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Nutritional supplementation with myo-inositol in growing mice specifically augments mandibular endochondral growth

Yuki Yamaguchi, Hiroyuki Kanzaki*, Yutaka Miyamoto, Kanako Itohiya, Sari Fukaya, Yuuta Katsumata, Yoshiki Nakamura

Department of Orthodontics, Tsurumi University School of Dental Medicine, Yokohama, Japan

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ABSTRACT

Introduction: The purpose of this study was to examine growth-promoting effects of myo-inositol nutritional supplementation on the mandible in experimental animals.

Methods: Mice were fed on diets that contained various concentration of myo-inositol for 3 to 12 weeks. The length of the mandible, maxilla, and femur were measured on μCT images. The mandible and tibia were examined histologically and immunohistochemically. The effects of myo-inositol on cell proliferation and chondrocytic differentiation were examined using ATDC5 cells.

Results: Myo-inositol supplementation had no effects on body weight, length, and maxilla and femur lengths. However, the length of mandible and the thickness of the mandibular condylar cartilage (MCC) were increased by myo-inositol supplement. Microarray analysis revealed that Pik3cd was highly expressed in MCC as compared to that in the cartilage of the tibial growth plate, which was confirmed by real-time RT-PCR and immunohistochemistry. ATDC5 cells also highly expressed Pik3CD. Myoinositol induced increases in cell proliferation and chondrocytic differentiation in ATDC5 cells. The addition of a PIK3CD inhibitor blocked the induction of cell proliferation by myo-inositol in ATDC5 cells.

Conclusions: Nutritional supplementation with myo-inositol in growing mice augmented mandibular endochondral growth without any systemic effects. The specific promotion of mandibular growth by myoinositol is primarily dependent on the specific intensive expression of PIK3CD in the MCC.

1. Introduction

Mandibular retrognathism is associated with insufficient growth of the mandible [1], which results in a small and retruded mandible [2]. Mandibular retrognathism sometimes causes respiratory difficulty due to narrowed airways [3], temporomandibular joint disorders due to the increased burden on the temporomandibular joint [4], declined chewing function [5], and maxillofacial deformity with aesthetic problems [6].

In the treatment of mandibular retrognathism, surgical mandibular advancement is usually performed in adult patients [7]. In growing patients, the promotion of mandibular growth is performed by functional appliances [8]. However, the effects of functional appliance are not always effective in daily practices [9]. Therefore, a more effective, reliable, and safer therapeutic method for mandibular growth promotion is greatly needed.

Growth of the mandible is mainly dependent on endochondral ossification [10]. Endochondral ossification is influenced by several factors, such as genetics [11], hormones [12] and nutrition [13]. Various growth factors such as growth hormone (GH) [14], fibroblast growth factor (FGF) [15], bone morphogenetic protein (BMP) [16] and insulin-like growth factor-1 (IGF-1) [14] [15] [16] are involved in endochondral ossification. In particular, IGF-1 has been reported to be involved in growth through regulation of endochondral ossification [15], regulating growth in limb bones and in the mandible [16] [17] [18]. Expression of both IGF-1 and IGF-1R genes were increased in the mandibles of the non-deviated side in a mouse model with functional lateral deviation of the mandible [19]. Furthermore, it was reported that local administration of IGF-1 to the unilateral mandibular fossa induced an increase in the thickness of unilateral mandibular cartilage through unilateral endochondral ossification [20].

As to the relationship between nutrition and growth, supplementing diets with myo-inositol has been reported to promote weight gain in experimental animals [21]. Mice lacking myo-inositol synthase and inositol monophosphatase had extreme mandibular retrognathism [22], while supplementing myo-inositol to maternal mice rescued

* Corresponding author at: Department of Orthodontics, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan.
E-mail address: kanzaki-h@tsurumi-u.ac.jp (H. Kanzaki).

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mandibular retrognathism in newborn mice [22]. These findings suggest that myo-inositol supplementation may promote endochondral growth in the mandible.

In this study, we hypothesized that dietary supplementation with myo-inositol promotes mandibular growth in growing mice via augmentation of endochondral growth in mandibular condylar cartilage. We examined the promotional effects of myo-inositol on mandibular growth in animal experiments and further investigated the promotional mechanism in cell culture experiments.

2. Materials and methods

2.1. Chemicals

Myo-inositol was purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Laboratory MR stock powder (myo-inositol content: 9.7 mg/kg) was purchased from Sankyo Labo Service Corporation, Inc., (Tokyo, Japan).

2.2. Experimental animals

The protocol for animal experiments was reviewed and approved by the Institutional Animal Care and Use Committee of Tsurumi University school of dental medicine (No. 28A029), and animal experiments were performed in compliance with the Regulation for Animal Experiments and Related Activities at Tsurumi University school of dental medicine. The animals used in our experiments were three-week-old male BALB/c mice (CLEA Japan, Inc., Tokyo, Japan).

2.3. Experimental procedure

Thirty-one mice were divided into the following three groups: control (n = 10), low concentration (n = 11), and high concentration group (n = 10). Mice in the control group were fed a normal powder diet (myo-inositol content: 9.7 mg/kg), the low concentration group was fed a powder diet + myo-inositol (6.6 g/kg), and the high concentration group was fed a powder diet + myo-inositol (66 g/kg). The average food intake of mice was around 3 g/day/mice. All mice were started to feed the above diet at weaning (3 weeks-old) and fed until 12 weeks old and weighed once a week.

2.4. Micro-computed tomography analysis

Mice were anesthetized by intraperitoneal injection with a mixture containing medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol-tartrate (5 mg/kg) when 12 weeks old, and subsequently scanned with a microCT (microCT) system (inspeXio SMX-225 CT; Simadzu Corp., Kyoto, Japan). The tube voltage was set at 170 kV with a constant 70 mA current. After reconstitution, the DICOM data were rendered into three-dimensional images using OsiriX 64bit (Newton Graphics, Sapporo, Japan) and analyzed with multi-planar reconstruction (MPR) three screens setting (XY-plane, YZ-plane, and ZX-plane). The following reference points for measurements on the mandibular bone, maxilla, and femur were set: the condylar maximal projecting point of the mandibular angle (Cd), the mesial alveolar bone apex of the mandibular first molar (Al) (Fig. 1A and D), the distal alveolar bone apex of the maxillary third molar (Mu 3), the palatal alveolar bone apex of the maxillary incisor (Iu) (Fig. 1B and D), trochanteric fossa (Tf), and patellar surface of femur (Ps) (Fig. 1C). Cd to Al, Mu3 to Iu, Tf to Ps, and ((Cd - Al) - (Cd - Iu)) were measured as the length of the mandible, maxilla, femur, and the maxillo-mandibular differential [23] (Fig. 1). The smaller maxillo-mandibular differential indicates the smaller mandible relative to the maxilla.

