate layer of the urothelium were exposed to the outside milieu, they started to differentiate into umbrella cells within days after damage and that is the mechanism by which the urothelial barrier function is maintained. Thus, we speculate that urothelial cells somehow receive information from their surrounding environment. The (molecular) identity of the signaling mechanism has not yet been identified. We would like to propose that acid or osmolarity change is recognized by Trpm7 in intermediate or basal urothelial cells, and this might trigger their differentiation into mature umbrella cells. Future studies should reveal factors for umbrella cell differentiation and involvement of Trpm7 in this process.

Some TRP channels have been reported to form a complex with other signaling proteins and/or cytoskeletal proteins. Because there is no evidence showing the involvement of Trpm7 channel activity with the in vivo phenotype, other possibilities cannot be excluded. Trpm7 might contribute to the intercellular junction formation as a scaffolding protein. In cancer cells, it has been reported that Trpm7 associates with myosin and phosphorylates it by its kinase domain in the C terminus, which is involved in intercellular junction formation (12). TRPV4 has been reported to be involved in intracellular junction formation, directly associating with E-cadherin and β-catenin, which are the major players of adherens junction (35). Future work using channel-dead or kinase-dead Trpm7 knock-in mice might be suitable to address the question whether the channel or kinase activity of Trpm7 is involved in the in vivo phenotype.

**Author Contributions**—M. W. and Y. S. designed the study and performed the experiments. K. U. analyzed the data. N. M. and K. M. provided technical assistance and contributed to the electron microscopy study. K. U., S. M., H. K., and M. T. contributed to the data discussion. M. W., Y. S., and M. T. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.
Functional Impairment of Urothelial Trpm7-Knock-out Bladder

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