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C-terminus of PIEZO1 governs Ca^{2+} influx and intracellular ERK1/2 signaling pathway in mechanotransduction

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ABSTRACT

Cells sense and respond to extracellular mechanical stress through mechanotransduction receptors and ion channels, which regulate cellular behaviors such as cell proliferation and differentiation. Among them, PIEZO1, piezo-type mechanosensitive ion channel component 1, has recently been highlighted as a mechanosensitive ion channel in various cell types including mesenchymal stem cells. We previously reported that PIEZO1 is essential for ERK1/2 phosphorylation and osteoblast differentiation in bone marrow-derived mesenchymal stem cells (BMSCs), induced by hydrostatic pressure loading and treatment with the PIEZO1-specific activator Yoda1. However, the molecular mechanism underlying how PIEZO1 induces mechanotransduction remains unclear. In this study, we investigated that the role of the C-terminus in regulating extracellular Ca^{2+} influx and activating the ERK1/2 signaling pathway. We observed the activation of Fluo-4 AM in the Yoda1-stimulated human BMSC line UE7T-13, but not in a calcium-depleted cell culture medium. Similarly, Western blotting analysis revealed that Yoda1 treatment induced ERK1/2 phosphorylation, but this induction was not observed in calcium-depleted cell culture medium. To investigate the functional role of the C-terminus of PIEZO1, we generated HEK293 cells stably expressing the full-length mouse PIEZO1 (PIEZO1-FL) and a deletion-type PIEZO1 lacking the C-terminal intracellular region containing the R-Ras-binding domain (PIEZO1-ΔR-Ras). We found that Yoda1 treatment predominantly activated Flou-4 AM and ERK1/2 in PIEZO1-FL-trasfected cells but neither in PIEZO1-ΔR-Ras-transfected cells nor control cells. Our results indicate that the C-terminus of PIEZO1, which contains the R-Ras binding domain, plays an essential role in Ca^{2+} influx and activation of the ERK1/2 signaling pathway, suggesting that this domain is crucial for the mechanotransduction of osteoblastic differentiation in BMSCs.

1. Introduction

PIEZO1, piezo-type mechanosensitive ion channel component 1, is located on the cell membrane and regulates cellular mechanotransduction by inducing Ca^{2+} influx in response to extracellular stimuli [1,2]. PIEZO1 has been recognized as a potential target for the development of new therapies for various diseases such as cardiovascular disease and genetic diseases, as well as for regenerative medicine.

The human *PIEZO1* gene, located on chromosome 16, contains 41 exons and encodes a protein consisting of 2521 amino acids. In mice, *Piezo1* is located on chromosome 8, contains 38 exons, and produces a protein consisting of 2546 amino acids. These amino acid sequences identity of 81%. The amino acid domain structure of the PIEZO family is highly conserved between humans and mice, consisting of more than 30 transmembrane domains with a unique PIEZO motif in the center and an R-Ras-binding domain at the C-terminus [3,4]. Based on observations

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made using cryoelectron microscopy and functional characterization, PIEZO1 is thought to be trimerized on the plasma membrane, forming a propeller-like structure with three spreading blades surrounding a central ion-conducting pore [5]. This blade-like structure consists of multi-transmembrane domains responsible for mechanosensing. Additionally, a unique structure connects the blade and the central ion-conducting pore, controlling the pore size, and is thought to play a lever-like role in controlling the delicate mechanical sensing function of PIEZO1 [5]. When mechanically stimulated, PIEZO1 opens the central pore gate, allowing selective permeation of Ca^{2+} from the extracellular space into the cell [6–9]. However, the molecular mechanism by which PIEZO1 transduces mechanical stimuli into intracellular signals has not been fully elucidated.