2.5. Measurement of body length

A standardized photograph of the whole body was taken at 12 weeks old. The height of the control group and the high concentration group were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). A straight line was drawn between the ears and the distance from the midpoint to the tail was measured as the body length.

2.6. Tissue preparation and histological analysis

Mice were euthanized by cervical dislocation at 12 weeks old, and the mandible and tibia were excised and fixed with 4% paraformaldehyde in PBS overnight. The specimen was decalcified with 10% ethylenediamine tetraacetic acid in PBS for two weeks at 4 °C and embedded in paraffin. Serial sections (7 μm thick) were then prepared. After deparaffinization, sections were stained with hematoxylin and eosin dyes, and the thickness of the mandibular condylar cartilage (MCC) was measured.

2.7. Immunohistochemical analysis

After deparaffinization, the sections were incubated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity, then treated with normal goat serum (Abcam, Cambridge, MA). The sections were incubated with anti-collagen type II antibody (diluted 100-fold; AbFrontier Co., Ltd. CA, USA) or anti-PI3 Kinase p110 delta antibody (diluted 200-fold; Abcam, Cambridge, MA) in Can Get Signal® immunostain immunoreaction enhancer solution (TOYOBO CO., LTD., Osaka, Japan). After washing, the sections were incubated with peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA), flooded with DAB solution (Vector Laboratories), and evaluated with a microscope (BZ-9000; Keyence Co., Osaka, Japan).

2.8. Vital staining

For the bone apposition labeling study, calcein (Wako Pure Chemical Industries, Ltd.) was injected intraperitoneally (16 mg/kg BW) into seven-week-old experimental mice, followed by xylenol orange (Wako Pure Chemical Industries, Ltd.) injection (50 mg/kg BW) at eight weeks. Mice were euthanized one week after the xylenol orange injection, and the mandibles were excised and serial undecalcified frozen sections were prepared [24]. The distances between calcein (green fluorescence) and xylenol orange (red fluorescence) were measured using a fluorescent microscope (BZ-9000; Keyence Co., Osaka, Japan).

2.9. Laser capture microdissection (LCM)

The method used for LCM has been described in detail [24] [25]. The microdissected samples from the surface of the MCC and the tibial growth plate (5 sections, 140 μm^2) were used for RNA extraction.

2.10. Microarray analysis

RNA samples of the MCC and tibia growth plate from five-week-old mice were collected and analyzed by Whole Mouse Genome Microarray 4x44K (Agilent Technologies, Santa Clara, CA). Whole gene expression levels in MCC were compared with the tibial growth plate.

2.11. Cells and cell culture

The chondrocyte cell line, ATDC5 was obtained from Riken Bioresource Center (Tsukuba, Japan). ATDC5 was cultured in DMEM/Ham's F-12 with L-Gln and Sodium Pyruvate, without HEPES, containing 10% fetal bovine serum and supplemented with antibiotics

