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Molecular expression of Mg^{2+} regulator TRPM7 and CNNM4 in rat odontoblasts

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ABSTRACT

Objective: Magnesium, the second most abundant cation in cellular fluid, is critical for mineralization of hard tissues. Among the molecules involved in cellular Mg^{2+} homeostasis, functional impairment of Mg^{2+} permeable ion channel TRPM7 or Mg^{2+} transporter CNNM4 have been found to result in severe hypomineralization of the enamel and dentin. However, molecular expressions of TRPM7, CNNM4 and their respective homologues have not been fully investigated in adult odontoblasts.

Design: Expressions of TRPM6, TRPM7, CNNM1, CNNM2, CNNM3, CNNM4 were screened in acutely dissociated rat odontoblasts by single cell RT-PCR. Among these candidates, expression levels of TRPM7 and CNNM4 were compared along the odontoblast layer by immunohistochemical analysis. Finally, the coexpression pattern of TRPM7 and CNNM4 in subcellular regions was examined by immunocytochemical analysis.

Results: ScRT-PCR revealed high expression rate of TRPM7 and CNNM4 in odontoblasts, with CNNM4 detected almost exclusively in TRPM7-positive odontoblasts. However, CNNM2 and CNNM3 were detected in only a small population of odontoblasts, and TRPM6 and CNNM1 were not detected even in the pulp tissue. Immunohistochemical analysis revealed higher CNNM4 expression in the apical odontoblast layer than the coronal area, in contrast to the ubiquitous expression of TRPM7. Lastly, immunocytochemical analysis revealed colocalization of CNNM4 with TRPM7 in the odontoblastic process.

Conclusions: CNNM4 and TRPM7 may serve as main Mg^{2+} regulators in odontoblasts, possibly with selective involvement of CNNM4 in apical dentin formation or mineralization. Colocalization of TRPM7 and CNNM4 in the odontoblastic process suggest functional coupling of these two molecules to maintain Mg^{2+} homeostasis.

1. Introduction

Magnesium, the second most abundant cation in cellular fluids, is crucial for concerted cellular function (Romani, 2006). Mg^{2+} is not only an important cofactor for enzyme activity, but it also regulates the permeability of various channels and participates in hard tissue metabolism (Bigi et al., 1992; Nowak, Bregestovski, Ascher, Herber, & Prochiantz, 1984; Numata, Shimizu, & Okada, 2007; Rude & Gruber, 2004). Cellular Mg^{2+} homeostasis in mammalian cells is maintained by Mg^{2+} influx and efflux, which are mediated by Mg^{2+} regulators such as Mg^{2+} permeable channels and Mg^{2+} transporters (Quamme, 2010; Romani, 2006; Schlingmann, Waldegger, Konrad, Chubanov, & Gudermann, 2007). TRPM6 and TRPM7 ion channels, which are permeable to Mg^{2+}, have been found to regulate cellular Mg^{2+} level to maintain Mg^{2+} homeostasis in intestinal epithelial cells, distal convoluted tubule cells, osteoblasts, etc (Abed & Moreau, 2009; Schlingmann et al., 2002; Schmitz et al., 2003). Similarly, Mg^{2+} transporter CNNMs have been demonstrated to maintain Mg^{2+} homeostasis by mediating either Mg^{2+} influx or efflux (Ferre, Hoenderop, & Bindels, 2011).

Disruption in Mg^{2+} homeostasis caused by dietary Mg^{2+} deficiency results in odontoblast atrophy and hypomineralization of the dentin (Bernick & Hungerford, 1964; Irving, 1940). Dysfunctions of cellular Mg^{2+} regulators such as TRPM7 or CNNM4 expressed in odontoblasts have been also found to affect dentin mineralization (Luder, Gerth-Kahlert, Ostertag-Benzinger, & Schorderet, 2013; Nakano et al., 2016; Parry et al., 2009). For example, functional impairment of TRPM7 resulted in dentin hypomineralization due to inhibition of alkaline phosphatase activity by insufficient intracellular Mg^{2+} supply (Nakano et al., 2016). Mutation of CNNM4, a Mg^{2+} transporter which mediates

