

# Ca<sup>2+</sup>/Mg<sup>2+</sup> homeostasis-related TRPM7 channel mediates chondrocyte hypertrophy via regulation of the PI3K-Akt signaling pathway

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**Abstract.** Chondrocytes are specialized cells that form cartilage tissue, and are able to respond to their osmotic environment and exercise important roles in endochondral ossification via undergoing proliferation, hypertrophy and apoptosis. The transient receptor melastatin potential 7 (TRPM7) cation channel can modulate the intracellular and extracellular levels of Ca<sup>2+</sup> and Mg<sup>2+</sup>, and therefore the cellular osmotic environment. However, the molecular pathways involved in TRPM7-mediated signal transduction have yet to be elucidated. In the present study, the expression and functionality of TRPM7 were investigated during chondrocyte proliferation and hypertrophy. The ATDC5 mouse cell line was employed and cellular viability was evaluated using the MTT assay, whereas hypertrophy was monitored via evaluating the expression of chondrogenic marker genes and the activity of alkaline phosphatase (ALP). Gene expression of TRPM7 appeared slightly upregulated during the proliferative stages of chondrocyte development, and significantly upregulated during the hypertrophic stages, suggesting the importance of Ca<sup>2+</sup>/Mg<sup>2+</sup> homeostasis for chondrocyte growth. Low extracellular Ca<sup>2+</sup>/Mg<sup>2+</sup> levels significantly reduced the expression of type X collagen, Indian hedgehog homolog (Ihh) and matrix metalloproteinase (MMP)-13 genes, as well as ALP activity; however, cell viability remained unaffected. Conversely, the

gene expression levels of TRPM7 appeared upregulated in ATDC5 cells under low extracellular Ca<sup>2+</sup> or Mg<sup>2+</sup> conditions. Silencing TRPM7 expression during the chondrocyte differentiation period also reduced type X collagen, Ihh and MMP-13 gene expression, and ALP activity. Furthermore, the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt signaling pathway was activated following TRPM7 overexpression, and inhibited following TRPM7 silencing. Notably, the actions of TRPM7 on chondrocyte hypertrophy were abolished through the inhibition of PI3K-Akt signaling. The present results suggested that TRPM7 may be involved in Ca<sup>2+</sup>/Mg<sup>2+</sup> homeostasis during chondrocyte hypertrophy, and contribute to endochondral ossification via interacting with the PI3K-Akt signaling pathway.

## Introduction

Endochondral ossification is a complex cellular process that is involved in bone formation and fracture healing, particularly in the growth of long bones (1-3). During endochondral ossification, chondrocytes serve a crucial role, and have been reported to participate throughout the processes of ossification by undergoing proliferation, hypertrophy and apoptosis (4). When endochondral ossification is initiated, mesenchymal cells condense and differentiate into chondrocytes, forming an initial cartilage callus. Chondrocytes are able to proliferate rapidly and secrete type II collagen, aggrecan and other cartilage-specific extracellular matrix proteins. Highly proliferative chondrocytes then stop dividing and markedly increase their volume, thus becoming hypertrophic (5). Before they become apoptotic, hypertrophic chondrocytes can secrete type X collagen and matrix metalloproteinase (MMP)-13, to alter the matrix composition and promote its mineralization by calcium carbonate, resulting in the formation of endochondral ossifications (6-8).

The volume increase of chondrocytes during hypertrophy is regulated by several factors, such as fluctuations in the osmotic environment and other biophysical parameters (9). Alterations in extracellular Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations can alter the osmotic environment and cause chondrocytes to respond to osmotic stress via increasing their volume. The

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osmotic stimulation of chondrocytes can trigger cytoplasmic  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  signals, which may be involved in gene expression, volume regulation, cellular metabolism and other cellular processes (10). The response of chondrocytes to the extracellular environment has been extensively investigated (3,4,6); however, the molecular mechanisms through which the cells respond to the environmental alterations have yet to be elucidated.

The transient receptor potential melastatin (TRPM) channels form a subfamily of the TRP family of cation channels, which are expressed in mammalian cells and serve key roles in transcellular  $\text{Ca}^{2+}$  transport (11). TRPM7, which is one of the eight TRPM members, consists of a cation channel and a protein kinase. Similar to TRPM2 and TRPM6, TRPM7 possesses a catalytic domain in the long C-terminus, which can spontaneously activate the entry of divalent cations, including  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (12,13). In addition, the TRPM7 channel has been associated with cellular proliferation, migration and survival, and may be implicated in the regulation of bone formation (12). However, the molecular mechanisms underlying the implication of the TRPM7 channel in chondrocyte response to the extracellular environment and hypertrophy have yet to be elucidated.

The present study aimed to investigate the expression and function of TRPM7 in hypertrophic chondrocytes. The putative role of TRPM7 in the modulation of extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentration was investigated during chondrocyte hypertrophy. Its actions in chondrocyte hypertrophy and subsequent ossification were also explored. Finally, the regulatory effects of TRPM7 activation on phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt signaling were evaluated during chondrocyte growth and hypertrophy.

