CCAAT/Enhancer-binding Protein β Regulates the Repression of Type II Collagen Expression during the Differentiation from Proliferative to Hypertrophic Chondrocytes

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CCAAT/Enhancer Binding Protein β Regulates the Repression of Type II Collagen Expression during the Differentiation from Proliferative to Hypertrophic Chondrocytes

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Running title: *Regulation of Col2a1 by C/EBPβ during chondrocyte differentiation*

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Background: CCAAT/enhancer binding protein β (C/EBPβ) promotes hypertrophic differentiation of chondrocytes.
Results: C/EBPβ directly represses the expression of type II collagen by interacting with its intronic enhancer.
Conclusion: C/EBPβ biphasically function to repress genes characteristic of proliferative chondrocytes, while stimulating genes expressed by hypertrophic chondrocytes.
Significance: C/EBPβ is a key regulator to trigger phenotypic conversion from proliferative to hypertrophic chondrocytes.

ABSTRACT

CCAAT/enhancer binding protein β (C/EBPβ) is a transcription factor that promotes hypertrophic differentiation by stimulating type X collagen and matrix metalloproteinase 13 during chondrocyte differentiation. However, the effect of C/EBPβ on proliferative chondrocytes is unclear. Here, we investigated whether C/EBPβ represses type II collagen (COL2A1) expression and is involved in the regulation of sex-determining region Y-type high mobility group box 9 (SOX9), a crucial factor for transactivation of *Col2a1*. Endogenous expression of C/EBPβ in the embryonic growth plate and differentiated ATDC5 cells were opposite to those of COL2A1 and SOX9. Overexpression of C/EBPβ by adenovirus vector in ATDC5 cells caused marked repression of *Col2a1*. The expression of *Sox9* mRNA and nuclear protein was also repressed, resulting in decreased binding of SOX9 to the *Col2a1* enhancer as shown by a ChIP assay. Knockdown of C/EBPβ by lentivirus expressing shRNA caused significant stimulation of these genes in ATDC5 cells. Reporter assays demonstrated that C/EBPβ repressed transcriptional activity of *Col2a1*. Deletion and mutation analysis showed that the C/EBPβ core responsive element was located between +2144 and +2152 bp within the *Col2a1* enhancer. EMSA and ChIP assays also revealed that C/EBPβ directly bound to this region. *Ex vivo* organ cultures of mouse limbs transfected with C/EBPβ showed that the expression of COL2A1 and SOX9 were reduced upon ectopic C/EBPβ expression. Together, these results indicated that C/EBPβ represses the transcriptional activity of *Col2a1* both directly, and indirectly through modulation of *Sox9* expression. This consequently promotes the phenotypic conversion from proliferative to hypertrophic chondrocytes during chondrocyte differentiation.

The sequential differentiation process of chondrocytes is observed not only in skeletal formation, but also in fracture healing and development of osteophytes in osteoarthritis (OA) (1-4). Chondrogenesis initiates when mesenchymal cells condense and differentiate into proliferative chondrocytes that synthesize cartilage-specific extracellular matrix (ECM)

including type II collagen (COL2A1) and aggrecan (ACAN). Thereafter, chondrocytes change morphology to become hypertrophic chondrocytes and convert their gene expression profile. They stop expressing COL2A1 and ACAN and start expressing type X collagen (COL10A1), matrix metalloproteinase-13 (MMP13), and vascular endothelial growth factor (VEGF). Finally, osteoblasts migrate into the cartilage, a process that is accompanied by vascular invasion and apoptosis of hypertrophic chondrocytes, and completes formation of bone. In contrast to the developing cartilage of the growing bone, chondrocytes in adult articular cartilage do not pursue the hypertrophic differentiation process and maintain the characteristic ECM structure consisting of COL2A1 and ACAN. However, phenotypic conversion to hypertrophic chondrocytes is also observed in degenerative articular cartilage of OA (2).

This sequential differentiation process is tightly regulated by various transcription factors (1, 3). Sex-determining region Y-type high mobility group box 9 (SOX9) is an essential transcription factor for chondrogenesis during mesenchymal condensation and chondrocyte proliferation (5). In humans, its malfunction causes a severe chondrodysplasia called campomelic dysplasia, which is characterized by severe malformations of cartilage-derived structures. *Sox9* knockout mice showed no aggregation of chondroblasts and subsequent expression of ECM (6). Furthermore, SOX9 was reported to directly bind and activate cartilage-specific regulatory elements of *Col2a1*, resulting in its cartilage-specific gene expression (7, 8). While SOX9 is indispensable for chondrogenesis, it was also reported to work as a negative regulator of hypertrophic differentiation (9). Several studies have reported that Wingless-type MMTV integration site (Wnt) (10), bone morphogenetic protein 2 (BMP2) (11), parathyroid hormone-related protein (PTHrP) (12), interleukin 1β (IL-1β) (13) and Runt-related transcription factor 2 (RUNX2) (14) can regulate *SOX9* expression or activity in arthritic chondrocytes or during chondrocyte differentiation.

