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Brief Reports: TRPM7 Senses Mechanical Stimulation Inducing Osteogenesis in Human Bone Marrow Mesenchymal Stem Cells

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Key Words. Marrow stromal stem cells • Mesenchymal stem cells • Calcium flux • Differentiation and mechanosensation

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotential stem cells residing in the bone marrow. Several studies have shown that mechanical stimulation modulates MSC differentiation through mobilization of second messengers, but the mechanism of mechanotransduction remains poorly understood. In this study, using fluorescence and laser confocal microcopy as well as patch-clamp techniques, we identified the transient receptor potential melastatin type 7 (TRPM7) channel as the key channel involved in mechanotransduction in bone marrow MSCs. TRPM7 knockdown completely abolished the pressure-induced cytosolic Ca^{2+} increase and pressure-induced osteogenesis. TRPM7 directly sensed membrane tension, independent of the cytoplasm and the integrity of cytoskeleton. Ca^{2+} influx through TRPM7 further triggered Ca^{2+} release from the inositol trisphosphate receptor type 2 on the endoplasmic reticulum and promoted NFATc1 nuclear localization and osteogenesis. These results identified a central role of TRPM7 in MSC mechanical stimulation-induced osteogenesis. STEM CELLS *2015;33:615–621*

INTRODUCTION

Loading-induced bending of bones generates strain gradients in the medullary cavity, resulting in the development of hydrostatic pressure and fluid flow-induced shear stress, which may directly stimulate mesenchymal stem cells (MSCs) within the marrow [1]. Accumulating evidence suggests that mechanical stimuli, such as pressure [2], shear stress [3], and stretch [4], modulate MSC differentiation through mobilization of second messengers, such as inositol trisphosphate (IP_3) [5] and Ca^{2+} [6]. Spontaneous Ca^{2+} oscillations and stimulation induce a cytosolic Ca^{2+} increase, which has been well defined to regulate the expression level of transcription related genes [7, 8]. In the process of mechanical stimulation-induced MSC differentiation through Ca^{2+} mobilization, the mechanism by which mechanical stimulation induces intracellular Ca^{2+} increase is still unclear. Characterization of ionic currents in MSCs shows that Ca^{2+} entry through the plasma membrane is mainly mediated by store operated Ca^{2+} channels, with a little contribution from voltage-gated Ca^{2+} channels [9–11]. The transient receptor potential channels (TRP channels) that reside in the plasma membrane are nonselective cation channels and thought to be candidates for mechanosensation [12, 13]. We tested the hypothesis that TRP channels sensed mechanical stimulation and mediated the Ca^{2+} influx, resulting in osteogenesis in human bone marrow MSCs.

MATERIALS AND METHODS

Human bone marrow MSCs used in this study were the same as in our previous study [14]. Compressive force was applied on the MSCs seeded in 35-mm culture dishes as previously described [15]. Statistical analysis was performed using GraphPad Prism (Version 5.0) software. All data were expressed as mean ± SEM. Detailed materials and methods are available in Supporting Information.

RESULTS AND DISCUSSIONS

Pressure Induces an Increase in Cytosolic Ca^{2+} in MSCs

In order to investigate whether mechanical stimulation could induce cytosolic Ca^{2+} increase, we measured the intracellular Ca^{2+} response to pressure stimulation in MSCs loaded with fura-2 AM. We found that cytosolic Ca^{2+} was significantly increased by pressure loading (Supporting Information Fig. S1A). A pressure gradient test further showed that the cytosolic Ca^{2+} increase depended on the