## Materials and methods

**Cell culture.** Mouse chondrogenic ATDC5 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DME/F12; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) hybrid medium, supplemented with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1X penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5%  $\text{CO}_2$  atmosphere. To induce chondrogenesis, ATDC5 cells were cultured in the aforementioned medium supplemented with 1X Insulin-Transferrin-Selenium (ITS; Gibco; Thermo Fisher Scientific, Inc.). To study the role of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in chondrocyte hypertrophy, cells were incubated for 14 days in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free DME/F12 (Merck KGaA) medium, containing basal 1.4 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  (Control), 0.4 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  (low  $\text{Ca}^{2+}$ ), or 1.4 mM  $\text{Ca}^{2+}$  and 0.1 mM  $\text{Mg}^{2+}$  (low  $\text{Mg}^{2+}$ ).

**Cell transfection.** Small interfering (si)RNA targeting TRPM7 mRNA (TACGTCCAAGGTCGGGCAGGAAGA) and a TRPM7 plasmid (accession: NM\_017672, [https://www.ncbi.nlm.nih.gov/nucleotide/NM\\_017672](https://www.ncbi.nlm.nih.gov/nucleotide/NM_017672)) were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). Cells grown to ~80% confluence were transfected twice a

week with TRPM7 siRNA, non-targeting negative control siRNA (TACGTCCAAGGTCGGGCAGGAAGA) (Takara Biotechnology Co., Ltd.), or the TRPM7 plasmid in addition to the control vector (Takara Biotechnology Co., Ltd.) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the delivery agent, to maintain silencing of TRPM7 expression for 14 days. Transfection complexes were prepared according to the manufacturer's protocol.

**Cell treatment.** ATDC5 cells were transfected with TRPM7-plasmid, followed by treatment with or without the Akt inhibitor MK2206 (200 nM, KinaseChem Co., Ltd., Pontypriid, UK) or the PI3K inhibitor LY294002 (200 nM, AbMole Bioscience, Shanghai, China) for 14 days. Then the expression of p-Akt, total Akt protein, type X collagen, Ihh and MMP-13 protein respectively were evaluated.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA samples were reverse transcribed using PrimeScript™ RT Master Mix (Perfect Real Time; Takara Biotechnology Co., Ltd.). The mRNA expression levels of type X collagen, Indian hedgehog homolog (Ihh) and MMP-13 were assessed using the CellAmp™ RT-PCR for Direct Prep kit (Real Time) & Protein Analysis (Takara Biotechnology Co., Ltd.) by SYBR® Green I probe. The primers used were as follows: Type X collagen (sense 5'-TTCATGGGATGTTTTATGCTGAACG-3', antisense 5'-TTTAGGTCCTTGGGGTCCCATTATTC-3'), Indian hedgehog homolog (Ihh) (sense 5'-GCTCTGGCTGCGATTCTCACACG-3', antisense 5'-CAGAGACTCCGCCCATGACAGCA-3'), MMP-13 (sense 5'-AGAAGTCTACAGTGACCTCCACAGTT-3', antisense 5'-GACTCTCACAATGCGATTACTCC-3'), GAPDH (sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCCTGTTGCTGTA-3'). qPCR was performed under the following conditions: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 1 min, at 63°C for 2 min, and at 72°C for 1 min, and then a final annealing step at 72°C for 10 min in a reaction volume of 20  $\mu\text{l}$  with 1  $\mu\text{l}$  RT Products, 10  $\mu\text{l}$  2X Master Mix (Takara Biotechnology Co., Ltd.), 1  $\mu\text{l}$  sense primer, 1  $\mu\text{l}$  antisense primer and 7  $\mu\text{l}$  ddH<sub>2</sub>O. All PCR analyses were performed in triplicate. The relative expression levels of each gene were normalized to GAPDH and were calculated using the  $2^{-\Delta\Delta C_t}$  method (14).

**Western blot analysis.** Cell lysates were prepared using 200  $\mu\text{l}$  lysis buffer containing 20 mmol/l HEPES, 25 mmol/l  $\text{MgCl}_2$ , 5 mmol/l KCl, 0.5% (v/v) complete protease inhibitor and Triton X-100. Debris was removed by centrifugation at 12,000  $\times$  g for 10 min at 4°C. Protein concentrations were determined using the bicinchoninic acid method, with bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) as the standard. Equal amounts of protein (80  $\mu\text{g}$ ) were separated by 8% SDS-PAGE (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 h in blocking buffer at room temperature and incubated overnight at 4°C with the following primary antibodies: Rabbit anti-collagen II (ab34712; 1:2,000), anti-aggrexin (ab36861; 1:1,000), anti-collagen X (ab195220; 1:1,000), anti-MMP-13