CCAAT/enhancer binding proteins (C/EBP) are a family of basic leucine zipper transcription factors with 6 members as follows: C/EBPα, β, δ, ε, γ, and ζ. Among them, C/EBPβ (encoded by *CEBPB*) was first identified as a nuclear protein that bound to the IL-1β response element in the IL-6 promoter region (15) and it has subsequently been reported to regulate various genes involved in cell differentiation, proliferation, survival, immune function, tumor invasiveness and progression (16-19). Previously, it was reported that C/EBPβ, in response to IL-1β, down-regulated cartilage-derived retinoic acid-sensitive protein (*Cd-rap*), a small secreted protein expressed in cartilage throughout chondrogenesis and in mature chondrocytes (20). C/EBPβ induced by pro-inflammatory cytokines such as IL-1β and tumor necrosis factor α (TNF-α) directly binds to the MMP13 and MMP3 promoter regions and stimulates their expression in chondrocytes, resulting in degradation of cartilage in arthritis (21, 22). Furthermore, it was reported that hypertrophic differentiation of chondrocytes was delayed in C/EBPβ knockout mice through transactivation of cell cycle factor p57 (23). This was associated with decreased expression of *Col10a1* (24) and suggested that C/EBPβ plays an important role in promoting hypertrophic differentiation of chondrocytes. C/EBPβ is also involved in the hypertrophic changes of articular chondrocytes in OA (23). Although these studies showed that C/EBPβ stimulates genes expressed in hypertrophic chondrocytes, it is not fully understood whether C/EBPβ influences expression of genes characteristic of proliferative chondrocytes.

Here, we investigated the direct involvement of C/EBPβ in regulating ECM of proliferating chondrocytes, specifically *Col2a1*, during chondrocyte differentiation. We also investigated the regulation of *Sox9* expression by C/EBPβ, which indirectly leads to the regulation of *Col2a1* during chondrocyte differentiation.

EXPERIMENTAL PROCEDURES

Immunohistochemistry- With local ethics committee approval, tissue samples of growth plate were obtained from mouse embryos (E16.5). For the immunoperoxidase method, the DAKO Envision kit (DAKO, Glostrup, Denmark) was used. Deparaffinized sections (3

μm thickness) were treated with hyaluronidase (Sigma Aldrich, St. Louis, MO) (25 mg/ml in sodium acetate buffer, pH5.5, 0.85% NaCl) for 30 minutes to stain ECM. Each section was subjected to antigen retrieval by microwaving in 10 mM citrate buffer (sodium citrate, pH 6.0) for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 30 minutes. The specimens were placed in blocking reagent for 30 minutes and incubated overnight at 4°C with primary antibodies. The samples were further incubated with secondary antibodies for 30 minutes and then a colorimetric reaction was carried out with 3,3´-diaminobenzidine and 0.02% H_2O_2, followed by counterstaining with hematoxylin. For immunofluorescent staining, Alexa Fluor 488 and 568 (Invitrogen, Carlsbad, CA) were used as secondary antibodies and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories; Burlingame, CA). The primary antibodies were as follows: C/EBPβ (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, SOX9 (AB5535; Millipore, Billerica, MA) diluted 1:2000, type II Collagen (LB-1297; LSL Biolafitte, St. Germain en lay, France) diluted 1:800, type X Collagen (LB-0092; LSL Biolafitte) diluted 1:800, MMP13 (ab39012; Abcam, Cambridge, MA) diluted 1:100, RUNX2 (AP7735a; Abgent, San Diego, CA) diluted in 1:100, β-galactosidase (200-4136; Rockland, Gilbertsville, PA) diluted 1:1000 and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) diluted 1:1000.

Cell culture- ATDC5 cells, a mouse chondrogenic cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) / Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS). To induce chondrogenic and hypertrophic differentiation, sub-confluent cultures were changed to medium containing 1% ITS (insulin–transferrin–selenium) Universal Culture Supplement Premix reagent (BD Biosciences, San Jose, CA). Rat chondrosarcoma (RCS) cells and SW1353, human chondrosarcoma cells, were cultured in DMEM with 10% FBS. Primary chondrocytes were isolated from the rib cages and sternums of 1-day-old mice as previously described (25) and cultured in DMEM with 10% FBS.

Virus vectors- Adenovirus vectors expressing C/EBPβ-LAP or LacZ control were kindly provided by Dr. Hiroshi Sakaue (Kobe University, Kobe, Japan) (26). Liver-enriched activator protein (LAP) is one of the isoforms of C/EBPβ, which carries a trans-activator domain (18, 27). ATDC5 cells were transfected with these vectors and differentiated for 2 weeks with ITS. Stable ATDC5 cell lines were generated with lentivirus vectors expressing short hairpin RNA (shRNA) for *Cebpb* (TRCN0000231411) (Sigma Aldrich) or control. ATDC5 cells, selected with puromycin (2 μg/ml), were differentiated for 2 weeks.

RNA extraction, quantitative real-time RT-PCR and semi-quantitative RT-PCR- Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (0.5 μg) was reverse-transcribed using the Prime script RT reagent kit (Takara Bio, Shiga, Japan) to make single-stranded cDNA. Quantitative real-time RT-PCR was performed with the Light Cycler 2.0 System (Roche) using SYBR Premix Ex Taq II (Takara Bio). The primers were as follows: for *Cebpb*, 5'- ACGACTTCCTCTCCGACCTCT -3' (forward) and 5'- CGAGGCTCACGTAACCGTAGT -3' (reverse); for *Col2a1*, 5'- CGAGTGGAAGAGCGGAGACT -3' (forward) and 5'- AACTTTCATGGCGTCCAAGGT -3' (reverse); for *Acan*, 5'- GAAGAGCCTCGAATCACCTG -3' (forward) and 5'- ATCCTGGGCACATTATGGAA -3' (reverse); for *Sox9*, 5'- GAGGCCACGGAACAGACTCA -3' (forward) and 5'- CAGCGCCTTGAAGATAGCATT -3' (reverse); for *Col10a1*, 5'- TAAGAACGGCACGCCTACGA -3' (forward) and 5'- TGATTGCACTCCCTGAAGCC -3' (reverse); for *Mmp13*, 5'- CACAGCAAGCCAGAATAAAG -3' (forward) and 5'- CACACATCAGTAAGCACCAAG -3' (reverse); for *Runx2*, 5'- AACCACAGAACCACAAGT -3' (forward) and 5'- AAATGACTCGGTTGGTCT -3' (reverse); for *Col1a1*, 5'- AGACATGTTCAGCTTTGTGGAC -3' (forward) and 5'- GCAGCTGACTTCAGGGATG -3' (reverse); and for *18S*, 5'- GTAACCCGTTGAACCCCATT -3' (forward)

