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Homeoprotein Hex is expressed in mouse developing chondrocytes

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Endochondral ossification is a complex process involving the formation of cartilage and the subsequent replacement by mineralized bone. Although the proliferation and differentiation of chondrocytes are strictly regulated, the molecular mechanisms involved are not completely understood. Here, we show that a divergent-type homeobox gene, *hematopoietically expressed homeobox gene* (*HEX*), is expressed in mouse chondrogenic cell line ATDC5. The expression of Hex protein drastically increased during differentiation. The chondrogenic differentiation-enhanced expression of Hex protein was also observed in chondrocytes in the tibia of embryonic day 15.5 (E15.5) mouse embryos. The localization of Hex protein in the chondrocytes of the tibia changed in association with maturation; namely, there was Hex protein in the cytoplasm near the endoplasmic reticulum (ER) in resting chondrocytes, which moved to the nucleus in prehypertrophic chondrocytes, and thereafter entered the ER in hypertrophic chondrocytes. These results suggest Hex expression and subcellular localization are associated with chondrocyte maturation.

Keywords: cartilage formation/chondrocyte/hematopoietically expressed homeoprotein (Hex)/homeobox gene/nucleocytoplasmic transport.

Abbreviations: BMP, bone morphogenic protein; BRE, BMP responsive element; DAPI, 4′,6-diamidino-2-phenyindole; E, embryonic days; ER, endoplasmic reticulum; *HEX*, *hematopoietically expressed homeobox gene*; GST, glutathione *S*-transferase; Kap7, karyopherin/importin 7; NES, nuclear export signal; NLS, nuclear localization signal; PB, sodium phosphate buffer; PBS, phosphate-buffered saline; *Prh*, *proline-rich homeobox* gene; RIPA, radioimmunoprecipitation assay.

Bone formation is an essential process for skeletal development and tissue patterning (*1*). Bone formation consists of endochondral ossification and involves the formation of cartilage and the subsequent replacement by mineralized bone (e.g. tibiae, femora and rib), and membranous ossification (e.g. skull). Since cartilage matrix is synthesized and secreted from chondrocytes, differentiation and maturation of chondrocytes are critical events for bone formation. Chondrocytes are derived from aggregated mesenchymal cells, actively secrete the cartilage-specific matrix proteins and then form cartilage primordia. Subsequently, chondrocytes mature into resting, proliferating and hypertrophic chondrocytes from the centre of the primordia. The hypertrophic chondrocytes switch the type of the matrix to mineralizing matrix by the deposition of calcium and finally undergo programmed cell death. Mineralized matrix is replaced by trabecular bone by osteoblasts and osteoclasts from invaded blood vessels (*2*, *3*). Although the differentiation to chondrocytes and the subsequent mineralization are strictly regulated, the molecular mechanisms involved are not completely understood.

Hematopoietically expressed homeobox protein (Hex), also known as proline-rich homeodomain protein (Prh), is a transcription factor belonging to divergent-type homeoprotein containing DNA-binding homeodomain (*4–9*). Hex is a key molecule in normal morphogenesis through the regulation of cell proliferation and differentiation in hematopoietic cells, vascular system, liver, thyroid, heart, pancreas and skin during development (*9*). Conventional Hex knock-out mice showed anterior truncation of the brain, and liver and thyroid dysplasia, and then died around E10.5 (*10*, *11*). In hematopoiesis, Hex is required for the differentiation of hemangioblasts into hematopoietic progenitors and the repression of hemangioblast proliferation (*12*, *13*). Hex is essential for normal liver morphogenesis by promoting the differentiation of hepatic endoderm into hepatoblasts through the transition to a pseudostratified epithelium, and by the activation of several liver-specific genes such as hepatocyte nuclear factors (*10*, *14–16*). Exogenous Hex expression in embryonic chick skin leads to proliferation resulting in induction of feather buds via Wnt signal or elongation of scales (*17–19*). In terms of molecular mechanism, Hex plays multiple roles in a context-dependent manner as transrepressor, transactivator and post-translational regulator. For example, Hex acts as a transrepressor of the *ESM-1* gene in endothelial cell lines EOMA and

HUVEC, thyroglobulin gene in thyroid cell lines, and *Goosecoid* gene in *Xenopus* embryo and ES cells (*20*–*22*). Hex activates the transcription of L-type pyruvate kinase gene in human hepatocarcinoma cell line Hep G2 and human primary cultured hepatocytes (*15*). On the other hand, as a post-transcriptional regulator, Hex eIF-4E dependently inhibits the export of cyclin D1 mRNA into the nucleus in leukemia cell lines U937 and K562 (*23*). Despite its significance, the expression and the physiological role of the divergent-type homeobox gene involved in chondrogenesis remain unclear. Because the aggregation of mesenchymal cells, which is the first step of endochondral ossification, started at E12.5, the phenotype of *HEX* knock-out mice in skeletal development is still unknown.

We report here that *HEX* mRNA is expressed in chondrocytes and that the expression increases along with chondrocyte differentiation. The cellular localization of Hex protein shuttles between the cytoplasm and the nucleus during differentiation. These results suggest that Hex acts as a transcription factor at prehypertrophic stage.

Materials and Methods

Cell culture

Mouse chondrogenic cell line ATDC5 was obtained from the Riken Cell Bank (Tsukuba, Japan). ATDC5 cells were maintained as described previously (*20*, *21*). The cells were cultured in the maintenance medium consisting of Dulbecco's modified Eagle medium/Ham's F-12 (1 : 1) containing 5% fetal bovine serum (FBS), 10 μg/ml human transferrin (Roche Applied Bioscience, Basel, Switzerland) and 30 nM sodium selenite (Sigma-Aldrich, Inc., St Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air. For chondrogenic induction, the cells were cultured in the maintenance medium supplemented with 10 μg/ml bovine insulin (Sigma-Aldrich, Inc.), and the medium was replaced every other day. Subsequently, for induction of hypertrophy followed by mineral deposition, the culture medium was switched to alpha-modified Eagle medium containing 5% FBS plus 10 μg/ml insulin, 10 μg/ml transferrin and 30 nM sodium selenite and the CO_2 concentration was shifted to 3% to facilitate mineralization in culture after 3 weeks.