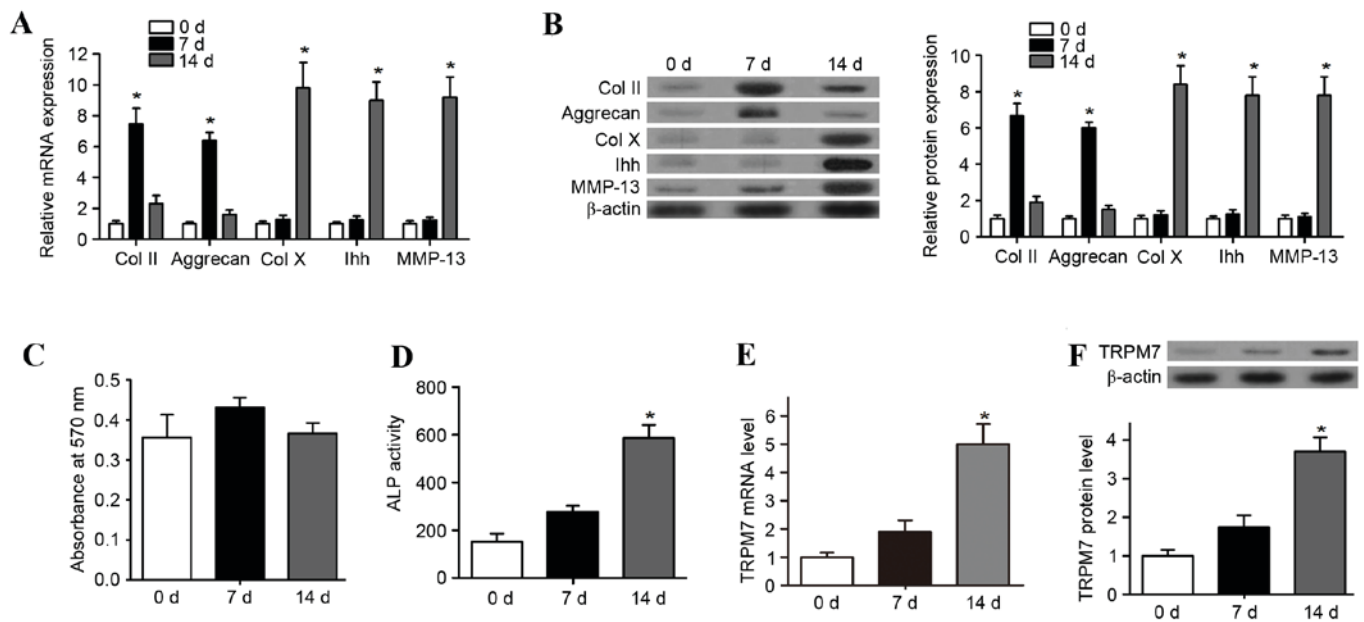


Figure 1. ATDC5 cell proliferation and hypertrophy. Cells were grown to confluence (day 0) or were further incubated for 7 or 14 days in differentiation medium. (A) mRNA and (B) protein expression levels of chondrogenic markers were determined using RT-qPCR and western blot analysis. (C) Cellular proliferation was assessed using the MTT assay. (D) ALP activity was measured and presented as  $\text{nmol pnp} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$ . (E) mRNA and (F) protein expression levels of TRPM7 were determined using RT-qPCR and western blot analysis ( $n=5/\text{group}$ ). Data are expressed as the mean  $\pm$  standard error of the mean. \* $P<0.05$  vs. day 0. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ALP, alkaline phosphatase; TRPM, transient receptor potential melastatin; Col II, type II collagen; Col X, type X collagen; Ihh, Indian hedgehog homolog; MMP, matrix metalloproteinase.

(ab75606; 1:2,000), anti-phosphorylated (p)-Akt (ab8932; 1:500), anti-total Akt (ab185633; 1:500), mouse anti-p85 (ab86714; 1:1,000; all from Abcam, Cambridge, UK), rabbit anti-Ihh (184624; 1:1,000), anti-TRPM7 (217938; 1:2,000; Merck KGaA) and mouse anti- $\beta$ -actin (8226; 1:3,000; ABClonal Biotech Co., Ltd., Cambridge, MA, USA). Secondary antibodies conjugated with horseradish peroxidase (Rabbit Anti-Mouse IgG; ab6728; 1:2,000; Goat Anti-Rabbit IgG; ab150077; 1:2,000; Abcam) were incubated with the blots for 1 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK). The blots were semi-quantified using the ImageQuant<sup>TM</sup> software version 7.0 (GE Healthcare Life Sciences).

**Alkaline phosphatase (ALP) activity.** ALP activity was assessed as previously described (15). Briefly, cell lysates were incubated with diethanolamine buffer (pH 10.0) containing 10 mM para-nitrophenyl phosphate (PNPP), 0.9 M diethanolamine and 2 mM  $\text{MgCl}_2$ , at  $37^\circ\text{C}$  for 30 min. The reaction was terminated with 1 M NaOH. The kinetics of PNPP hydrolysis were measured at 415 nm.