and 5'- CCATCCAATCGGTAGTAGCG -3' (reverse). Data were corrected for expression of the housekeeping gene *18S*. Semi-quantitative RT-PCR was performed with a thermal cycler using Ex Taq (Takara Bio). The primers were as follows: for *Cebpb*, 5'-AGTACAAGATCCGGCGCGAG -3' (forward) and 5'- TGCTTGAACAAGTTCCGCAG -3' (reverse); for *Col2a1*, 5'-CGAGTGGAAGAGCGGAGACT -3' (forward) and 5'- AACTTTCATGGCGTCCAAGGT -3' (reverse); for *Sox9*, 5'-CGAACGCACATCAAGACGA -3' (forward) and 5'- AGGTGAAGGTGGAGTAGAGGC -3' (reverse); and for *18S*, 5'-GTAACCCGTTGAACCCCATT -3' (forward) and 5'- CCATCCAATCGGTAGTAGCG -3' (reverse).

Western blot- Whole cell lysates were extracted from cells using M-RIPA buffer (Sigma Aldrich). Nuclear extracts were isolated using Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). Cell lysates were electrophoresed in 4-12% gradient polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL). After blocking in Tris-buffered saline-Tween containing 3% non-fat milk, the membranes were incubated with primary antibody against C/EBPβ (C-19; Santa Cruz Biotechnology) diluted 1:500, SOX9 (AB5535; Millipore) diluted 1:1000, or Flag (F4042; Sigma Aldrich) diluted 1:1000 in blocking reagent at room temperature for 1 hour. We also used ACTIN (MAB1501; Millipore) and LAMIN A/C (H-110; Santa Cruz Biotechnology) antibodies as internal loading controls. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted in blocking reagent was added and incubated at room temperature for 1 hour. The immunoreactivity of the blots was detected using ECL Prime (Amersham) and photographed with Ez Capture MG (ATTO, Tokyo, Japan). The band densities were evaluated by densitometric scan using CS Analyzer (ATTO).

Chondrocyte differentiation assay- ATDC5 cells were cultured for 3 weeks after transfection with adenovirus vectors. The ATDC5 cells were washed three times with PBS then fixed with 4% formaldehyde for 10 minutes. Alcian blue staining was performed with 0.3% Alcian blue 8GS (Sigma Aldrich) in 0.1 N HCl. After staining overnight, the cells were washed with distilled water three times.

Plasmid preparation and reporter assay- Mouse *Col2a1* sequences spanning -952 to +73 and +2038 to +2678 (including promoter and intron 1) were subcloned into the pGL-4.10 (luc2) vector (Promega, Madison, WI). Deletion sequences of promoter and enhancer were also generated in various combinations using PCR. Mutation constructs were made with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). RCS or SW1353 cells were seeded one day before transfection in 24-well tissue culture plates at 3×10^4 cells/cm^2. 0.4μg of reporter plasmids were co-transfected with expression vectors like pCMV-LAP (an expression vector of rat C/EBPβ) and an A-C/EBP vector tagged with Flag (a dominant-negative C/EBP expression vector kindly provided by Dr. Charles R. Vinson) (28) using Lipofectamine Plus reagent (Invitrogen). Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility shift assay (EMSA) - Nuclear protein was extracted from ATDC5 cells that had been transfected with C/EBPβ using Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Complementary oligonucleotides were end-labeled with the Biotin 3' End DNA Labeling Kit (Thermo Scientific), then annealed to obtain double-stranded oligonucleotides. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). Twenty fmol of biotin-labeled probes were incubated with nuclear protein in 1x binding buffer (including 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly(dI-dC)) at room temperature for 20 minutes. For competition experiments, the cold probes were added at a 200-fold molar excess. For antibody interference experiments, the nuclear extract and 1 μl of C/EBPβ antibody (Santa Cruz Biotechnology) were pre-incubated for 1 hour at 4 ℃. Binding samples were subjected to electrophoresis in a 6% DNA Retardation gel (Invitrogen) and run in 0.5x TBE buffer at 100V

for 1 hour, then transferred to a positively charged membrane (Invitrogen) and cross-linked. Detection was performed using streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate. The oligonucleotides were as follows: wild-type, 5'-CAGATGGGCTGAAACCCTGCC -3' (sense) and 5'- GGCAGGGTTTCAGCCCATCTG -3' (antisense); and mutant, 5'-CAGATGGGCTGACCCCCTGCC -3' (sense) and 5'- GGCAGGGGGTCAGCCCATCTG -3' (antisense).

Chromatin immunoprecipitation (ChIP) assay- ChIP assays were performed with a ChIP Assay kit (Millipore). ATDC5 cells were differentiated for 3 weeks to induce hypertrophic differentiation or cultured for 4 days after transfection with adenovirus vectors expressing C/EBPβ or LacZ control. The ATDC5 cells were fixed with 4% formaldehyde and sonicated. For immunoprecipitation experiments, C/EBPβ antibody (Santa Cruz Biotechnology), SOX9 antibody (Millipore), and normal rabbit IgG (Santa Cruz Biotechnology) were used. Primers used in PCR were as follows: amplified between +2003 and +2198 bp for the *Col2a1* enhancer including the C/EBPβ binding site, between +2136 and +2292 bp for the *Col2a1* enhancer including the SOX9 binding site, and between -1891 and -1611 bp as a negative control.

