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Expression patterns of βig-h3 in chondrocyte differentiation during endochondral ossification

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Abbreviations: Col X, type X collagen; PC, perichondrium; PH, prehypertrophic chondrocytes; PO, periosteum; βig-h3, TGF-β-induced gene

Abstract

βig-h3 is a TGF-β-induced extracellular matrix protein which is expressed in many tissues including bones and cartilages. In previous reports, we showed that βig-h3 mediates cell adhesion and migration and, especially in bones, negatively regulates the mineralization in the end stage of endochondral ossification. Here, to elucidate the expression pattern and role of βig-h3 in chondrocyte differentiation, ATDC5 chondrocytes and embryonic and postnatal mice were used for *in vitro* differentiation studies and *in vivo* studies, respectively. βig-h3 was strongly induced by the treatment of TGF-β1 and the expression level of βig-h3 mRNA and protein were highly expressed in the early stages of differentiation but decreased in the late stages in ATDC5. Furthermore, the patterns of TGF-β1, -β2, and -β3 mRNA expression were concurrent with βig-h3 in ATDC5. βig-h3 was deeply stained in perichondrium (PC), periosteum (PO), and prehypertrophic chondrocytes (PH) through the entire period of endochondral ossification in mice. βig-h3 was mainly expressed in PC and PH at embryonic days and obviously in PH in postnatal days. These results suggest that βig-h3 may play a critical role as a regulator of chondrogenic differentiation in endochondral ossification.

Keywords: cell differentiation; chondrocytes; osteogenesis; transforming growth factor-β

Introduction

Bone formation in vertebrates is classified into intramembranous and endochondral ossification (Olsen *et al*., 2000). The flat bones, which are found in the skull and mandible, are formed by intramembranous ossification whereas most other bones are produced by endochondral ossification arising from a cartilaginous template. During the endochondral ossification process, mesenchymal cells differentiate into chondrocytes following that the chondrocytes differentiate into the proliferative, prehypertrophic, hypertrophic and, finally calcified chondrocytes. *In vivo* and *in vitro* differentiation of chondrocytes is primarily characterized by the expression of the extracellular matrix (ECM) proteins including collagens (Metsaranta *et al*., 1992; Nakata *et al*., 1993), proteoglycans (Goetinck, 1991), and non-collagenous glycoproteins (Chen *et al*., 1995). With many reports for ECM proteins regulating chondrocyte differentiation, it has been suggested that ECM proteins may be one of the most important controllers for endochondral ossification.

βig-h3 is an ECM protein that is first identified in a human lung adenocarcinoma cell line by treatment of TGF-β1 (Skonier *et al*., 1992) and is strongly induced by TGF-β in various types of cells (Skonier *et al*., 1994; LeBaron *et al*., 1995; Thapa *et al*., 2007). βig-h3 is ubiquitously detected in normal tissues such as the heart, kidneys, liver, and skin and involved in cell adhesion, growth and migration, tumorigenesis, wound healing, and apoptosis, indicating that it may have an important function throughout the body (Skonier *et al*., 1992; 1994; Kim *et al*., 2000a; 2002; 2003; Park *et al*., 2004; Lee *et al*., 2006; Jung *et al*., 2007). During mouse embryogenesis, βig-h3 is mainly expressed in bones, cartilages and other mesoderm-derived organs (Schorderet *et al*., 2000). Our recent study has demonstrated the involvement of βig-h3 in endochondral ossification showing the inhibitory effect and molecular mechanisms on osteoblast differentiation (Kim *et al*., 2000b; Thapa *et al*., 2005). Members of the TGF-β superfamily are important regulators of chondrocyte differentiation during endochondral ossification. Several studies

report that TGF-β localizes in hypertrophic chondrocytes and vascular structure (Thorp *et al*., 1992), and TGF-β mediated differentiation of hypertrophic chondrocytes is required for the maintenance of the articular cartilage (Yang *et al*., 2001) and is involved in apoptosis (Gibson *et al*., 2001). These reports imply that βig-h3 induced by TGF-β may be involved in endochondral ossification. However, the precise expression pattern and function of βig-h3 in chondrocyte differentiation during endochondral ossification still remains obscure.