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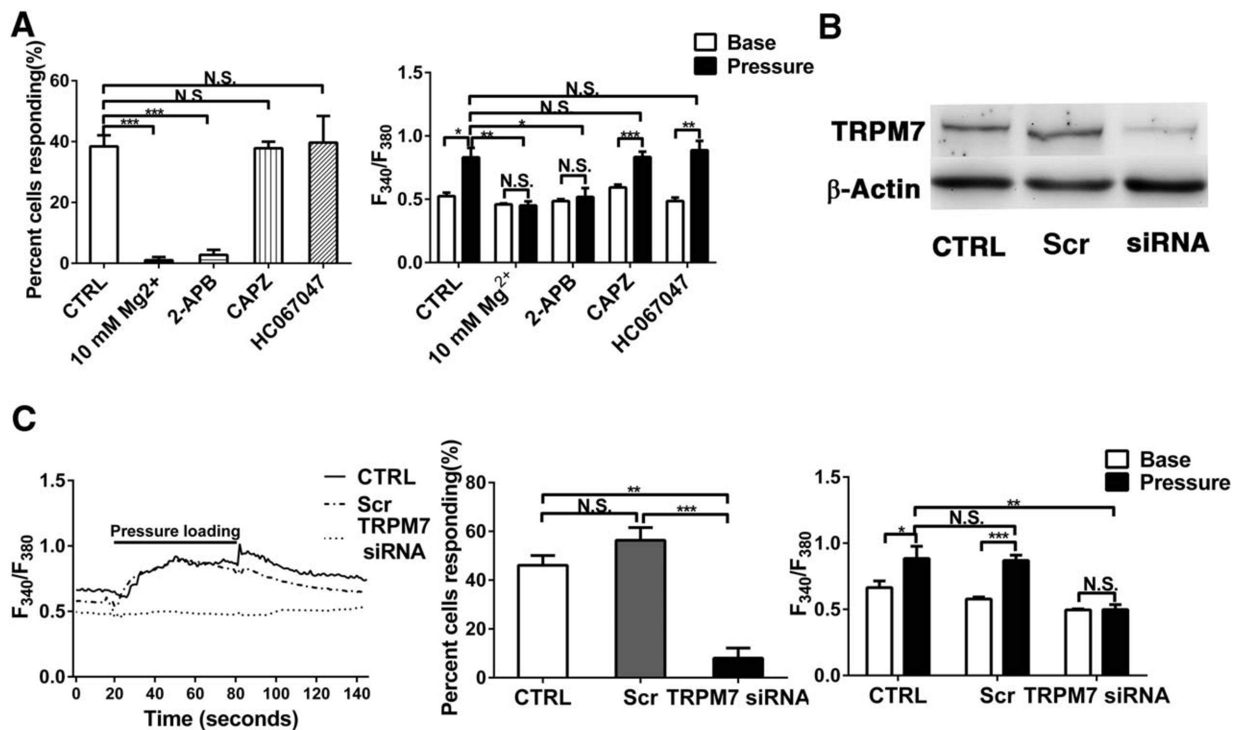

Figure 1. TRPM7 mediates the pressure-induced Ca^{2+} influx in mesenchymal stem cells. **(A):** The pressure-induced cytosolic Ca^{2+} increase was inhibited by 10 mM Mg^{2+} or 100 μM 2-APB, but not CAPZ or HC067047. Left panel shows the percentage of cells responding to pressure. The percentages of cells responding to pressure in the CTRL group, 10 mM Mg^{2+} group, 100 μM 2-APB group, CAPZ group, and HC067047 group are 38.41% ± 3.66%, 1.04% ± 1.04%, 2.75% ± 1.66%, 37.81% ± 2.17%, and 39.70% ± 8.74%, respectively. Right panel shows the peak ratio of F340/F380 during the pressure loading term. The peak ratios for the CTRL group, 10 mM Mg^{2+} group, 100 μM 2-APB group, CAPZ group, and HC067047 group are 0.83 ± 0.08, 0.45 ± 0.03, 0.52 ± 0.07, 0.83 ± 0.04, and 0.89 ± 0.07, respectively (CTRL = vehicle control, *, <.05; **, <.01; and ***, <.001, more than three independent experiments). **(B):** Western blot confirming TRPM7 knockdown by siRNA. **(C):** TRPM7 knockdown abolished the pressure-induced cytosolic Ca^{2+} increase. Left panel shows the ratio of F340/F380 over time. The ratio of the CTRL group and Scr group after pressure loading increased from 0.66 ± 0.05 and 0.58 ± 0.01 to 0.88 ± 0.09 and 0.87 ± 0.04, respectively. The pressure induced rise in Ca^{2+} was abolished with TRPM7 knockdown. Middle panel shows the percentage of cells responding to pressure. The percentages of cells responding in the CTRL group, Scr group, and siRNA group are 46.09% ± 4.04%, 56.35% ± 5.21%, and 8.00% ± 4.14%, respectively. Right panel shows the peak ratio of F340/F380 during the pressure loading term. The peak ratios for the CTRL and Scr group are 0.88 ± 0.09 and 0.87 ± 0.04, respectively. While no obvious change in TRPM7 knockdown group (CTRL = blank control, Scr = scrambled siRNA control, * < .05; **, < .01; and ***, < .001, more than three independent experiments). Abbreviations: CAPZ, capsazepine; TRPM7, transient receptor potential melastatin type 7.