Ras is a member of the small G-protein family and functions as a small GTPase. Ras activates several intracellular signaling pathways [10]. Activation of Ras leads to downstream signal transduction from membrane receptors to various effectors, ultimately regulating cellular processes such as cell proliferation, differentiation, and apoptosis [11,12]. One of the critical effectors of Ras is Raf1. The Ras/Mitogen-Activated Protein Kinase (MAPK) signaling pathway is a well-established MAPK pathway. In this pathway, the activation occurs sequentially in the order of Ras/Raf1/MEK1/ERK [13–15]. Both intracellular Ca^{2+} influx and MAPK signaling activation are crucial for the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblasts [16–18]. We have previously demonstrated that PIEZO1 plays an important role in cell fate determination in human bone marrow- and human dental pulp-derived mesenchymal stem cells [19,20]. In UE7T-13 cells, a human BMSC line, knockdown of *PIEZO1* gene expression using *PIEZO1* siRNA suppressed the phosphorylation of ERK1/2, bone morphogenetic protein 2 (*BMP2*) expression, and osteogenic differentiation by hydrostatic pressure loading or stimulation with Yoda1. Additionally, treatment with the MEK inhibitor U0126 or the Ras inhibitor FTS suppressed *BMP2* expression induced by hydrostatic pressure or Yoda1 stimulation. These findings suggest that the R-Ras--binding domain of PIEZO1 is important in the mechanotransduction signaling of PIEZO1 [19]. However, the role of the R-Ras binding domain in the C-terminus of PIEZO1 remains unclear.

In this study, we investigated that the role of the PIEZO1 C-terminus in the regulation of Ca^{2+} influx and activation of the ERK1/2 signaling pathway. Here, we showed that the R-Ras-binding domain at the C-terminus may play an essential role in the mechanotransduction of osteoblastic differentiation in BMSCs.

2. Material and methods

2.1. Cell and cell culture

The human bone marrow-derived mesenchymal stem cell line UE7T-13 cells and the human embryonic kidney cells 293 cells (HEK293) were maintained with α-modified minimum essential medium (α-MEM) (Gibco) containing 10% fetal bovine serum (BioWest), 100 units/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B (Gibco) at 37 °C with 5% CO_2.

2.2. Calcium imaging

Cells were seeded at a density of 1×10^6 cells in 35-mm collagen I-coated dishes. Fluo-4 AM was applied to cells according to the manufacturer's instructions (Dojinsha). The cells were stimulated with either Hanks' balanced salt solution (HBSS) (Wako) containing 5 μM Yoda1 (Cayman Chemical), 10 μM ionomycin (Adipogen Life Sciences), or the same amount of dimethyl sulfoxide (DMSO) as the negative control. The fluorescence emission was measured using a ChemiDoc imaging system (Bio-Rad).

2.3. Western blotting analysis

Cells were seeded in 60-mm cell culture dishes at 60% confluence. The cells were incubated with serum-free α-MEM at 37 °C for 1 h. Subsequently, the cells were treated with 5 μM Yoda1 or the same amount of DMSO for 1 min in α-MEM or calcium-free medium S-MEM (Gibco). Following treatment, the cells were washed with phosphate-buffered saline containing 1 mM sodium vanadate (Na_3VO_4) and lysed in ice-cold Sigma CellLytic™ M reagent supplemented with Complete Mini Protein Inhibitor Cocktail tablets (Roche) and PhosSTOP™ (Roche Diagnostics) for 5 min. The lysed cells were centrifuged at 12,000 rpm for 5 min at 4 °C, and the protein concentration of each sample was measured with BCA assay regent (Thermo Fischer Scientific). The samples were denatured in LDS sample buffer with sample reducing agent (Invitrogen) and loaded onto a NuPAGETM 4–12% Bis-Tris Protein Gels (Invitrogen), with 10 μg of lysate protein being applied to each lane. After SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membranes and blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (Bio-Rad Laboratories, Inc.). Membranes were then immunoblotted with primary antibodies targeting ERK1/2, phospho-ERK1/2, CaMKII, phospho-CaMKII,β-ACTIN, and V5 (Cell Signaling) and subsequently incubated with Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling). Finally, the proteins were visualized using an ECL kit (Cytiva). The blot images were acquired using an Amersham Imager 600 (Cytiva). ImageJ 1.48v (National Institutes of Health, Bethesda, MD, USA) was used to quantify the band intensities.