The present study was designed to investigate βig-h3 expression patterns during endochondral ossification. βig-h3 was dramatically induced by TGF-β1 in ATDC5, mouse embryonic carcinoma derived cell line which is frequently used for the *in vitro* model of chondrocyte differentiation (Akiyama *et al*., 1997). We analyzed the expression pattern of βig-h3 and chondrogenic marker genes during chondrocyte differentiation and examined the βig-h3 expression in long bones during mouse development. Thus, the importance of βig-h3 mediating chondrogenic differentiation was indicated.

Materials and Methods

Cell culture

The mouse embryonic carcinoma derived chondrocyte cell line, ATDC5 was cultured in a 1:1 mixture of DMEM and Ham's F-12 (DMEM/F12) medium (Invitrogen corp., Carlabad, CA) supplemented with 5% FBS (Invitrogen), 10 μg/ml human transferrin (Roche, Germany), and 30 nM sodium selenite (Sigma, St. Louis, MO). To examine the expression level of βig-h3 induced by TGF-β1 treatment, ATDC5 cells were plated at a density of 2×10^5 cells/well in 6-well culture dishes, grown to 80% confluency, and then treated for 48 h with various concentrations (0, 0.2, and 1 ng/ml) of recombinant TGF-β1 (R&D systems, Minneapolis, MN). Cultured media were collected, lyophilized, and analyzed by western blotting. To investigate the βig-h3 expression during differentiation, ATDC5 cells were seeded in 6-well culture dishes at a density of 6×10^4 cells/well and cultured in media additionally supplemented with 10 μg/ml of human insulin (Lilly, Indianapolis, IN). After 21 days, the culture medium was switched to minimal essential medium-alpha (α-MEM, Invitrogen) and grown at 3% CO_2 to facilitate hypertrophy and mineralization of cells. On days 4, 7, 14, 21, 28, 35, and 42, culture media and cells were harvested for western and northern blot analysis.

Western blot analysis

The harvested culture media were lyophilized prior to western blotting. Three μg of each sample was mixed with a 2× sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiled for 5 min. Then, the samples were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, UK). The membrane was incubated for 2 h at room temperature (RT) with anti-mouse βig-h3 antiserum (diluted 1:2,000 in PBS) and reacted with peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:2,000, Amersham). The blot was then identified by the enhanced chemiluminescence (ECL) system (Amersham).

Total RNA isolation and northern blot analysis

Total RNA was isolated from cultured ATDC5 cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Ten μg of total RNA was fractionated on 1% agarose gel containing formaldehyde, transferred onto a nylon membrane (Amersham) by capillary method using 20× sodium chloride sodium citrate buffer (SSC, pH 7.0), and cross-linked to membranes by UV irradiation (UV Stratalinker 1800, Stratagene, La Jolla, CA). DNA probes were labeled with 30 μCi [α-^{32}P] dCTP using a random priming Megaprime DNA labeling Kit (Amersham). The hybridization was performed at 68°C for 3 h using ExpressHyb hybridization solution (Clontech, Mountain View, CA). After hybridization, the membranes were sequentially washed with 2× SSC/0.05% SDS and 0.1× SSC/0.1% SDS solutions to remove nonspecific bound probes and then exposed to X-ray film (AGFA) for 3 days at -70°C before development to detect hybridization signals.

Immunohistochemistry

To analyze the expression pattern of βig-h3, femora from ICR mice of embryonic day 13.5 (E13.5) and E15.5, and postnatal day 7 (P7) and P14 (Korea Biolink, Korea) were removed and fixed in 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer for 16 to 42 h at 4°C. After fixation, the femora from postnatal mice were decalcified in 10% EDTA, pH 7.4 for 2 to 20 days. Then, specimens were dehydrated through ethanol series, embedded in paraffin, and cut into 5 μm sections. Deparaffinized sections were quenched in 1% H_2O_2 in methanol, washed in PBS (pH 7.8), and blocked with normal goat serum for 2 h at RT. The

sections were incubated overnight at 4°C with rabbit anti-mouse βig-h3 antiserum diluted 1:2000 in PBS. After washing in PBS, the sections were incubated with biotinylated-anti-rabbit IgG antibody for 1 h at RT, reacted for 1 h with VETASTAIN *elite* ABC Reagent (Vector Laboratories, Burlingame, CA) and then developed using DAKO Liquid DAB$^+$ Substrate-Chromogen System (DAKO, Denmark). Counterstaining was performed with 1% methyl green in ddH_2O. Non-immune rabbit IgG was used as a control of immunohistochemistry (IHC).