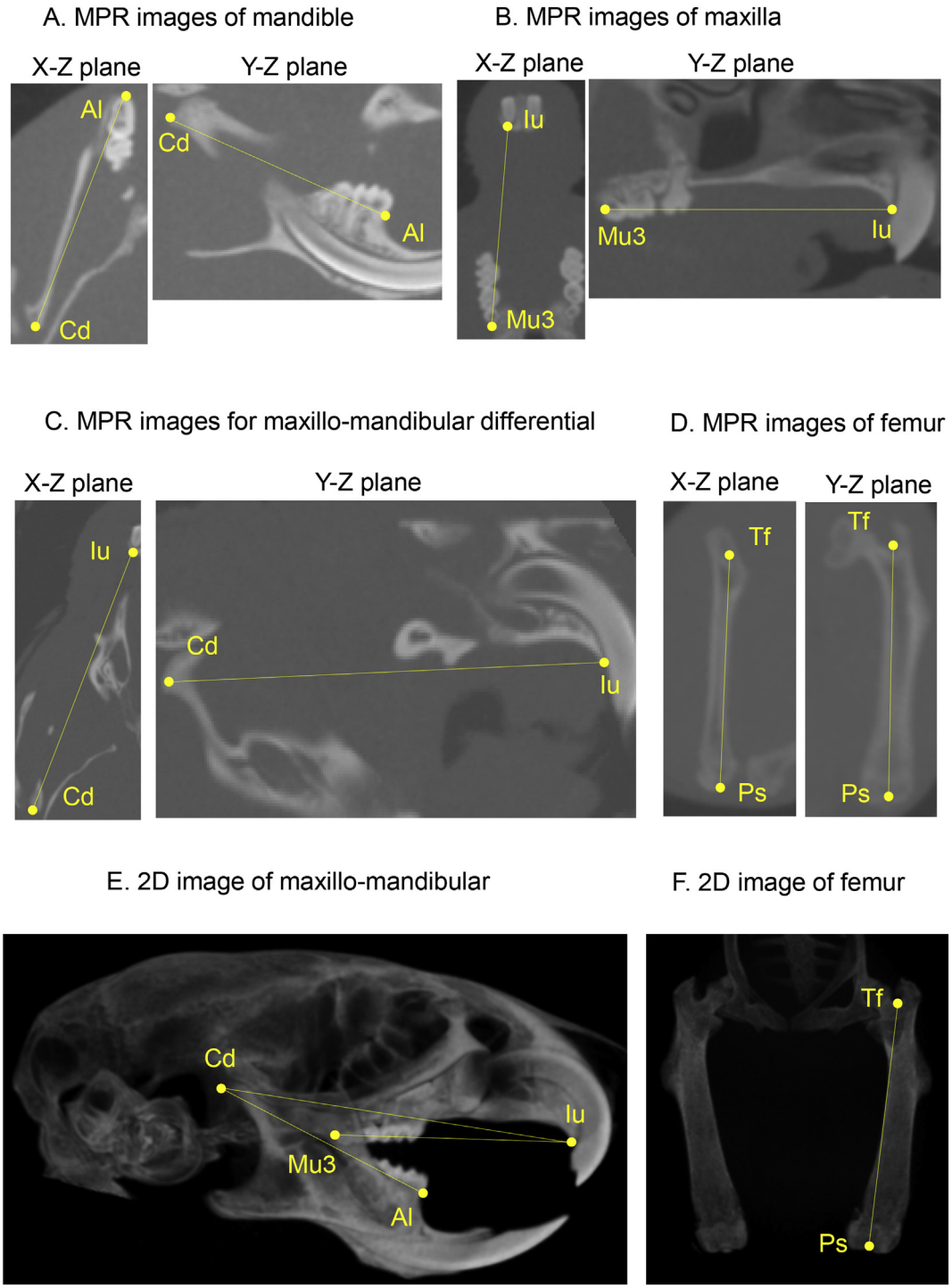

Fig. 1. MicroCT analysis.
A. MPR images of mandible. The X-z and the y-z planes are shown. Reference points of the maximal projecting point of the condylar (Cd) and the mesial alveolar bone apex of the mandibular first molar (Al) were set.
B. MPR image of maxilla. The X-z and the y-z planes are shown. Reference points of the distal alveolar bone apex of the maxillary third molar (Mu 3) and the palatal alveolar bone apex of the maxillary incisor (Iu) were set.
C. MPR image of mandibular condyle and maxilla. The X-z and y-z planes are shown. Reference points of Cd and Iu were set.
D. MPR image of femur. The X-z and y-z planes are shown. Reference points of the trochanteric fossa (Tf) and the patellar surface of femur (Ps) were set. The distance of each reference points were measured.
E and F. Representative 2D image of maxilla-mandibular (E) and femur (F) are shown. Each reference point describe above are depicted.

(100 U/mL of penicillin and 100 μg/mL of streptomycin).

The protocol for obtaining the mice primary chondrocyte from mandibular condylar cartilage was performed according to Ting's method [26] with minor modification. Briefly, the mandible was excised from three-week-old male BALB/c mice, and MCC were dissected with sharp scalpel. Dissected MCC were washed with PBS thrice to eliminate the contamination of blood cells, were subsequently minced, were resuspended in DMEM/Ham's F-12, with L-Gln and

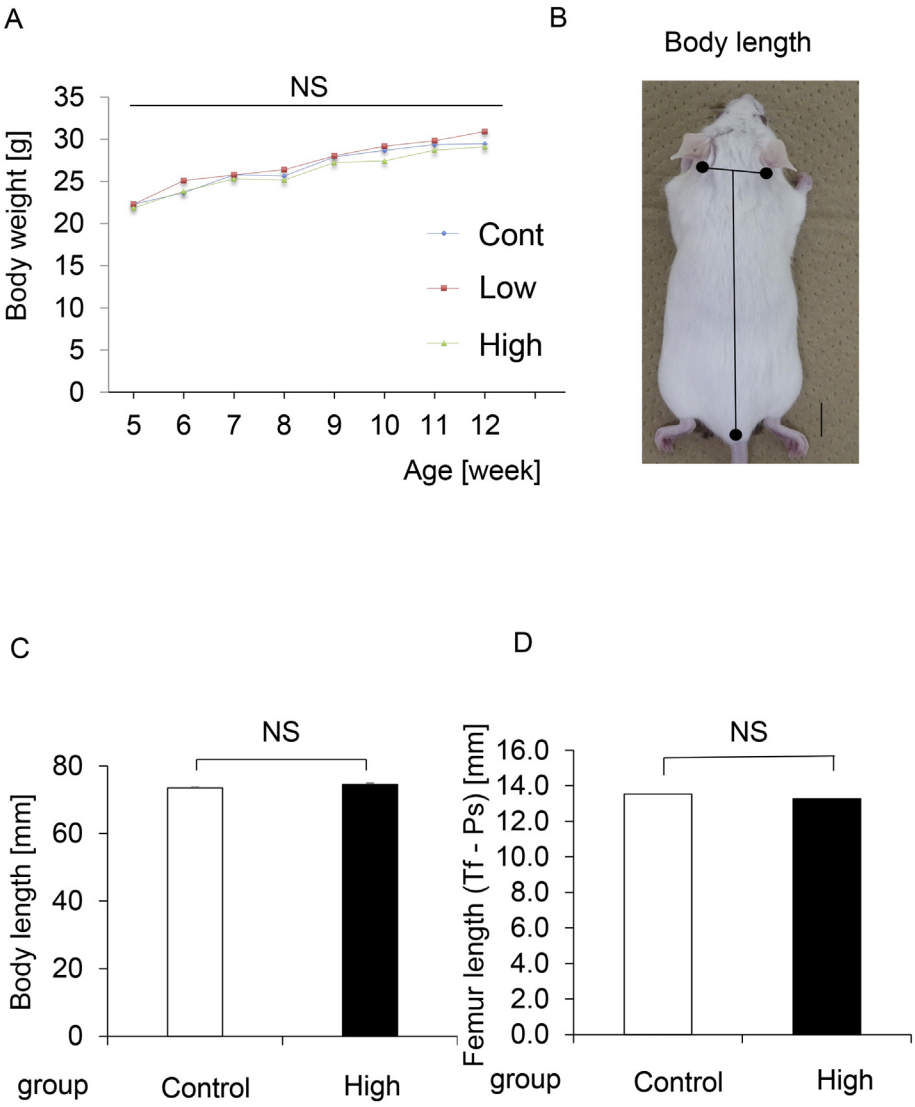

Fig. 2. Myo-inositol did not promote systemic growth.
A. Average body weight of the control (circle, n = 5), low concentration (triangle, n = 5), and high concentration (square, n = 5) group are shown. NS: not significantly different between groups.
B. Representative photographs of the mice. A straight line was drawn between the ears and the distance from the midpoint to the tail was measured as the mouse body length. Bar: 10 mm.
C. Body length of the control and high concentration group are shown. NS: not significantly different between the groups.
D. Length of the femur of the control and high concentration group are shown. NS: not significantly different between groups.

Sodium Pyruvate, without HEPES (Nacalai tesque, Kyoto, Japan), containing 20% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) supplemented with antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), and were plated on cell culture dish. The outgrown cells from the minced MCC were used as the primary chondrocyte from mandibular condylar cartilage (PMC).