pressure force. As the peak free Ca^{2+} concentration and the percentage of responding cells reached a plateau at both 2 g/cm^2 and 3 g/cm^2 (Supporting Information Fig. S1A), we selected the 2 g/cm^2 pressure stimulation for further studies.

The Transient Receptor Potential Melastatin Type 7 Channel Mediates the Pressure-Induced Ca^{2+} Influx

The cytosolic Ca^{2+} increase induced by pressure was completely abolished in Ca^{2+}-free bath solution (Supporting Information Fig. S1B). Also, a nonspecific TRP channel antagonist Ruthenium Red (RR, 10 μM) blocked the cytosolic Ca^{2+} increase induced by 2 g/cm^2 pressure (Supporting Information Fig. S1B). The extracellular Ca^{2+} dependence and RR sensitivity of the cytosolic Ca^{2+} increase indicated a TRP channel-mediated Ca^{2+} influx.

To determine which type of TRP channel mediated the pressure-induced Ca^{2+} influx, we applied pharmacological inhibitors of different TRP channels. Capsazepine (20 μM), a selective TRPV1 blocker, and HC-067047 (10 μM), a selective TRPV4 blocker, did not inhibit the pressure-induced cytosolic Ca^{2+} increase. However, high Mg^{2+} (10 mM) and 2-APB (100 μM) eliminated the pressure-induced rise in cytosolic Ca^{2+} (Fig. 1A). Since it is widely accepted that sensitivity to inhibition by Mg^{2+} and 2-APB is characteristic of transient receptor potential melastatin type 7 (TRPM7), we further used RNA interference to knockdown TRPM7 (Fig. 1B). The control and scrambled siRNA groups exhibited comparable responses to pressure stimulation. However, in the TRPM7 siRNA group, both the peak free Ca^{2+} concentration and the percentage of responding cells decreased to basal levels (Fig. 1C). These data demonstrated that TRPM7 was responsible for the pressure-induced Ca^{2+} influx, thereby transducing mechanical stimuli to intracellular signals.

The TRPM7 Channel Independently Senses the Mechanical Stimulation

Generally, mechanosensitive channels may sense mechanical stimuli through either membrane tension or intracellular accessory proteins [16]. Mechanosensitive channel currents induced by mechanical stimuli, such as stretch and suction [17, 18], are effective indicators of channel opening. Therefore, except for pressure loading, we applied a 3-μm stretch or a 3-cm H_2O suction to activate TRPM7. Stretch induced a rise in cytosolic Ca^{2+} levels, and suction induced the membrane currents (cell-attached model) in the control and scrambled groups, but not in the TRPM7 knockdown group