In situ hybridization

In situ hybridization on sections of mouse femora aged P7, P14, and P21 was performed by using [^{35}S] uridine triphosphate (UTP)-labeled riboprobes as described previously (Park *et al*., 2001). Antisense and sense riboprobes for βigh3 digested with *Eco*RV or *Bam*HI were produced by T7 and T3 RNA polymerase, respectively. A type X collagen (ColX) probe was prepared as previously described (Inada *et al*., 1999). [^{35}S] UTP-labeled riboprobes that adjusted to 5×10^4 cpm/μl were denatured and placed in each slide which was digested with 10 μg/ml proteinase K. Hybridization was carried out overnight in a humidified box at 52°C, followed by high stringency washes with 50% formamide and 20 mM DTT at 65°C. For autoradiography, the dehydrated slides were dipped into photographic emulsion (Kodak NTB-2, Eastman Kodak, Rochester, NY), dried, and exposed for 2-3 weeks at 4°C. The slides were then developed (Kodak D-19, Eastman Kodak), fixed (Kodak Unifix, Eastman Kodak), counterstained with hematoxylin, and mounted with DePeX (BDG). The tissue morphology and *in situ* hybridization result were examined under the bright field and dark field of the fluorescence microscope, respectively.

Results

Expression of βig-h3 mRNA and protein during *in vitro* differentiation of ATDC5 cells

As βig-h3 is known to be induced by TGF-β in many different cells, we examined whether TGF-β induces the expression of βig-h3 in ATDC5, a mouse embryonic carcinoma derived cell line. ATDC5 cells were treated with various concentrations of TGF-β1 for 48 h and cultured media were then harvested to analyze the βig-h3 expression by western blotting (Figure 1A). Even