Animals

All animal experiments were carried out in accordance with the guidelines of the Committee of Animal Care and Experiments of Teikyo University. Pregnant ddY mice were purchased from Sankyo Labo Service (Tokyo, Japan). The pregnant mice were anesthetized with diethyl ether and the tibiae of embryos were isolated.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was prepared from ATDC5 cells using Trizol LS reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the instruction manual. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Life Technologies Corporation) using poly(dT)$_{20}$ primer. Primers for quantitative RT–PCR were designed by Primer Express Software (Life Technologies Corporation). The sequences of primers were as follows: *HEX*, 5′-GCCAAGATGT TACAGCTCAGTGA-3′ and 5′-GCTCGGCGATTCTGAAAC C-3′; *β-actin*, 5′-TCCCTCACCCTCCCAAAAG-3′ and 5′-CATGG ACGCGACCATCCT-3′; *COL2A1*, 5′-AAAACTGGTGGAGCAG CAAGA-3′ and 5′-AGCCACCGTTCATGGTCTCT-3′ and *COL10A1*, 5′-GCTCCAATTGCCCAATGC-3′ and 5′-AAGGAC GAGTGGACGTACTCAGA-3′. The PCR was performed with SYBR Premix EX Taq II (Takara Bio Inc., Shiga, Japan) by Applied Biosystems 7500 Real-Time PCR Systems. The results were analysed by accessory software in real-time PCR. The amounts of *HEX*, *COL2A1* and *COL10A1* transcripts were normalized by that of β-actin.

In situ *hybridization*

In situ hybridization was carried out as described previously (*18*). Mouse *HEX*, *COL2A1 and COl10A1* cDNA fragment, which was a template of *in vitro* transcription, was amplified by PCR. The primers were as follows: *HEX*, 5′-AAAAAGAATTCTGGCCGCCGC CTACGGCCCC-3′ and 5′-TTTTTGTCGACCCCCTCGATGTCC ACCTCCT-3′; *COL2A1*, 5′-AAAAAGAATTCGCTGGGCTGCC GGGTCTCC-3′ and 5′-TTTTTGTCGACGCCTTCTCGTCATAC CCTCC-3′; *COL10A1*, 5′-AAAAAGAATTCCTGGGGGGTGGT GAGGGGGT-3′ and 5′-CCCCCGTCGACTATTCTTCCACTCG ATTGAA-3′. Subsequently, the amplified products were cloned into pBluescript SK at *Eco*RI and *Sal*I sites. The probes were synthesized using DIG RNA labelling kit (Roche Diagnostics, Mannheim, Germany) according to the instruction manual.

Transfection

The full-length cDNA fragment encoding mouse *HEX* was amplified by PCR and cloned into pcDNA3.1 (Life Technologies Corporation) at *Kpn*I and *Sal*I sites. The sequences of primers containing a restriction enzyme site were 5′-AAAAAAAAGGTACCATGCAGTTCCC GCACCC-3′ and 5′-GGGGGGTCGACTGTCATCCAGCATTAA AG-3′. The clone was sequenced to confirm the absence of mutations. The plasmid was transfected to ATDC5 cells by lipofection using Lipofectamine LTX supplemented with Plus reagent (Life Technologies Corporation), according to the manufacturer's manual.

Antibodies

Site-specific antibodies against mouse Hex amino (N-) terminus (M1-R138) or carboxyl (C-) terminus (Q198-G271) were prepared in rabbit and rat, respectively, as described previously (*19*). Antigenic polypeptides were prepared as recombinant glutathione S-transferase (GST) fusion proteins from *Escherichia coli* BL21, according to the manufacturer's manual of GST fusion system (GE Healthcare UK Ltd, Buckinghamshire, UK). The cDNA fragments coding those regions of Hex protein were amplified by PCR and cloned into pGEX-2 T (GE Healthcare) at *Bam*HI and *Eco*RI for the N-terminus, and *Bam*HI and *Sma*I for the C-terminus. The sequences of primers containing a restriction enzyme site were as follows: N-terminal region, 5′-AAAAAGGATCCATGCAGTTCCCG CACCC-3′ and 5′-GGGGAATTCTTAGTGCAGAGGTCGCTG G-3′ and C-terminal region, 5′-GGAGGATCCCTGAAACAGAA ACAGGAGAATCCT-3′ and 5′-CCGATGAAATTACGACCTAC TACTTAAGGGA-3′. The clone was sequenced to confirm the absence of mutations. The GST and Hex polypeptide fusion proteins were immunized to female NZW rabbit (11 weeks old) or Wistar rat (7 weeks old) using standard methods.

Western blotting

Western blotting was performed as described in Ref. (*19*). Whole-cell lysate from ATDC5 cells was prepared with SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 10% glycerol, 5% mercaptoethanol and 4.2% SDS). The lysate was boiled for 5 min following sonication, and the protein (5 μg each) was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% acrylamide gel. The proteins in the polyacrylamide gel were blotted to polyvinylidene difluoride membrane, and blocked in TBS-T (Tris-buffered saline, 20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and 0.1% Tween-20) containing 5% fat-free milk overnight at 4°C. The membrane was reacted with anti-Hex antibodies at 10,000-fold dilution in Can Get Signal signal-enhancer solution (Toyobo, Osaka, Japan) for 1 h, and washed three times with TBS-T. Subsequently, horseradish peroxidase-conjugated secondary antibodies were applied. Signals were detected by enhanced chemiluminescence (GE Healthcare), according to the manufacturer's manual.

Immunoprecipitation

Whole-cell lysate from 10^7 cells was prepared by incubation with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0) for 15 min on ice, followed by homogenization.

The N-terminus of anti-Hex rabbit polyclonal antibodies was added to the lysate at 250-fold dilution overnight at 4°C under agitation. The antibodies were bound to Protein A-Sepharose (GE Healthcare) at 4°C for 1 h. After the formation of the complex, the beads were washed with RIPA buffer three times and the complex was eluted using 50 mM glycine–HCl, pH 3.0. The eluate was analysed by western blotting using the C-terminus of anti-Hex rat polyclonal antibodies described above.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min followed by incubation in PBS containing 0.1% Triton X-100 for 30 min, and blocked in PBS containing 2% BSA (blocking buffer) for 1 h. The cells were reacted with anti-Hex antibodies at 2,000-fold dilution in blocking buffer overnight at 4°C. The samples were washed four times with PBS and reacted with the secondary antibodies, which were Alexa Fluor 568-conjugated anti-rat IgG antibodies (1 μg/ml, Life Technologies Corporation). The samples were washed four times with PBS, and the immunoreaction was observed under a fluorescent microscope (Olympus Corporation, Tokyo, Japan). For immunostaining of the mouse embryonic tibia, it was fixed with 4% paraformaldehyde in PBS overnight and a frozen section (6 μm thick) was prepared. Subsequent steps were the same as described above. To absorb anti-Hex antibodies with antigenic GST-Hex polypeptide, the antibodies (finally diluted 2,000-fold) and GST-Hex fusion protein or GST (400 μg each) were incubated overnight at 4°C. The absorbed antibodies were used for immunostaining as primary antibodies.

Histological procedure

For Alcian blue staining, the cells were fixed with ice-cold methanol for 5 min at −20°C and stained with 0.1 M HCl containing 0.1% Alcian blue overnight. Alizarin red staining was performed according to the method of Dahl (*22*). The cells were fixed with 95% ethanol for 20 min at −20°C followed by staining with 1% Alizarin red S for 5 min.