All cells were cultured at 37 °C in a 5% CO_2 incubator. Cells were cultured in the presence or absence of myo-inositol (0, 1, 10, or 100 μM). In some experiments, cells were stimulated with BMP4 (100 ng/mL) to induce chondrocytic differentiation.

2.12. Cell counting

ATDC 5 were plated on 6-well plates (1.1×10^3 cells/cm^2 density) and cultured three days with or without myo-inositol. PMC were plated on 48-well plates (0.5×10^4 cells/cm^2 density) and cultured five days with or without myo-inositol. The cells were then detached with 250 or 500 μL of trypsin-EDTA solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and suspended in 0.25 or 0.5 mL of PBS. The number of cells was counted using a hemocytometer (Funakoshi Co., Ltd., Tokyo, Japan).

2.13. Cell proliferation assay

For cell proliferation assays, Alamar Blue Cell Viability Reagent (Thermo Fisher Scientific, Waltham, MA) was used according to the manufacturer's instructions. The fluorescence intensity (excitation 545 nm/emission 590 nm) was measured using Synergy HTX (BioTek Japan, Tokyo, Japan).

2.14. Phosphoinositide 3-kinase (PI3K) inhibition experiment

Cells were cultured with Hybridoma-SFM (Thermo Fisher Scientific, Waltham, MA) for phosphatidylinositol 3-kinase catalytic deltapolypeptide (PIK3CD) inhibition experiments.

CAL-101 (Chemescene LLC, Monmouth Junction, NJ) was used as a PI3K (PIK3CD)-selective inhibitor. ATDC 5 were plated on 6-well plates (1.1×10^3 cells/cm^2 density) and cultured three days with or without myo-inositol and CAL-101 (0.1 μM). PMC were plated on 48-well plates (0.5×10^4 cells/cm^2 density) and cultured five days with or without myo-inositol and CAL-101 (0.1 μM). Cell proliferation was then analyzed using the cell counting and Alamar Blue assay.

2.15. RNA extraction and Reverse Transcription (RT)

RNA from microdissected samples were extracted using the RNeasy

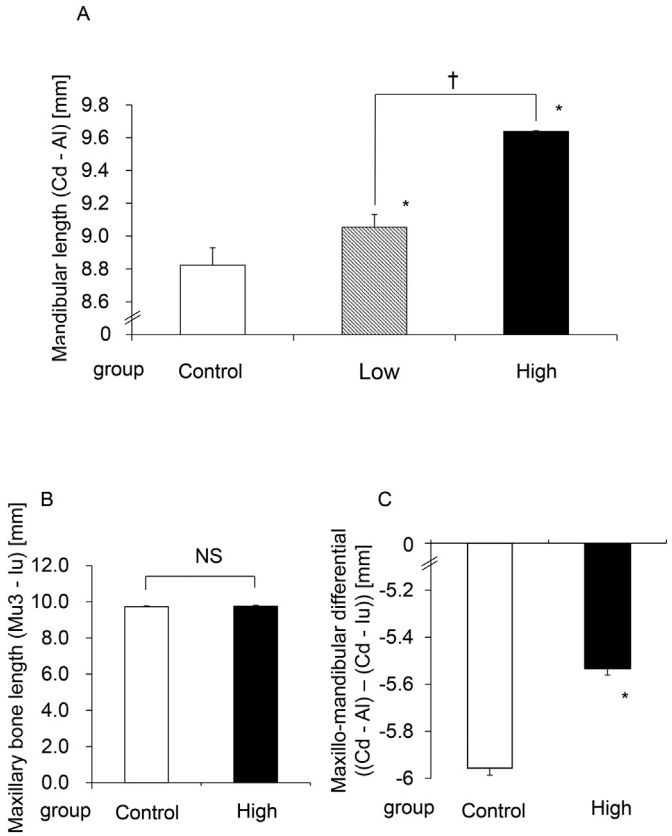

Fig. 3. Myo-inositol promoted growth of the mandible but not maxilla.
A. The length of the mandible (Cd - Al) of the control (n = 5), low concentration (n = 5), and high concentration (n = 5) group are shown. *: $p < 0.05$ versus control. †: $p < 0.05$ between the groups.
B. The length of maxilla (Mu3 – Iu) of the control and high concentration group are shown. NS: not significantly different between groups.
C. The maxillo-mandibular differential (Cd - Al) - (Cd - Iu) of the control and high concentration group are shown. *: $p < 0.05$ versus control.

Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse transcribed using iScript cDNA Supermix (Bio-Rad Laboratories, Hercules, CA). RNA from cultured cells were extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Co., St Louis, MO) according to the manufacturer's instructions. After measuring the RNA concentration, equal amounts of RNA (500 ng) were reverse transcribed and cDNA was diluted five-fold with Tris-EDTA buffer.

2.16. Real-time RT-PCR analysis

Real-time RT-PCR was performed using SsoFast EvaGreen-Supermix (Bio-Rad, Laboratories) on a CFX connect Real-Time PCR System (Bio-Rad Laboratories). Fold changes of genes of interest were calculated by using the −ΔΔCT method with ribosomal protein S18 (RPS18) as a reference gene. Primer sequences for mouse aggrecan (*Aggrecan*), mouse collagen type II (*ColII*), mouse collagen type X (*ColX*), *Pik3cd*, and *RPS18* were as follows:

Aggrecan:
(Forward) 5′-CCTGCTACTTCATCGACCCC-3′,
(Reverse) 5′-AGATGCTGTTGACTCGAACCT-3′
ColII:
(Forward) 5′-GGGAATGTCCTCTGCGATGAC-3′,
(Reverse) 5′-GAAGGGGATCTCGGGGTTG-3′
ColX:
(Forward) 5′-TTCTGCTGCTAATGTTCTTGACC-3′,
(Reverse) 5′-GGGATGAAGTATTGTGTCTTGGG-3′
Pik3cd:
(Forward) 5′-GTAAACGACTTCCGCACTAAGA-3′,
(Reverse) 5′-GCTGACACGCAATAAGCCG-3′
RPS18:
(Forward) 5′-AGTTCCAGCACATTTTGCGAG-3′,
(Reverse) 5′-TCATCCTCCGTGAGTTCTCCA-3′

2.17. Statistical analysis

All data are presented as the mean ± standard error. Multiple comparisons were performed using Tukey's test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Myo-inositol exhibited no promotional effects on systemic growth

Firstly, the body weight and length of the mice were examined to determine whether myo-inositol has any systemic influence (Fig. 2A). There was no statistically significant difference in body weight at each time point among the three groups during the experiment. Average body lengths at 12 weeks old were 73.4 ± 0.2 mm and 74.5 ± 0.4 mm in the control and high concentration group, respectively, and the difference was not statistically significant (Fig. 2B and C). The average lengths of the femur in each group measured by μCT were 13.5 ± 0.00 mm and 13.2 ± 0.01 mm, respectively, with no significant difference between the groups (Fig. 2D). These results suggest that myo-inositol has no promotional effect on whole body growth.