**MTT assay.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate ATDC5 cell viability as previously described (16). Briefly, cells were washed with PBS and then incubated with MTT (0.5 mg/ml) in PBS containing glucose (5.5 mM) and  $\text{CaCl}_2$  (11 mM), for 1 h at  $37^\circ\text{C}$ . After dissolving the formazan crystals with DMSO, absorbance was measured at 570 nm.

**Statistical analysis.** The statistical significance of the difference between groups was assessed by Student's t-test for

pair-wise comparisons or one-way analysis of variance. A Bonferroni correction was used to adjust for multiple testing. Data are expressed as the mean  $\pm$  standard error of the mean. All experiments were performed independently at least three times.  $P<0.05$  was considered to indicate a statistically significant difference. The analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

**TRPM7 is upregulated during chondrocyte hypertrophy.** Hypertrophic chondrocytes serve a key role in the process of endochondral ossification during bone formation and fracture healing. To determine the role of TRPM7 channels in chondrocyte proliferation and hypertrophy, the expression of TRPM7 in the ATDC5 murine chondrogenic cell line was investigated in response to treatment with ITS. The effects of ITS treatment on ATDC5 cellular viability were evaluated using the MTT assay and by assessing the mRNA expression levels of type II collagen and aggrecan. Chondrocyte hypertrophy was investigated by assessing the mRNA expression levels of the chondrogenic markers type X collagen, Ihh and MMP-13 at the indicated time points (17). ITS differentiation treatment significantly increased the levels of type II collagen and aggrecan within the first 7 days of culture (Fig. 1A and B). Following 14 days of treatment, the expression levels of type X collagen, Ihh and MMP-13 were significantly increased; conversely, the levels of type II collagen and aggrecan, as well as cellular viability, had almost returned to baseline (Fig. 1C). In agreement with the aforementioned results, ITS treatment increased ALP activity in chondrocytes by day 14 of treatment, indicating that chondrocytes were in the hypertrophy phase