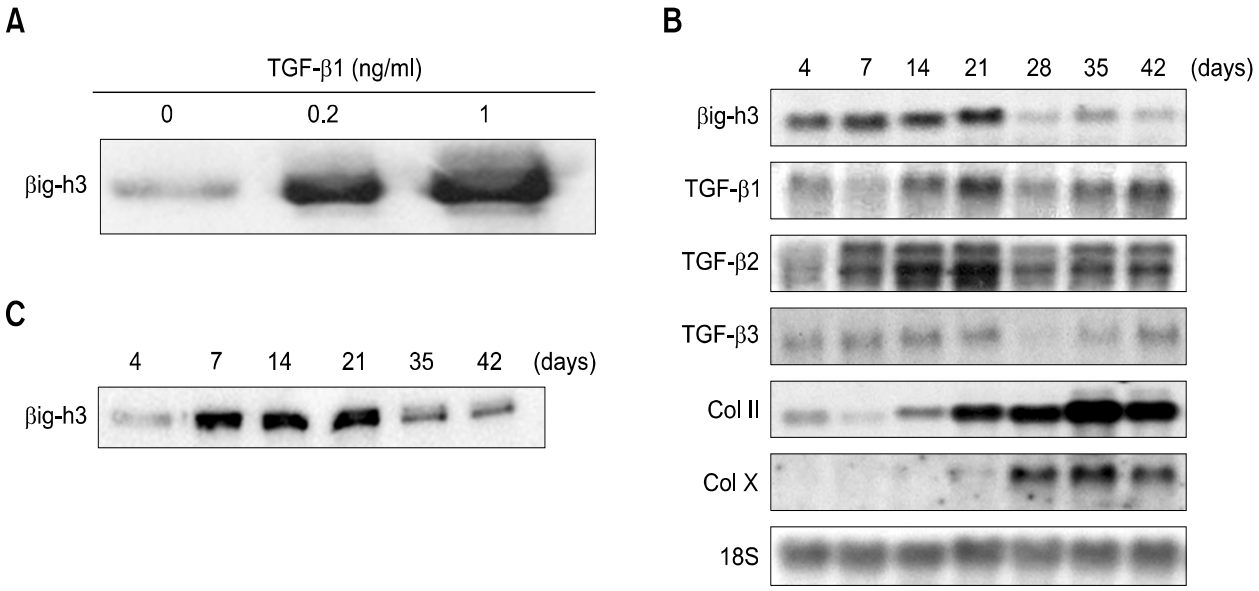

Figure 1. Expression of βig-h3 in differentiated ATDC5 cells. (A) TGF-β1-induced βig-h3 expression in ATDC5 cells. The ATDC5 cells were seeded in 6-well culture dishes at a density of 2×10^5 cells per well and treated with the indicated concentrations of TGF-β1. After incubation for 48 h, βig-h3 expression from culture media was analyzed by western blotting. (B) The mRNA expression of βig-h3 and marker genes during chondrogenic differentiation of ATDC5 cells. Total RNA was extracted from ATDC5 cells on indicated differentiation times and northern blotting was performed using specific probes for βig-h3, TGF-β1, -β2, -β3, collagen type II (ColII), and X (ColX). The consistency of RNA loading in each lane was evaluated by reprobing the membrane with 18S rRNA probe. (C) The expression of βig-h3 protein during chondrogenic differentiation of ATDC5 cells. ATDC5 cells were seeded at a density of 6×10^4 cells/well in 6-well dishes and differentiated for 42 days. On indicated time periods, cultured media were harvested and the expression levels of βig-h3 protein were determined by western blotting.

though the endogenous βig-h3 level was quite low in ATDC5 cells, the expression of βig-h3 was induced by TGF-β and the level of induced βig-h3 protein was prominent even at a low concentration of 0.2 ng/ml of TGF-β1. To investigate the expression level of βig-h3 during chondrocyte differentiation, ATDC5 cells were differentiated into chondrocytes for 42 days under the insulin-induced culture condition. Time-dependent changes with cartilage nodule formation were verified in differentiated ATDC5 cells by alcian blue staining (data not shown). To determine the expression level of βig-h3 and marker genes during *in vitro* chondrogenic differentiation, northern blot analysis was performed at an indicated time point from differentiated ATDC5 cells (Figure 1B). Up-regulation of βig-h3 expression was observed in an early time of *in vitro* differentiation and was sustained for 3 weeks prior to a rapid decrease at late stages of differentiation. Although the expression patterns of βig-h3 were similar to TGF-β mRNA, unlike βig-h3, a weak expression of TGF-β1 and -β2 was still detected in the late stages of differentiation. Type II and X collagen, which are marker genes of chondrocyte differentiation, were also observed in differentiating ATDC5 cells. During *in vitro* differentiation, ATDC5 cells proliferated and formed cartilage nodules with an expression of type II collagen (ColII) and, subsequently, hypertrophied and synthesized type X collagen (ColX) in the late stages of differen-