Immunoelectron microscopic analysis

The pre-embedding gold enhancement immuno-gold method was performed as described previously (*23*). The cryosection of embryonic tibia was prepared as described for indirect immunofluorescence assay. The section was permeabilized with 0.1 M sodium phosphate buffer (PB), pH 7.4, containing 0.25% saponin for 30 min, and blocked by incubation for 30 min in PB containing 0.005% saponin, 10% BSA, 10% normal goat serum and 0.1% cold-water fish skin gelatin. The section was exposed overnight to anti-Hex rat polyclonal antibodies (1/1,000 dilution) in the blocking solution. After washing with PB containing 0.005% saponin, the section was incubated with colloidal gold (1.4 nm diameter, Nanoprobes Inc., NY, USA)-conjugated goat anti-rat IgG antibodies in the blocking solution. The section was then washed with PB and fixed with 1% glutaraldehyde in PB for 10 min. After washing with PBS containing 50 mM glycine, with PBS containing 1% BSA, and with MilliQ water, gold labelling was intensified with a gold enhancement kit (GoldEnhance EM, Nanoprobes Inc.) for 5 min at room temperature according to the manufacturer's instructions. After washing with MilliQ water, the section was post-fixed in 1% OsO_4 containing 1.5% potassium ferrocyanide in PB for 60 min at room temperature, and then washed again with MilliQ water. The section was dehydrated in a series of graded ethanol solutions and embedded in epoxy resin. After the epoxy resin hardened, the cover slip was removed from it. Ultra-thin sections were cut horizontally to the cryosection and doubly stained with uranyl and lead citrate. Samples were analysed with an H7600 electron microscope (Hitachi, Tokyo, Japan).

Knock-down of HEX gene

HEX knock-down ATDC5 cell line was established by pSilencer siRNA expression system (Life Technologies Corporation) according to the manual. Briefly, the oligonucleotides encoding short hairpin RNA (shRNA) inhibiting *HEX* gene expression, 5′-GATCCGA AGTTCGAGACTCAGAAATTCAAGAGATTTCTGAGTCTCG AACTTCTTA-3′ and 5′-AGCTTAAGAAGTTCGAGACTCAGA AATCTCTTGAA TTTCTGAGTCTCGAACTTCG-3′, were annealed and subsequently cloned into pSilencer4.1-CMV neo at *Hind*III and *Bam*HI sites. For the establishment of the clone stably expressing *HEX* shRNA, the plasmid-encoding *HEX* shRNA was transfected to ATDC5 cells by lipofection using Lipofectamine LTX supplemented with Plus reagent. The cells were selected with 300 μg/ml G418 (Sigma-Aldrich), and then the G418-resistant colonies were picked up and propagated. As a control, scramble sequence shRNA-expressing clones were also established. Positive clone was judged in terms of the amount of *HEX* mRNA measured by quantitative RT–PCR.

Results

Hex is expressed in chondrocyte-like cells induced from ATDC5 cells

As a model for chondrogenesis and subsequent mineral deposits, we used mouse chondrogenic cell line ATDC5 similar to mesenchymal cells (*20*, *21*, *24*). By treatment with 10 μg/ml insulin in the maintenance medium, the cells form nodule-like aggregates and differentiate into chondrocytes, which can be confirmed by Alcian blue staining (Fig. 1; *24*, *28*). Furthermore, cultivation in the mineralizing medium with insulin under 3% CO_2 for additional 3 weeks can differentiate mineralizing chondrocytes from the chondrogenic ATDC5 cells, which were positive for Alizarin red staining (Fig. 1; *25*). With the additional cultivation of ATDC5 cells in the maintenance medium with insulin after 3 weeks, the cells were negative for Alizarin red staining (data not shown; *21*).

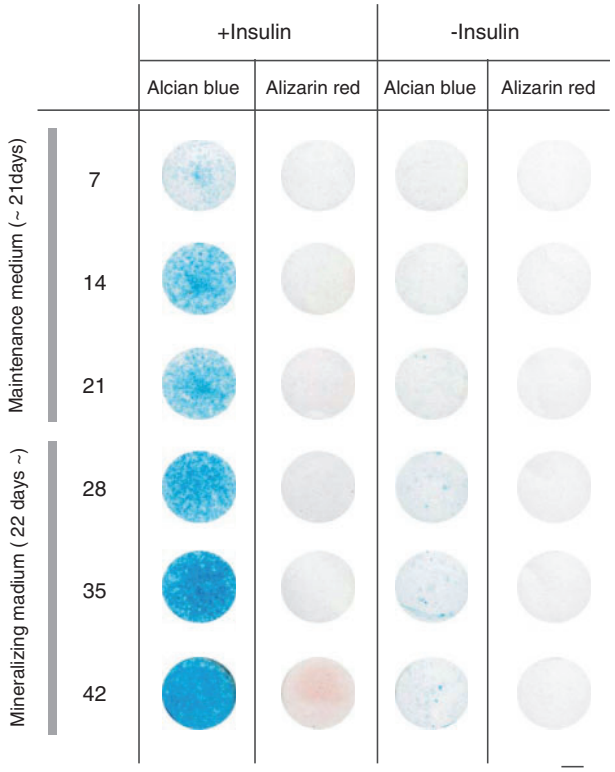

Fig. 1 Differentiation of ATDC5 cells into chondrocyte-like cells by treatment with insulin. ATDC5 cells were cultured in the presence or absence of 10 μg/ml bovine insulin for the indicated days. The cells were cultured in the maintenance medium under 5% CO_2 for initial 3 weeks, and subsequently the culture condition was switched to mineralizing medium under 3% CO_2. The cells were stained with Alcian blue (AB) and Alizarin red (AR) at the indicated days. The culture condition and staining method are precisely described in 'Materials and Methods' section.

To study the expression and quantification of *HEX* mRNA in chondrocyte differentiation and mineralization, we performed quantitative RT–PCR analysis of induced ATDC5 cells by insulin. *HEX* transcript was detected from uninduced ATDC5 cells, and the amount of *HEX* transcript in ATDC5 cells significantly increased during culture in the maintenance medium under 5% CO_2 for 28 days (Fig. 2A).