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Mg^{2+} efflux (Funato et al., 2014), has been revealed to be the cause of Jalili syndrome, a recessively inherited disease causing amelogenesis imperfecta accompanied with mineralization deficiency of the dentin (Luder et al., 2013; Parry et al., 2009). Although these phenotypic alterations seem to clearly indicate the important role of Mg^{2+} regulators in odontoblasts, molecular expressions of Mg^{2+} permeable channels and Mg^{2+} transporters are yet to be fully investigated in adult odontoblasts.

In the present study, we thus sought to investigate the molecular expression of TRPM7 and CNNM4 in odontoblasts, and further screened the closely related homologues of TRPM7 and CNNM4, TRPM6 (Bates-Withers et al., 2011; Chubanov et al., 2004) and CNNM1 ~ 3 (Wang et al., 2004), respectively. Our single cell RT-PCR and immunocytochemical analysis results indicate that CNNM4 and TRPM7 may serve as the main Mg^{2+} regulators in odontoblasts, suggesting functional coupling between two molecules for the regulation of Mg^{2+} homeostasis.

2. Materials and methods

2.1. Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. Male Sprague Dawley rats (Orient Bio Inc.) of 8 ~ 10 weeks old (n = 7) were used for the experiments. The animals were housed 3 per cage at a temperature-controlled room (23 ± 1 °C, 12 h/12 h light/dark cycle) and maintained with pellet diet and tap water *ad libitum*. The study conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for preclinical studies.

2.2. Acute dissociation of odontoblasts

Acute dissociation of odontoblasts was performed as previously described (Guo, Berry, Somerman, & Davidson, 2000; Lee et al., 2017; Won et al., 2018; Yeon et al., 2009). Rats were anesthetized with isoflurane and sacrificed by cervical dislocation. The upper and lower incisors were extracted within 2 min after cervical dislocation and were immediately cooled in ice-cold extracellular saline (ECS) of the following composition (in mM); 140 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES; and 10 glucose; 290–310 mOsm, pH 7.3–7.4 adjusted with NaOH. After removing the soft tissue surrounding the teeth, the teeth were sectioned transversely into 2 mm pieces with a rotating low speed diamond disk. During the above procedure, ice-cold extracellular saline (ECS) was constantly supplied onto teeth to prevent drying or overheating of the tissue. The pulp contents in the teeth sections were gently pulled out with forceps and treated with 3 mL enzyme cocktail containing collagenase IA (3 mg/mL) and dispase (0.22 mg/mL) in Ca^{2+} and Mg^{2+} free ECS for 25 min at 37 °C. After incubation, the sections were transferred to ice-cold ECS and gently triturated with a Pasteur pipette. After the first trituration, the suspension was collected in a separate tube and ECS was added to the remaining tissue and trituration was repeated. The suspension was added to the separate tube and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in ECS and placed on coverslips coated with poly-D-lysine (Sigma) and Cell-tak (Corning). The odontoblasts were kept at 3–5 °C and used within 6 h after plating.

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from the pulp was isolated using the RNeasy mini kit (Qiagen). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by M-MLV reverse transcriptase (Invitrogen) according to manufacturer's protocols. Primers (Bioneer) used are listed in Table 1 (GAPDH, TRPM6, TRPM7, CNNM1, CNNM2, CNNM3, CNNM4; outer primers, DSPP; inner primer). PCR amplification was done by AccupowerTM Hotstart PCR Premix (Bioneer).