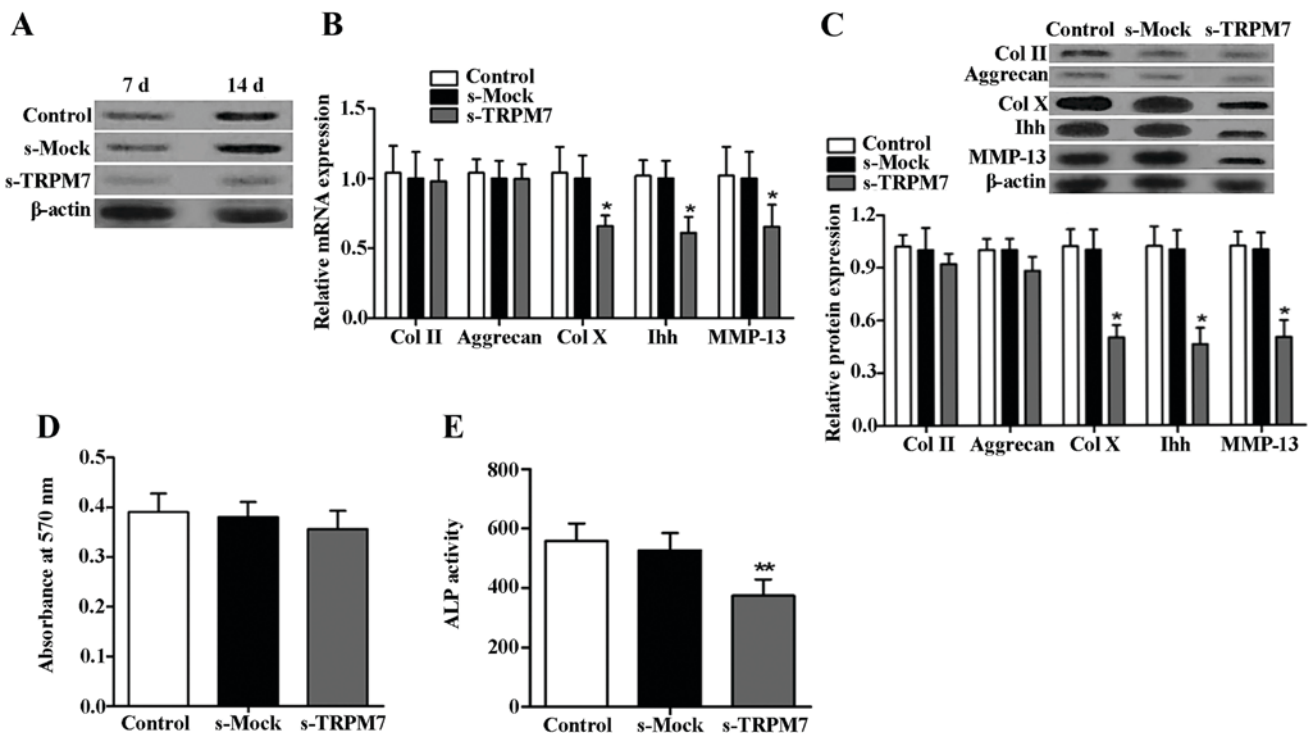


Figure 2. Effects of TRPM7 silencing on ATDC5 cell proliferation and hypertrophy. Cells were cultured in basal medium (Control) or in differentiation media and transfected s-TRPM7 or s-Mock twice a week for 14 days. (A) TRPM7 protein expression levels were assessed on days 7 and 14 using western blot analysis. (B) mRNA and (C) protein expression levels of chondrogenic markers following 14 days of culture were determined using reverse transcription-quantitative polymerase chain reaction and western blot analysis. (D) Cellular proliferation was assessed using the MTT assay. (E) ALP activity was measured and presented as nmol pnp.mg<sup>-1</sup>.h<sup>-1</sup> (n=5/group). Data are expressed as the mean  $\pm$  standard error of the mean. \*P<0.05, \*\*P<0.01 vs. s-Mock group. TRPM, transient receptor potential melastatin; s, small interfering RNA; ALP, alkaline phosphatase; Col II, type II collagen; Col X, type X collagen; Ihh, Indian hedgehog homolog; MMP, matrix metalloproteinase.

(Fig. 1D). Furthermore, the gene and protein expression levels of TRPM7 were evaluated during chondrocyte hypertrophy. TRPM7 expression appeared slightly upregulated in chondrocytes on day 7, whereas it was significantly upregulated following 14 days of differentiation treatment (Fig. 1E and F).

**TRPM7 silencing inhibits chondrocyte hypertrophy.** siRNA targeting TRPM7 (s-TRPM7) was employed to investigate whether TRPM7 is involved in chondrocyte proliferation and hypertrophy. ATDC5 cells were transfected with s-TRPM7 or non-targeting siRNA (s-Mock) twice a week to silence TRPM7 expression (Fig. 2A). Compared with the control and s-Mock groups, mRNA and protein expression levels of type X collagen, Ihh and MMP-13 were significantly reduced in ATDC5 cells transfected with s-TRPM7, whereas the levels of type II collagen and aggrecan (Fig. 2B and C), as well as cellular viability (Fig. 2D), remained unaffected. In addition, silencing TRPM7 gene expression significantly reduced ALP activity in ATDC5 cells following 14 days of culture (Fig. 2E). These results suggested that TRPM7 expression is essential for the development of chondrocyte hypertrophy, although TRPM7 may not be a key regulator of chondrocyte proliferation.

**Effects of low Ca<sup>2+</sup> and Mg<sup>2+</sup> levels on TRPM7 expression and chondrocyte hypertrophy.** Since the TRPM7 channel is Ca<sup>2+</sup>- and Mg<sup>2+</sup>- permeable, the effects of low extracellular Ca<sup>2+</sup> and Mg<sup>2+</sup> levels were assessed on chondrocyte proliferation and

hypertrophy. A significant reduction in type X collagen, Ihh and MMP-13 mRNA and protein levels was observed under conditions of low extracellular Ca<sup>2+</sup> or Mg<sup>2+</sup> (Fig. 3A and B). ALP activity was also reduced under low extracellular Ca<sup>2+</sup> or Mg<sup>2+</sup> culture conditions, whereas cell viability remained unaffected, thus suggesting that extracellular Ca<sup>2+</sup> or Mg<sup>2+</sup> depletion did not abolish ATDC5 cell viability, but inhibited their hypertrophy to a certain extent (Fig. 3C and D). Conversely, TRPM7 mRNA and protein levels in ATDC5 cells were upregulated under low extracellular Ca<sup>2+</sup> or Mg<sup>2+</sup> culture conditions, thus suggesting a role for the channels in cellular responses to the altered extracellular environment (Fig. 3E and F).

**PI3K-Akt signaling is involved in TRPM7 regulation during chondrocyte hypertrophy.** PI3K-Akt signaling has been reported to be involved in chondrocyte growth and differentiation, and TRP channels have been demonstrated to regulate PI3K-Akt signaling in epithelial cells (11). To investigate the mechanism underlying the modulatory role of TRPM7 during chondrocyte hypertrophy, protein expression levels of the PI3K p85 subunit and Akt were investigated using western blot analysis. Overexpression of TRPM7 (Fig. 4A) upregulated the levels of p85 and total Akt protein in ATDC5 cells. In addition, levels of p-Akt increased in parallel with total Akt in proliferating chondrocytes and were maintained in hypertrophic chondrocytes (Fig. 4B and C). Conversely, silencing the expression of TRPM7 downregulated the expression of p85, total Akt and p-Akt in ATDC5 cells (Fig. 4D).