Ex vivo organ culture- Tibias were isolated from hind limbs of E14.5 mouse embryos and cultured in organ culture medium (29). One day after dissection, each tibia obtained from identical mouse embryos were transfected with adenovirus vectors expressing C/EBPβ or LacZ control and cultured at 37℃ in a humidified 5% CO_2 incubator for 4 days. Simultaneously, tibias dissected from different embryos were cultured without transfection of adenovirus vectors for 4 days. Safranin O and immunofluorescent staining was performed. Histological analysis was repeated at least twice for each sample from six pairs of limbs, respectively.

Statistical analysis- Data are reported as mean ± S.D. of three independent experiments, each performed in duplicate. Statistical analysis was performed using statistical software JMP 9 (SAS Institute, Inc. Cary, NC). The Mann-Whitney U-test was used for two-group comparisons. $p < 0.05$ was considered statistically significant.

RESULTS

Expression patterns of C/EBPβ, COL2A1 and SOX9 during chondrocyte differentiation- To confirm the expression pattern of C/EBPβ and chondrocyte differentiation markers *in vivo*, immunohistochemistry of mouse embryos was performed (Figure 1A). Consistent with previous reports, SOX9 was strongly expressed from proliferative to pre-hypertrophic chondrocytes, but not by hypertrophic chondrocytes. COL2A1 was expressed in chondrocytes from the proliferative zone then gradually decreased towards the hypertrophic zone. C/EBPβ was weakly detected in proliferative chondrocytes, but strongly expressed by pre-hypertrophic and hypertrophic chondrocytes where it co-localized with COL10A1. These results indicated an opposite expression pattern between C/EBPβ and COL2A1 and SOX9 during chondrocyte differentiation.

To investigate the relationship of expression patterns between C/EBPβ and various chondrocyte differentiation markers *in vitro*, ATDC5 cells were used. To confirm that ATDC5 cells possess the ability of chondrogenic and hypertrophic differentiation, the expression of each marker was compared between ATDC5 cells (at the 14th day) and primary chondrocytes obtained from mouse ribs and sternums cartilage. The mRNA expression profile of these differentiation markers in ATCD5 cells was similar to that in primary chondrocytes (Table 1), indicating that ATDC5 cells represent the chondrocytic nature. During differentiation of ATDC5 cells, expression of *Col2a1* mRNA gradually increased to a maximum at the 14th day and then decreased (Figure 1B). This pattern was consistent with *Sox9* mRNA expression. In contrast, Western blot analysis of nuclear proteins extracted from ATDC5 cells revealed that C/EBPβ was elevated at a later stage, similar to *Col10a1* mRNA expression (Figure 1B, C). These findings suggested that C/EBPβ could be involved in the repression of *Col2a1* and *Sox9* during hypertrophic differentiation of chondrocytes.

C/EBPβ represses expression of Col2a1 and

Acan, while stimulating expression of hypertrophic markers- To investigate the effect of C/EBPβ on chondrocyte differentiation markers, ATDC5 cells were transfected with adenovirus vectors expressing C/EBPβ or LacZ control and the cells were subsequently differentiated. Hypertrophic markers such as *Col10a1* and *Mmp13* as well as *Runx2*, which is known to be a major regulator of chondrocyte maturation and osteoblast differentiation (30), were significantly increased along with ectopic *Cebpb* expression (Figure 2A). These findings confirmed that C/EBPβ promotes hypertrophic differentiation of chondrocytes. Conversely, the expression of both *Col2a1* and *Acan* was significantly repressed in ATDC5 cells transfected with C/EBPβ at all stages of differentiation (Figure 2B). The expression of *Col1a1* was decreased at a later stage during differentiation of ATDC5 cells (Figure 1B), and was also reduced by overexpression of C/EBPβ (Figure 2A), demonstrating that repression of *Col2a1* and *Acan* is not the cause of chondrocyte dedifferentiation towards fibroblastic phenotype. These results were further confirmed by alcian blue staining of ATDC5 cells cultured for 3 weeks (Figure 2C). These findings indicate that C/EBPβ may biphasically function to repress the expression of *Col2a1* and *Acan*, while stimulating hypertrophic markers during hypertrophic differentiation of chondrocytes.

C/EBPβ indirectly repressed expression of Col2a1 through interacting with SOX9- We investigated the effect of C/EBPβ on the expression of *Sox9* because SOX9 is known to be a crucial transcription factor for activating *Col2a1*. The expression of *Sox9* mRNA was significantly repressed by C/EBPβ at all stages of differentiation along with ectopic *Cebpb* expression (Figure 3A). Western blot analysis also revealed that SOX9 proteins extracted from both cytoplasm and nucleus were strongly repressed by C/EBPβ (Figure 3B). Furthermore, a ChIP assay using ATDC5 cells transfected with adenovirus vectors revealed that the binding of SOX9 to *Col2a1* intronic enhancer was decreased by overexpression of C/EBPβ (Figure 3C). These results indicated that C/EBPβ may repress the expression of *Col2a1* through interacting with SOX9 during hypertrophic differentiation of chondrocytes.

C/EBPβ knockdown by shRNA increased expression of chondrocyte differentiation markers- To further examine the relationship between C/EBPβ and chondrocyte differentiation markers, shRNA-transfected ATDC5 cells targeting *Cebpb* were differentiated. Nuclear extracts and mRNA expression of C/EBPβ were effectively reduced by shRNA compared to control (Figure 4A, C). The expression of *Col10a1* was reduced at a later stage (Figure 4A), while the expression of *Col2a1* and *Acan* was significantly increased by C/EBPβ knockdown along with chondrocyte differentiation (Figure 4B). The expression of *Sox9* mRNA was significantly increased on the 4th day in ATDC5 cells transfected with shRNA (Figure 4B). Furthermore, nuclear protein of SOX9 was markedly increased on the 4th day and this was also confirmed by densitometric scan (Figure 4C, D). Together, these findings suggest that C/EBPβ is involved in repression of differentiation markers at the endogenous level during hypertrophic differentiation of chondrocytes.