2.4. Plasmid construction and transfection

To create a full-length *Piezo1* expression vector (PIEZO1-full), a 7641 bp mouse *Piezo1* gene without a stop codon in the cording sequence (CDS) was inserted into the pcDNA-DEST40 vector. In contrast, to create a *Piezo1* expression vector lacking the R-Ras-binding domain (PIEZO1-ΔR-Ras), a region from the start codon to 6375 (2123 residues), excluding the amino acid sequence corresponding to the R-Ras-binding domain (2126–2544 residues) of PIEZO1, was inserted into the same vector; an empty vector was used as a control. HEK293 cells were transfected with 2 μg each DNA using Lipofectamin™ LTX (Invitrogen) as per the manufacturer's instructions. For stable transfection, G418 (400 μg/ml) selection was initiated 48 h after transfection and continued for two weeks. Transfected pools were maintained with 40 μg/ml G418. Genetic recombination experiments were approved by the Institute for Genome Research, Tokushima University (2019-100). Note that Yoda1 binding to PIEZO1-ΔR-Ras cells was not inhibited, as the binding site of Yoda1 is considered to be the amino acid region from residues 1961 to 2063 [21].

2.5. Statistics

Data presented in Figs. 1, 2A and 3B, 3C, and 4A represent the results of three independent experiments with similar outcomes. In Figs. 2B and 4B, data were obtained from three independent experiments, and the error bars indicate the standard deviations. Statistical analysis was carried out using the Student's t-test, with p-values less than 0.05 (*) considered statistically significant.

3. Results

3.1. Calcium dynamics by PIEZO1 activator Yoda1 treatment

Yoda1 is a small molecule compound that activates PIEZO1, a mechanosensitive selective cation channel that leads to Ca^{2+} influx in cells [22]. Our previous study showed that stimulation of UE7T-13 cells with 5 μM Yoda1 increased the expression of *BMP2* and promoted calcified nodule formation [19]. Therefore, we first examined whether 5 μM Yoda1 affected the intracellular Ca^{2+} dynamics in UE7T-13 cells. To