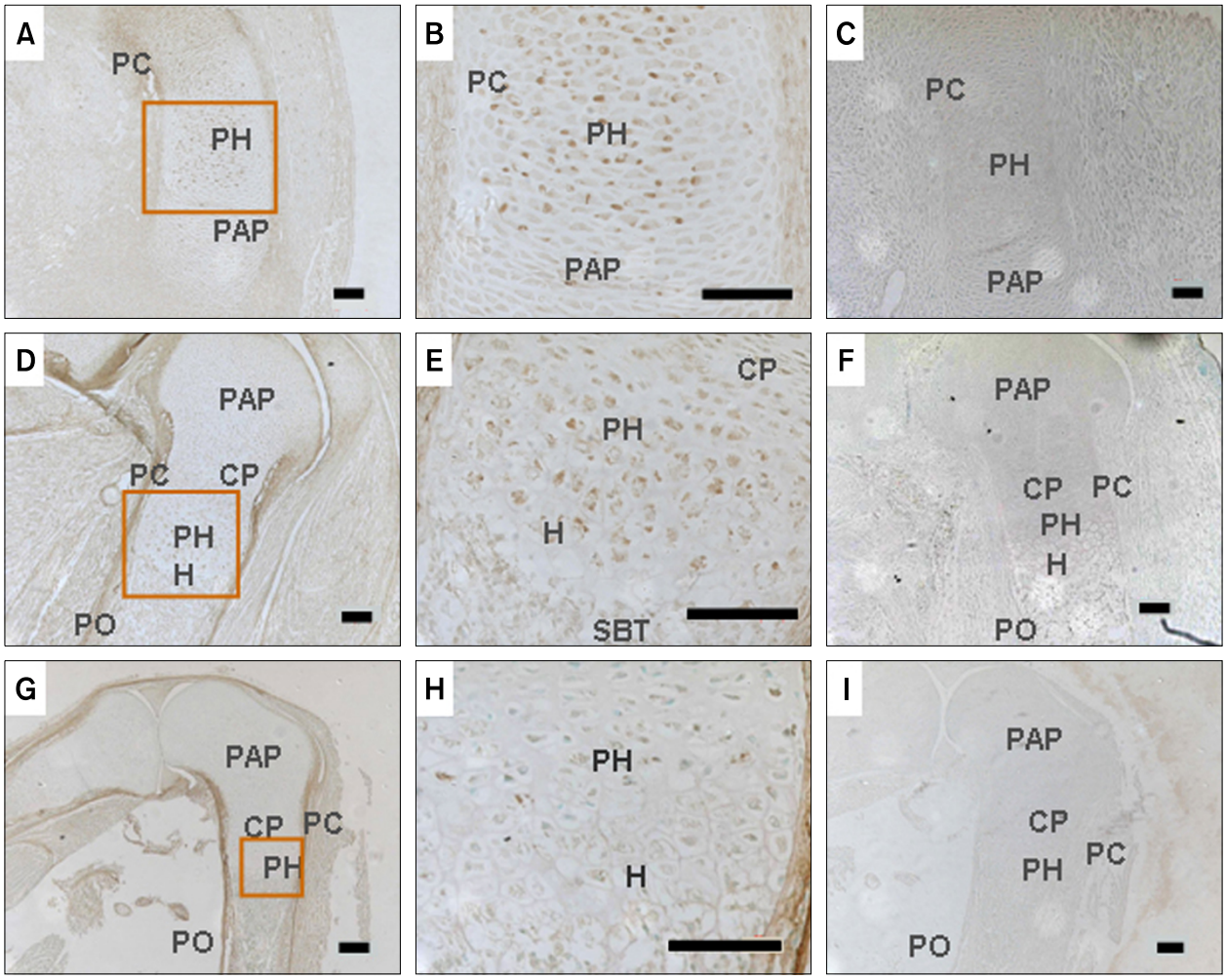

Figure 2. Immunohistochemical detection of βig-h3 protein in the femur of mouse embryos at embryonic periods. Paraffin sections from femoral bone of ICR mouse embryos at embryonic day 13.5 (E13.5) (A), E15.5 (D), and E18.5 (G) were prepared to detect βig-h3 protein with rabbit anti-mouse βig-h3 antibody as described in "Materials and Methods". The sections were treated with rabbit anti-mouse βig-h3 antibody (A, D, G) or non-immune rabbit IgG (C, F, I). B, E, and H were higher-magnification images corresponding to small rectangles of A, D, and G, respectively. CP, columnar proliferating chondrocytes; H, hypertrophic chondrocytes; PAP, periarticular proliferating chondrocytes; PC, perichondrium; PH, prehypertrophic chondrocytes; PO, periosteum; SBT, spongy bone trabeculae; Scale bar, 50 μm (A-C); 100 μm (D-I).

tiation (Figure 1B). Corresponding to mRNA expression, protein expression of βig-h3 was also highly increased at an early stage and maintained at a high level for 3 weeks of *in vitro* differentiation of ATDC5 cells. Then, its expression was dramatically decreased in subsequent days (Figure 1C). This finding indicates that the up-regulation of βig-h3 expression may be important for an early stage of chondrocyte differentiation.

βig-h3 protein expression in developing mouse femur

To clarify the expression pattern of βig-h3 protein during endochondral ossification, an immunohistochemical study was performed in the mouse femur from embryonic (E) to postnatal (P) day. Periarticular proliferating chondrocytes (PAP) and prehypertrophic chondrocytes (PH) were observed in cartilage condensation of the limb buds at E13.5 of mouse development. At this time, intensive expression of βig-h3 was observed in PH differentiated from the PAP and perichondrium (PC) (Figure 2, A and B). Long bone at E15.5 of mouse has primary ossification center in which spongy bone trabeculae (SBT) and medullary cavity are observed. At E15.5, βig-h3 was widely expressed in many kinds of cells, intensively in the PH and PC, and weakly in periosteum (PO), which were shown in the femur of early embryonic development (Figure 2, D and E). The expression of βig-h3 in the PC and PO was maintained in all subsequent stages of the endochondral ossification (Figure 2G and H). No βig-h3 expression was observed in normal rabbit IgG as a negative control (Figure 2C, F, and I).