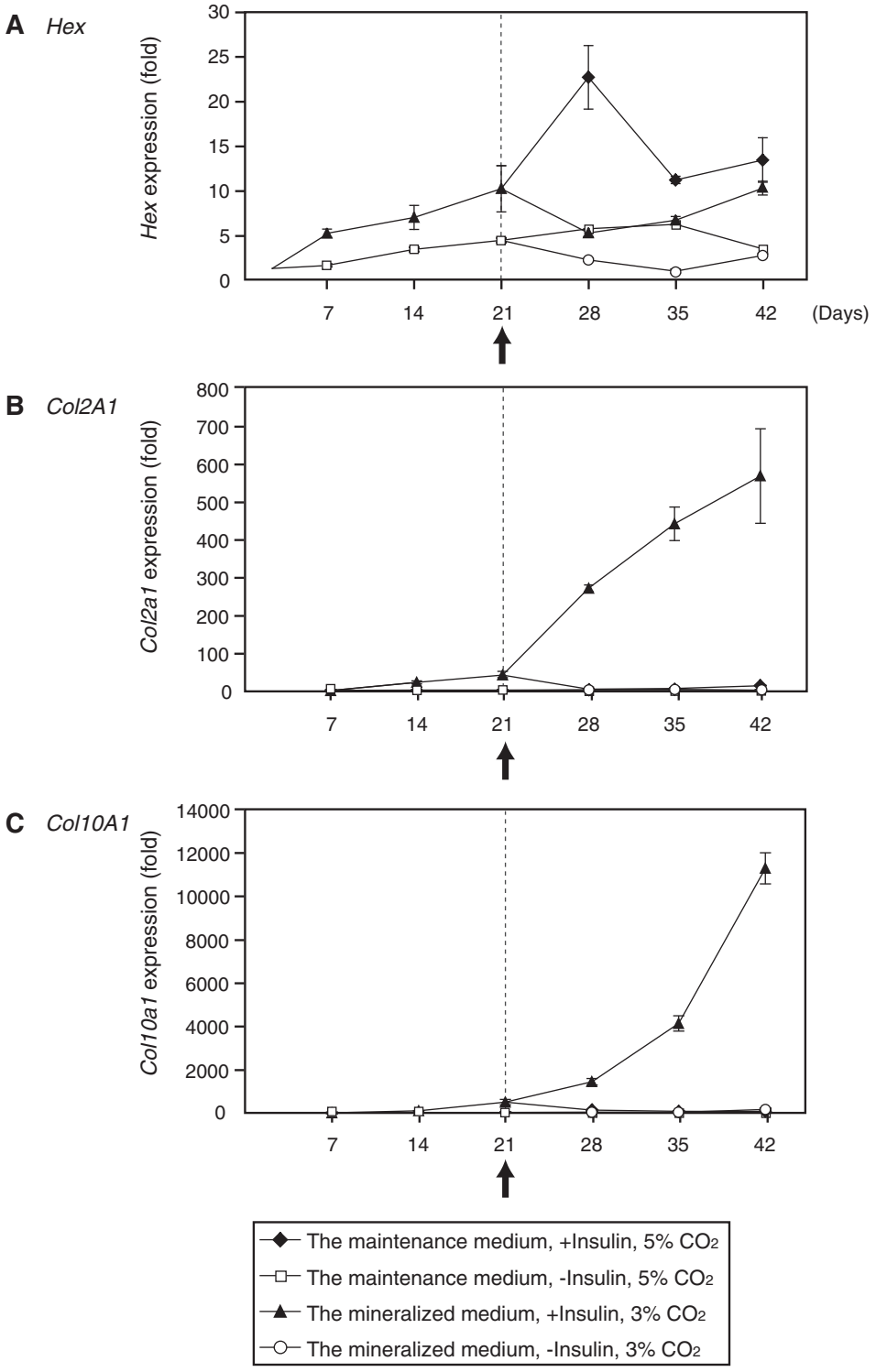

Fig. 2 Chondrogenic differentiation enhances the expression of *HEX* mRNA. The amount of *HEX* (A), *COL2A1* (B) or *COL10A1* (C) transcript was determined by quantitative RT–PCR analysis. The cells were cultured in the maintenance medium under 5% CO_2 for initial 3 weeks with (filled rhomboids) or without insulin (open squares). Arrow indicates the timing when the culture condition was switched. Filled triangles and open circles are in the cultivation in the mineralizing medium under 3% CO_2, respectively. The detail of the culture condition is described in 'Materials and Methods' section. These results were normalized by β-actin and were compared with those of cultured cells without insulin for 3 days. The results are shown as means ± SD, $n = 3$.

The elevation of *HEX* expression transiently decreased by switching the culture condition to facilitate mineralization after 21 days. *COL2A1,* which encodes the type II collagen α (I) chain, and *COL10A1,* which encodes the type X collagen α (I) chain, are marker genes of resting and proliferating chondrocytes and hypertrophic chondrocytes, respectively. Transcripts of *COL2A1* and *COL10A1* drastically increased in the mineralizing condition (Fig. 2B and C). It was noted that the levels of *COL2A1* and *COL10A1* expression were eliminated in the maintenance medium under 5% CO_2 after 21 days.

Subsequently, we analysed the expression of Hex protein in differentiated ATDC5 cells. We prepared the antibodies against the N-terminus and the C-terminus of Hex, and checked their specificity. Both antibodies specifically detected the single ~38 kDa polypeptide in uninduced ATDC5 cells transfected with exogenous Hex by western blotting while the polypeptide was not detected in the lysate transfected with vector alone (Fig. 3A and B). It migrated to a position corresponding to 38 kDa although Hex protein is predicted to be 30 kDa (Fig. 3A and B). This result is similar to those in previous reports, where Hex polypeptide was detected as 35–37 kDa (mouse: 271 amino acids) and 40 kDa (human: 270 amino acids) by western blotting (*29*, *30*). Immunohistochemistry using the antibodies against C-terminus of Hex also showed high specificity (Fig. 3C, upper panels). We note that exogenous Hex protein in uninduced ATDC5 cells was predominantly localized in the nucleus (Fig. 3C, lower panels).