3.2. Myo-inositol specifically promoted the growth of the mandible

MicroCT analysis revealed that the average mandibular length (Cd - Al) of each group at 12 weeks old were 8.82 ± 0.00 mm, 9.05 ± 0.00 mm, and 9.63 ± 0.00 mm in the control, the low concentration, and the high concentration group, respectively (Fig. 3A). There were statistically significant differences between the control and the myo-inositol groups. In addition, the length of the high concentration group was significantly larger than that in the low concentration group.

Average maxillary bone lengths were 9.72 ± 0.05 mm and 9.75 ± 0.05 mm in the control and the high concentration group, respectively, with no statistically significant difference between the groups (Fig. 3B).

Average maxilla-mandibular differential ((Cd-Al) – (Cd-Iu)) were -5.95 ± 0.11 mm and - 5.53 ± 0.08 mm in the control and the high concentration group, respectively, with statistically significant difference between the groups (Fig. 3C).

These results indicated that supplementation of diet with myo-inositol dose-dependently promoted mandibular growth specifically.

3.3. Myo-inositol promoted the growth and differentiation of MCC

Histological analysis revealed that MCC thickness in the control and the high concentration group were 82.67 ± 2.64 μm and 100.34 ± 5.45 μm, respectively, which showed significant differences between the groups (Fig. 4A, B and C).

Immunohistochemical staining for chondrocytic differentiation marker, Col2 were performed (Fig. 4D–G). Many Col2 positive cells were identified in the high concentration group (Fig. 4G), and a few Col2 positive cells were identified even in the cartilage in the control group (Fig. 4F).

These results suggest that myo-inositol promoted the growth and differentiation of the MCC.

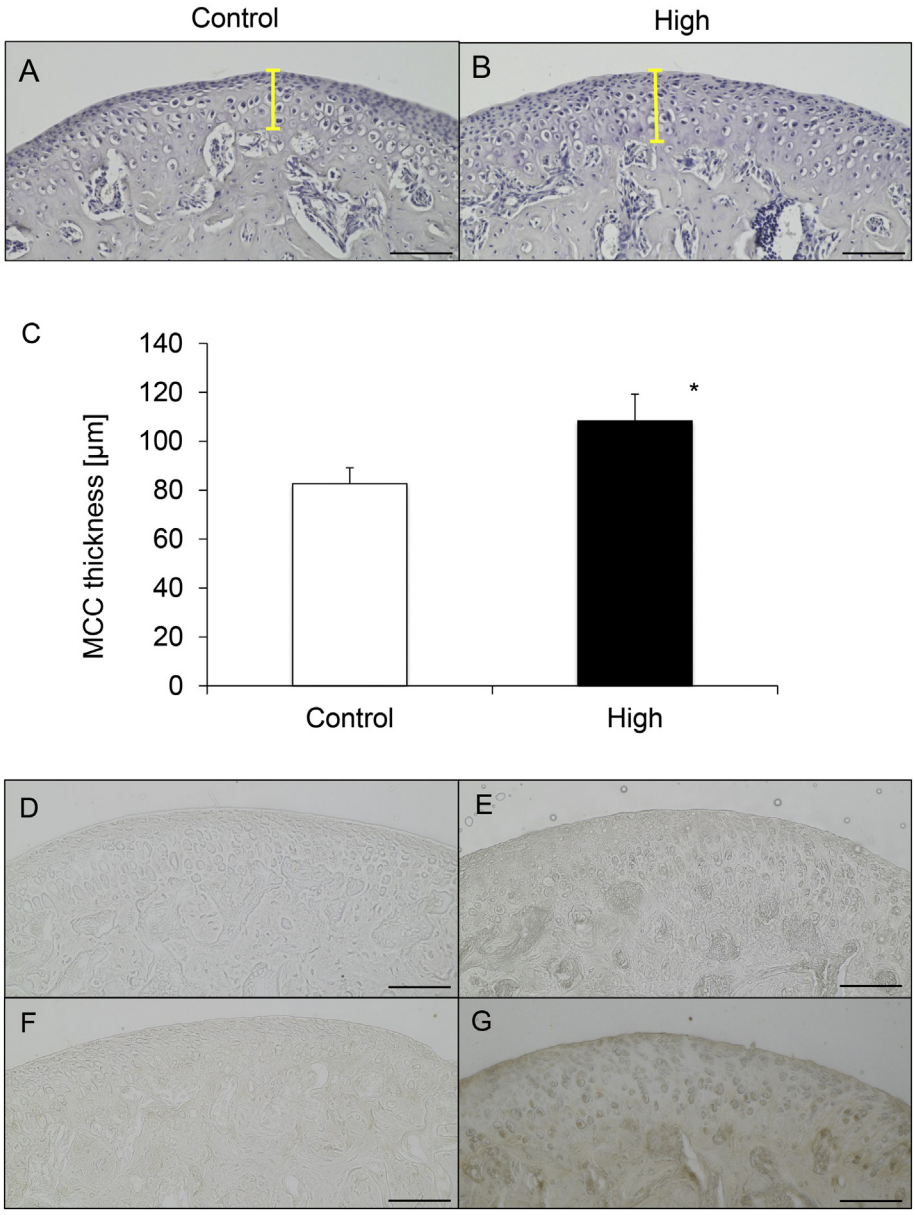

Fig. 4. Myo-inositol promoted growth of the MCC.
A, B. Representative photographs of H-E stained sections of the MCC of the control and high concentration group are shown. The thickness of the MCC is shown by the yellow scale. Bar: 100 μm.
C. The thickness of the MCC in the control and high concentration group are shown. Mean value of three sections/mice, three mice, total nine sections are shown. *: $p < 0.05$ versus control.
D–G. Immunohistochemical staining for *ColII* of the control and high concentration group. Representative photographs of the staining without primary antibody of the section of the control (D) and high concentration group (E) are shown. Representative photographs of the staining with primary and secondary antibody of the section of the control (F) and high concentration group (G) are shown. Bar: 100 μm.