C/EBPβ repressed transcriptional activity of Col2a1 in chondrocytes- We confirmed that the expression of *Col2a1* is regulated by C/EBPβ. However, it is unclear whether C/EBPβ directly regulates the expression of *Col2a1* because SOX9 could be involved in this regulation. To investigate the direct regulation of *Col2a1* by C/EBPβ, a *Col2a1* reporter construct was generated (Figure 5A). We used two chondrocytic cell lines, RCS and SW1353 cells. RCS cells constitutively express *Sox9*, leading to stronger expression of *Col2a1* than SW1353 cells. On the other hand, SW1353 cells strongly express endogenous *CEBPB* compared with RCS cells (Figure 5B). Luciferase activity of *Col2a1* reporter construct in RCS cells was significantly higher than in SW1353 cell (Figure 5C), indicating that the validity of this reporter construct. *Col2a1* promoter activity was down-regulated by C/EBPβ in a dose-dependent manner even in RCS cells that express *Sox9* (Figure 5D). In contrast, A-C/EBP, which inhibits binding of C/EBP family members to specific binding sites by forming a heterodimeric complex (28), reversed the down-regulation of *Col2a1* promoter activity

caused by C/EBPβ in a dose-dependent manner (Figure 5E). A-C/EBP also increased *Col2a1* promoter activity in a dose-dependent manner in SW1353 cells (Figure 5F).

C/EBPβ directly down-regulated Col2a1 through its intronic enhancer- To identify the C/EBPβ response element in the *Col2a1* gene, deletion and mutation analyses were performed. C/EBPβ was reported to recognize T(T/G)NNGNAA(T/G) as a binding sequence (15), and we found two conserved C/EBPβ binding motifs within 1 kb of the *Col2a1* promoter (Figure 6A). Although *Col2a1* promoter activity gradually decreased along with deletion of a series of 5′ promoter elements, transcriptional repression by C/EBPβ was observed for all deletion constructs (Figure 6A). This indicated that the functional C/EBPβ binding element was located within 640 bp of the *Col2a1* enhancer.

Next, we generated deletion constructs of the enhancer combined to the shortest promoter, which did not contain C/EBP binding motifs, to investigate the function of C/EBPβ on the enhancer element. A reporter assay revealed that only the reporter construct, which included the first intron sequence between +2038 and +2249 bp, was down-regulated by C/EBPβ (Figure 6B), suggesting that there were functional C/EBPβ binding sites in this construct. We identified one conserved C/EBPβ binding motif within this enhancer sequence. A mutation, from GNAA to GNCC, which is essential for C/EBPβ binding, was introduced (Figure 6B). An inhibition of luciferase activity by C/EBPβ was not observed in this mutation construct (Figure 6B). These results suggest that C/EBPβ bound to the binding element located between +2144 bp to +2152 bp in the *Col2a1* enhancer. To verify the direct binding of C/EBPβ to this element in the *Col2a1* enhancer, EMSA was performed (Figure 6C). C/EBPβ bound strongly to the wild-type probe, but binding to the mutant probe was weak. Non-labeled wild-type probe inhibited the binding of C/EBPβ to labeled wild-type probe, but non-labeled mutant probe could not block it. Supershift was observed by addition of a C/EBPβ antibody indicating the specificity of C/EBPβ binding. A ChIP assay also confirmed that C/EBPβ bound to the enhancer region of *Col2a1* (Figure 6D). These results suggest that C/EBPβ directly represses transcriptional activity of *Col2a1* by interacting with the enhancer region.

Ectopic expression of C/EBPβ represses the expression of COL2A1 and SOX9 in ex vivo organ culture- Finally, we performed an *ex vivo* organ culture of mouse tibias (Figure 7). Genes expression of tibias which had been transfected with the adenovirus vector expressing LacZ control were equivalent to those of tibias without transfection, indicating that the LacZ-infected tibias were the proper controls for C/EBPβ-infected tibias. Immunofluorescent staining with a β-gal antibody showed that transfection using adenovirus vector was efficiently performed and increased expression of C/EBPβ induced by the infection was also confirmed. Although the transfection of adenovirus vector expressing LacZ slightly reduced the expression of COL2A1 compared with non-treated tibias, it was strongly reduced in C/EBPβ-infected tibias along with the ectopic expression of C/EBPβ. The expressions of COL10A1, RUNX2 and MMP13 were misexpressed through the tibias which were transfected with C/EBPβ, compared with LacZ control. Forced expression of C/EBPβ may lead the ectopic expression of these genes even in the regions that do not show the morphological hypertrophy because C/EBPβ is reported as a direct regulator of them. Moreover, the expression of SOX9 was also decreased and restricted to a small upper area of the growth plate by overexpression of C/EBPβ, similar to the expression of COL2A1. Together, these results further confirmed that C/EBPβ could be involved in regulation of phenotypic conversion from proliferative to hypertrophic chondrocytes by repressing the genes characteristic of proliferative chondrocytes during chondrocyte differentiation.

DISCUSSION

Endochondral ossification is tightly regulated by various factors. During this process, differentiation from proliferative to hypertrophic chondrocytes is accompanied by transition of ECM gene expression from *COL2A1* to *COL10A1*. C/EBPβ has been reported to promote hypertrophic differentiation by transactivation of cell cycle factor p57 (23) and

it stimulates expression of hypertrophic markers such as *COL10A1* and *MMP13* (24). However, the regulation of genes characteristic of proliferative chondrocytes by C/EBPβ remains to be elucidated. The present study is the first to show that C/EBPβ represses transcriptional activity of *Col2a1* both directly and indirectly through modulation of SOX9 expression during hypertrophic differentiation of chondrocytes. We also report that *Acan* is repressed along with SOX9 and that *Runx2* is stimulated along with *Col10a1*.