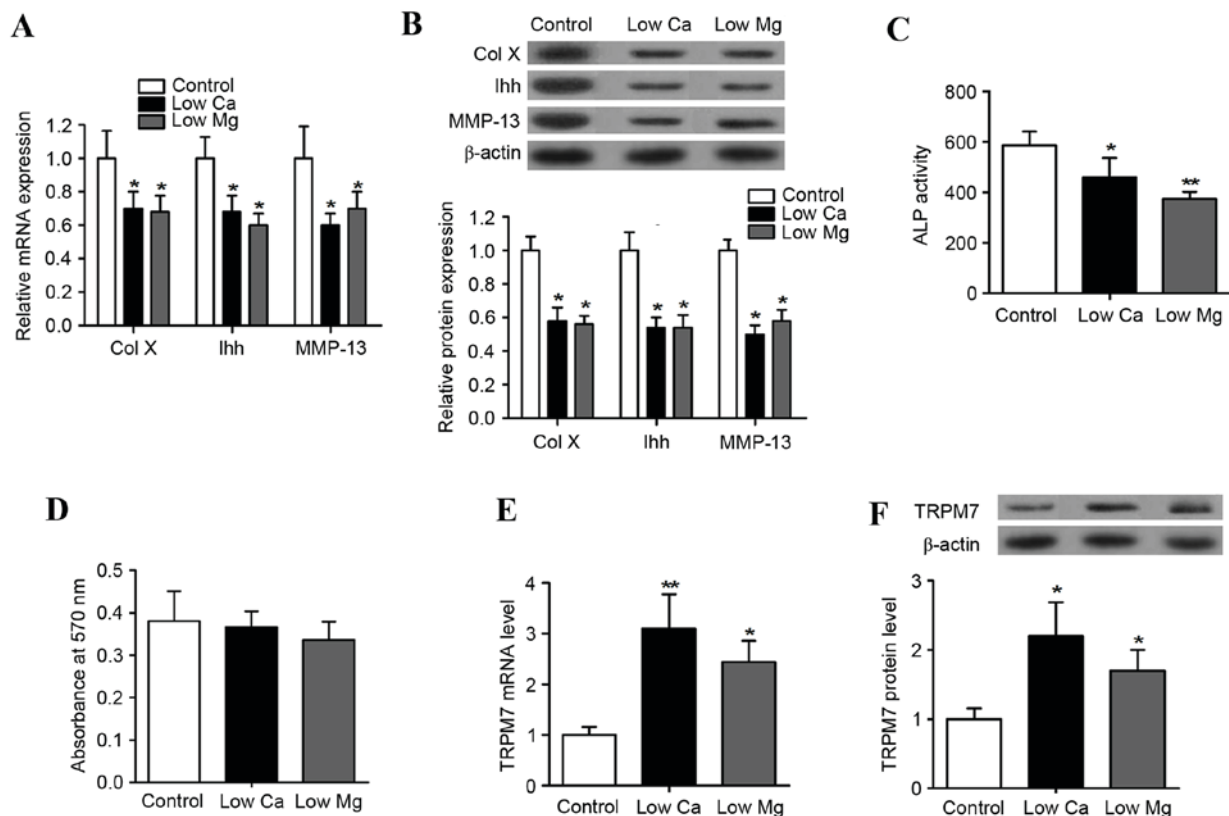


Figure 3. Effects of reduced extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  levels on ATDC5 cell viability and hypertrophy. After confluence was achieved, cells were cultured for 14 days with medium containing 1.4 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  (Control), 0.4 mM  $\text{Ca}^{2+}$  and 0.8 mM  $\text{Mg}^{2+}$  (low  $\text{Ca}^{2+}$ ), or 1.4 mM  $\text{Ca}^{2+}$  and 0.1 mM  $\text{Mg}^{2+}$  (low  $\text{Mg}^{2+}$ ). (A) mRNA and (B) protein expression levels of chondrogenic markers were determined by RT-qPCR and western blot analysis. (C) ALP activity was measured and presented as nmol pnp.mg<sup>-1</sup>.h<sup>-1</sup>. (D) Cellular viability was assessed using the MTT assay. (E) mRNA and (F) protein expression levels of TRPM7 were determined using RT-qPCR and western blot analysis (n=5/group). Data are expressed as the mean  $\pm$  standard error of the mean. \*P<0.05, \*\*P<0.01 vs. Control group. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TRPM, transient receptor potential melastatin; Col II, type II collagen; Col X, type X collagen; Ihh, Indian hedgehog homolog; MMP, matrix metalloproteinase.

To investigate the role of PI3K-Akt signaling in chondrocyte proliferation and hypertrophy, the Akt inhibitor MK2206 and the PI3K inhibitor LY294002 were administered to cultured ATDC5 cells. Administration of the inhibitors abolished Akt phosphorylation (Fig. 5A). The TRPM7-induced enhancement of type X collagen, Ihh and MMP-13 expression in ATDC5 cells was inhibited following treatment with MK2206 or LY294002 (Fig. 5B), thus suggesting that PI3K-Akt signaling may be involved in TRPM7-mediated regulation of chondrocyte hypertrophy.