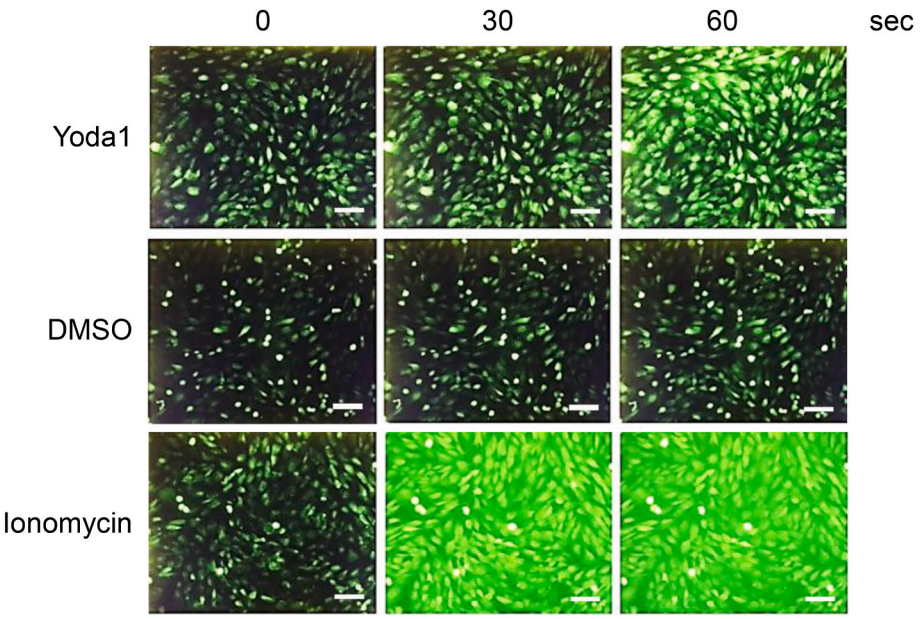

Fig. 1. Yoda1, a PIEZO activator, alters calcium dynamics in UE7T-13. UE7T-13 cells were treated with Fluo-4 AM-containing medium for 45 min, followed by the addition of 5 μM Yoda1. Images were captured every 10 s for 180 min. Dimethyl sulfoxide (DMSO) and ionomycin (10 μM) were used as negative and positive controls, respectively. A scale bar of 100 μm is shown in the figure. The experiment was repeated three times to confirm the reproducibility of the results; the figure shows representative images.

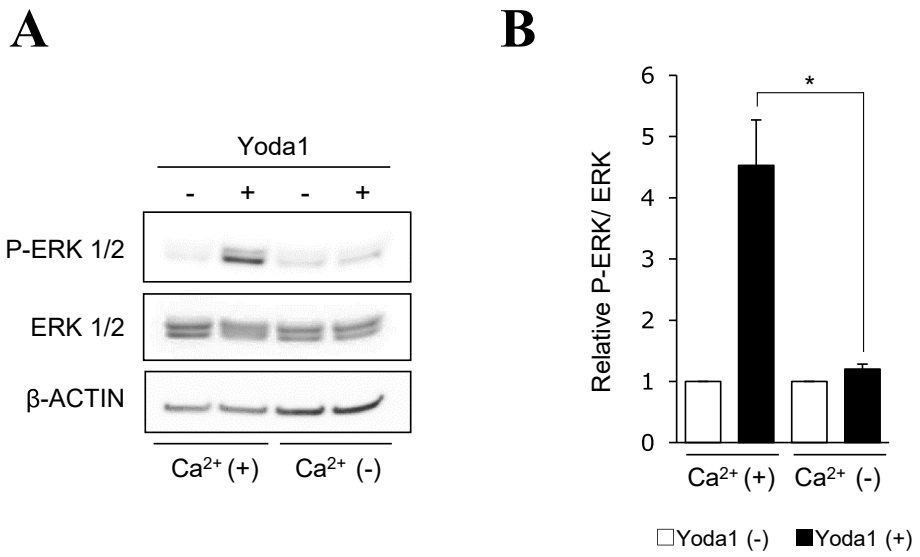

Fig. 2. Yoda1-induces ERK1/2 phosphorylation is extracellular Ca^{2+} ion-dependent in UE7T-13. (A) UE7T-13 cells were cultured in media containing or lacking Ca^{2+}, and deprived of serum. The cells were then stimulated with 5 μM Yoda1 for 1 min and subjected to Western blotting analysis using anti-phospho-ERK1/2, anti-ERK, and anti-β-ACTIN antibodies. (B) Digital images of the bands were quantified using NIH ImageJ software. Results from three independent experiments were combined for evaluation. The data are presented as the mean (±S.E.M.), and statistical significance was determined as *$p < 0.05$.

test hypothesis, we used the intracellular calcium indicator Flou-4 AM and observed the cells using fluorescence microscopy. We found that the intracellular fluorescence intensity of UE7T-13 cells gradually increased from 20 s after 5 μM Yoda1 stimulation and significantly increased at 30 s and 60 s after stimulation compared to the negative control (DMSO stimulation) (Fig. 1). The fluorescence intensity peaked approximately 120 s after stimulation and then decreased (data not shown). Ionomycin, a calcium ionophore, was used as a positive control. Taken together, these findings suggest that Yoda1 activates PIEZO1, leading to a significant increase in intracellular Ca^{2+} levels in UE7T-13 cells, which may play an essential role in promoting osteoblast differentiation of bone marrow-derived mesenchymal stem cells (BMSCs).