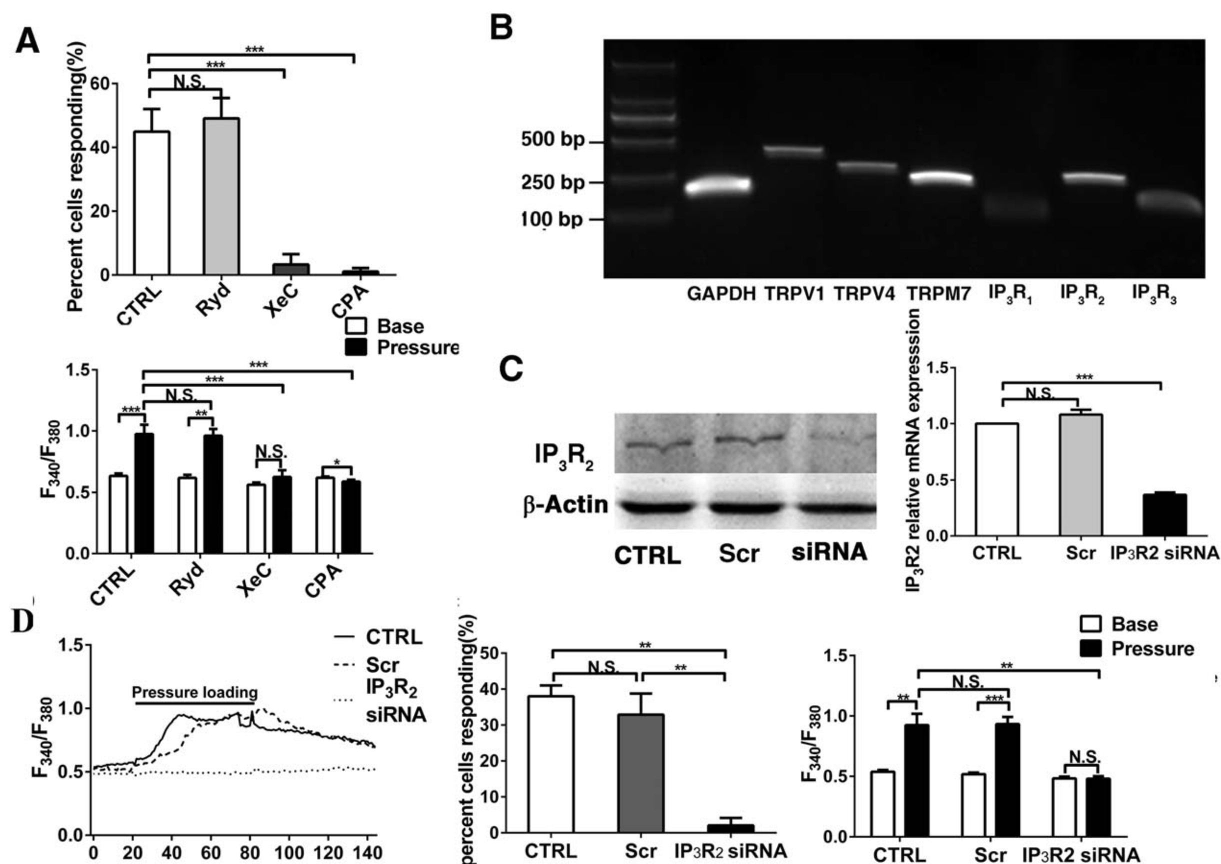

Figure 2. IP_3R2 mediates pressure-induced intracellular Ca^{2+} release. **(A):** CPA or Xestospongin C inhibited the pressure-induced cytosolic Ca^{2+} elevation, while ryanodine did not. Upper panel shows the percentage of cells responding to pressure. The percentages of responding cells in the CTRL group, Ryanodine group (Ryd), Xestospongin C (XeC) group, and CPA group are 44.95% ± 7.09%, 49.08% ± 6.40%, 3.25% ± 3.25%, and 1.08% ± 1.08%, respectively. Lower panel shows the peak ratio of F340/F380 during the pressure loading term. The peak ratios for CTRL group, Ryanodine (Ryd) group, Xestospongin C (XeC) group, and CPA group are 0.98 ± 0.08, 0.97 ± 0.06, 0.63 ± 0.06, and 0.59 ± 0.01, respectively (CTRL = vehicle control, *, $<$ .05; **, $<$ .01; and ***, $<$.001, more than three independent experiments). **(B):** Messenger RNA expression levels of IP_3R subtypes. **(C):** Knockdown of IP_3R2 by siRNA was confirmed at the protein and mRNA levels. **(D):** IP_3R2 knockdown prevented the pressure-induced Ca^{2+} elevation. Left panel shows the ratio of F340/F380 over time. The ratio of the CTRL group and Scr group after pressure loading increased from 0.54 ± 0.01 and 0.52 ± 0.01 to 0.92 ± 0.09 and 0.93 ± 0.06, respectively. The pressure induced rise in Ca^{2+} was inhibited with IP_3R2 knockdown. Middle panel shows the percentage of cells responding to pressure. The percentages of cells responding in the CTRL group, Scr group, and siRNA group are 38.03% ± 2.98%, 32.90% ± 5.86%, and 2.07% ± 2.07%, respectively. Right panel shows the peak ratio of F340/F380 during the pressure loading term. The peak ratios for the CTRL and Scr group are 0.92 ± 0.09 and 0.93 ± 0.06, respectively. While no obvious change was found in IP_3R2 knockdown group (CTRL = blank control, Scr = scrambled siRNA control, *, $<$.05; **, $<$.01; and ***, $<$.001, more than three independent experiments). Abbreviations: CPA, cyclopiazonic acid; IP_3R2, inositol trisphosphate receptor type 2.