We used ATDC5 cells as a model system of chondrocyte differentiation, because ATDC5 cells represent equivalent expression of chondrocyte differentiation markers like *Col2a1*, *Acan* and *Sox9* with primary chondrocytes (Table 1). The expressions of hypertrophic markers such as *Mmp13*, *Runx2* and *Cebpb* were higher in primary chondrocytes. Because the primary chondrocytes was obtained from mouse ribs and sternums, they might contain more hypertrophic chondrocytes than ATDC5 cells at the 14th day. Meanwhile, the expression of *Col1a1* was higher in ATDC5 cells. ATDC5 cells show chondrogenic and hypertrophic differentiation in nodules and undifferentiated ATDC5 cells remain fibroblastic morphology around nodules. Subsequently, the cells form numerous nodules and hypertrophic differentiation of ATDC5 cells progresses (31). Thus, mix of the cells at various differentiation stages may cause higher expression of *Col1a1* in ATDC5 cells than in primary chondrocytes. Together, it is considered that ATDC5 cell line is one of the useful models to study chondrogenic differentiation and hypertrophic transformation of chondrocytes *in vitro*.

A more significant repression of SOX9 protein compared to its mRNA by C/EBPβ was observed (Figure 3A, B). One possible mechanism is that SOX9 protein is degraded post-transcriptionally. In fact, it was reported that ubiquitination of SOX9 protein determined its transcriptional activity (14, 32). A significant decrease of SOX9 nuclear protein resulted in down-regulation of *Col2a1* (Figure 3C). These findings suggest that indirect repression of *Col2a1* through interaction with *Sox9* is another mechanism by which C/EBPβ regulates *Col2a1* during hypertrophic differentiation of chondrocytes. Although this study did not explore the inhibitory mechanism of *Acan* during hypertrophic differentiation of chondrocytes, the repression of *Sox9* by C/EBPβ may also be involved in regulating the expression of *Acan* because SOX9 is known to stimulate *ACAN* (33).

Although a reporter assay and EMSA revealed that C/EBPβ directly bound to an intronic enhancer region of *Col2a1* in order to repress its transcription, a point mutation of the C/EBPβ binding site within the *Col2a1* intronic enhancer also decreased *Col2a1* luciferase activity (Figure 6B). Moreover, the *Col2a1* reporter was no longer active when the enhancer sequence was deleted despite the existence of binding sites for SOX trio (Figure 6B). One possible reason is that this sequence may be essential for another unknown transcription factor, which enhances transactivation of *Col2a1*, and a competition in binding between the unknown factor and C/EBPβ may be the mechanism of the repression of *Col2a1* by C/EBPβ. Further studies will be necessary to examine the detailed mechanism of the interaction between C/EBPβ and other transcriptional factors.

Meanwhile, it is interesting that a functional C/EBPβ binding site is located in the *Col2a1* enhancer because the site is located nearby the binding elements for SOX trio. SOX9 was reported to interact with the co-activators cAMP response element-binding protein (CBP) and p300 and regulate *Col2a1* through histone acetylation around its enhancer region (34). A previous report also demonstrated that *Cd-rap*, a cartilage-specific ECM molecule, was activated by SOX9 and that CBP and p300 were involved in this mechanism by inhibiting binding of C/EBPβ to *Cd-rap* (35). Considering these reports, it is possible that C/EBPβ represses *Col2a1* by competing with SOX9 binding via co-activators like CBP and p300 in the *Col2a1* enhancer region.

Regulation of *Col2a1* occurs not only during skeletal development, but also in arthritic articular cartilage. C/EBPβ is known to be induced by pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, and it is expressed in arthritic cartilage (15, 20, 22). It has been suggested that C/EBPβ also plays a role in the

repression of *COL2A1* in response to inflammatory signals (20). Some other transcription factors including c-Fos, c-Jun, Jun B, and Egr-1, have been reported to be induced by IL-1β and involved in the down-regulation of *COL2A1* during OA (36). It was also reported that Egr-1 inhibited interactions between CBP, Sp1, and TATA-binding proteins by interfering with Sp1 binding to the *COL2A1* proximal promoter (37). ESE-1 was also reported to repress *COL2A1* by Egr-1 through binding to the *COL2A1* promoter (38). In *Col2a1* regulation by C/EBPβ, C/EBPβ may also interact with these co-factors, consequently inhibiting initiation of mRNA transcription in response to the inflammatory signal.

This study also showed that exogenous C/EBPβ stimulated expression of hypertrophic markers such as *Col10a1* and *Mmp13,* but also *Runx2*, even in the early differentiation stages of ATDC5 cells (Figure 2A). RUNX2 directly binds to the *COL10A1* promoter and enhances its transcription (39). RUNX2 is also reported to regulate *MMP13* and osteocalcin expression synergistically with C/EBPβ (24, 40). Our results imply that C/EBPβ may enhance expression of *Runx2* to further promote hypertrophic differentiation of chondrocytes.

It was reported that hypertrophic chondrocytes also appear in OA articular cartilage (2). Chondrocyte hypertrophy occurs in association with the expression of RUNX2 and MMP13 in a mechanically-induced OA model (41) and C/EBPβ is also observed in the OA model (23, 24). However, the reason for the hypertrophic changes of articular chondrocytes in OA is not fully understood. Since pro-inflammatory cytokines such as IL-1β are involved in early OA (42), C/EBPβ may transmit the inflammatory signal to trigger hypertrophic conversion by repressing *Col2a1* and stimulating *Runx2*, *Col10a1* and *Mmp13* expression.