It was observed that βig-h3 expression was still retained in PC and PO of the long bones on day 7 after birth. (Figure 3A-C). βig-h3 was intensively expressed in the PH of the epiphysis in which secondary ossification center was observed. At P14, the strong expression of βig-h3 was continuously present in the PH, PC, and PO (Figure 3E-G). With the development of secondary ossification, βig-h3 was strongly expressed in the PH and newly formed spongy bone trabeculae (SBT) but weakly expressed in H. The expression of βig-h3 was constantly observed for several weeks after birth. At 5 weeks of age, βig-h3 was still

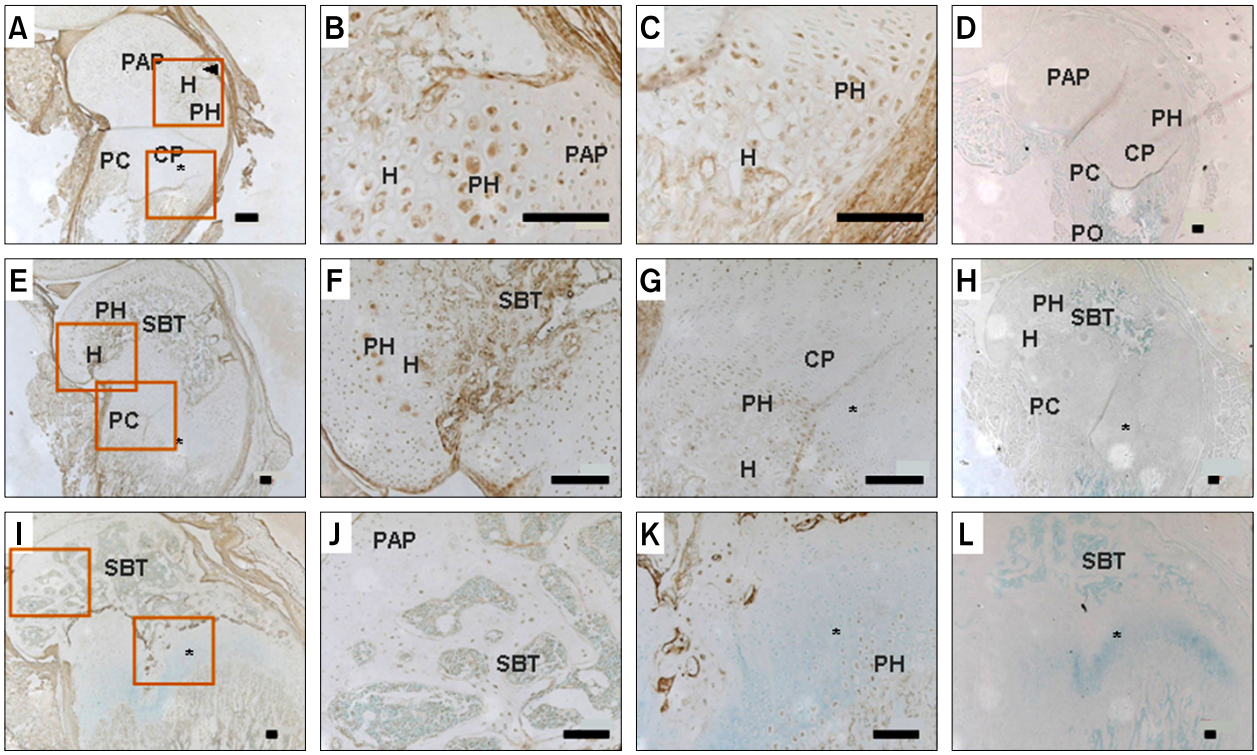

Figure 3. Immunohistochemical analysis of βig-h3 protein in mouse femur at postnatal periods. βig-h3 protein in mouse femoral bone at postnatal day 7 (P7) (A), P14 (E), and P35 (I) were detected with rabbit anti-mouse βig-h3 antibody as described in "Materials and Methods". The paraffin sections were treated with rabbit anti-mouse βig-h3 antibody (A, E, I) or non-immune rabbit IgG (D, H, L). Images in column 2 and 3 were higher-magnification images corresponding to small rectangles in column 1. CP, columnar proliferating chondrocytes; H, hypertrophic chondrocytes; PAP, periarticular proliferating chondrocytes; PC, perichondrium; PH, prehypertrophic chondrocytes; PO, periosteum; SBT, spongy bone trabeculae; arrowhead, starting point of secondary ossification center; asterisk, epiphyseal plate; Scale bar, 100 μm.

expressed in the PH, PC, and PO including ECM surrounding chondrocytes (Figure 3, I-K). Interestingly, the strong expression of βig-h3 was detected at the newly formed SBT of the secondary ossification center and in a region that was invaded by vascular structures containing osteoblasts, osteoclasts, and red marrow cells in the confines of epiphyseal plate. βig-h3 expression was not shown in normal rabbit IgG as a nonimmune control (Figure 3D, H, and L).