## Discussion

Chondrocyte proliferation, hypertrophy, secretion of extracellular matrix proteins, apoptosis and mineralization are involved in endochondral ossification (18). Since the volume increase of chondrocytes, which is involved in their proliferation and hypertrophy, has been reported to be influenced by alterations in the osmotic environment and  $\text{Ca}^{2+}$  influx (10), the roles of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and the TRPM7 cation channel were investigated during chondrocyte hypertrophy. ATDC5 cells are a well-known *in vitro* model of chondrocyte differentiation. The results of the present study demonstrated that the expression of type X collagen was increased in ATDC5 cells undergoing differentiation. Ihh signaling from prehypertrophic

chondrocytes is implicated in the control of chondrocyte maturation. St-Jacques *et al* demonstrated that Ihh signaling regulated the proliferation and differentiation of chondrocytes and was essential for bone formation (19). In the present study, Ihh gene expression in ATDC5 cells was also increased during differentiation. In addition, the expression of MMP-13 and the activity of ALP were upregulated in ATDC5 cells, confirming the efficiency of the treatment used to promote cellular proliferation and hypertrophy. Notably, TRPM7 gene expression was increased during chondrocyte differentiation, suggesting a critical role for the channel in chondrocyte maturation.

The regulation of chondrocyte growth has been associated with the osmotic environment of cells. Alterations in osmotic pressure can initiate intracellular signaling cascades leading to alterations in chondrocyte volume (10). Previous studies have revealed that the  $\text{Ca}^{2+}$ -permeable transient receptor potential vanilloid type 4 channel was present in chondrocytes, and mediated their responses to hypo-osmotic stress via regulating extracellular and intracellular  $\text{Ca}^{2+}$  levels (9,10). Since the TRPM7 channel is also  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -permeable, its effects on  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$  homeostasis were investigated during chondrocyte proliferation and maturation. The present results demonstrated that varying cation concentrations during chondrocyte differentiation were associated with the growth status. Low extracellular  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  levels significantly reduced

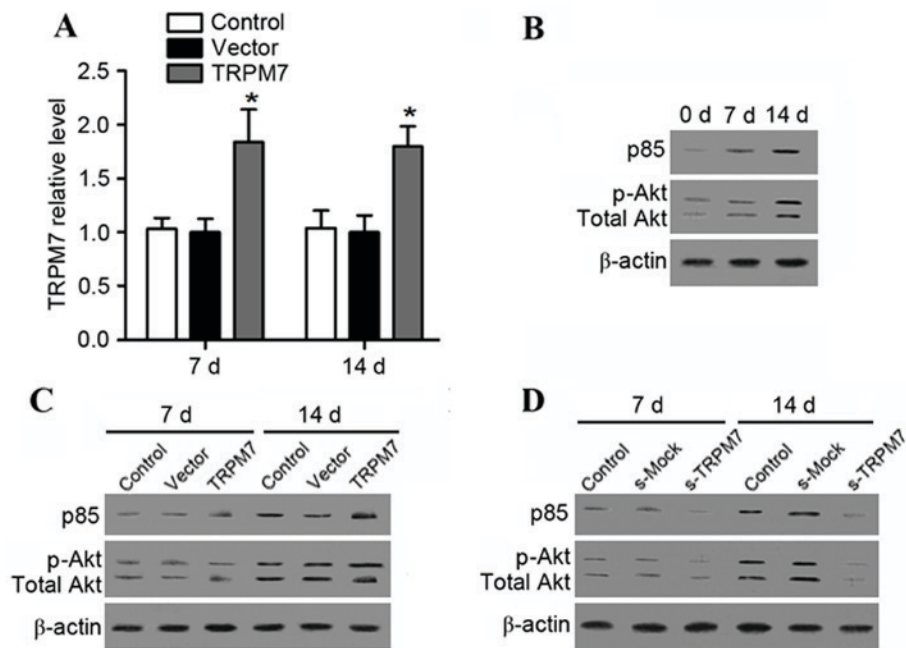


Figure 4. TRPM7 activated PI3K-Akt cascades to regulate chondrocyte hypertrophy. ATDC5 cells were cultured in basal medium (Control) or in differentiation media and were transfected with TRPM7-plasmid or control vector, or with s-TRPM7 or s-Mock twice a week for 14 days. (A) TRPM7 plasmid increased TRPM7 expression on days 7 and 14 as revealed by reverse transcription-quantitative polymerase chain reaction ( $n=5$ /group). Data are expressed as the mean  $\pm$  standard error of the mean. \* $P<0.05$  vs. Vector group. (B) Western blot analysis was used to determine the levels of PI3K and Akt in cells grown to confluence (day 0) or further incubated for 7 or 14 days in differentiation medium ( $n=5$ /group). (C) Western blot analysis was used to determine the levels of PI3K and Akt in cells transfected with TRPM7-plasmid or control vector on days 7 and 14 ( $n=5$ /group). (D) PI3K and Akt protein expression levels were measured in cells transfected with s-TRPM7 siRNA or s-Mock on days 7 and 14 ( $n=5$ /group). TRPM, transient receptor potential melastatin; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; s, small interfering RNA; p-, phosphorylated.