2.4. Single-cell reverse transcriptase polymerase chain reaction (scRT-PCR)

Single-cell reverse transcription polymerase chain reaction (scRT-PCR) was performed as previously described (number of animals used; n = 4, each used for separate experiments) (Lee et al., 2017; Won et al., 2017, 2018). The dissociated odontoblasts were collected with patch pipette with a tip diameter of about 10 μm. The collected cells were put into a polymerase chain reaction (PCR) tube containing 6 μl collection buffer consisted of 25 mM $MgCl_2$ (0.5 μl), 10 mM dNTP (1 μl), 100 ng/μl BSA (1 μl), 50 μM Oligo$(dT)_{20}$ (1 μl), Random hexamer (1 μl), RNaseOUT (0.7 μl). After collection, the tubes were added with 5 μl reverse transcription reagents containing 10x RT buffer (1 μl), 25 mM $MgCl_2$ (1.5 μl), 0.1 M DTT (1 μl), RNaseOUT (0.5 μl), Superscript III (1 μl) and then incubated 10 min at 25 °C, 90 min at 50 °C, then 5 min at 85 °C for cDNA synthesis. All PCR amplifications were performed with nested primers (Bioneer, information listed in Table 1). The first round of PCR was performed in reaction buffer (25 μl) containing 10x PCR buffer (2.5 μl), 50 mM $MgCl_2$ (0.75 μl), 10 mM dNTP (0.5 μl), 0.1 μM outer primers, cDNA (1 μl), 5 U/μl Platinum Taq (0.2 μl). For the second round, the first round reaction buffer (25 μl) was used except outer primers and cDNA were substituted with 0.1 μM inner primers and 1 μl of first round product. All scRT-PCR agents were purchased from Invitrogen unless stated otherwise.

2.5. Immunohistochemistry

Rats were anesthetized with a lethal dose of sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 0.01 M PBS followed by 4% paraformaldehyde in 0.01 M PBS. The upper and lower incisors were extracted and post-fixed at 4C° overnight. The fixed specimens were washed in 0.01 M PBS and decalcified in 10% EDTA solution (pH 7.4) at 4C° for 3 weeks and fresh EDTA solution was newly supplied onto the specimen twice a week. The decalcified teeth were cryoprotected with 30% sucrose in PBS for 3 days and frozen for cryosection. Frozen teeth were longitudinally sectioned into 10 μm thickness and mounted onto glass slides by thaw-mount method. For 1st antibody treatment, the specimens were treated with goat anti-TRPM7 (1:500; Ab729, Abcam (Kwon, Baek, Park, Chung, & Oh, 2014; Won et al., 2018)) and rabbit anti-CNNM4 (1:500; HPA017732, Sigma), diluted in 1% NDS PBST for overnight at 4 °C. Primary antibody was omitted for negative control (data not shown). For 2nd antibody treatment, the specimens were treated with Alexa Fluor 488 donkey anti-goat (1:200; 305-165-045, Jackson) and Cy3 donkey anti-rabbit (1:200; 711-165-152, Jackson) diluted in 1% NDS PBST for 1 h at RT. The specimens were counterstained with DAPI (Sigma) and coverslipped with mounting media (Vector Laboratories, Inc.). The specimens were visualized with a confocal microscope (LSM700; Carl Zeiss). Single plane images were acquired with a 20x objective (320.09 x 320.09 μm, 512 × 512 pixels) and raw images were used for fluorescence intensity profile (intensity was profiled every 0.625 μm along the drawn axis). The representative images were exported in TIFF format. Images were processed with Zen software (Carl Zeiss).

2.6. Immunocytochemistry

Odontoblasts plated on glass coverslips were fixed with 4% paraformaldehyde (Biosesang) for 15 min at room temperature (RT). For blocking and permeabilization, 5% normal donkey serum (NDS) and 0.1% Triton X in 0.01 M phosphate buffer solution (PBS, 0.1% Triton X in PBS; PBST) was used for 1 h at RT. For 1st antibody treatment, the specimens were treated with goat anti-TRPM7 (1:500; Ab729, Abcam (Kwon et al., 2014; Won et al., 2018) and rabbit anti-CNNM4 (1:500; HPA017732, Sigma), diluted in 1% NDS PBST for overnight at 4 °C. Primary antibody was omitted for negative control (data not shown).