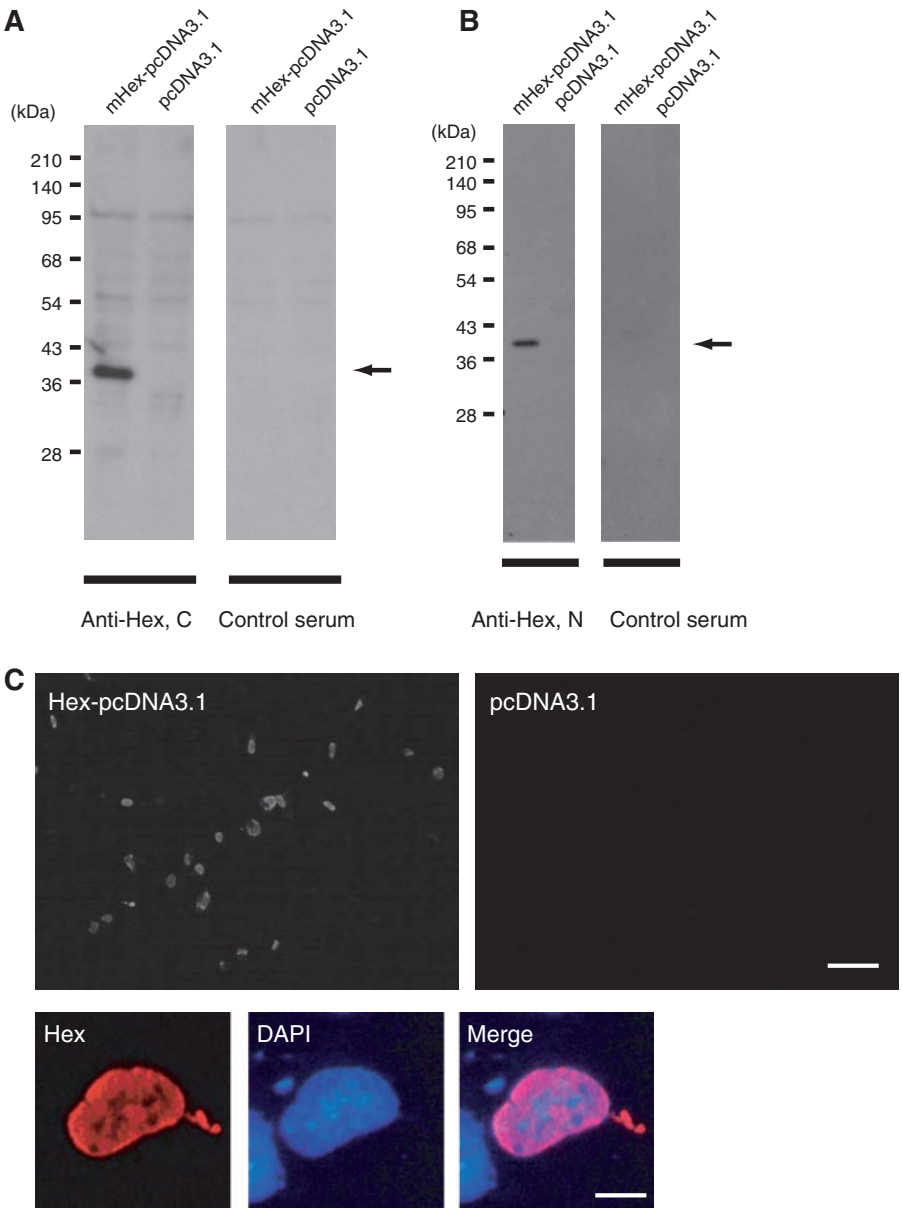

Fig. 3 Specificity of anti-Hex antibodies. (A and B) Western blotting. Whole-cell lysate of ATDC5 cells transfected with Hex was separated by SDS–PAGE and immunoblotted with anti-Hex, C terminus rat polyclonal antibodies (A), or anti-Hex, N terminus rabbit polyclonal antibodies (B). Blotting with control serum on the same blot is also shown. The arrow indicates Hex polypeptide. (C) Immunostaining of undifferentiated ATDC5 cells that overexpressed Hex alone with anti-Hex rat polyclonal antibodies. Scale bar of the upper panels = 100 μm. The lower panels are enlarged images of a cell in the upper panel. The nucleus of the cell was visualized with 4′,6-diamidino-2-phenyindole (DAPI). Scale bar = 10 μm.

These results indicated that the antibodies specifically recognized Hex protein. Next, we examined the detection of endogenous Hex protein by immunoprecipitation with anti-Hex, N-terminus rabbit polyclonal antibodies followed by immunoblotting using anti-Hex, C-terminus rat polyclonal antibodies. An immunoreactive polypeptide of ~38 kDa was faintly detected from the immunoprecipitant with anti-Hex antibodies from the lysate of mineralized ATDC5 cells, like hypertrophic chondrocytes (Fig. 4A). Neither the lysate from the cells cultured for 21 days in maintenance medium with or without insulin nor that cultured in the mineralizing condition without insulin for 42 days gave a positive result. We failed to detect immunoreaction by western blotting with both antibodies in whole-cell lysate prepared from mineralizing ATDC5 cells for