(Fig. 3A first, second, and third panel and Fig. 3B). To further clarify the mechanism of TRPM7 activation in MSCs, we used inside-out patch clamp to record the membrane currents induced by suction. The typical square currents with similar amplitude as previously reported [17, 18] were recorded in both control and scrambled siRNA groups, but not in the TRPM7 siRNA group (Supporting Information Fig. S2A). The cytoskeleton is an important intracellular element mediating mechanosensation [19]. Although pretreatment with cytochalasin D (cytoD, 1 μM) for 3 hours resulted in severe disruption of the cytoskeleton, suction-induced membrane currents were still recorded (Supporting Information Fig. S2B), indicating that the pressure-induced TRPM7 activation was cytoskeleton independent. These data indicated that the pressure-induced TRPM7 activation was independently through membrane tension or bending, independent of intracellular signals. This finding was consistent with the heterologously expressed TRPM7 in HEK293T cells [20].

The Inositol Trisphosphate Receptor Type 2 Mediates the Pressure-Induced Release of Ca^{2+} from the Endoplasmic Reticulum

Extracellular Ca^{2+} and intracellular Ca^{2+} stores are the two main sources for cytosolic Ca^{2+} elevation. In MSCs, fluid flow-induced cytosolic Ca^{2+} increase involves intracellular Ca^{2+} stores and IP_3 [3], indicating that the IP_3 receptor (IP_3R) may play an essential role. However, the exact IP_3R subtype mediating Ca^{2+} release is still unknown. In our model, when cyclopiazonic acid (CPA) (10 μM) was applied to block endoplasmic reticulum (ER) Ca^{2+} pumps and deplete the intracellular Ca^{2+} stores, the pressure-induced cytosolic Ca^{2+} increase was significantly inhibited (Fig. 2A). Since the ryanodine receptor (RyR) and the IP_3R are two main Ca^{2+} release channels on the ER, we applied ryanodine (10 μM) and XestosponginC (5 μM) separately to block the RyR and IP_3R, respectively. We found that blocking the IP_3R but not the RyR abolished the

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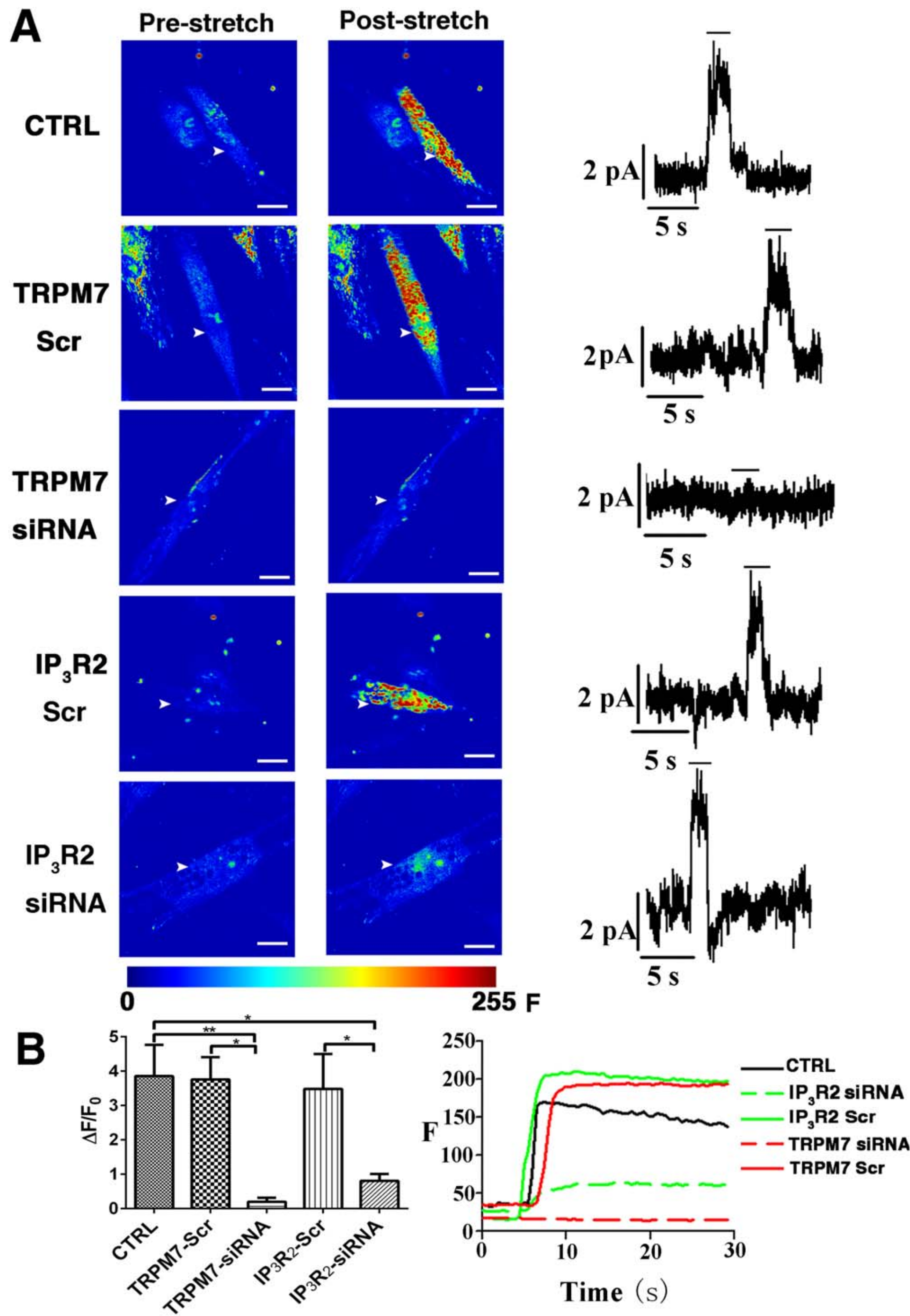