3.2. Requirement of extracellular Ca^{2+} in PIEZO1-induced ERK1/2 phosphorylation

Western blot analysis showed that 1-min stimulation of UE7T-13 cells with 5 μM Yoda1 resulted in detectable ERK1/2 phosphorylation, an intracellular signaling pathway important for the differentiation of BMSCs into osteoblasts (Fig. 2A and B). To clarify whether the phosphorylation of ERK1/2 by Yoda1 stimulation was dependent on extracellular Ca^{2+} influx, we examined whether phosphorylation of ERK1/2 could be induced by Yoda1 in the absence of extracellular Ca^{2+}. Yoda1 failed to induce ERK1/2 phosphorylation in a Ca^{2+}-free medium (Fig. 2A and B). These results indicate that Ca^{2+} influx is required for the

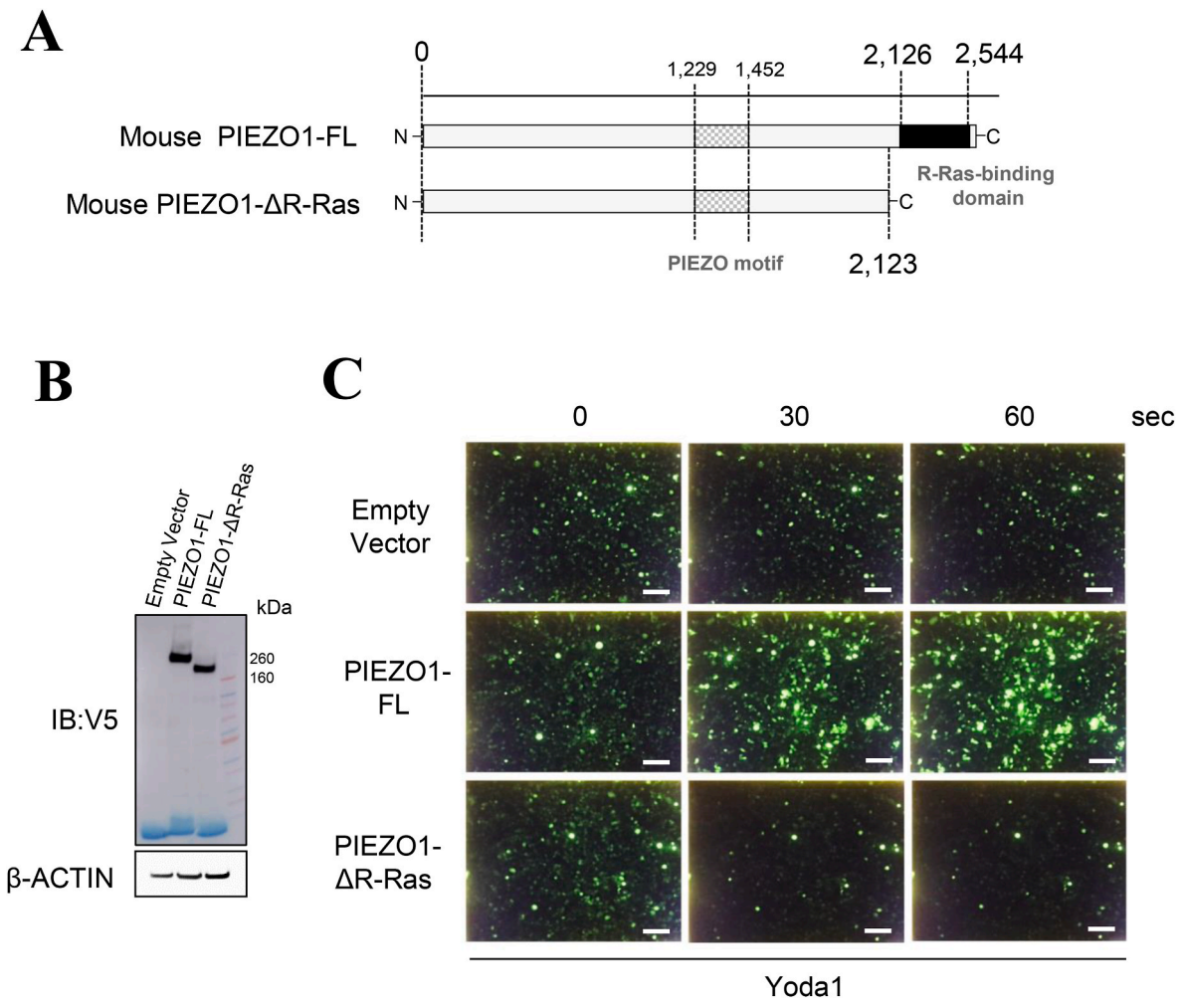

Fig. 3. R-Ras binding domain of PIEZO1 C-terminus is important for calcium dynamics. (A) Domain scheme of Piezo1 full-length construct (PIEZO1-FL) and deletion construct of the R-Ras binding domain of Piezo1 (PIEZO1-ΔR-Ras) are shown, with the R-Ras binding domain indicated by a black box at the C-terminus of Piezo1. (B) HEK293 cells were stably transfected with the empty vector, the PIEZO1-FL, or the PIEZO1-ΔR-Ras expression vector. Stable transfectants were analyzed by Western blotting analysis using anti-V5 and anti-β-ACTIN antibodies. (C)The empty vector-, the PIEZO1-FL-, or the PIEZO1-ΔR-Ras-transfected cells were incubated with Fluo-4 AM and then stimulated with 5 μM Yoda1. Images were captured every 10 s for 180 min. A scale bar of 100 μm is shown in the figure. To ensure the reproducibility of the results, the experiment was repeated three times, and the figure shows representative images.

phosphorylation of ERK1/2 via PIEZO1 activation in BMSCs.

3.3. *R-Ras binding domain of the C-terminus of PIEZO1 for mechanotransduction*