3.4. Myo-inositol induced endochondral bone formation

Vital staining revealed that the distance between fluorescent labelings in the high concentration group was significantly larger than that in the control group (Fig. 5A–C). These results indicate that myo-inositol promoted endochondral bone formation.

3.5. Pik3cd was strongly expressed in the MCC

We then investigated why myo-inositol showed a specific promotional effect on the MCC. Microarray analysis revealed that genes encoding enzymes related to myo-inositol metabolism exhibited differences in expression between MCC and the tibial growth plate (Table 1). Among them, *Pik3cd* (gene ID: 18707) was highly expressed in the MCC and confirmed with real-time RT-PCR. *Pik3cd* was highly expressed in MCC when compared to the tibial growth plate (Fig. 6A). Expression of *Pik3cd* in ATDC5 and PMC was significantly higher than that in the tibial growth plate (Fig. 6A).

Intense immunoreaction to PIK3CD was observed in MCC in immunohistochemistry (Fig. 6E), but this reaction was not observed in growth plate cartilage (Fig. 6D). Together, PIK3CD was specifically expressed in the MCC. As ATDC5 and PMC expresses certain extent of Pik3CD expression, we used both cells for in vitro experiments.

3.6. Myo-inositol promoted cell proliferation in vitro

To investigate how myo-inositol specifically promoted growth of the MCC, cell culture experiments using ATDC5 and PMC were performed by comparing cell numbers in myo-inositol-treated and -untreated cells. The number of ATDC5 in control and myo-inositol-treated cells were $1.9 \pm 0.15 \times 10^5$ cells/mL and $2.9 \pm 0.2 \times 10^5$ cells/mL, respectively, and the difference was statistically significant (Fig. 7A). Cell proliferation assays using Alamar Blue in ATDC5 also showed that the promotion of cell proliferation by myo-inositol was dose-dependent (Fig. 7B). We further examined the effect of myo-inositol on chondrocyte proliferation using primary chondrocyte obtained from mandibular condylar cartilage. Similar to the results of ATDC5, the number of PMC in control and myo-inositol-treated cells were $4.0 \pm 1.9 \times 10^4$ cells/mL and $8.5 \pm 2.4 \times 10^4$ cells/mL, respectively, and the difference was statistically significant (Fig. 7C). These results

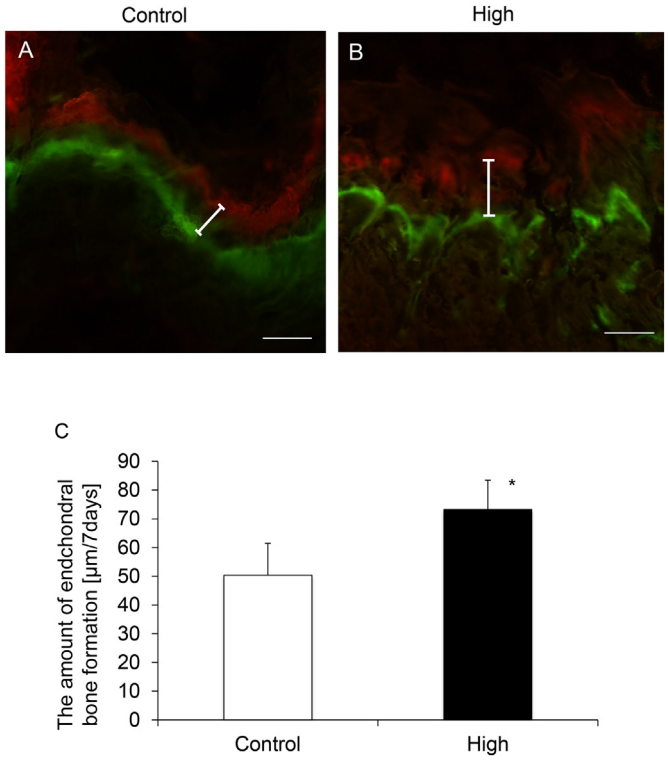

Fig. 5. Myo-inositol promoted endochondral bone formation.
A, B. Representative photographs of the subchondral bone of the control (A) and high concentration group (B) are shown. The distance between fluorescent labelings are indicated by the white scale. Bar: 100 μm.
C. The distance between the green fluorescence to the red fluorescence of the control and high concentration group are shown. Mean value of three sections/mice, three mice, total nine sections are shown. *: $p < 0.05$ versus control.

Table 1
Pik3cd was strongly expressed in MCC.

Gene	Expression ratio (MCC/tibial growth plate cartilage)
Pik3ca	471.2
Pik3cb	4.1
Pik3cg	5.4
Pik3cd	2057.8

indicate that myo-inositol promoted chondrocyte proliferation in vitro.

3.7. Myo-inositol induced chondrocytic differentiation in vitro

To further analyze the effect of myo-inositol on chondrocytic differentiation of ATDC5, the expression of chondrocyte-related genes *Aggrecan*, *ColII*, and *ColX* were examined using real-time RT-PCR. Compared with the control, myo-inositol significantly up-regulated the expression of *Aggrecan*, *ColII* and *ColX*, which were similar to the results of *BMP4*. (Fig. 8A–C). These results suggest that the effects of myo-inositol on the chondrocytes were similar to those of *BMP4*, and myo-inositol-promoted chondrocytic differentiation.

3.8. PI3K inhibition reduced the promotional effect of myo-inositol

To clarify the role of PIK3CD in the myo-inositol-mediated promotion of endochondral growth, a selective PIK3CD inhibitor was used. The addition of the inhibitor completely blocked myo-inositol-mediated cell proliferation in both ATDC5 and PMC (Fig. 9A and B). These results suggest that the promotion of endochondral growth by myo-inositol depends on PIK3CD signaling.