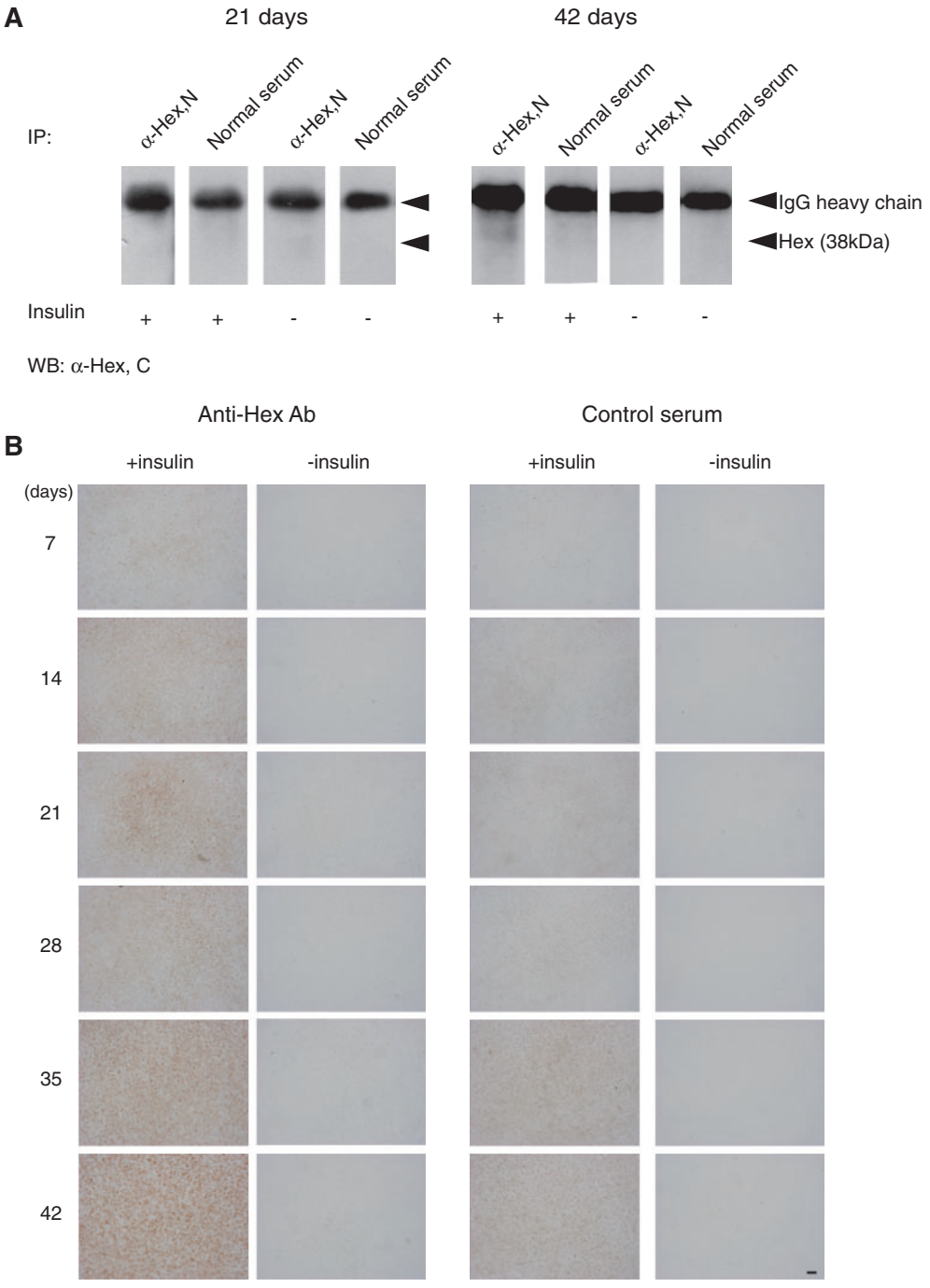

Fig. 4 Differentiation-enhanced expression of Hex protein in ATDC5 cells. (A) Immunoprecipitation. ATDC5 cells were differentiated into hypertrophic chondrocyte-like cells as described in 'Materials and Methods' section. Whole cell lysate of the cells was immunoprecipitated with anti-N terminus of Hex rabbit polyclonal antibodies, followed by immunoblotting with anti-C terminus of Hex rat polyclonal antibodies. (B) Immunohistochemistry. The cells were cultured in the maintenance medium under 5% CO_2 for 21 days followed by in the mineralizing medium under 3% CO2 for additional 21 days. The samples were stained with anti-C terminus of Hex rat polyclonal antibodies (left panels). The results of staining of control serum (right panels) are also shown. Scale bar = 10 μm.

42 days (data not shown). Because the expression level of Hex protein was low in the ATDC5 cells, we could not detect it by western blotting, as was shown for other homeoproteins (*31*). Immunohistochemistry also detected the up-regulation of Hex protein in the ATDC5 cells upon stimulation by insulin (Fig. 4B, left panels). On the other hand, the immunoreaction in the uninduced cells was under the detection limit and the immunoreaction using control serum was not detected (Fig. 4B, right panels). We established the ATDC5 clone stably expressing shRNA repressing *HEX* expression (Supplementary Fig. S1A). Compared with the clone expressing scramble shRNA induced by treatment with insulin for 21 days, weakened immunoreaction with anti-Hex was detected in the clones expressing HEX shRNA (Supplementary Fig. S1B). These results indicated that Hex protein is expressed in differentiated ATDC5 cells, and the anti-Hex antibodies recognize endogenous Hex protein by immonohistochemistry.

Hex protein moves between nucleocytoplasm during differentiation in mouse tibia

To examine whether Hex is expressed in chondrocytes *in vivo*, we analysed the tibiae of mouse embryos at E15.5, the stage where the differentiation and maturation of chondrocytes are in progress. The stage of chondrocytes in the cartilage is distinguishable by morphological criteria. From the end of the cartilage primordium, the rounded cells are resting chondrocytes, the flattened and aligned cells that form columnar layers parallel to the long axis are proliferating chondrocytes, and the cells that increase in volume followed by shrinkage are hypertrophic chondrocytes. *In situ* hybridization with *HEX* antisense probe to the tibia revealed that *HEX* mRNA was predominantly detected in proliferating chondrocytes, and the weak signal was also detected in resting chondrocytes (Fig. 5A, upper panel). Hybridizing with sense probe, very weak signal was detected in resting and proliferating cells (Fig. 5A, lower panel). The signals of *COL2A1* and *COL10A1* were indicated for resting and proliferating chondrocytes, and hypertrophic chondrocytes, respectively (Fig. 5B and C). These results indicated that *HEX* transcript in embryonic tibia was predominantly expressed in resting and proliferating chondrocytes. Next, we examined the expression and the subcellular localization of Hex protein by immunohistochemistry in the tibiae of mouse embryos. The immunoreaction with anti-Hex antibodies in the tibia was detected in chondrocytes, which increased during chondrocyte maturation (Fig. 6A). The immunoreaction was eliminated by pre-absorption with antigenic protein (Fig. 6B). The intracellular localization of Hex protein shifted from cytoplasm to nucleus, and thereafter to cytoplasm during maturation (Fig. 7A). Immunoelectron microscopy revealed Hex immunoreactivity localized in the cytoplasm near the rough ER in resting chondrocytes and localized in the cytoplasm and the nucleus at the proliferating stage, and predominantly localized in the nucleus in pre-hypertrophic chondrocytes (Fig. 7B). In hypertrophic chondrocytes, the immunoreactivity of Hex mainly localized in swollen ER, and in the nucleus

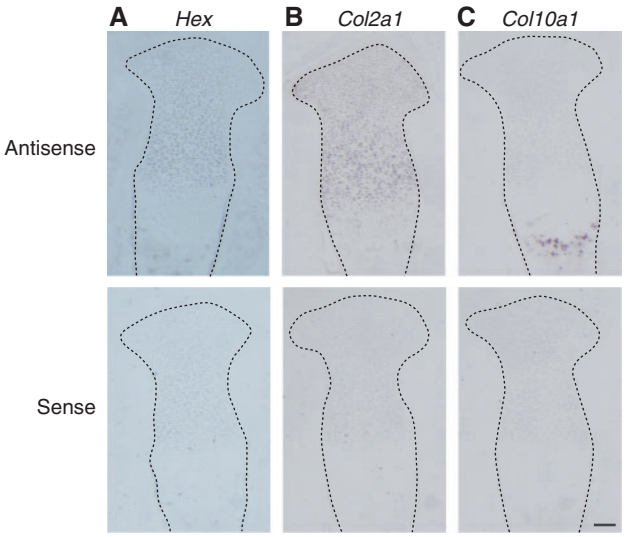

Fig. 5 Expression of *HEX* mRNA in cartilage in mouse tibia as revealed by *in situ* hybridization. The digoxigenin-labelled antisense (upper panels) or sense riboprobe (lower panels) to *HEX* (A), *COL2A1* (B) and *COL10A1* mRNA (C) was hybridized. Dotted line indicates the outer boundary of the cartilage primodium. Scale bar = 100 μm.

in a small portion of cells (Fig. 7A and B, data not shown). We did not explain its physiological significance in this stage because the disrupted cell structure obviously showed cell death. In the control experiment in which normal rat serum was used instead of primary antibody, immunoreactivity was scarcely observed (Supplementary Fig. S2). Finally, we concluded that Hex protein is expressed and shuttles between the cytoplasm and the nucleus in differentiating chondrocytes of mouse.