Localization of βig-h3 and marker gene of chondrocyte differentiation in developing mouse femur

To elucidate the expression of βig-h3 in chondrocytes during endochondral ossification, the localization of βig-h3 expression was compared to ColX, which is a marker for the hypertrophic zone. *In situ* hybridization was performed by using [^{35}S] UTP-labeled βig-h3 and ColX riboprobes on femoral sections of postnatal mice (Figure 4). Prominent expression of βig-h3 transcripts were detected in chondrogenic tissue destined for cartilage or bone formation. With the maturation of chondrocytes, a surge of βig-h3 expression occurred in the prehypertrophic and early hypertrophic chondrocytes, but not in the ColX positive hypertrophic zone. Sense probes did not show any significant signal with these tissues (data not shown).

Discussion

Endochondral bone ossification is an important process responsible for bone growth in the vertebrate skeleton, mainly in long bones during skeletal development. This is a complex process which requires the sequential formation and degradation of cartilaginous structures serving as templates for the developing bones. Precartilage condensation observed at the first stage of endochondral ossification is composed of undifferentiated mesenchymal stem cells (MSCs), which are multipotential to differentiate, and specific precartilage extracellular matrix molecules (ECMs), which regulate the differentiation of chondrocytes (Tacchetti *et al*., 1992; Hall and Miyake, 1995). Various ECMs in precartilage condensation have been understood to play important roles in the control of endochondral ossification.

βig-h3, an ECM protein, is expressed in many tissues, including the heart, kidneys, liver, and cartilage (Skonier *et al*., 1992; LeBaron *et al*., 1995; Hashimoto *et al*., 1997). During the chick embryogenesis, βig-h3 was localized at the precartilage condensation of limb buds and highly expressed in the prehypertrophic in the vertebrae, indicating that this protein may have an important role in cartilage development (Ohno *et al*., 2002). During mouse development, βig-h3 expression was high in pre-chondrocytic mesenchymal cells at