Figure 3. TRPM7-mediated Ca^{2+} influx triggers IP_3R2 to amplify the cytosolic Ca^{2+} increase. **(A):** TRPM7 knockdown abolished the stretch-induced cytosolic Ca^{2+} elevation and the suction-induced membrane currents. IP_3R2 knockdown inhibited the stretch-induced global Ca^{2+} elevation but did not influence the local Ca^{2+} elevation or the suction-induced membrane currents. Left column shows cells prior to stretch (white arrow). Middle column shows cells after stretch (white arrow) (scale bar = 20 μm). Right column shows the suction-induced membrane currents (black bar indicate suction period). Stretch experiments were performed in more than 12 cells and suction experiments were performed in more than 7 cells. **(B):** Left histogram shows the quantification of the increased fluorescence ratio after stretching. The increased fluorescence ratios for the CTRL group, TRPM7-Scr, TRPM7-siRNA, IP_3R2-Scr, and IP_3R2-siRNA are 3.85 ± 0.90, 3.76 ± 0.65, 1.13 ± 0.12, 3.48 ± 1.02, and 0.81 ± 0.20, respectively. Right graph shows the time course of representative tracings of the stretch-induced response in five groups (CTRL = blank control, Scr = scrambled siRNA control *, $<.05$ and **, $<.01$). Abbreviations: IP_3R2, inositol trisphosphate receptor type 2; TRPM7, transient receptor potential melastatin type 7.

pressure-induced cytosolic Ca^{2+} increase (Fig. 2A). In order to identify the exact IP_3R subtype mediating Ca^{2+} release, we analyzed the expression of all three IP_3R subtypes and found that inositol trisphosphate receptor type 2 (IP_3R2) exhibited the highest expression level (Fig. 2B). Therefore, RNA interference was used to knockdown IP_3R2 (Fig. 2C). After pressure