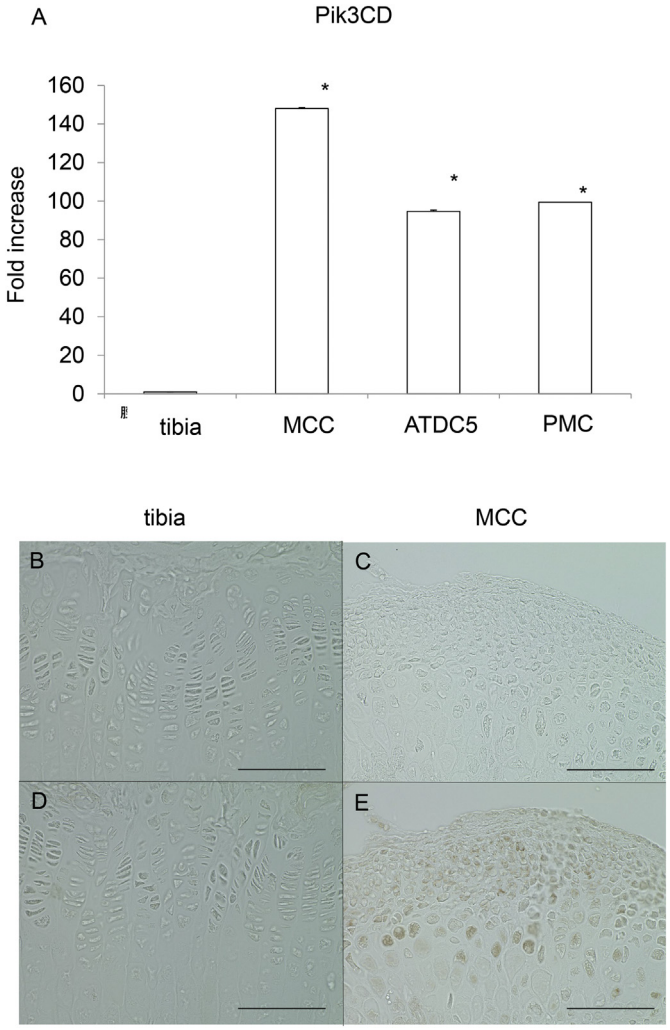

Fig. 6. PIK3CD was highly expressed in MCC.
A. Fold change of the gene expression level of PIK3CD in tibial growth plate cartilage, MCC, ATDC5 and PMC are shown. *: $p < 0.05$ versus tibia.
B–E. Immunohistochemical staining for PIK3CD on tibial growth plate cartilage and MCC. Representative photographs of the staining without primary antibody of the section of tibial growth plate (B) and MCC (C) are shown. Representative photographs of the staining with primary and secondary antibody of the section of tibial growth plate (D) and MCC (E) are shown. Bar: 100 μm.

4. Discussion

This is the first report on the specific promotion of mandibular growth by nutritional supplementation. Myo-inositol evidently showed a promotional effect on mandibular growth in mice, though this promotional effect was not observed on body weight, body length, maxilla, and femur. In past report [21], myo-inositol induced weight gain but not in this study. In the report, control diet contains no myo-inositol with chemical treatment, though the control diet we used contained quite small amount of myo-inositol. We presumed that this difference in the amount of myo-inositol in the control diet caused the difference in the response of body weight between the report and ours. Nutritional supplementation with myo-inositol has the potential to specifically promote endochondral growth in the mandible without any systemic influence.

Since mandibular growth depends mainly on endochondral growth in the MCC [27], the effect of myo-inositol on the growth of the chondrocyte cell line was examined using chondrocyte cell line ATDC5 and primary chondrocyte obtained from MCC. Myo-inositol augmented both chondrocyte proliferation and differentiation, and consequently

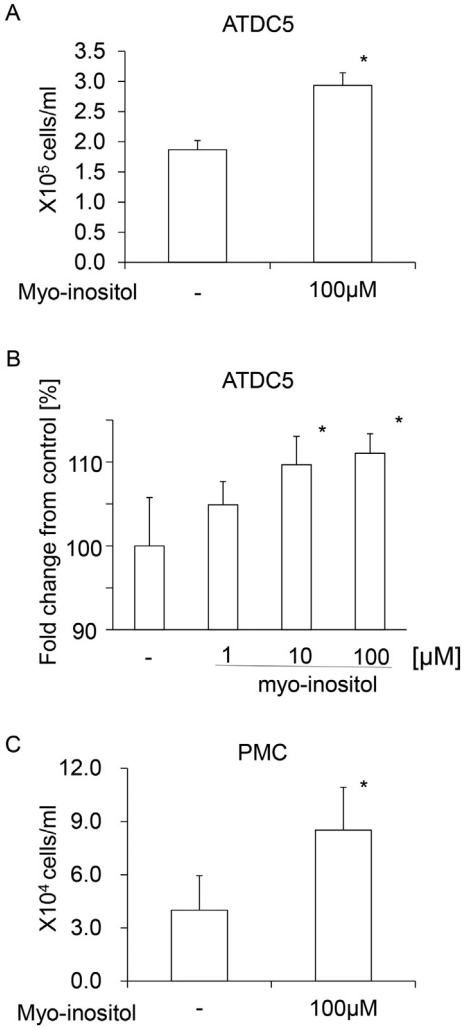

Fig. 7. Myo-inositol facilitated cell proliferation.
A. Number of ATDC5 on day 5 in the control cells and the cells cultured with 100 μM of myo-inositol are shown. *: $p < 0.05$ between the groups (n = 3).
B. The results of the cell proliferation assay using Alamar Blue of the ATDC5 treated with the various concentration of myo-inositol are shown. *: $p < 0.05$ versus control (n = 3).
C. Number of PMC on day 3 in the control cells and the cells cultured with 100 μM of myo-inositol are shown. *: $p < 0.05$ between the groups (n = 6).