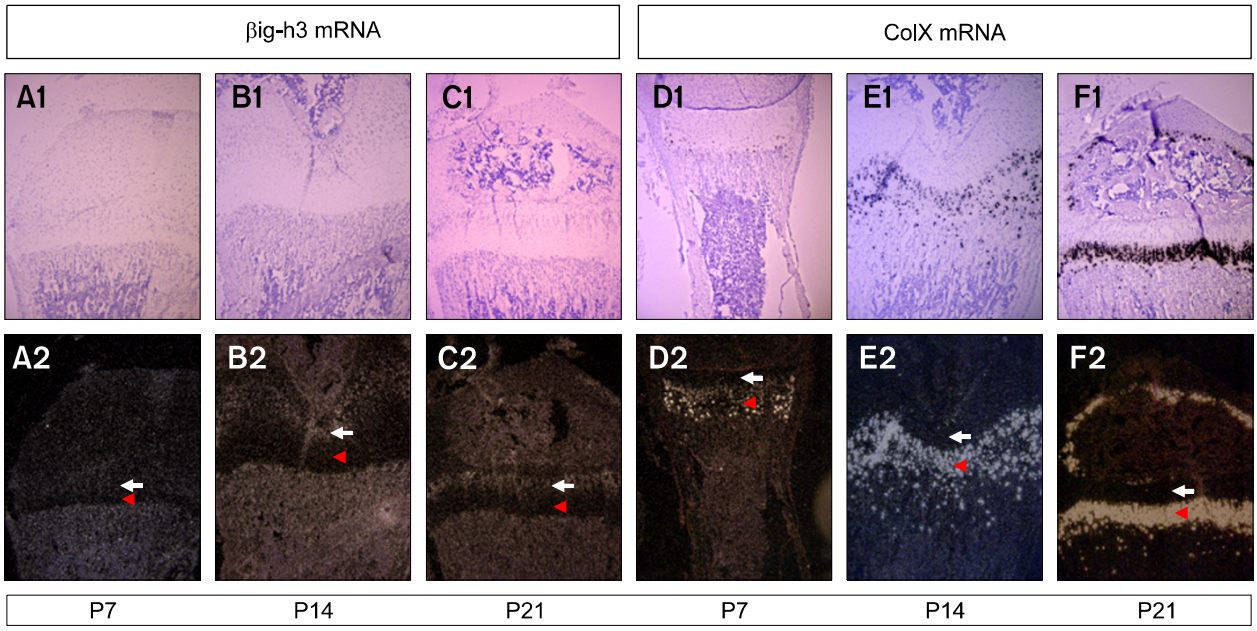

Figure 4. *In situ* hybridization of βig-h3 (A, B, C) and ColX (D, E, F) mRNA in the mouse femur at postnatal day 7 (P7), P14, and P21. Radioactive antisense probes were hybridized to the postnatal developing femur resulting in βig-h3-positive structures identified in prehypertrophic chondrocytes (white arrow) whereas ColX-positive structures identified in hypertrophic chondrocytes (red arrowhead). The tissue morphology was examined under the bright field (row 1) and *in situ* hybridization signal was observed under the dark field of the fluorescence microscope (row 2).

E12.5, and continuously observed during the cartilaginous formation (Ferguson *et al*., 2003). In particular, βig-h3 transcripts appeared abundant in proliferating chondrocytes, whereas they appeared much less frequently in hypertrophic chondroctyes. Corresponding to the previous reports of βig-h3 transcripts, this study showed the expression pattern of βig-h3 protein during mouse development. *In vivo* histological analysis of this study explained that the expression of βig-h3 protein was abundant in prehypertrophic chondrocytes, perichondrium, and periosteum from embryonic to postnatal growth, but not in hypertrophic chondrocytes. In comparison to TGF-β1 expression restricted to the proliferative and upper hypertrophic zones in chondrocytes (Horner *et al*., 1998), this result suggests that βig-h3 may have some critical roles related to the function of TGF-β during chondrocyte differentiation and endochondral ossification. Furthermore, this present study with the expression pattern of proteins is more appropriate than transcripts to elucidate the role of βig-h3 as a functional protein in endochondral ossification.

Mouse embryonic carcinoma derived chondrogenic cell line, ATDC5 cells are frequently used for *in vitro* models of chondrogenesis (Akiyama *et al*., 1997). In the presence of insulin, ATDC5 cells proliferate and differentiate to form cartilage nodules, and then express type II collagens, aggrecans, and parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptors. With further differentiation, ATDC5 cells are hypertrophied and finally express type X collagens (Shukunami *et al*., 1996, 1997). Finally, mineralized ECM is generated at the last stages of ATDC5 cell differentiation. TGF-β is important to control chondrocyte and osteoblast differentiation, and in particular, it regulates *in vitro* chondrogenic differentiation in ATDC5 cells with the increased expression of fibronectin isoforms (Han *et al*., 2005). Here the pattern of βig-h3 expression during chondrocyte differentiation in ATDC5 cells was explained. The *in vitro* differentiation study of ATDC5 cells showed that βig-h3 expression was highly increased at the early stages of differentiation, followed by an abrupt decrease at the late stages. Moreover, the expression pattern of βig-h3 was concurrent with the pattern of TGF-β1, -β2, and -β3 during ATDC5 cells differentiation. We also observed the chondrocyte maturation in the condition of βig-h3 overexpression which is on βig-h3-coated culture plates or on TGF-β-induced βig-h3, resulting in the only a little increase of chondrocyte maturation (data not shown). Although this *in vitro* data has begun to clarify the role of βig-h3 in chondrocyte maturation, further *in vivo* studies with βig-h3-overexpressed animals will continue to elucidate the function of βig-h3 in chondrocyte maturation.

Taken together, this study suggests that βig-h3 is mainly induced by TGF-β1 at the prehypertrophic chondrocytes and may mediate the function of TGF-β during endochondral ossification. This implies more exactly the role of βig-h3 mediated by the function of TGF-β in prehypertrophic - hypertrophic differentiation during endochondral ossification.

Acknowledgments

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