Discussion

Here, we show that Hex is expressed in mouse chondrocytes and its protein is transported between the cytoplasm and the nucleus during differentiation of chondrocytes in embryonic tibia. This is the first evidence for this derived from precise analysis. However, the physiological function of Hex remains unclear.

The differentiation of chondrocytes was shown to be regulated by complex and harmonious cascades of transcription factors (*1*). *HEX* expression dramatically up-regulated in ATDC5 cells in the chondrogenic differentiation-inducing culture condition for 28 days compared with the cultivation in mineralization-inducing condition (Fig. 2A). This result suggests that the transcription of *HEX* gene is mainly induced during differentiation to early (resting and proliferating) chondrocytes rather than mineralizing (hypertrophic) chondrocytes, which is in good agreement with the result of *in situ* hybridization (Fig. 5). Now we cannot clearly explain the reason why *HEX* expression pattern is biphasic in the mineralizing condition. We expect that the transcription of *HEX* gene is regulated by different transcription factors in the chondrogenic culture condition and the mineralizing condition because *HEX* gene expression is controlled in a tissue-dependent

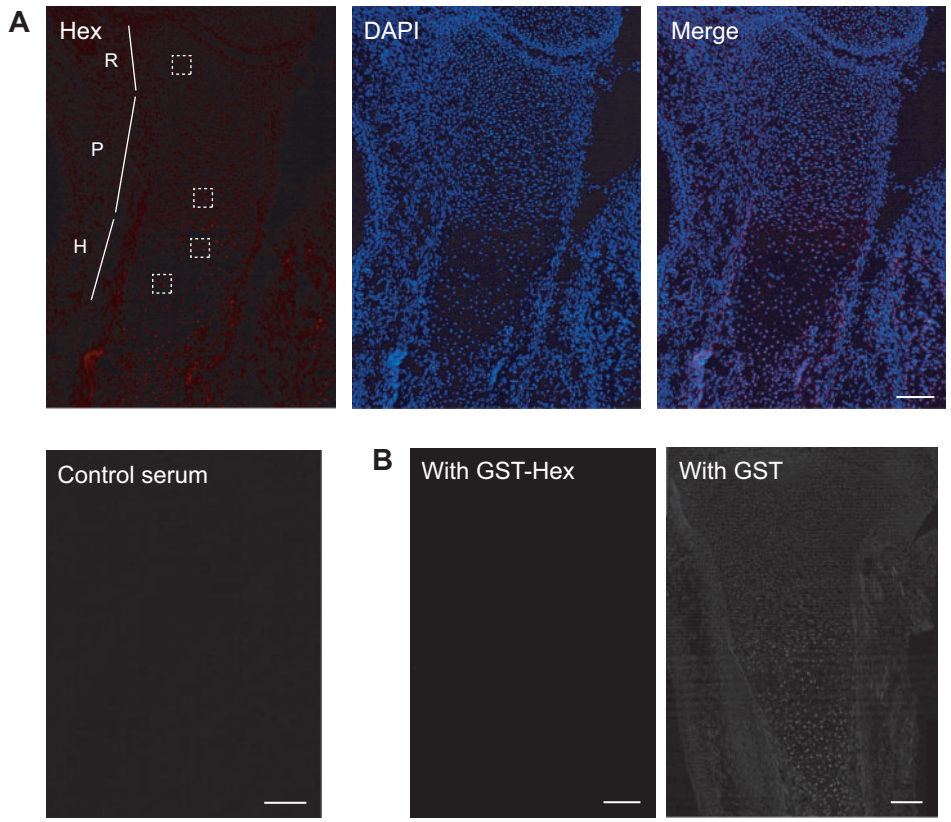

Fig. 6 Expression of Hex protein in the tibia of E15.5 embryonic mouse. The frozen section of the embryonic mouse hindlimb at E15.5 was stained with anti-Hex, C-terminus rat polyclonal antibodies (A). R, resting chondrocytes; P, proliferating chondrocytes; H, hypertrophic chondrocytes. (B) Pre-absorption of anti-Hex antibodies by the antigenic peptide for immunohistochemical staining. Anti-Hex antibodies were absorbed by incubation with GST-Hex (left panel) or GST (right panel) and were used as primary antibodies. Scale bar = 100 μm.

transcription factors (*9*). For instance, the promoter region of *HEX* gene is bound to SP1 and SP3 in liver and hematopoietic cell line (*32*, *33*). Furthermore, *HEX* gene is activated by binding of HNF3 and GATA-4 to the promoter region in liver cells (*34*). *HEX* has a blood-islet specific enhancer in the first intron (*35*, *36*). In thyroid cells, *HEX* gene is up-regulated by homeoproteins TFF and Pax (*37*, *38*). It is interesting that the upstream region of avian *HEX* gene contains a bone morphogenic proteins (BMPs) responsive element (BRE), therefore, the transcription of *HEX* gene in avian lateral endoderm at an early developmental stage is up-regulated by BMPs in an autocrine manner (*39*). This is an issue to resolve in the future.

We showed that Hex protein moved between the nuclei and the cytoplasm, depending on maturation in the tibia of mouse (Fig. 7). Indeed, Hex has a potential nuclear localization signal (NLS) in residues 189–195, and a nuclear export signal (NES) in residues 171–184, with both motifs localized in the homeodomain (*29*, *40*, *41*). These findings suggest that the function of Hex as a transcription factor is controlled by nucleocytoplasmic transport in a differentiation stage-specific manner. Therefore, Hex might mainly act in prehypertrophic stage because of its nuclear localization although *HEX* mRNA is dominantly expressed in proliferating chondrocytes (Figs 2 and 5–7). It is reported tissue- and cancer-specific subcellular localizations of Hex protein (*23*, *42*, *43*). For example, Hex protein is associated with promyelocytic leukemia (PML) body in the nucleus in U937 and K562, whereas Hex is localized in the nucleoplasm and the cytoplasm in Hep G2 cells. It is of interest how nucleocytoplasmic transport of Hex protein is regulated upon chondrocyte differentiation. We have two hypotheses about the mechanism controlling the localization of Hex protein: one is the interaction with the nuclear import factor which is expressed in a specific stage of chondrocyte maturation; the other is the post-translational modification of Hex protein regulating interaction with the nuclear-import factor. For the former case, Hex might be imported to the nucleus with the specific subtype of importin-α expressed in pre-hypertrophic stage. Importin-α is the molecule that acts as a classical NLS receptor and is classified into three subtypes, which recognize distinct cargoes. In ES cells, the expression of importin-α subtype is switched from α1 to α5 depending on neural differentiation, and forced expression of importin-α5 triggers neural differentiation (*44*). Hex is imported into the nucleus depending on interaction with karyopherin/importin 7 (Kap7) in Hep G2 cells (*41*). Kap7 might be expressed and import Hex in the nucleus in prehypertrophic stage. In the latter case, we suspect that Hex may be modified by phosphorylation. S163 and S177 of Hex protein were phosphorylated and the phosphorylation of these sites is necessary to bind DNA in