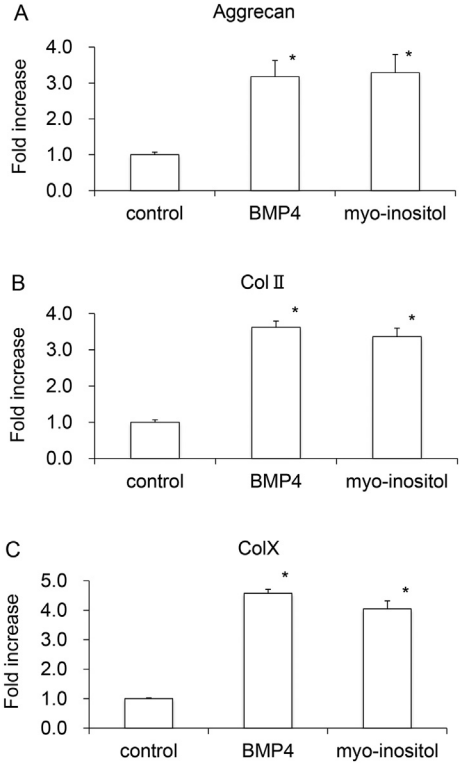

Fig. 8. Myo-inositol induced chondrocytic differentiation.
A. Fold change of the gene expression level of *Aggrecan* in control, BMP4-treated, and 100 μM of myo-inositol-treated ATDC5 on day 3 are shown. *: $p < 0.05$ versus control (n = 3).
B. Fold change of the gene expression level of *ColII* in control, BMP4-treated, and 100 μM of myo-inositol-treated ATDC5 on day 3 are shown. *: $p < 0.05$ versus control (n = 3).
C. Fold change of the gene expression level of *ColX* in control, BMP4-treated, and 100 μM of myo-inositol-treated ATDC5 on day 3 are shown. *: $p < 0.05$ versus control (n = 3).

augmented the production of cartilage matrix components such as aggrecan, collagen types II and X, which resulted in the promotion of mandibular endochondral growth. These results indicate that myo-inositol induces mitotic phase in cells [28].

Myo-inositol is a precursor of phosphatidylinositol (PI) [29], and intracellular myo-inositol is synthesized from glucose or supplemented by external uptake [30] [31] [32]. In this study, supplementation with myo-inositol would augment intracellular myo-inositol leading to increased intracellular PI, which activates signaling pathways for cell proliferation and differentiation.

PI3K is classified into three classes [33] with class I consisting of PIK3CA (p110-α), PIK3CB (p110-β), PIK3CG (p110-γ) and PIK3CD (p110-δ). These act as key enzymes to produce PI, a second messenger for intracellular signaling after PI3K [33]. Class I PI3K is responsible for the production of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in the cell membrane [34], and PIP3 induces recruitment of proteins that promote cell proliferation [35]. Our results indicate that PIK3CD was highly expressed in MCC as compared with that in the cartilage of the tibial growth plate. PIK3CD inhibition in ATDC5 and PMC clearly demonstrates that myo-inositol-mediated augmentation of cell proliferation is dependent on PIK3CD. These findings support in vivo myo-inositol-mediated promotion of endochondral growth specifically in MCC is due to specific intense expression of PIK3CD in MCC. We measured the lengths of femurs because femur is the representative another site of endochondral ossification. Our results indicate that Pik3cd was highly expressed in MCC as compared with that in the cartilage of the tibial growth plate.

As to the reason why other sites of endochondral ossification were not affected by myo-inositol supplementation, we presumed the difference of Pik3cd expression is dependent of the phenomenon. Pik3cd inhibition in ATDC5 and PMC cells, which extensively expresses Pik3cd, clearly demonstrates that myo-inositol-mediated augmentation of cell proliferation is dependent on Pik3cd. Together, we presumed that specific promotion of endochondral growth in MCC would be dependent on the specific intense expression of Pik3cd in MCC.

Growth hormone is known to promote growth of the shaft bone [36]. This growth promotion is dependent on IGF-1 up-regulation [37], which promotes cell division in the target organs [38]. Administration of growth hormone has been clinically used for children with short stature, though it requires repeat injections [39]. It was previously reported that local unilateral IGF-1 injection in TMJ successfully augmented unilateral endochondral growth of the mandible [20]. However, a high IGF-1 blood level was recently reported to positively correlate with cancer incidence in prostate cancer, breast cancer, and colorectal cancer [40]. Systemic administration of IGF-1 for the promotion of mandibular growth would be difficult.

Myo-inositol has been used in living organisms as a water-soluble

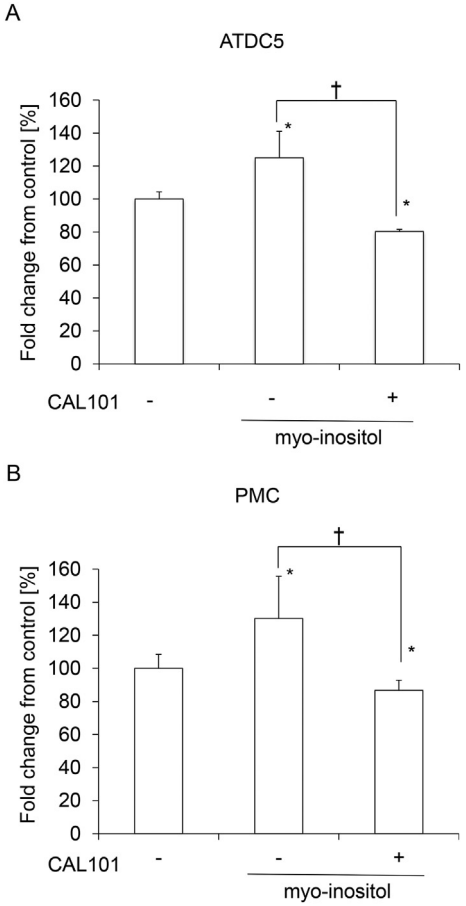

Fig. 9. PIK3CD inhibition reduced the growth-promoting effect of myo-inositol. A–B. Results of cell proliferation assays on ATDC5 (A; n = 3) and PMC (B; n = 6) treated with 100 μM of myo-inositol and Pik3cd inhibitor, CAL101, are shown. *: $p < 0.05$ versus control. †: $p < 0.05$ between the groups.

vitamin-like substance [41]. Myo-inositol is contained in numerous foods such as fruits, beans, grains, and nuts, hence, is usually supplied in foods [42]. Our results clearly demonstrate that myo-inositol supplementation successfully, specifically, and safely augmented endochondral growth of the mandible in experimental mice. Water-soluble vitamins are excreted in the urine in cases of excessive intake, which results in fewer incidences of excessive symptoms [42]. The properties of myo-inositol support its safety and future clinical use. Reinforcement of the specific nutritional ingredients would be a potent therapeutic option for promoting mandibular growth in growing patients.

5. Conclusion

We are the first to discover that nutritional supplementation with myo-inositol in growing mice specifically augments mandibular endochondral growth without any systemic influence. This specific promotion of mandibular growth is due to the unique intense expression of PIK3CD in the MCC.

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