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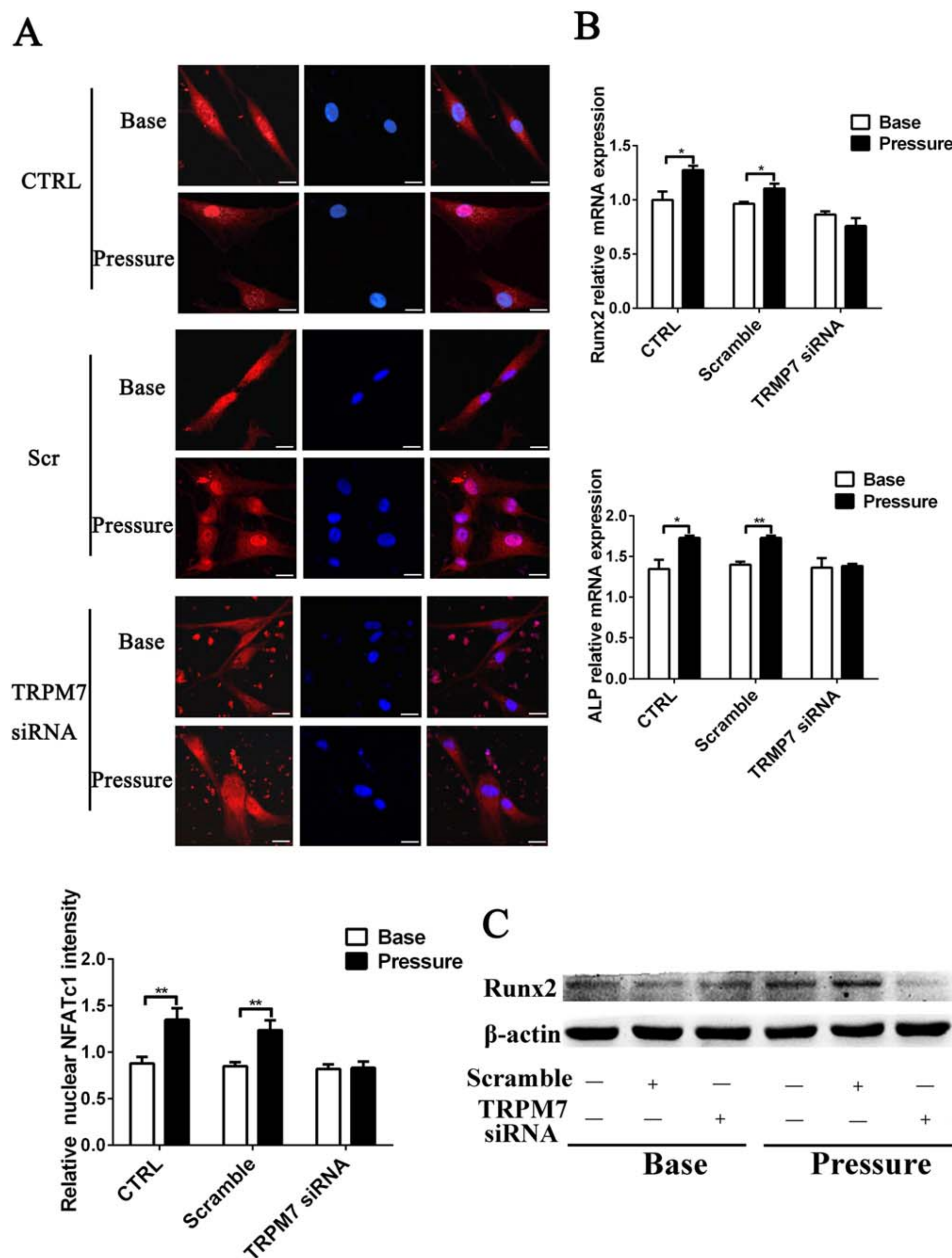

Figure 4. Pressure-induced NFATc1 nuclear localization and osteogenesis are dependent on TRPM7. **(A):** Immunofluorescence of NFATc1. The red staining is NFATc1, the blue staining is DAPI, and the third column shows the merged images. Pressure induced nuclear localization of NFATc1 in the control and scrambled group, but not in the TRPM7 knockdown group. The histogram is the quantification of the nuclear NFATc1 fluorescence intensity relative to that of the fluorescence intensity in the cytoplasm. **(B):** Real-time PCR of Runx2 and ALP mRNA expression. Pressure induced an upregulation in both genes, which were inhibited by TRPM7 knockdown. **(C):** Western blot shows an increase in Runx2 protein expression after pressure, which was prevented by TRPM7 knockdown. Abbreviations: ALP, Alkaline phosphatase; TRPM7, transient receptor potential melastatin type 7.

loading, the peak free Ca^{2+} concentration of cells with IP_3R2 knockdown remained at basal levels and the percentage of cells responding to pressure was significantly lower compared to the control and scrambled groups (Fig. 2D).