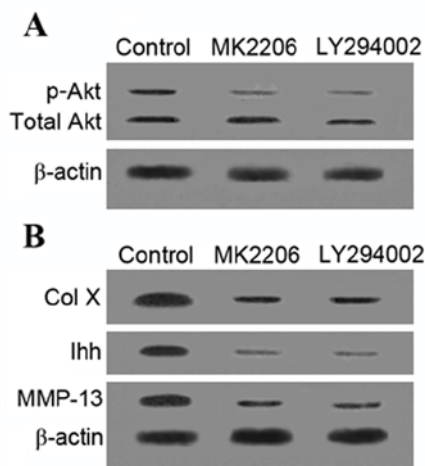


Figure 5. Akt signaling was inhibited by MK2206 and LY294002. (A) ATDC5 cells were transfected with TRPM7-plasmid, followed by treatment with or without (Control) the Akt inhibitor MK2206 (200 nM) or the PI3K inhibitor LY294002 (200 nM) for 14 days. Western blot analysis was used to assess p-Akt and total Akt protein expression levels. (B) Cells were transfected with TRPM7-plasmid, followed by treatment with or without (Control) MK2206 or LY294002 (200 nM) for 14 days. Western blot analysis was used to assess type X collagen, Ihh and MMP-13 protein expression levels. TRPM, transient receptor potential melastatin; p-, phosphorylated; Col X, type X collagen; Ihh, Indian hedgehog homolog; MMP, matrix metalloproteinase.

the production of type X collagen and the gene expression of Ihh and MMP-13. Similar effects were produced on ALP activity. Conversely, TRPM7 gene expression in ATDC5 cells was upregulated under low extracellular  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  culture

conditions, thus suggesting that the TRPM7 channel may contribute to the response of chondrocytes to altered extracellular conditions. Notably, low extracellular  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  levels appeared to have no effect on ATDC5 cell viability, suggesting that the differentiation and hypertrophy of ATDC5 cells was not abolished but rather reduced to a certain extent under low  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  conditions.

Silencing the expression of TRPM7 reduced the expression of type X collagen, Ihh and MMP-13 in ATDC5 cells within 14 days of culture. Similarly, ALP activity was also reduced. Conversely, levels of type II collagen and aggrecan remained unaffected by TRPM7 silencing during ATDC5 cell proliferation, suggesting that TRPM7 expression, although essential for chondrocyte hypertrophy, may not be critical for chondrocyte proliferation. In addition, osteoblast cells arise from osteoprogenitor cells located in the bone marrow, and their proliferation and differentiation has been implicated in bone formation and remodeling (20-22). It has been reported that intracellular  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  homeostasis, maintained through TRPM7 expression, was important for osteoblast proliferation, migration and differentiation (12). Therefore, it may be hypothesized that the TRPM7 channel is implicated in chondrocyte maturation, as well as osteoblast differentiation, and thus contributes to bone formation, particularly in long bones. Further studies are required to investigate this hypothesis.

The PI3K-Akt cascade is a key signaling pathway with pleiotropic functions, which has been implicated in critical differentiation pathways in various cell types, including chondrocytes, osteoblasts and myoblasts. Furthermore, mice lacking Akt1 and Akt2 demonstrated severely delayed

bone development (23). Therefore, it was hypothesized that PI3K-Akt signaling may be implicated in TRPM7 channel regulation during chondrocyte maturation and hypertrophy. The results of the present study demonstrated that the levels of the p85 regulatory PI3K subunit and Akt were increased during ATDC5 cell hypertrophy. Furthermore, the levels of p85, total Akt and p-Akt were upregulated following TRPM7 overexpression in ATDC5 cells, and downregulated following TRPM7 silencing. The TRPM7-induced enhancement of type X collagen, Ihh and MMP-13 expression was abolished following Akt and PI3K inhibition, thus suggesting that the TRPM7 channel may be implicated in chondrocyte hypertrophy during endochondral ossification via interacting with the PI3K-Akt signaling pathway.

In conclusion, the present results revealed that low extracellular levels of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , as well as TRPM7 silencing, downregulated the expression of type X collagen, Ihh and MMP-13, and delayed chondrocyte hypertrophy, thus suggesting that they may interfere with endochondral ossification. Through an interaction with PI3K-Akt signaling pathways, the TRPM7 channel may serve a crucial role in the regulation of chondrocyte proliferation and hypertrophy.

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