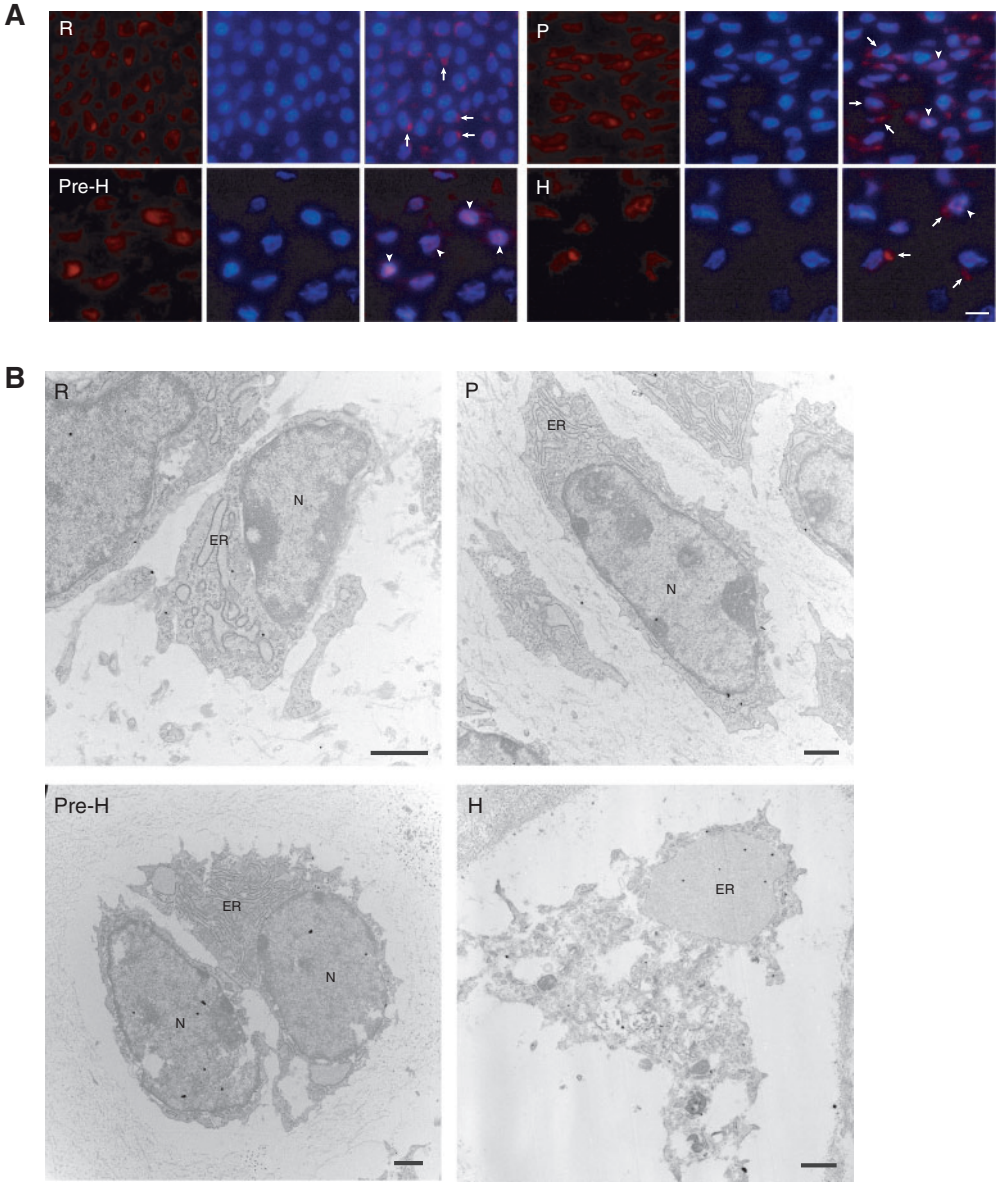

Fig. 7 Subcellular localization of Hex protein in the tibia of E15.5 embryonic mouse. A is shown at higher magnification for boxed areas in Fig. 6A. R, resting chondrocytes; P, proliferating chondrocytes; Pre-H, prehypertrophic chondrocytes; H, hypertrophic chondrocytes. Arrows and arrowheads indicate Hex immunoreaction in cytoplasm and nucleus, respectively. Scale bar = 10 μm. (B) Immunoelectron microscopic images of Hex protein in each stage of chondrocytes (B). N, nucleus; ER, endoplasmic reticulum. Scale bar = 1 μm.

K562 cell (*45*). Posphorylated Hex might interact with nuclear import factors. In terms of the localization of cytoplasm near ER membrane in resting chondrocytes, we suspect that Hex interacts with the ER membrane-associated protein in resting chondrocytes, and is then imported into the nucleus depending on maturation. For example, BBF2H7, an ER-resident basic leucine zipper transcription factor that has a transmembrane domain, is cleaved at transmembrane domain by regulated intramembranous proteolysis in response to ER stress, which is a physiological event in chondrogenesis for the synthesis of massive cartilage matrix, and located in the nucleus and then activates transcription of *Sec23a* gene to facilitate matrix secretion (*46*).

However, we could not resolve the physiological function of Hex in skeletal development. Although *HEX* knock-out mice are established, because of death around E10.5, the phenotype of the mice in skeletal development remains unclear (*10*, *11*). We found that *HEX* gene silence by RNA interference did not affect the expression of *COL2A1* and *COL10A1* genes in differentiating ATDC5 cells (R.M., unpublished observations). We speculate that Hex does not affect that the gene directly regulates chondrocyte differentiation but rather regulates a prehypertrophic chondrocyte-specific gene. We found that overexpression of *HEX* gene, whose protein localized in the nuclei, in uninduced ATDC5 cells caused cytotoxicity similarly to that in hematopoietic cells except T-cells (R.M. and A.O., unpublished observations, *47*). In K562 cells, *HEX* overexpression induced cell death (*48*). Hex might be associated with cell death after hypertrophy. Hence, we expect that this is the reason why the

expression level of Hex in cartilage remains low, which led to the detection of only a weak signal by immunoprecipitation (Fig. 4). Further studies, including analyses using the ATDC5 cells stably expressing *HEX* shRNA, are in progress in our laboratory.

Supplementary Data

Supplementary data are available at *JB* Online.

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Conflict of interest
None declared.

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