TRPM7 Activation Triggers IP_3R2-Mediated Ca^{2+} Release from the ER by Phospholipase C

The above data demonstrated that both TRPM7 and IP_3R2 were indispensable for the pressure-induced cytosolic Ca^{2+} increase. Furthermore, it was important to elucidate the relationship between the TRPM7-mediated Ca^{2+} influx and the IP_3R2-mediated Ca^{2+} release. Suction-induced single-channel currents were recorded by cell-attached patch clamp to indicate TRPM7 opening. Cytosolic Ca^{2+} signals were measured by confocal Ca^{2+} imaging technique with high space and time resolution. To avoid focus shifts in the process of confocal imaging, instead of pressure loading, a glass pipette was attached to the cell membrane and the pipette moved to induce membrane stretch. In the control and scrambled siRNA

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groups, membrane stretch and suction induced a rapid rise in global Ca^{2+} and a ~4 pA membrane current, respectively (Fig. 3A first, second, and fourth panel and Fig. 3B). In cells with IP_3R2 knockdown, the suction-induced membrane current had a similar amplitude as the control group, but membrane stretch only induced a slow and slight local Ca^{2+} rise (Fig. 3A fifth panel and Fig. 3B). In cells with TRPM7knockdown, stretch did not elicit a rise in Ca^{2+} and suction did not induce membrane currents (Fig. 3A third panel and Fig. 3B). These data indicated that the pressure-induced cytosolic Ca^{2+} increase was initiated by TRPM7-mediated Ca^{2+} influx and further amplified by IP_3R2-mediated Ca^{2+} release. TRPM7 has an alpha-kinase domain attached to its COOH-terminal tail [21]. The kinase interacts directly with multiple members of the phospholipase C (PLC) which can hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol trisphosphate (IP_3) activating the IP_3 receptor. Thus, we tried to block TRPM7 activation-induced IP_3R2-mediated Ca^{2+} release by the PLC specific inhibitor, U73122. When PLC was inhibited by U73122, stretch did not induce a rapid increase in global calcium concentration, but local calcium concentration was slightly increased. While a rapid global Ca^{2+} rise was induced in control group (Supporting Information Fig. S3). These results indicated that TRPM7-triggered IP3R2-mediated Ca^{2+} release was depended on PLC.

Pressure Induces TRPM7-Dependent Osteogenic Gene Expression

NFATc1 is activated by calcium signaling pathways and is an important transcription factor in osteoclast and osteoblast differentiation [22]. Activation of NFATc1 could promote osteoblast differentiation [23, 24]. When we applied pressure, NFATc1 nuclear localization was increased, consistent with another study [25]. In addition, the mRNA expression of bone-related genes Runx2 and Alkaline phosphatase (ALP) was upregulated and these changes were inhibited by TRPM7 knockdown (Fig. 4A, 4B). Since Runx2 is a key transcription factor in osteogenesis [26], the pressure-induced changes in Runx2 were also confirmed at the protein level (Fig. 4C).

These results demonstrated the central role of TRPM7 in conducting the mechanical stimulation-induced osteogenesis of MSCs. This proposal is indirectly supported by another study showing that cilia mediated fluid flow mechanostimulaiton in human MSCs, and increased osteogenic gene expressions [27]. Fluid flow was also reported to induce an increase in intracellular calcium in human MSCs [3]. Furthermore, TRP channels located in the cilia can transduce the mechanical stimulation to calcium signals [28].

Conclusions

Combining these reports with our results, we concluded that TRPM7 independently senses mechanical stimulation and mediates Ca^{2+} influx, which further triggers Ca^{2+} release from IP_3R2, increases NFATc1 nuclear localization and osteogenesis in human bone marrow MSCs.

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Author Contributions

E.X. and H.Q.Y: conception and design, collection and assembly of data, data analysis and interpretation and manuscript writing; Y.-H.G.: conception and design and manuscript writing; D.-H.D.: provision of study material; L.-H.H.: collection of data; Y.G.: collection and assembly of data; S.Q.W. and Y.Z.: conception and design, financial support, and final approval of manuscript. E.X. and H.Q.Y. contributed equally to this work.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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