Next, we aimed to clarify whether the R-Ras-binding domain (region from 2126 to 2544 in mouse PIEZO1) of the C-terminus of PIEZO1 was involved in the molecular mechanism of mechanotransduction induced by PIEZO1. We generated two constructs, including full length (PIEZO1-FL) and PIEZO1 lacking R-Ras binding domain (PIEZO1-ΔR-Ras) (Fig. 3A). Each of the expression vectors and an empty vector were transfected into human embryonic kidney 293 (HEK293) cells, and stable cell lines were created to express those genes. In these cells, we examined whether the PIEZO1-FL and PIEZO1-ΔR-Ras proteins were stably expressed. We confirmed that the Anti-V5 antibody detected the recombinant protein as a single band with the predicted molecular weight of the V5 fusion protein in each transfected cell line (Fig. 3B). To determine the effect of 5 μM Yoda1 stimulation on intracellular Ca^{2+} levels, the cells were examined using the Flou-4 AM system. The results showed that PIEZO1-FL-transfected cells showed significantly increased expression of the fluorescent dye by Flou-4 AM expression at 30 s and 60 s after Yoda1 stimulation, whereas PIEZO1-ΔR-Ras-transfected cells did not induce its expression (Fig. 3C). These results indicated that the C-terminus containing the R-Ras-binding region of PIEZO1 is important for Ca^{2+} influx.

To determine whether the R-Ras-binding domain of PIEZO1 was involved in the activation of ERK1/2 by Yoda1, PIEZO1-FL- and PIEZO1-ΔR-Ras-transfected cells were stimulated with 5 μM Yoda1 and the phosphorylation of ERK1/2 was investigated by Western blot analysis (Fig. 4A). PIEZO1-FL-transfected cells showed strong activation of ERK1/2 at 1 min after 5 μM Yoda1 stimulation. However, in PIEZO1-ΔR-Ras cells, the activation of ERK1/2 by Yoda1 was approximately 60% lower than that in PIEZO1-FL-transfected cells (Fig. 4B). These results indicate that PIEZO1 activates the ERK1/2 signaling pathway, and suggest that the R-Ras-binding domain at the C-terminus of PIEZO1 plays an important role as a molecular mechanism for the mechanotransduction by PIEZO1.