Finally, *ex vivo* organ cultures revealed that C/EBPβ overexpression markedly repressed the genes expressed by proliferative chondrocytes such as COL2A1 and SOX9 (Figure 7). MMP13 is known to be a major degrading enzyme of COL2A1 and we previously reported that C/EBPβ is a direct inducer of *MMP13* (22). Increase in the expression of MMP13 could be involved in the degradation of COL2A1 in C/EBPβ-infected tibias. Meanwhile, it is possible that the synthesis of COL2A1 was directly repressed by C/EBPβ, as *in vitro* experiments suggests. Furthermore, it was reported that the proliferative zone of C/EBPβ knockout mice was significantly enlarged compared to that of wild-type mice (23). These gain and loss of function experiments also confirmed that C/EBPβ could be involved in the regulation of phenotypic conversion from proliferative to hypertrophic chondrocytes. However, the difference of phenotype between C/EBPβ knockout mice and wild-type mice was slight and the dwarfism of C/EBPβ knockout mouse gradually disappeared as the mice grew up. Presumably other C/EBP families may compensate for the lack of C/EBPβ. In fact, we reported that not only C/EBPβ, but also C/EBPδ is induced during OA development (21) and C/EBPδ induced by cytokines also represses the transcriptional activity of *Cd-rap* (20). Although C/EBPβ could be a therapeutic target for OA, we have to create an inhibitor that specifically blocks C/EBP family activities in arthritic articular cartilage.

Together, our results suggest that C/EBPβ directly represses transcription of *Col2a1* during differentiation from proliferative to hypertrophic chondrocytes. In addition, C/EBPβ represses *Sox9* to indirectly inhibit transactivation of *Col2a1*. C/EBPβ also stimulates expression of *Mmp13*, *Col10a1* and *Runx2* and preliminary results obtained in our laboratory suggest that C/EBPβ also stimulates Indian hedgehog. Combined, these results suggest that C/EBPβ is a key regulator that triggers the phenotypic conversion from proliferative to hypertrophic chondrocytes by turning off genes characteristic of proliferative chondrocytes and turning on genes associated with hypertrophic chondrocytes. Therefore, C/EBPβ plays multiple roles in matrix degradation and hypertrophic differentiation of chondrocytes during bone development as well as in arthritic cartilage.

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FOOTNOTES
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The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; COL2A1, Type II collagen;COL10A1, Type X collagen; ACAN, Aggrecan; SOX9, Sex-determining region Y-type high mobility group box 9; MMP, Matrix metalloproteinase; RUNX2, Runt-related transcription factor 2; β-gal, beta galactosidase; OA, osteoarthritis; ECM, Extracellular matrix

FIGURE LEGENDS
FIGURE 1. Expression patterns of C/EBPβ, COL2A1 and SOX9 during chondrocyte differentiation. Upper limbs obtained from mouse embryos (E16.5) were subject to immunohistochemistry with COL2A1, SOX9, COL10A1 and C/EBPβ antibodies. Tissue stained with IgG is shown as a negative control. Hematoxylin was used as a counterstain. Red, green and blue bars indicate the proliferative, pre-hypertrophic and hypertrophic zones, respectively. Scale bar, 500 μm. Data are representative of two independent experiments performed in duplicate. (B) Time course of *Col2a1*, *Sox9*, *Col10a1* and *Col1a1* mRNA was measured by real-time RT-PCR in differentiating ATDC5 cells cultured for 4 weeks. Each value was normalized to *18S* in the same sample. The value of mRNA expression at each stage relative to that on the 4th day was indicated. Means ± S.D. of duplicates from three independent experiments are shown. (C) Time course of C/EBPβ protein was determined by Western blot of nuclear extracts of ATDC5 cells cultured for 4 weeks. Data are representative of two independent experiments performed in duplicate.
FIGURE 2. C/EBPβ repressed differentiation markers and stimulated hypertrophic markers in ATDC5 cells.
(A) ATDC5 cells were differentiated for 2 weeks after transfection with adenovirus vectors expressing C/EBPβ and LacZ control. Expression of *Cebpb*, *Col10a1*, *Mmp13*, *Runx2* and *Col1a1* mRNA was determined by real-time RT-PCR. Each value was normalized to *18S* in the same sample. The value of each mRNA expression relative to that of LacZ on the 4th day was indicated. Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. LacZ. (B) Real-time RT-PCR was performed to measure the expression levels of *Col2a1* and *Acan* mRNA. The same samples shown in (A) were used. Each value was normalized to *18S* in the same sample. The value of each mRNA expression relative to that of LacZ on the 4th day was indicated. Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. LacZ. (C) Alcian blue staining of ATDC5 cells 3 weeks after transfection with C/EBPβ and LacZ control.
FIGURE 3. C/EBPβ indirectly repressed transcriptional activity of *Col2a1* through modulating the expression of SOX9.

(A) Expression of Sox9 mRNA was measured by real-time RT-PCR. The same samples shown in Figure 2 were used. Each value was normalized to *18S* in the same sample. The value of each mRNA expression relative to that of LacZ on the 4th day was indicated. Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. LacZ. (B) Cytoplasmic and nuclear extracts were prepared from ATDC5 cells cultured for 4 days after transfection with adenovirus vectors. Western blot was performed using SOX9 and C/EBPβ antibodies. Data are representative of two independent experiments performed in duplicate. (C) A ChIP assay was performed using ATDC5 cells cultured for 4 days after transfection of adenovirus vectors. SOX9 antibodies were used to immunoprecipitate. Semi-quantitative RT-PCR was performed using primers that amplified between +2136 and +2292 bp to detect binding of SOX9 to the *Col2a1* enhancer. Data are representative of two independent experiments performed in duplicate.

FIGURE 4. Knockdown of C/EBPβ increased differentiation markers in ATDC5 cells.

(A) ATDC5 cells stably expressing shRNA for *Cebpb* were differentiated for 2 weeks. Expression of *Cebpb* and *Col10a1* mRNA was determined by real-time RT-PCR. Each value was normalized to *18S* in the same sample. The value of each mRNA expression relative to that of control on the 4th day was indicated. Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. control. (B) Real-time RT-PCR was performed to measure the expression levels of *Col2a1*, *Acan* and *Sox9* mRNA. The same samples shown in (A) were used. Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. control. (C) Western blot of nuclear extracts from stable ATDC5 cells was performed to investigate the expression of C/EBPβ and SOX9. (D) Densitometric scanning of SOX9 expression was performed. Each density of SOX9 was normalized with that of LAMIN A/C and the ratio by corrected densities of SOX9 to control on 4th day was calculated. Data are representative of two independent experiments performed in duplicate.

FIGURE 5. C/EBPβ repressed *Col2a1* promoter activity in chondrocytes.

(A) The *Col2a1* reporter construct containing -952 to +73 bp of the *Col2a1* promoter and +2038 to +2678 bp of the *Col2a1* enhancer. (B) Semi-quantitative RT-PCR was performed to determine the endogenous expression level of *Col2a1*, *Sox9* and *Cebpb* using mRNA isolated from SW1353 and RCS cells. Data are representative of two independent experiments performed in duplicate. (C) The *Col2a1* reporter construct was co-transfected into SW1353 and RCS cells. Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. SW1353. (D) The *Col2a1* reporter construct was co-transfected with pCMV-LAP and GFP into RCS cells. Western blot of cell lysates from transfected RCS cells was performed to confirm the expression of C/EBPβ. Means ± S.D. of duplicates from three independent experiments are shown. (E) The *Col2a1* reporter construct was co-transfected with 0.2μg of pCMV-LAP and various amounts of A-C/EBP into RCS cells. The expression of each vector was confirmed by Western blot. The Flag antibody was used to detect the expression of A-C/EBP. Means ± S.D. of duplicates from three independent experiments are shown. (F) The *Col2a1* reporter construct and A-C/EBP were co-transfected into SW1353 cells. The expression of A-C/EBP was confirmed by Western blot. Means ± S.D. of duplicates from three independent experiments are shown.

FIGURE 6. C/EBPβ directly down-regulated transcriptional activity of *Col2a1* through its enhancer region.

(A) A series of 5' *Col2a1* promoter deletion constructs were generated (left panel). Black and gray boxes indicate SOX9 binding elements reported by previous studies and conserved C/EBPβ binding motifs, respectively. These reporter constructs were co-transfected with 0.2 μg of pCMV-LAP or GFP into RCS cells, and a reporter assay was performed (right panel). Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. GFP. (B) Reporter constructs were generated, which combined -185 to +73 bp of the *Col2a1* promoter that had no conserved C/EBPβ binding motifs with various enhancer sequences (left panel). Black and gray boxes indicate the SOX9 binding element and the conserved C/EBPβ binding motif, respectively. A point mutation was also introduced to the C/EBPβ binding motif within the enhancer region. These reporter constructs were co-transfected with 0.2 μg of pCMV-LAP or GFP into RCS cells, and a reporter assay was performed

(right panel). Means ± S.D. of duplicates from three independent experiments are shown. $^{*}p < 0.05$ vs. GFP. (C) EMSA for specific binding of C/EBPβ to the *Col2a1* intronic enhancer. Consensus oligonucleotide (C), wild-type (Wt) and mutant (Mt) probes were incubated with nuclear extract from C/EBPβ-transfected ATDC5 cells. Competition and supershift experiments were also performed. Data are representative of two independent experiments performed in duplicate. (D) A ChIP assay for C/EBPβ using ATDC5 cells cultured for 3 weeks. Semi-quantitative RT-PCR was performed using primers as follows: enhancer region of *Col2a1* (from +2003 to +2198) and negative control (from -1891 and -1611 bp). Data are representative of two independent experiments performed in duplicate.

FIGURE 7. C/EBPβ repressed the expression of COL2A1 and SOX9 in *ex vivo* organ cultures.

Ex vivo organ culture of tibias dissected from E14.5 mouse embryos. Tibias were cultured for 4 days without adenovirus vectors (upper), or after transfection with adenovirus vectors expressing LacZ control (middle) and C/EBPβ (lower). Safranin O staining and immunofluorescent staining were performed to localize COL2A1, COL10A1, SOX9, C/EBPβ, RUNX2 and MMP13. β-galactosidase antibody (β-gal) was used to confirm the transfection efficiency of adenovirus vectors. DAPI was used as a counterstain. Red, green and blue bars indicate the proliferative, pre-hypertrophic and hypertrophic zones, respectively. Scale bar, 500 μm. Histological analysis was repeated at least twice for each sample from six pairs of limbs, respectively.

TABLE 1. Comparison of mRNA expression between primary chondrocytes and ATDC5 cells.
Quantitative real time RT-PCR was performed to compare the expression level of various genes using mRNA isolated from primary chondrocytes obtained from mouse ribs and sternums cartilage and ATDC5 cells at the 14th day. ΔCT values relative to the internal control gene (18s) were calculated. Means ± S.D. of duplicates from three independent experiments are shown.

	primary chondrocytes	ATDC5 cells
Col2a1	5.15±0.20	4.50±0.03
Acan	8.38±0.31	7.83±0.08
Sox9	11.31±0.20	10.17±0.03
Col10a1	11.67±0.43	11.85±0.29
Cebpb	15.99±0.15	17.37±0.18
Runx2	14.18±0.21	14.93±0.24
Mmp13	10.59±0.29	15.08±0.05
Col1a1	7.50±0.39	5.67±0.19