4. Discussion

Intracellular Ca^{2+} is an important secondary messengers that plays a role in various cellular functions, such as cell proliferation and differentiation [23,24]. This increase in intracellular Ca^{2+}concentration can occur through two mechanisms: (1) Ca^{2+} influx through calcium

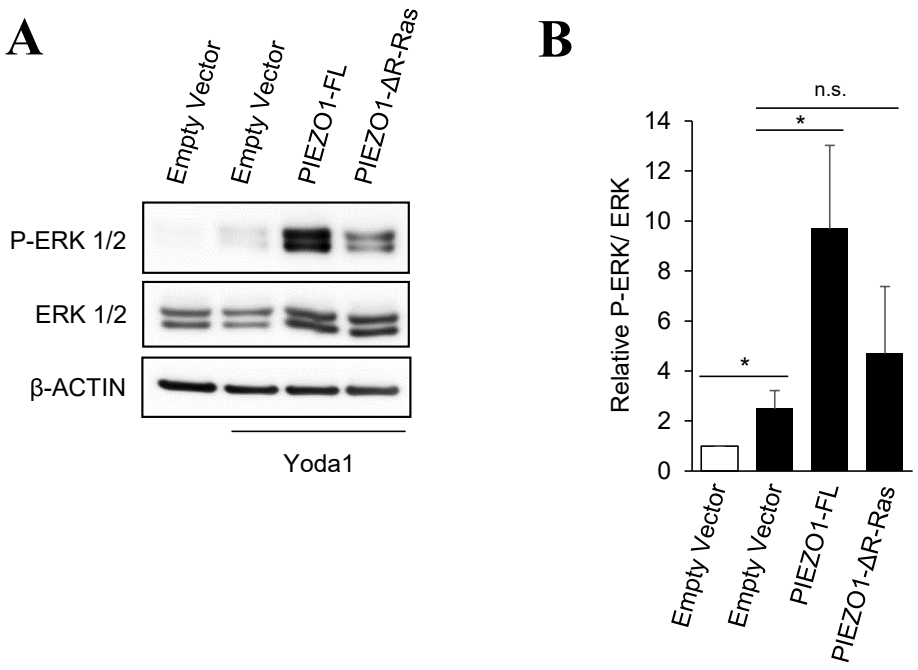

Fig. 4. ERK1/2 activation by PIEZO1 requires the R-Ras binding domain located in PIEZO1 C-terminus. (A) HEK293 cells stably transfected with the empty vector, the PIEZO1-FL, or the PIEZO1-ΔR-Ras expression vector, were stimulated with 5 μM Yoda1 for 1 min and subjected to Western blotting analysis using anti-phospho-ERK1/2, anti-ERK, and anti-β-ACTIN antibodies. (B) The bands in the digital images were quantified with NIH ImageJ software. The results of three independent experiments were pooled for evaluation. The data are presented as the mean (±S.E.M.), and statistical significance was determined as *$p < 0.05$. n.s., not significant.

channels in the plasma membrane, and (2) Ca^{2+} release from the endoplasmic reticulum (ER) triggered by the stimulation of receptors on the plasma membrane [24,25]. However, these are generally not independent systems; the plasma membrane and ER membrane are closely linked systems that work together to tightly regulate intracellular Ca^{2+}-concentrations and promote efficient cellular functions. Therefore, the same molecules involved in Ca^{2+} regulation could be expressed on both membranes. Previously, we have reported that pannexin 3 (Panx3), a member of the gap junction pannexin family acts as an adenosine triphosphate (ATP)-releasing hemichannel on the cell membrane, and is also expressed on ER membrane and regulates intracellular Ca^{2+} levels [26–29]. PIEZO1 is also expressed on the ER membrane and may be involved in Ca^{2+} release from the ER [30]. However, suppression of PIEZO1 expression using siRNA resulted in significantly reduced uptake of Ca^{2+} from the extracellular space [30]. Furthermore, depletion of ER-storage Ca^{2+} does not impact the elevation in intracellular Ca^{2+} levels induced by PIEZO1 activation [31]. In this study, we showed that activation of PIEZO1 by Yoda1 led to an increase in intracellular Ca^{2+} concentration, whereas the absence of extracellular Ca^{2+} inhibited the phosphorylation of ERK1/2 by PIEZO1 activation. These results suggested that the elevation of intracellular Ca^{2+} levels by PIEZO1 was primarily due to Ca^{2+} influx from the extracellular space.

Intracellular Ca^{2+} signals are often mediated by the Ca^{2+}-binding protein calmodulin (CaM), which plays a key role as a principal signal transducer. Activated CaMKII undergoes autophosphorylation and triggers a range of cellular responses. The ERK1/2 pathway is considered one of the downstream signaling molecules of CaMKII [32]. Therefore we investigated whether Yoda1 stimulation induced CaMKII phosphorylation in UE7T-13 cells. However, CaMKII phosphorylation was not detected by Western blotting analysis (data not shown). Hence, the ERK1/2 activation induced by PIEZO1 might be mediated by a calmodulin-independent signaling pathway in BMSCs.

Ras is a human proto-oncogene with GTPase activity that plays a crucial role in intracellular signaling pathways that regulate cell proliferation and differentiation [12,33]. H-Ras, K-Ras, and N-Ras are the prototype members of the Ras superfamily, and their mutations are associated with various human tumors [34]. R-Ras, which shares 55% sequence identity with H-Ras, also has a GTP binding site and effector Raf binding domain [35]. The R-Ras related subfamily comprises three members, R-Ras1(R-Ras), R-Ras2 (TC21), and R-Ras3 (M-Ras). M-Ras is strongly expressed in developing mouse bone and is involved in the osteoblast differentiation of BMSCs [36,37]. In the present study, we found that the R-Ras-binding region is important in ERK1/2 activation by PIEZO1. Indeed, PIEZO1-ΔR-Ras cells lacking the R-Ras binding region showed significantly suppressed Yoda1-induced phosphorylation of ERK1/2. In addition, our previous study indicated that FTS, a Ras inhibitor, suppressed ERK1/2-mediated *BMP2* expression via hydrostatic pressure and Yoda1 [19]. These results suggest that the R-Ras-binding domain of PIEZO1 directly activates ERK1/2 and may play a central role in mechanotransduction via PIEZO1.

Several studies have reported the involvement of PIEZO1 in bone formation [38–41]. Moreover, Piezo1 acts as a Ca^{2+} influx channel in response to mechanical stress or Yoda1 stimulation during the bone formation process [38,39,41]. However, the molecular mechanisms underlying Piezo1 activation and Ca^{2+} influx that promote osteoblast differentiation of BMSCs are not fully understood. In this study, we identified the R-Ras-binding domain of PIEZO1 as a crucial regulator of PIEZO1-mediated mechanotransduction, facilitating intracellular Ca^{2+} influx and activating the ERK1/2 signaling pathway. These results provide new insights into the molecular mechanisms of PIEZO1-mediated mechanotransduction and may have implications for the development of new therapeutic strategies for diseases involving mechanotransduction dysfunction.

Data availability statement

The datasets are available from the corresponding author upon reasonable request.

Author contributions

Asuna Sugimoto: Investigation, visualization, formal analysis,

writing-original draft; Kokoro Iwata: investigation, resources; Rika Kurogoushi: investigation; Manami Tanaka: investigation; Yumiko Nakashima: investigation; Yoshihito Yamakawa: investigation; Atsushi Oishi: investigation; Keigo Yoshizaki: vector construction; Satoshi Fukumoto: resources, data interpretation; Akihito Yamamoto: resources, data interpretation; Naozumi Ishimaru: resources, data interpretation; Tsutomu Iwamoto: conceptualization, methodology, writing-original draft, project administration, funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that might affect the research reported in this paper.

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